Original Article

Reprogramming of Human Pancreatic Organoid Cells into Insulin-Producing β-Like Cells by Small Molecules and *in Vitro* Transcribed Modified mRNA Encoding Neurogenin 3 Transcription Factor

(diabetes / organoid / reprogramming / synthetic mRNA / *in vitro* transcription / IVT / neurogenin 3 / β-cell / insulin / TGF / RepSox / small molecules)

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Abstract. Reprogramming of non-endocrine pancreatic cells into insulin-producing cells represents a promising therapeutic approach for the restoration of endogenous insulin production in diabetic patients. In this paper, we report that human organoid cells derived from the pancreatic tissue can be reprogrammed into the insulin-producing cells (IPCs) by the combination of *in vitro* transcribed modified mRNA encoding transcription factor neurogenin 3 and small molecules modulating the epigenetic state and signalling pathways. Upon the reprogramming, IPCs formed 4.6 ± 1.2 % of the total cells and expressed typical markers (insulin, glucokinase, ABCC8, KCNJ11, SLC2A2, SLC30A8) and transcription factors (PDX1, NEUROD1, MAFA, NKX2.2, NKX6.1, PAX4, PAX6) needed for the proper function of pancreatic β-cells. Additionally, we have revealed a positive effect of ALK5 inhibitor RepSox on the overall reprogramming efficiency. However, the reprogrammed IPCs possessed only a partial insulin-secretory capacity, as they were not able to respond to the changes in the extracellular glucose concentration by increasing insulin secretion. Based on the achieved results we conclude that due to the incomplete reprogramming, the IPCs have immature character and only partial properties of native human β-cells.

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Abbreviations: AMY – amylase, ANOVA – analysis of variance, CPEP – C-peptide, CHGA – chromogranin A, DAPI – 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride, ECAD – epithelial cadherin, EGF – epithelial growth factor, GCG – glucagon, GHRL – ghrelin, HI – human islets, HPRT – hypoxanthine phosphoribosyltransferase 1, INS – insulin, IPCs – insulin-producing cells, IVT – *in vitro* transcription, KRT19 – cytokeratin 19, n.s. – not significant, PCR – polymerase chain reaction, qRT-PCR– quantitative reverse transcription PCR, SEM – standard error of the mean, SOMA – somatostatin, TGF – transforming growth factor, w/o – without.

Introduction

Diabetes is a chronic metabolic disease caused by the loss of insulin-producing β-cells. β-Cells, located in the pancreatic tissue within the islets of Langerhans, provide strict regulation of the blood glucose level. The loss of β-cells results in insulin insufficiency, which can be treated by periodic application of exogenous insulin in the form of insulin injection or continuous delivery by an insulin pump. Despite the improvements in diabetes therapy, many patients cannot achieve a stable physiological glucose level and are at risk of life-threatening complications. Transplantations of a whole pancreas or isolated islets of Langerhans have shown that restoration of the insulin-producing tissue and subsequent normalization of the blood glucose level is an optimal therapeutic option for the treatment of diabetic patients (Shapiro et al., 2017). However, due to the limited supply of organ donors, this therapeutic approach is available only to a marginal number of diabetic patients. Therefore, the development of new alternative sources of insulin-producing cells (IPCs) is an ultimate goal in the field of β-cell replacement therapy.

One of the promising approaches for the restoration of endogenous IPCs is cellular reprogramming. It is

based on induced conversion of the cellular phenotype by ectopic over-expression of specific transcription factors (Heinrich et al., 2015). IPCs derived by reprogramming of various types of pancreatic cells, including acinar, ductal and endocrine non-β-cells, have already demonstrated the potential for treating diabetes in experimental animal models (Li et al., 2014; Wang et al., 2018; Furuyama et al., 2019). However, in all those cases the cell conversion was induced by viral vectors. Viral vectors possess the risk of integration into the host cell genome, mutagenesis and oncogenic transformation. This limits potential clinical application of such reprogramming approaches (Hacein-Bey-Abina et al., 2003; Cavazzana-Calvo et al., 2010). An alternative approach can be based on the application of non-viral vectors such as *in vitro* transcribed (IVT) mRNAs encoding specific reprogramming factors. This method has already been used for highly efficient generation of induced pluripotent stem cells (Warren et al., 2010) and for direct reprogramming into the neuronal and hepatocyte-like cell fates (Simeonov and Uppal, 2014; Goparaju et al., 2017; Kim et al., 2018).

From a clinical standpoint, the pancreatic duct cells seem to be an optimal cell type for the potential reprogramming into the IPCs. Firstly, the pancreatic ducts are accessible by the main pancreatic duct, so the reprogramming vectors can be delivered by intra-ductal injection (Wang et al., 2018). Moreover, pancreatic ducts contain a subpopulation of facultative progenitor cells. These are able to differentiate into the specialized acinar or endocrine cells under the pathophysiological conditions (Aguayo-Mazzucato and Bonner-Weir, 2018; Xu et al., 2008). Therefore, the pancreatic ductal cells are considered as a suitable cell source for the potential endogenous IPC reprogramming therapy.

A system for long-term *in vitro* cultivation of pancreatic duct-derived cells that can recapitulate the *in vivo* structure, genetic signature and functionality of the original tissue was recently developed (Huch et al., 2013; Loomans et al., 2018). This system is based on the cultivation of duct-derived cells containing a subpopulation of progenitor/stem-like cells. These cells form cyst-like oval structures, so-called 'organoids', upon embedding within the collagen and laminin-rich Matrigel matrix and subsequent culture in the optimized medium. This medium contains growth factors and small molecules that regulate several key signalling pathways, such as Wnt, transforming growth factor (TGF) β, mitogen-associated protein kinase (MAPK) and Notch pathways. This culture system promotes preservation and continuous expansion of the cell population derived from pancreatic ducts. It can be used as an *in vitro* model for studying the tissue-specific physiological and regenerative processes.

In our previous study, we have demonstrated that pancreatic acinar cell line AR42J can be reprogrammed into the IPCs using IVT-modified mRNAs encoding key transcription factors of β-cell differentiation (Koblas et al., 2016). In this paper, we demonstrate that human pancreatic organoid cells can be reprogrammed into the IPCs using IVT-modified mRNA encoding only the single transcription factor neurogenin 3 and a cocktail of small molecules modulating epigenetic modifiers and signalling pathways. The results of our study may provide a useful basis for potential development of therapeutic approaches aiming at reprogramming or regeneration of endogenous pancreatic β-cells in diabetic patients.

Material and Methods

Isolation of human CD133$^+$ pancreatic cells

All human primary tissues were obtained from Prodo Laboratories (Irvine, CA). The institutional review board approval for research use of human tissue was obtained from the Ethics Committees of the Institute for Clinical and Experimental Medicine and Thomayer Hospital in Prague. For the five donors used in this study (four males and one female), the mean donor age was 41.8 ± 10.1 years with BMI 27.9 ± 6.1 kg/m^2. The pancreatic tissue used in the study was derived from the non-diabetic organ donors only. The CD133$^+$ pancreatic cells were isolated from the islet-depleted cell fractions as published previously (Koblas et al., 2008).

Expansion and culture of human pancreatic organoids

Isolated CD133$^+$ pancreatic cells were diluted in the 'Expansion Medium' (10^7 cells/ml) consisting of Advanced DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with B27 supplement (w/o vitamin A) (Invitrogen), recombinant human (rh) EGF (50 ng/ml), rh R-spondin 1 (500 ng/ml), rh FGF10 (50 ng/ml), rh HGF (50 ng/ml), rh noggin (100 ng/ml) (all R&D Systems, Minneapolis, MN), 1.25 mM N-acetylcysteine, 10 mM nicotinamide (all Sigma-Aldrich, St. Louis, MO), prostaglandin E2 (3 μ M), CHIR99021 (5 μ M), SB-431542 (10 μ M), trichostatin A (20 nM) (all Cayman Chemical, Ann Arbor, MI). One hundred μl of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) was added to 100 μl of the cell suspension and the mixture was placed around the bottom rim of each culture well of 12-well plates (Sigma-Aldrich). After solidification at 37 °C for 30 min, each well was overlaid with 1 ml of Expansion Medium. Medium was changed every 1–3 days. The organoids were passaged each 7 to 10 days. The organoids were harvested from Matrigel using dispase solution (BD Biosciences) at 37 °C for 45 min and further dissociated into small pieces by Accutase solution (Sigma-Aldrich) at 20 °C for 20 min, followed by trituration. Dissociated organoids were then transferred to a fresh Matrigel culture system in a 1 : 2 split ratio. The remaining organoids or organoid-derived cells were used for further experiments or analysis.

Preparation of IVT NEUROG3 mRNA

In vitro transcription of NEUROG3 mRNA was performed as published previously (Koblas et al., 2016) using the DNA template containing the NEUROG3 complementary DNA (cDNA) sequence. The NEUROG3 coding region was derived by reverse transcription of mRNA isolated from human islet cells, using gene-specific primers and the AccuScript High-Fidelity 1st Strand cDNA Synthesis Kit (Agilent, Santa Clara, CA), according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of cDNA was performed using the same gene-specific primers and Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA), according to the manufacturer's instructions.

Culture and reprogramming of organoid-derived pancreatic cells

Pancreatic organoids were released from Matrigel, further dissociated by Accutase, washed with PBS and seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 100,000 cells per well/150 µl of the reprogramming medium (per well). The complex of IVT NEUROG3 mRNA with Messenger MAX transfection reagent (Life Technologies, Carlsbad, CA) was added to the appropriate wells. Culture plates were covered with a combination of human recombinant laminin 511, laminin 521 (Biolamina, Stockholm, Sweden) and fibronectin (BD Biosciences) prior to use.

The reprogramming medium was composed of Advanced DMEM/F12 supplemented with B27 supplement (with vitamin A), 2-mercaptoethanol (100 µM) (all Invitrogen), rh FGF7 (10 ng/ml), rh noggin (100 ng/ml), rh betacellulin (10 ng/ml) (all R&D systems), 1.25 mM N-acetylcysteine, 1 mM nicotinamide, human gastrin (20 ng/ml) (all Sigma-Aldrich) and the following small molecules: LY411575 (50 nM), BRD7552 (6 µM – days 0–7), LDN-193189 (100 nM), Gefitinib (1 µM – days 2–4), PD161570 (500 nM – days 7–15), CHIR 99021 (3 µM), CC-930 (1 µM), AS-1842856 (100 nM – days 5–15), R428 (2 µM – days 5–15), Aurora Kinase Inhibitor II (5 µM – days 5–15), glycyl-H 1152 (5 µM – days 5–15), PJ-34 (3 µM), LY2608204 (1 µM – days 5–15) and L-165041 (10 µM – days 7–15) (all Cayman Chemical). In addition, RepSox (10 µM), 5-aza-2'-deoxycytidine (1 µM - days 0–3), PP2 (10 µM - days 0–3), forskolin (10 µM), ISX-9 (20 µM), GSK126 (5 µM – days 0–3) (all Cayman Chemical) were added to the reprogramming medium according to the treatment group. Medium was changed every day during the first five days, followed by changes every third day.

IVT NEUROG3 mRNA transfection

mRNA transfection was carried out using the Lipofectamine MessengerMAX mRNA Transfection Reagent (Life Technologies). With Opti-MEM basal media (Life Technologies), synthetic mRNA was diluted to a concentration of 20 ng/µl and Lipofectamine MessengerMAX mRNA Transfection Reagent was diluted 33×. Diluted mRNA and transfection reagent were pooled 1 : 1 and incubated at room temperature for 5 min before being dispensed to the culture media at appropriate concentration.

Immunostaining

For immunostaining analyses, cultured organoids were harvested from Matrigel, washed with PBS and fixed with 4% paraformaldehyde for 1 h at 4 °C. Following fixation, the organoids were washed 3× in PBS, embedded in 1.5% agarose (Sigma-Aldrich), suspended in 30% sucrose (Sigma-Aldrich) solution in PBS overnight and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA) at –80 °C. Tissue sections were sectioned in 8 µm thickness. Immunostaining of tissue sections and cultured cells was performed as published previously (Koblas et al., 2016). The following antibodies were used for immunostaining: mouse anti-insulin (1 : 200) (Santa Cruz, Santa Cruz, CA), rabbit anti-C-peptide (1 : 100) (Cell Signaling, Danvers, MA), rabbit anti-glucagon (1 : 200) (Abcam, Cambridge, United Kingdom), rabbit anti-somatostatin (1 : 200) (Abcam), rabbit anti-ghrelin (1 : 200) (Abcam), mouse anti-E-cadherin (1 : 300) (Exbio, Prague, Czech Republic), mouse anti-cytokeratin 19 (1 : 500) (Exbio), rabbit anti-Ki67 (1 : 300) (Abcam), rabbit anti-chromogranin A (1 : 200) (NOVUS, Centennial, CO), rabbit anti-α-amylase (1 : 300) (Sigma-Aldrich), rabbit anti-PDX1 (1 : 100) (Abcam), rabbit anti-SOX9 (NOVUS), rabbit anti-NKX2.2 (NOVUS), rabbit anti-NKX6.1 (1 : 200) (Abcam), goat anti-NEUROD1 (1 : 200) (NOVUS), rabbit anti-MAFA (1 : 100) (Abcam), goat anti-PAX4 (1 : 100) (NOVUS), rabbit anti-PAX6 (NOVUS), sheep anti-neurogenin 3 (1 : 300) (NOVUS). The secondary antibodies were donkey anti-mouse, donkey anti-rabbit, donkey anti-goat, donkey anti-sheep IgG Alexa Fluor 555 or Alexa Fluor 647 (Invitrogen) at a 1 : 500 dilution. Cell nuclei were stained for 15 min at room temperature with NucBlue Fixed Cell ReadyProbes Reagent (Life Technologies) diluted 1 : 10 in PBS. Tissue sections and cell samples were quantified from at least ten visual fields (with 100× magnification) using the EVOS FL automatic cell counting tool (Life Technologies).

Isolation of cellular RNA

Total RNA was isolated using the RNeasy Mini Plus kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA was then treated for 1 h at 37 °C with Turbo DNase (Life Technologies), re-purified using RNA Clean & Concentrator-5 (Zymo Research, Irvine, CA), and quantitated using a Qubit fluorometer (Life Technologies).

qRT-PCR gene expression analysis

Gene expression analysis was performed as published previously (Koblas et al., 2016) using human gene-specific primers (IDT, Coralville, IA). Reactions and data

analysis were carried out using a QuantStudio 6 System (Life Technologies). All samples were assayed in triplicates. Fold changes in gene expression were determined using the ΔCT method, with normalization to *HPRT* expression. The sequences of primers are available upon request.

Human C-peptide content and glucose/KCl-stimulated secretion

Determination of human C-peptide content and glucose/KCl-stimulated C-peptide secretion of reprogrammed IPCs and human islets was performed as published previously (Koblas et al., 2016). In the samples from the glucose-stimulated C-peptide secretion assay and cell lysates, the C-peptide content was determined using the Ultrasensitive C-peptide ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions. All incubation steps were performed at 37 °C in a CO_2 incubator, and all solutions were equilibrated to 37 °C prior to use. For the C-peptide content analysis, the cells were lysed in RIPA buffer (Sigma-Aldrich). The DNA content of all samples was determined using a Qubit fluorometer.

Statistical analysis

Statistical analysis was performed using the PRISM software and two-tailed paired or unpaired Student's *t*-tests, or one-way ANOVA with Dunnett's post hoc test, as appropriate. The numbers of independent experiments performed are indicated in the text. Mean values are presented with standard error of the mean (SEM) in the format (mean ± SEM). P values of < 0.05 were considered to indicate statistically significant differences.

Results

Isolation and expansion of human pancreatic CD133$^+$ cells

CD133 positive (CD133$^+$) pancreatic cells were derived by immunomagnetic separation from the islet-depleted pancreatic tissue of organ donors (N = 5). On average, CD133$^+$ cells formed 8.2 ± 0.9 % of all pancreatic cells (N = 5). CD133$^+$ cells formed cyst-like structures (Fig. 1A), so-called 'organoids', within the first week upon embedding in the Matrigel matrix and cultivation in the culture medium containing prostaglandin E2, CHIR99021 (glycogen synthase kinase 3 inhibitor), SB-431542 (ALK5 inhibitor), trichostatin A (inhibitor of histone deacetylases) and growth factors R-spondin, noggin and epithelial growth factor (EGF). Organoids were self-organized into an oval single layer of epithelial cells mainly containing cytokeratin 19 (KRT19)-positive cells (94.7 ± 3.2 %)(Fig. 1B). The diameter of the organoids was in the range of 100–2,000 μm. Cells within the organoids were also positive for the markers of pancreatic ductal progenitor cells, transcription factors pancreatic and duodenal homeobox 1 (PDX1) and SRY (sex-determining region Y) box 9 (SOX9) (Fig. 1B). Organoids could be repeatedly passaged at least 20 times for a total period of five months (Fig. 1C), without any loss in proliferative capacity and changes in the cellular phenotype (Fig. 1D). The average cell doubling time was 72 ± 9 h, depending on the frequency of passages (once every 7–10 days). The proliferation marker Ki-67 was detected in 21.4 ± 3.1 % cells as determined after 1- and 5-month culture (N = 5), without any detectable difference in Ki67 positivity during the cultivation period.

Immunostaining revealed that organoid cells maintained an apical-basolateral polarization, as evidenced by the detection of the CD133 marker at the apical side of the organoids (Fig. 1B). Additionally, the epithelial cell marker epithelial cadherin (E-cadherin) was detected on the membrane side of the organoid cells (Fig. 1B). Analysis of gene expression by quantitative reverse transcription PCR (qRT-PCR) confirmed that organoids expressed transcription factors PDX1 and SOX9 (Fig. 3B). Moreover, the expression of pancreatic ductal progenitor cell marker NK 6 homeobox 1 (NKX6.1), transcription factor ISL LIM homeobox 1 (ISL1), potassium voltage-gated channel component (KCNJ11) and glucose transporter solute carrier family 2 member 2 (SLC2A2) was also detected (Fig. 3B). However, organoid cells were negative for endocrine progenitor cell marker neurogenin 3 (NEUROG3), pan-endocrine cell marker chromogranin A (CHGA) and β-cell marker insulin (INS), suggesting a preserved ductal character of organoid cells (Fig. 1B, Fig. 3B).

Induction of the endocrine character in human pancreatic organoid cells by the IVT-modified neurogenin 3 mRNA

Neurogenin 3 transcription factor initiates differentiation of all pancreatic endocrine cells. As the organoid cells were negative for this transcription factor, we tested whether the ectopic expression of neurogenin 3 can induce conversion of organoid cells into the cells with endocrine character. For that purpose we used temporal over-expression, based on the repeated transfections of IVT-modified NEUROG3 mRNA into the organoid cells. In order to allow the mRNA transfection of organoid cells, we had to change the culture conditions from Matrigel-based three-dimensional cell culture system into a standard two-dimensional planar cell culture surface. After screening various culture conditions it was revealed that organoid-derived cells can be further cultured on a cell culture surface coated with a combination of extracellular-matrix proteins laminin 521, laminin 511 and fibronectin. The transfer of dissociated organoid cells onto the 2D planar cell culture surface allowed efficient transfection by IVT-modified NEUROG3 mRNA, as revealed by immunostaining for the NEUROG3 transcription factor. NEUROG3 expression was dose-dependent, with the maximal expression rate achieved at a concentration of 1.5 μg IVT-modified

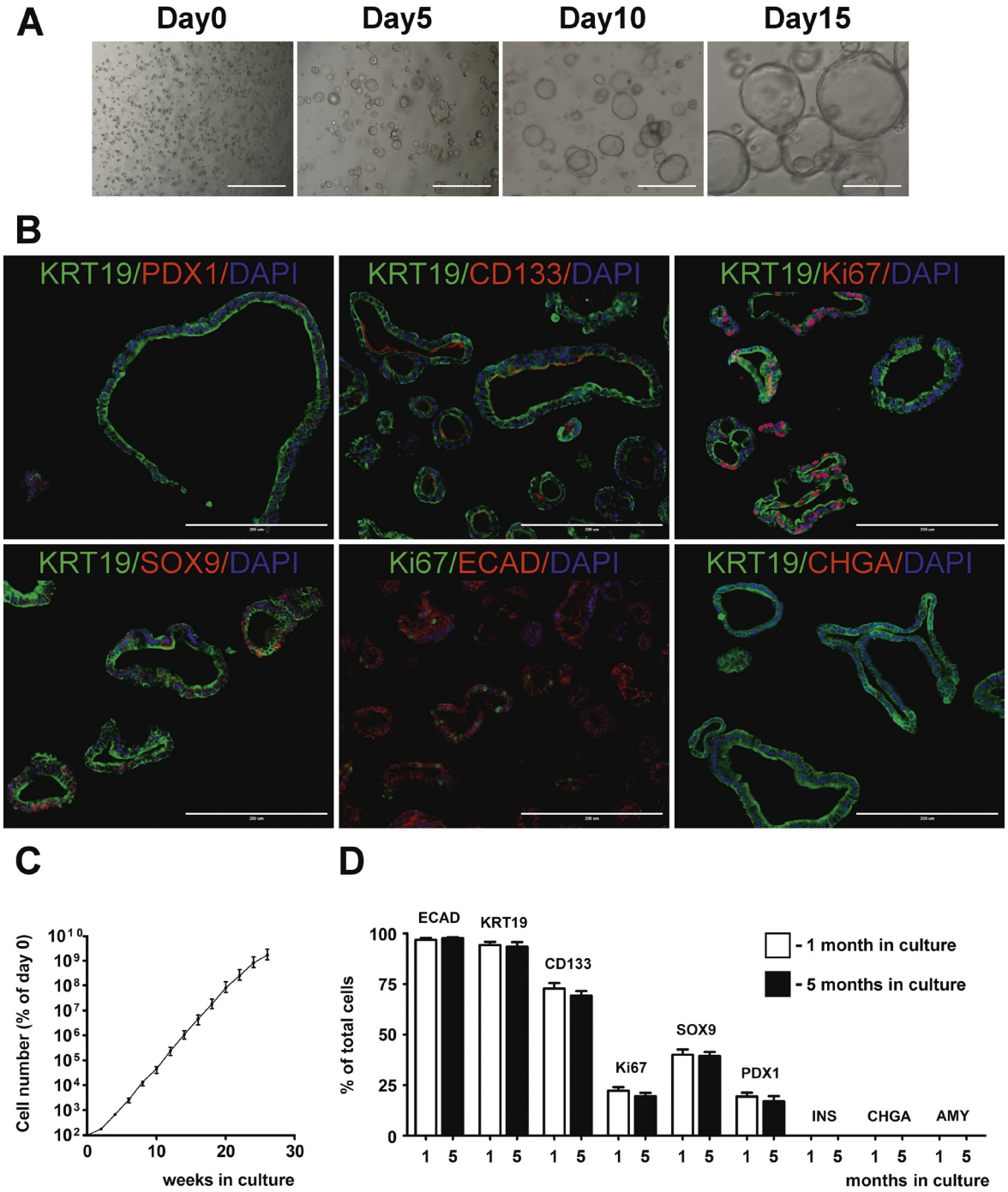

Fig. 1. Characterization of human organoids derived from CD133^{+} pancreatic cells

A: Bright-field images of organoid development and growth within the first 15 days of cultivation in a 3D Matrigel-based culture system. Scale bar = 500 µm

B: Immunostaining for the ductal cell marker cytokeratin 19 (KRT19) (green) and transcription factor PDX1 (red) – top left panel; KRT19 (green) and stem cell marker CD133 (red) – top middle panel; KRT19 (green) and proliferation marker Ki67 (red) – top right panel; KRT19 (green) and transcription factor SOX9 (red) – bottom left panel; Ki67 (green) and epithelial cell marker E-cadherin (ECAD) (red) – bottom middle panel; KRT19 (green) and pan-endocrine marker chromogranin A (CHGA) (red) – bottom right panel. Cell nuclei were stained by DAPI (blue). Scale bar = 200 µm

C: Growth curve of pancreatic organoids during the 5-month culture period. Cell numbers were counted from the total cell samples derived at each passage. The expansion curve is representative of five donors. Data represent means ± SEM.

D: Characterization and quantification of organoid cells during the 5-month culture period by immunostaining for typical markers of epithelial cells (ECAD), ductal cells (KRT19), proliferation (Ki67), pancreatic progenitor cells (CD133), (PDX1), (SOX9), β-cells (INS), endocrine cells (CHGA) and exocrine cells (AMY). Immunostaining was performed after one (white bars) and five (black bars) months of organoid culture. Organoids derived from five donors were analysed. At least 10 organoids per sample were quantified. Data represent means ± SEM.

NEUROG3 mRNA/ml media (Fig. 2). At that dose, NEUROG3 was expressed by 37.1 ± 5.4 % cells (N = 10). The transfection of organoid cells with IVT-modified NEUROG3 mRNA repeated daily for four days followed by subsequent cultivation for another 11 days induced endogenous expression of key endocrine and β-cell-specific transcription factors, such as neuronal differentiation 1 (NEUROD1), NK2 homeobox 2 (NKX2.2), paired box 4 (PAX4) and paired box 6 (PAX6) (Fig. 3B). The expression of pan-endocrine marker CHGA (Fig. 4B) and genes important for the proper function of pancreatic β-cells such as prohormone processing enzymes proprotein convertase subtilisin/kexin type 1 (PCSK1) and proprotein convertase subtilisin/kexin type 2 (PCSK2), sulphonylurea receptor 1 protein (ABCC8) and solute carrier family 30 member 8 (SLC30A8) was also induced.

Moreover, the expression of PDX1 and NKX6.1 transcription factors and potassium channel component KCNJ11 was increased in comparison with the expression level of the original organoids and untreated control samples (N = 10). In addition, NEUROG3 over-expression also induced expression of ghrelin (GHRL) (4.6 ± 1.5 % of all cells) and somatostatin (SOMA) (5.1 ± 1.6 % of all cells) hormones (Fig. 4B), albeit in the case of somatostatin at a lower level in comparison with that of adult human islets (Fig. 3B). Insulin or glucagon transcripts were not detected by qRT-PCR analysis (Fig. 3B). Our results indicate that the repeated transfection of IVT-modified NEUROG3 mRNA into the organoid cells can induce their reprogramming into the cells with endocrine character, however, only that of pancreatic somatostatin-producing δ-cells and ghrelin-producing ε-cells.

Reprogramming of pancreatic organoid cells into the insulin-producing cells

Although the IVT-modified NEUROG3 mRNA was able to induce the endocrine character in pancreatic organoid-derived cells, the limited expression of only ghrelin and somatostatin hormones suggested that further stimulation is needed in order to derive IPCs. NEUROG3 is active only during the early phase of pancreatic endocrine cell differentiation, while further differentiation into the specific endocrine subtypes is regulated by other transcription factors. Therefore, we performed screening of small molecules and cell culture conditions that can activate further reprogramming into the β-like cell phenotype.

With the aim to induce or increase the endogenous expression of β-cell-specifying transcription factors we tested small molecules that had already been used either for the differentiation of pluripotent stem cells into the β-cells or for the reprogramming of various cell types into the endocrine or neuronal cell fates (Afrikanova et al., 2011; Dioum et al., 2011; Rezania et al., 2014; Koblas et al., 2016; Fontcuberta-PiSunyer et al., 2018). These compounds mainly target signalling pathways or induce epigenetic changes important for the activation of gene expression. The combinatorial screening revealed that a combination of IVT-modified NEUROG3 mRNA with the following small molecules efficiently induced reprogramming of organoid cells into the IPCs: RepSox (TGF β type I activin like kinase receptor ALK5 inhibitor); 5-aza-2'-deoxycytidine (5-aza-CdR) (inhibitor of DNA methyltransferases); PP2 (Src family kinase inhibitor); forskolin (adenylyl cyclase activator); ISX-9 (Ca^{2+} influx activator); GSK126 (EZH2 inhibitor). This set of six small molecules is hereafter referred to as RAPFIG small molecules. While the epigenetic modifiers (GSK126 and 5-aza-CdR) were used only during the first three days – in order to modulate the epigenetic state of the cells during the ectopic NEUROG3 over-expression – the other small molecules, with the exception of PP2, were used during the entire reprogramming procedure. PP2 was used only on days 1–3 of reprogramming.

As revealed by the qRT-PCR analysis, the combination of IVT-modified NEUROG3 mRNA and RAPFIG small molecules induced expression of the MAF bZIP A

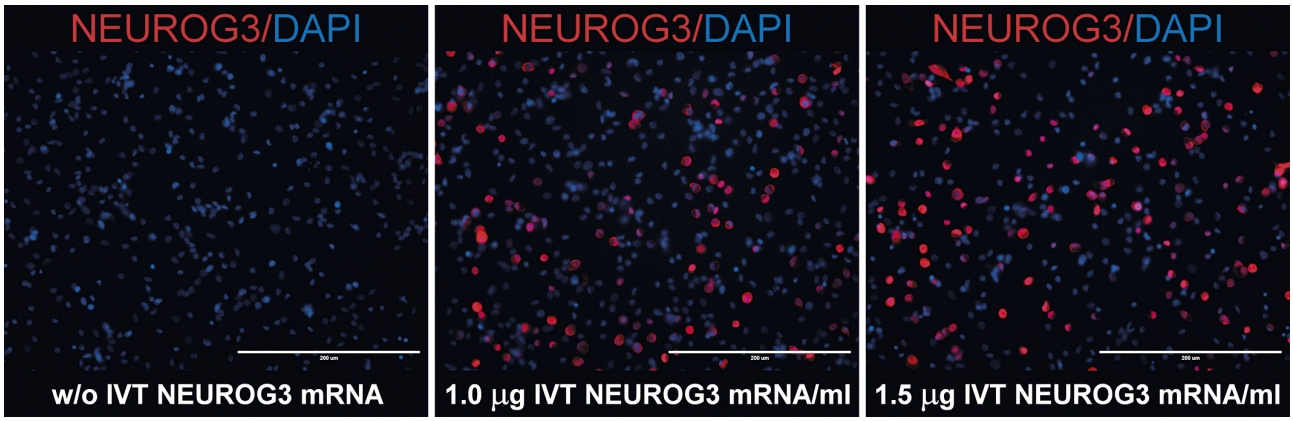

Fig. 2. Detection of neurogenin 3 expression in cells derived from pancreatic organoids after the transfection with IVT NEUROG3 mRNA. Dose-dependent expression of neurogenin 3 (red) after the transfection of organoid-derived cells with IVT NEUROG3 mRNA at doses of 0 (left panel), 1,000 (middle panel) and 1,500 (right panel) ng/ml media as determined by immunostaining 20 h post-transfection. Cell nuclei were stained by DAPI (blue). Scale bar = 200 μm

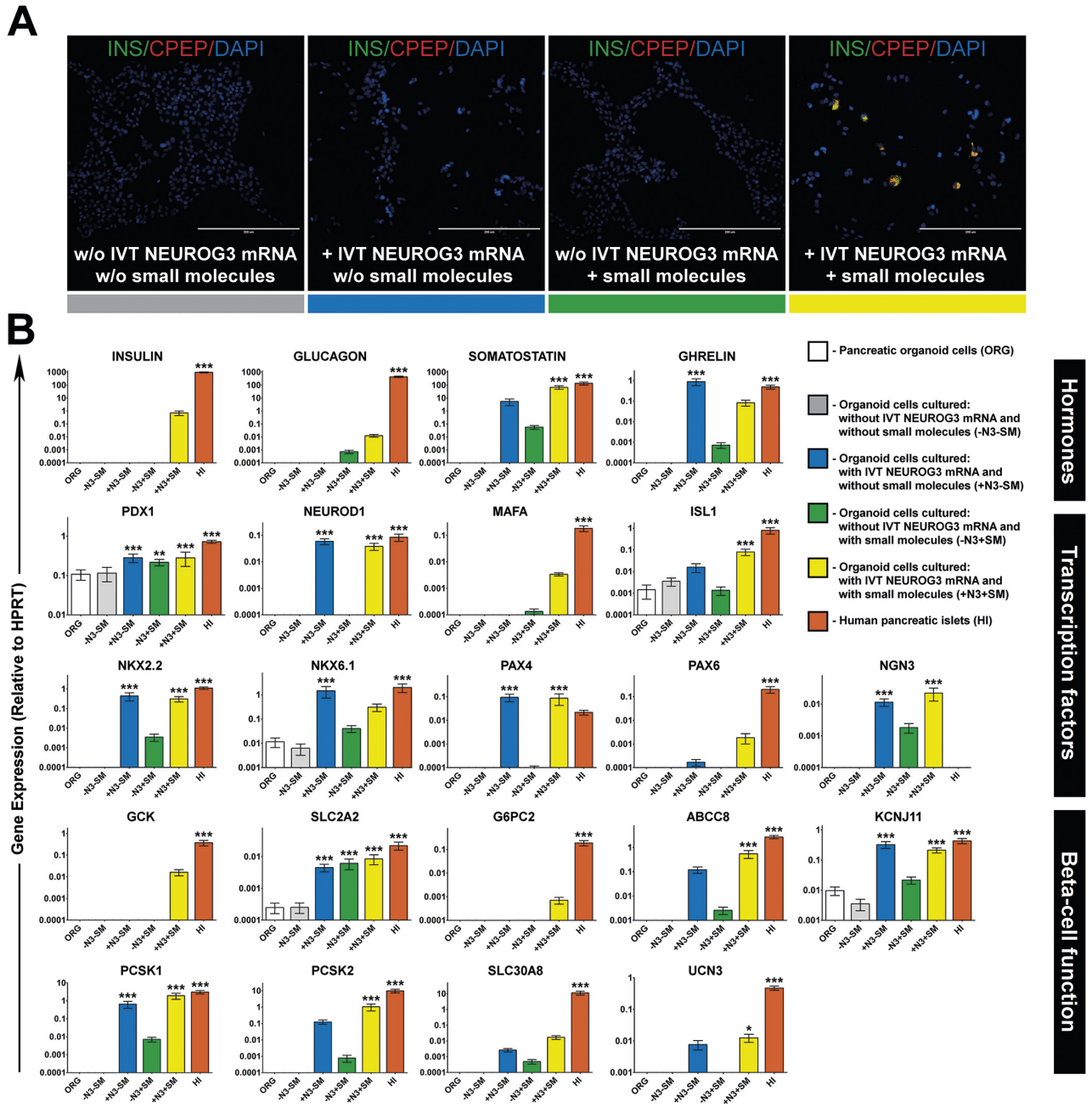

Fig. 3. Reprogramming of pancreatic organoid-derived cells into the IPCs by the combination of IVT NEUROG3 mRNA and small molecules

A: Immunostaining for insulin (INS) (green) and C-peptide (CPEP) (red) in the organoid-derived cell samples cultured: without IVT NEUROG3 mRNA and without small molecules (first panel); without IVT NEUROG3 mRNA and with small molecules (second panel); with IVT NEUROG3 mRNA and without small molecules (third panel); with IVT NEUROG3 mRNA and with small molecules (fourth panel). Note that co-staining of insulin and C-peptide in the same cell gives yellow signal. Cell nuclei were stained by DAPI (blue). Scale bar = 200 μm

B: Gene expression analysis of the cultured pancreatic organoids; IPCs reprogrammed by the IVT NEUROG3 mRNA and small molecules; control groups and adult human islets. The expression of pancreatic hormones (insulin, glucagon, somatostatin, ghrelin), key transcription factors of pancreatic β-cells (PDX1, NEUROD1, MAFA, NKX2.2, NKX6.1, PAX6, ISL1), β-cell functionality markers (GCK, PCSK1, PCSK2, ABCC8, KCNJ11, SLC2A2, SLC30A8, G6PC2) and β-cell maturation marker (UCN3) was determined. Expression of each gene was normalized to *HPRT*. Organoid cells and treatment groups (N = 5 biological replicates, each with two technical replicates), human islets (N = 2 biological replicates, each with two technical replicates). Gene expression analysis of different treatment groups was performed using the samples derived at the end of the treatment period (day 15), for organoid cells, 3-month-old organoids were used. One-way ANOVA with Dunnett's post hoc test was performed to compare organoids, human islets and all treatment groups with the control untreated group (without IVT NEUROG3 mRNA and without small molecules), where *P < 0.05, ** P < 0.01, ***P < 0.001.

(MAFA) transcription factor, glucokinase (GCK), islet-specific glucose-6-phosphatase catalytic subunit 2 (G6PC2), and glucagon and insulin genes in IPCs (Fig. 3B). Additionally, up-regulation of ISL1 and PAX6 transcription factors, somatostatin, and functional genes such as *SLC2A2*, *SLC30A8*, *PCSK1*, *PCSK2* and *ABCC8* was also detected. On the other hand, the expression of the ghrelin hormone and NKX6.1 transcription factor was down-regulated in comparison to the expression level of these genes in organoid cells reprogrammed only with the IVT-modified NEUROG3 mRNA, i.e., reprogrammed without the RAPFIG small molecules. Production of insulin (4.6 ± 1.2 % of all cells), somatostatin (25.3 ± 5.6 % of all cells) and ghrelin (2.1 ± 1.2 % of all cells) (Fig. 4B) at the protein level was confirmed by the immunostaining for these hormones (Fig. 4A). The average number of reprogrammed IPCs was 4.6 ± 1.2 % of all cells (Fig. 4B). Additionally, positive co-staining for insulin and C-peptide (CPEP) was detected, demonstrating the capability of reprogrammed IPCs to process prohormone proinsulin into a fully mature insulin and C-peptide by the endopeptidases PCSK1 and PCSK2 (Fig. 4A). Negative immunostaining for glucagon confirmed qRT-PCR results, as the expression of glucagon at the mRNA level was lower in comparison with the other pancreatic hormones (Fig. 3B). Following the reprogramming period, no cell was positive for proliferation marker Ki-67 as revealed by immunostaining (data not shown). Moreover, the total cell number was 58.9 ± 12.7 % lower in the group treated with the combination of IVT-modified NEUROG3 mRNA and small molecules in comparison with the control untreated group.

Furthermore, the immunostaining revealed co-expression of insulin with several key transcription factors important for the β-cell-specific expression programme (e.g., PDX1, NKX6.1, NEUROD1, NKX2.2, PAX4) (Fig. 6A). However, the expression of PDX1 and NKX6.1 was relatively heterogeneous. The expression of MAFA and PAX6 transcription factors at the protein level was not detected at all. In order to determine whether the reprogrammed IPCs express insulin alone or in combination with other pancreatic hormones – which is the characteristic of immature β-cells – we performed double-immunostaining for a combination of insulin with glucagon, somatostatin, or ghrelin hormones. We did not detect any insulin-positive cells that co-expressed glucagon or ghrelin. However, co-expression of insulin with somatostatin was detected in a subpopulation of reprogrammed IPCs, revealing the immature character of these cells (Fig. 4A). Moreover, co-staining for insulin and cytokeratin 19 also detected double-positive cells, although the intensity of cytokeratin 19 staining in IPCs was significantly lower than in the insulin-negative cells.

These results revealed that the combination of IVT-modified NEUROG3 mRNA and small molecules targeting signalling pathways and epigenetic modifiers can induce reprogramming of pancreatic organoid cells into the IPCs. These cells are able to produce and process insulin. However, the insufficient induction of MAFA and PAX6 transcription factors, and co-expression of insulin and somatostatin hormones, suggest that these cells are not fully reprogrammed into the mature β-like cells.

The role of small molecules in the reprogramming of pancreatic organoid cells into the insulin-producing cells

Following optimization of the reprogramming protocol, we determined the effect of RAPFIG small molecules on the reprogramming process in more detail. We chose the most potent compounds according to their effect on insulin gene expression, and determined their influence on the gene expression profile of the reprogrammed IPCs. Systematic removal of individual small molecules from the reprogramming medium revealed that only RepSox (ALK5 Inhibitor II) was crucial for the induction of insulin expression by the reprogrammed IPCs. Omission of RepSox from the reprogramming medium significantly reduced the expression of somatostatin, ABCC8, G6PC2, PCSK2, SLC30A8 and transcription factors ISL1, MAFA and PAX6, while the expression of insulin and glucagon hormones was not detected at all (Fig. 5). The exclusion of other small molecules from the reprogramming medium had no such a significant effect on the expression profile of IPCs.

Interestingly, the cultivation of organoid cells in the basal medium containing only the RAPFIG small molecules, without the ectopic over-expression of NEUROG3, was also able to induce the endocrine character. Small molecules up-regulated expression of SLC2A2 and PDX1 and induced expression of PCSK1, PCSK2, ABCC8, SLC30A8, transcription factors NKX2.2 and MAFA**,** and ghrelin, glucagon and somatostatin hormones in comparison to the basal medium not containing the RAPFIG small molecules (Fig. 3B).

It is important to note that in addition to the RAPFIG small molecules, the basal reprogramming medium also contained other small molecules such as LY411575 (γ-secretase inhibitor); BRD7552 (PDX1 transcription factor inducer); LDN-193189 (ALK2 and ALK3 inhibitor); Gefitinib (EGFR inhibitor); PD161570 (FGFR1 inhibitor); CHIR 99021 (GSK-3 inhibitor); CC-930 (JNK inhibitor); AS-1842856 (FoxO1 inhibitor); R428 (Axl kinase inhibitor); Aurora Kinase Inhibitor II; glycyl-H 1152 (Rho-kinase (ROCK) inhibitor); PJ-34 (inhibitor of poly (ADP-ribose) polymerases); LY2608204 (glucokinase activator) and L-165041 (PPARβ/δ agonist). Therefore, the overall combination of all the small molecules, and not only the RAPFIG compounds, has a positive and synergic effect on the improved reprogramming efficiency.

In this study, we also tested the effect of other inhibitors of epigenetic modifiers, such as EPZ5676, UNC0638, BIX01294 and SBHA. None of them had any significant effect on the improvement of the reprogramming efficiency (data not shown).

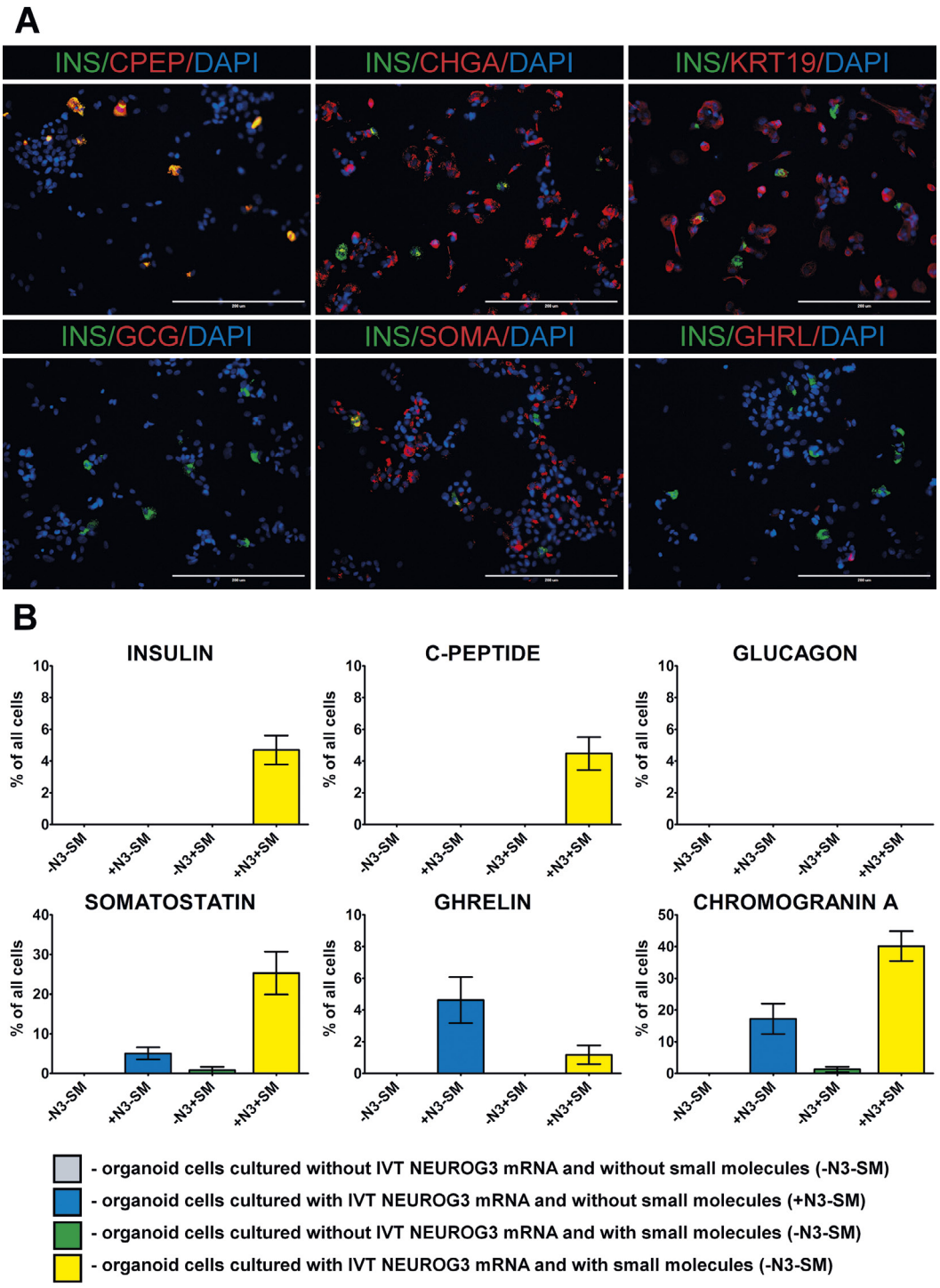

Fig. 4. Characterization of IPCs derived by the reprogramming of pancreatic organoid cells

A: Immunostaining for insulin (green) and C-peptide (red) – top left panel; insulin (green) and pan-endocrine marker chromogranin A (red) – top middle panel; insulin (green) and ductal cell marker cytokeratin 19 (red) – top right panel; insulin (green) and glucagon (red) – bottom left panel; insulin (green) and somatostatin (red) – bottom middle panel; insulin (green) and ghrelin (red) – bottom right panel. Note yellow cells that reveal co-expression of insulin with C-peptide, chromogranin A, somatostatin and cytokeratin 19 by the subpopulation of reprogrammed IPCs. Cell nuclei were stained by DAPI (blue). Scale bar = 200 μm

B: Quantification of specific cell types in different treatment and control groups at the end of the reprogramming procedure (day 15). Immunostaining for β-cell-specific markers insulin (INS – green) and C-peptide (CPEP – red), α-cell-specific marker glucagon (GCG – red), δ cell-specific marker somatostatin (SOMA – red), ε-cell-specific marker ghrelin (GHRL – red), pan-endocrine marker chromogranin A (CHGA – red) and ductal cell-specific marker cytokeratin 19 (KRT19 – red). Organoid-derived cells from five donors with two technical replicates per each treatment group were analysed. At least 1,000 counted cells per sample were quantified. Data represent means ± SEM.

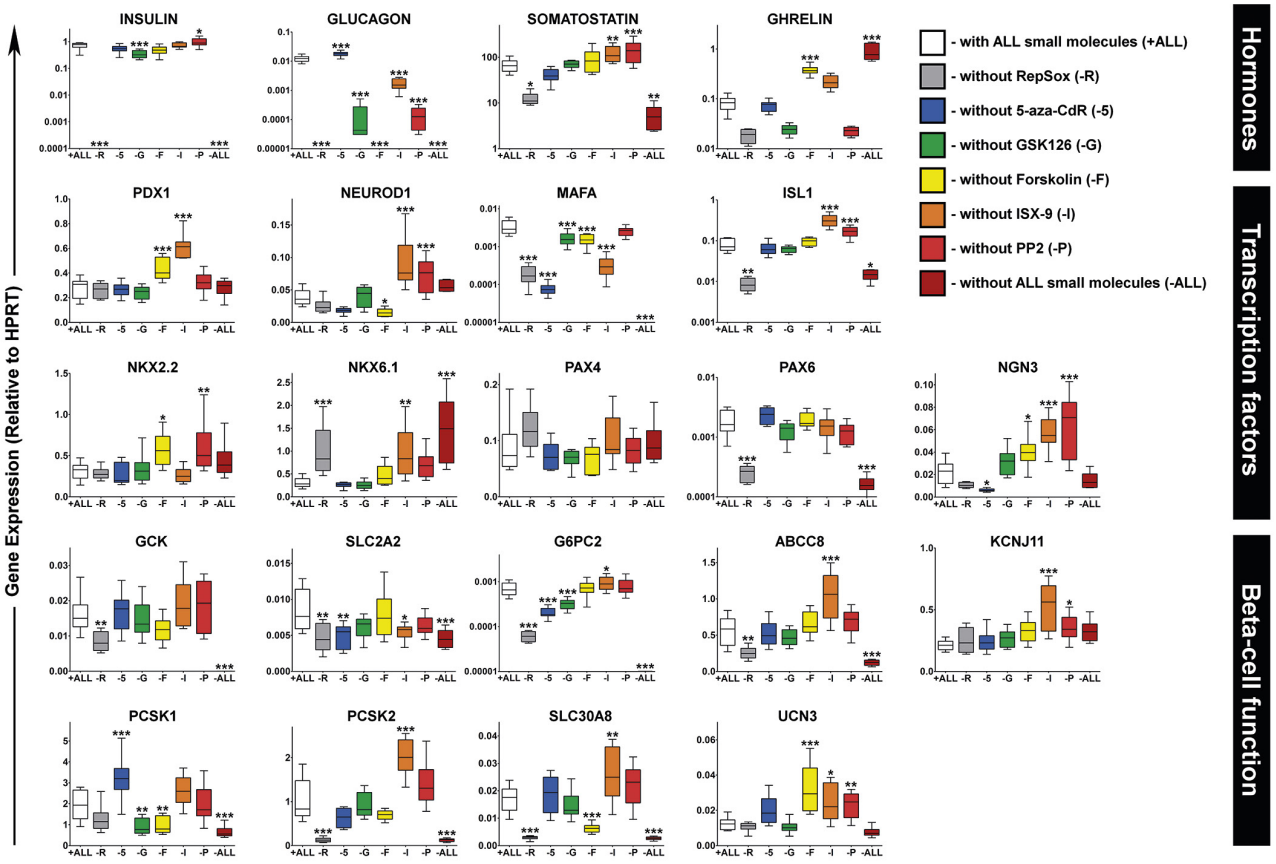

Fig. 5. The effect of individual small molecules on the gene expression profile of organoid-derived cells after the reprogramming procedure. Omission of each small molecule from the reprogramming medium is indicated as follows: w/o RepSox (-R), w/o 5-aza-2'-deoxycytidine (-5), w/o GSK126 (-G), w/o forskolin (-F), w/o ISX-9 (-I), w/o PP2 (-P), w/o all RAPFIG small molecules (-ALL). Gene expression analysis of different treatment groups was performed using the samples derived at the end of the treatment period (day 15) (N = 5 biological replicates, each with two technical replicates). Data are presented as box and whisker plots. Expression of each gene was normalized to *HPRT*. One-way ANOVA with Dunnett´s post hoc test was performed to compare all treatment groups with the control group (medium containing all small molecules), where *P < 0.05, ** P < 0.01, ***P < 0.001.

Functional properties of reprogrammed IPCs

The key role of pancreatic β-cells is to regulate the blood glucose level by glucose-stimulated insulin secretion. Therefore, we tested the potential of reprogrammed IPCs to respond to the changes in the extracellular glucose concentration by the glucose-stimulated C-peptide secretion test. Exogenous insulin from the culture medium can be absorbed by the cells and potentially may give false positive results in glucose-stimulated insulin secretion tests. As part of the pro-insulin molecule, C-peptide is a direct by-product of insulin biosynthesis and is released from β-cells together with insulin in equimolar amounts. Therefore, detection of C-peptide is a more reliable method for determination of the capacity of cells to produce and secret insulin/C-peptide of endogenous origin. Although the reprogrammed IPCs were able to secrete C-peptide under the basal and increased glucose levels, they were not able to respond to an increased extracellular glucose concentration (3 vs. 20 mmol/l) by increasing the C-peptide secretion (Fig. 6B). In order to determine whether the insufficient glucose responsiveness was caused by the deficiencies at the level of β-cell-specific glucose sensing-mechanisms or in the following steps of the insulin secretory mechanism, we also tested the response of the reprogrammed IPCs to the changes in the extracellular potassium concentration (5 vs. 30 mmol/l). Interestingly, IPCs responded to the increase in the extracellular potassium chloride (KCl) concentration by 3.2-fold increase in C-peptide secretion in comparison with the C-peptide secretion at the basal KCl level (Fig. 6B). It compares well with the human islet secretory response to the depolarization by 30 mM KCl, which induced a 5.2-fold increase in the C-peptide secretion (Fig. 6B).

Based on these results we assume that the reprogrammed IPCs developed only some mechanisms responsible for the glucose-stimulated insulin secretion. Therefore, further improvements in the glucose-sensing mechanism need to be achieved in order to establish the functional glucose-stimulated insulin secretory capacity.

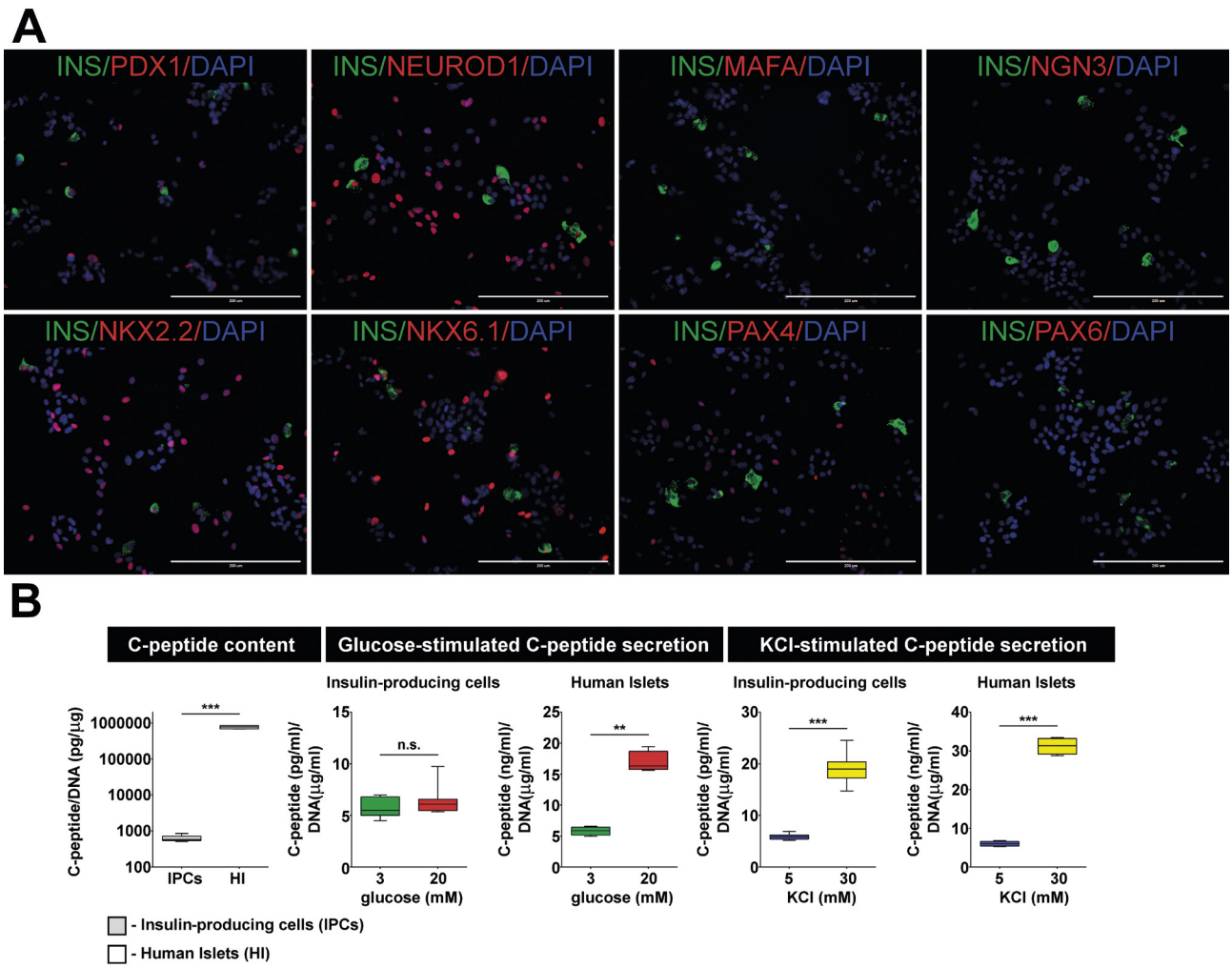

Fig. 6: Characterization and functional properties of reprogrammed IPCs

A: Immunostaining for insulin (green) and key transcription factors of pancreatic β-cells: PDX1 (red) – top first panel, NEUROD1 (red) – top second panel, MAFA (red) – top third panel, NKX2.2 (red) – bottom first panel, NKX6.1 (red) – bottom second panel, PAX4 (red) – bottom third panel, PAX6 (red) – bottom fourth panel, in reprogrammed IPCs. Cell nuclei were stained by DAPI (blue). Scale bar = 200 µm

B: C-peptide content of reprogrammed IPCs and human islets, normalized to the total amount of DNA in each sample. Data are presented as box and whisker plots (IPCs N = 5 biological replicates, each with two technical replicates; human islets N = 2 biological replicates, each with two technical replicates). Statistical analysis was performed using an unpaired two-tailed *t*-test, ***P < 0.001.

Glucose-stimulated C-peptide secretion of reprogrammed IPCs and human islets was determined by sequential 60-min incubation under static conditions at low (3 mmol/l) and high (20 mmol/l) glucose concentration. The effect of depolarizing agent KCl on C-peptide secretion was determined by sequential 60-min incubation under static conditions at low (5 mmol/l), followed by high (30 mmol/l) KCl concentration, with 3 mmol/l glucose in each. C-peptide concentration in the media was normalized to the total amount of cellular DNA content in each sample. Data are presented as box and whisker plots (IPCs N = 5 biological replicates, each with two technical replicates; human islets N = 2 biological replicates, each with two technical replicates). Statistical analysis was performed using a paired two-tailed *t*-test. Asterisks indicate statistical significance: **P < 0.01, ***P < 0.001, n.s. - not significant.

Discussion

In the current study we demonstrate that human pancreatic organoid cells of ductal origin can be reprogrammed into IPCs by the combination of IVT-modified mRNA encoding transcription factor neurogenin 3 and small molecules modulating the epigenetic state and signalling pathways of the reprogrammed cells. The reprogrammed IPCs were able to process prohormone proinsulin into the mature insulin and C-peptide. In addition, the IPCs expressed some of the key genes and transcription factors needed for the proper function of pancreatic β-cells. Nevertheless, the immature character of the reprogrammed IPCs and insufficient glucose-stimulated insulin secretion revealed an incomplete conversion to the fully functional β-like cells. In addition,

we also revealed that pancreatic organoid cells can be reprogrammed toward the other pancreatic endocrine cell fates by temporal ectopic over-expression of the neurogenin 3 transcription factor.

The key role of neurogenin 3 transcription factor for β-cell differentiation has already been demonstrated by many *in vivo* as well as *in vitro* studies. During their development, all pancreatic endocrine cells are derived from neurogenin 3-expressing endocrine progenitors (Gradwohl et al., 2000). Moreover, ectopic over-expression or stabilization of neurogenin 3 induces ductal-to-β-cell trans-differentiation, although only under the *in vivo* conditions (Sancho et al., 2014; Vieira et al., 2018). The *in vitro* reprogramming of pancreatic ductal cells into the IPCs requires ectopic expression of additional transcription factors that direct further differentiation into the β-cell phenotype (Lee et al., 2013). In our study, neurogenin 3 over-expression induced massive conversion of pancreatic organoid cells into chromogranin A-positive cells, suggesting a ductal-to-endocrine cell fate conversion that was confirmed by the detection of ghrelin and somatostatin hormones. This result is in agreement with previous reports revealing the role of neurogenin 3 in the pancreatic endocrine cell differentiation and reprogramming. It was reported that neurogenin 3 over-expression in pancreatic duct-derived cells activates endogenous expression of endocrine transcription factors such as NKX2.2, NEUROD1, INSM1, PAX4 and RFX6 (Swales et al., 2012; Lee et al., 2013). Likewise, transfection of organoid-derived cells with the IVT NEUROG3 mRNA induced expression of NEUROD1, NKX2.2 and PAX4 transcription factors in our study. However, temporal neurogenin 3 over-expression and induction of several endocrine transcription factors was not sufficient to induce the conversion into IPCs.

Further reprogramming into the IPCs was promoted by a combination of small molecules modulating the epigenetic state and signalling pathways of the reprogrammed cells. Based on our screening we identified a group of six small molecules that, in combination with IVT NEUROG3 mRNA, were capable of further conversion into the IPCs. By withdrawing each individual molecule from the six-molecule pool, we identified RepSox (TGF β type I activin receptor-like kinase ALK5 inhibitor) as the most important one. The addition of RepSox into the small molecule cocktail significantly increased the expression of somatostatin and transcription factors ISL1, MAFA and PAX6. However, most importantly, RepSox induced expression of insulin and glucagon genes. Our results are in agreement with the RepSox role in the differentiation of pluripotent stem cells into the β-cells. During the late stages of stem cell differentiation, this small molecule dose-dependently up-regulates the expression of insulin, glucagon, somatostatin and MAFA transcription factor (Rezania et al., 2014). Moreover, an animal model revealed that inhibition of TGF β receptor II signalling in pancreatic ductal cells allows duct-to-β-cell conversion after partial pancreatectomy (El-Gohary et al., 2016). This suggests an important role of TGF signalling in the differentiation and cell fate conversion into the β-cell fate.

In our reprogramming protocol, we focused on the $CD133^+$ pancreatic cells. These cells were previously used as a cell source for reprogramming into the IPCs using adenoviral vectors encoding PDX1, NEUROG3, MAFA and PAX6 transcription factors (Lee et al., 2013). The $CD133^+$ pancreatic cells have been widely characterized and are considered as one of the potential pancreatic progenitor cell types (Koblas et al., 2008; Dorrell et al., 2014; Jin et al., 2016; Aguayo-Mazzucato and Bonner-Weir, 2018). In the human pancreas, PDX1, SOX9 and NKX6.1 transcription factors are expressed by the tip and trunk progenitors that give rise to the ductal and endocrine cells during embryonic development (Jennings et al., 2013). The expression of these transcription factors by the pancreatic $CD133^+$ organoid cells suggests that these cells may have a phenotype similar to the trunk pancreatic progenitors. Therefore, based on these progenitor-like properties, $CD133^+$ pancreatic cells can be considered as a cell type suitable for the reprogramming into the IPCs.

It is an important aspect of our study that we have not used a viral-based reprogramming approach. Viral vectors can persist in the reprogrammed cells for a long time and provide persistent ectopic expression of the key transcription factors such as PDX1 and MAFA, which are important for the maintenance of the β-cell phenotype and insulin expression (Lee et al., 2013; Li et al., 2014). The optimal reprogramming strategy should activate a new cell type-specific expression programme that is maintained and persists even when the ectopic expression of reprogramming factors is discontinued. As the IVT-modified NEUROG3 mRNA allows temporal over-expression of neurogenin 3 only during the early phase of the reprogramming procedure, it can be assumed that the newly acquired cell fate of IPCs was achieved by the accomplished reprogramming and not by the persistent expression of the reprogramming factor(s).

Another benefit of our protocol is that the application of IVT-modified mRNA does not bear the risk of potential insertional mutagenesis and oncogenic transformation, unlike viral-based reprogramming approaches. Additionally, it allows controlled temporal expression of the reprogramming factors, based on the duration of repeated IVT mRNA transfections. Moreover, the expression level of the encoded protein can be partially regulated by the amount of applied IVT mRNA. In this regard, it is important to note that neurogenin 3 has to be expressed at a sufficiently high level in order to induce the cell fate conversion into the endocrine character (Wang et al., 2010; Lee et al., 2013). The massive conversion of pancreatic organoid cells into the chromogranin A-positive cells induced by IVT NEUROG3 mRNA in our study suggests that the expression level of neurogenin 3 was sufficient to induce the ductal-to-endocrine cell fate conversion.

On the other hand, a key obstacle of the mRNA-based approach is a significant dependence of the mRNA delivery efficiency on the cell type and culture conditions. The overall efficiency of our reprogramming protocol is partially restricted by the limited transfection of organoid-derived cells, as only 37.1 ± 5.4 % cells were efficiently transfected with the IVT-modified mRNA. Moreover, after the first week of the reprogramming procedure, the transfection efficiency significantly deteriorated, limiting further application of IVT-modified mRNAs during the late stages of reprogramming. Although the cationic lipid-based transfection reagent used in our study is highly efficient in the delivery of IVT mRNAs into the immortalized cell lines (Koblas et al., 2016; Leontovyc et al., 2017), the transfection efficiency of this reagent is partially limited in the case of several types of primary cells. Therefore, further improvement in the reprogramming efficiency may be achieved by the development and application of a transfection reagent that would allow more efficient delivery of IVT mRNA into the primary cells, including human pancreatic organoid-derived cells.

Although our protocol was able to induce conversion of duct-derived cells into the insulin-producing β-like cells, these cells possessed an immature β-cell phenotype. Firstly, the reprogrammed IPCs expressed only 0.7 ± 0.4 % of the insulin transcripts compared to that of adult human islets, assuming that human β-cells form more than 50 % of the islet cell mass, while our reprogrammed IPCs represented only 4.6 ± 1.2 % of the total cell mass. In addition to insulin, the reprogrammed IPCs co-expressed somatostatin, and upon the glucose challenge were not able to respond by increasing insulin secretion. IPCs derived by our reprogramming protocol partially resemble β-like cells derived by the differentiation of human embryonic stem cells using the early differentiation protocols. These cells also had a limited insulin expression capacity in comparison with adult human islets, were polyhormonal, glucose unresponsive and displayed reduced expression of some of the key β-cell transcription factors (Rezania et al., 2012; Bruin et al., 2014; Hrvatin et al., 2014).

The entire mechanism of glucose-stimulated insulin secretion by pancreatic β-cells is based on the following steps. An increase in the extracellular glucose concentration leads to the augmented glucose metabolism and consequent increase in the ATP levels. This triggers closure of ATP-sensitive potassium (KATP) channels. Membrane depolarization caused by the closure of KATP channels activates opening of voltage-sensitive calcium channels and subsequent calcium-induced insulin secretion (Maechler and Wollheim, 2001). Because the reprogrammed IPCs were able to release insulin upon direct depolarization by KCl, the defect(s) in the glucose-stimulated insulin secretion are probably caused by the insufficiently developed glucose-sensing mechanism. Indeed, the expression level of glucokinase – a key enzyme determining increased ATP production in response to the elevated glucose level – was significantly decreased in reprogrammed IPCs in comparison with adult human islets. In addition, the expression of G6PC2, one of the β-cell-specific metabolic components, was not expressed at a sufficient level. Therefore, we can conclude that the reprogrammed IPCs were not glucose responsive, as some of the components of the β-cell-specific glucose-sensing mechanisms were not induced sufficiently.

Based on the achieved results we suppose that further up-regulation of PAX6 and MAFA transcription factors can significantly improve the outcomes of our protocol. The reduced expression levels of PAX6 and MAFA may explain the limited insulin expression, as both of these transcription factors participate in the insulin gene expression. Moreover, MAFA and PAX6 also regulate expression of genes encoding the key elements of the glucose-sensing mechanism (Gosmain et al., 2012; Nishimura et al., 2015). Thus, the reduced expression of both these transcription factors can partially explain the insufficient glucose responsiveness of the reprogrammed IPCs.

Finally, it may be concluded that understanding the mechanisms underlying differentiation or reprogramming of pancreatic progenitor cells into the insulin-producing β-like cells is an essential task for the development of therapeutic approaches aiming at regeneration of endogenous pancreatic β-cells in the diabetic patients.

Our study provides a proof of concept that organoid cells derived from pancreatic tissue can be reprogrammed toward the β-like cell phenotype using a non-integrative reprogramming approach. This approach is based on the application of IVT-modified mRNA encoding pro-endocrine transcription factor neurogenin 3 and small molecules modulating the epigenetic state and signalling pathways. However, additional studies are needed to determine whether the pancreatic organoid-derived IPCs can be further converted into the fully functional and mature β-like cells. Thus, the optimization of the reprogramming protocol, medium composition and culture conditions is warranted for improvement of the reprogramming efficiency and function of IPCs.

Conflict of interest

There is no conflict of interest.

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