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# Time-efficient strategies in human iPS cell-derived pancreatic progenitor differentiation and cryopreservation: advancing towards practical applications

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## Abstract

**Background** Differentiation of patient-specific induced pluripotent stem cells (iPS) helps researchers to study the individual sensibility to drugs. However, differentiation protocols are time-consuming, and not all tissues have been studied. Few works are available regarding pancreatic exocrine differentiation of iPS cells, and little is known on culturing and cryopreserving these cells.

**Methods** We differentiated the iPS cells of two pediatric Crohn's disease patients into pancreatic progenitors and exocrine cells, adapting and shortening a protocol for differentiating embryonic stem cells. We analyzed the expression of key genes and proteins of the differentiation process by qPCR and immunofluorescence, respectively. We explored the possibility of keeping differentiated cells in culture and freezing and thawing them to shorten the time needed for the differentiation. We analyzed the cell cycle of undifferentiated and differentiated cells by flow cytometry.

**Results** The analysis of mRNA levels of key pancreatic differentiation genes *PDX1* and pancreatic amylase indicate that iPS cells were successfully differentiated into pancreatic exocrine cells with expression of *PDX1* (one way ANOVA  $p < 0.0001$ ), and the two isoforms of amylase (one way ANOVA  $p < 0.05$ ) significantly higher in exocrine cells in comparison to iPS cells. Differentiation efficiency was also confirmed by immunofluorescence analysis of *PDX1* and amylase. We confirmed the possibility of shortening the time necessary for obtaining pancreatic cells without losing differentiation efficiency. Pancreatic progenitors and exocrine cells were maintained in culture and cryopreserved. Interestingly, the stemness marker *OCT4* resulted significantly lower after subculturing ( $OCT4$   $p < 0.001$ ; one-way ANOVA) and after freezing and thawing procedures ( $p < 0.05$ , one-way ANOVA) suggesting a reduction of undifferentiated stem cells leading to a purer population of pancreatic progenitor cells. Also, the stemness marker *NANOG* resulted lower after passaging, corroborating this result.

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**Conclusions** In this work, we optimized the generation of patient-specific pancreatic differentiated cells and laid the foundation for creating a bank of patient-specific pancreatic lines exploitable for tailored pharmacological assays.

**Trial registration** The study was approved by the Ethical Committee of the Institute of Maternal and Child Health IRCCS Burlo Garofolo, with approval number 1556 (internal ID RC 44/22).

**Keywords** Human induced pluripotent stem cells, Pancreatic progenitors, Pancreatic exocrine cells, Patient-specific model, Crohn's disease.

## Background

Human induced pluripotent stem cells (iPS cells) have been widely used in various research fields since their discovery in 2006 [1]. This is principally due to their ability to differentiate, under appropriate stimulation, in almost all cells of the human body, becoming an important tool for regenerative medicine, disease modeling, and drug testing [2–4]. Particularly, this technology is important in the field of therapy personalization, creating the possibility to investigate the cellular and molecular mechanisms underlying the individual sensibility of patients to drugs.

In the last decade, pancreatic dysfunctions have been modeled using iPS cell differentiation. Indeed, in this context, different papers are available for obtaining pancreatic cells but they mainly focus on the endocrine part rather than the exocrine [5]. Here, we apply a modified version of the differentiation method developed by Shirasawa and colleagues [6] for obtaining pancreatic exocrine acinar cells. The method was initially developed for differentiating human embryonic stem cells (hESC) into pancreatic exocrine cells while we applied it for differentiating iPS cells of two pediatric patients with Crohn's disease. Indeed, around 5% of patients affected by inflammatory bowel diseases in treatment with thiopurines as immunosuppressants can develop pancreatitis, an idiosyncratic adverse reaction, with a higher incidence in the pediatric population [7]. Using patient-specific iPS cells and differentiated pancreatic exocrine cells it could be possible to shed light on the molecular mechanisms underlying this condition avoiding the development of this adverse effect.

The protocol consists of 4 different steps, leading to definitive endoderm, primitive gut tube cells, pancreatic progenitors, and mature exocrine cells formation in around 15 days of stimulation reducing by half the timing proposed previously without affecting the mRNA level of key differentiation genes or protein levels of important marker of differentiation [6].

One of the most time-consuming steps in this field of research is the time needed to obtain the final cell of interest (i.e. here pancreatic exocrine cells). Indeed, usually protocols for obtaining differentiated cells starting from human iPS cells require an overall long time of cell exposure to appropriate stimuli.

Therefore, beyond the time reduction of the protocol for differentiating human iPS cells in pancreatic cells, we focused on the development of a consistent strategy for maintaining in culture pancreatic progenitors and for freezing and thawing both pancreatic progenitors and exocrine cells obtained from pediatric Crohn's patient-derived iPS cells. Here, for the first time, we show that it is possible to freeze, thaw, and maintain in culture patient-specific pancreatic progenitors and exocrine cells and to re-plate and expand pancreatic progenitors without affecting mRNA and protein expression levels of key markers involved in pancreatic cell development. Moreover, we studied the cell cycle along the differentiation procedure to obtain information about the replication of differentiated cells only partially available in the literature.

Patient-specific exocrine cells and pancreatic progenitors are precious tools for researchers, giving them the possibility to perform pharmacological assays to study patient response to drugs and to predict adverse effects. The efficient generation of patient-specific differentiated cells will facilitate the research in personalized therapy and disease modeling. Unlike advances in the iPS cell field, in the last decade the differentiation in pancreatic exocrine cells has not been extensively investigated.

## Methods

### Samples

Human iPS cells used in this paper were obtained by reprogramming peripheral blood mononuclear cells (PBMCs) of two patients with Crohn's disease under pharmacological treatment. One of the two patients developed pancreatitis after thiopurine treatment as adverse effect. Briefly, PBMCs were isolated by adding an appropriate volume of Ficoll reagent (Sigma-Aldrich, Merck) to the blood of patients, and samples were centrifuged at 600 xg, 15 °C for 40 min. The intermediate phase, containing the PBMCs, was then separated, and cells were washed two times with sterile PBS. Cells were counted, frozen, and stored in liquid nitrogen until the moment of the reprogramming. Sendai virus-based vector was used, and the human iPS cells were characterized for genetic uniqueness, genomic integrity, pluripotency, and differentiation ability as reported elsewhere [8]. The analyses reported in this work were performed on one

clone per cell line. The study was approved by the Ethical Committee of the Institute of Maternal and Child Health IRCCS Burlo Garofolo, with approval number 1556 (internal ID of the study RC 44/22).

**Cell culture**

Human iPS cells were maintained in StemMACS iPS-Brew XF medium (Miltenyi Biotec) on diluted Matrigel (Corning, Life Sciences) coated plates (1:60 Matrigel-DMEM/F12 medium) to allow cell adhesion. Cell passage was performed after reaching 80% of confluence, determined by visual examination of the cultures. Human iPS cells were passed using a standard protocol, avoiding the complete break up of clusters formed. Cells were exposed to Y-27,632 (Rock inhibitor, Miltenyi Biotec) 10 μM for 24 h to facilitate cell adhesion. As a control, we used the normal healthy pancreatic ductal line H6C7 that was maintained in Keratinocyte SFM medium (Invitrogen), and the cells were subcultured weekly when reaching 80–90% of confluence using 0.5% of Trypsin-EDTA. Cell cultures were maintained according to standard procedures in a humidified incubator at 37 °C and with 5% CO<sub>2</sub>.

**Differentiation protocol**

The protocol was initially developed to differentiate human ESCs into pancreatic exocrine cells [6] and slightly modified for human iPS cell differentiation, reducing in particular the time needed for obtaining exocrine cells. This protocol is based on a 4 steps procedure: (1) differentiation of human iPS cells into definitive endoderm by activin A (100 ng/mL, Sigma-Aldrich) and CHIR99021 (3 μM, Sigma-Aldrich) for 4 days (stage I); (2) differentiation into primitive gut tube by fibroblast growth factor (FGF-7 50 ng/mL, Abnova) for 3 days (stage II); (3) differentiation into pancreatic progenitor cells by a combination of cyclopamine (0.25 μM, Sigma-Aldrich),

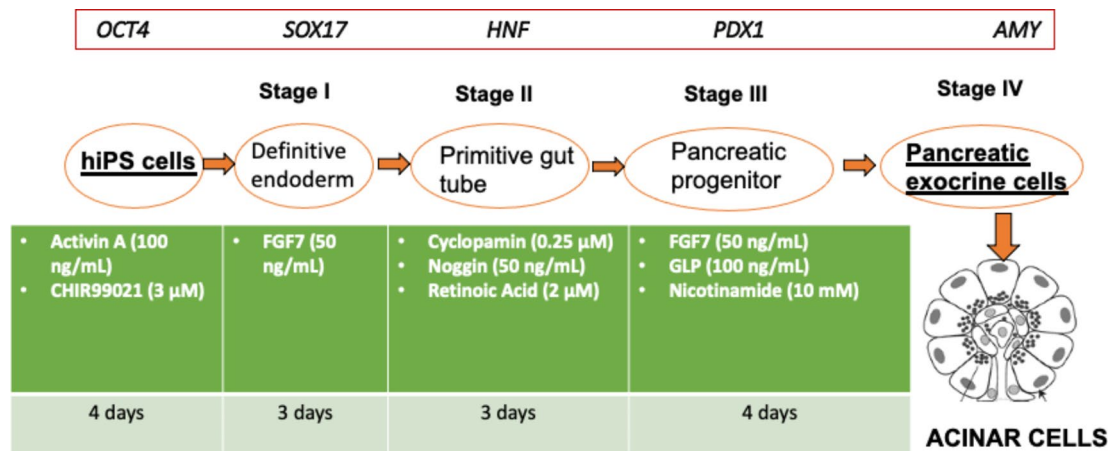
noggin (50 ng/mL, Invitrogen) and all-trans retinoic acid (2 μM, Sigma-Aldrich) (stage III) for 3 days; (4) differentiation into pancreatic exocrine cells by a combination of FGF-7 (50 ng/mL, Abnova), glucagon-like peptide 1 (100 ng/mL, RayBiotech) and nicotinamide (10 mM, Sigma-Aldrich) (stage IV) for 4 and 20 days. Cells were grown in RPMI 1640 medium (Sigma-Aldrich) with 1% of penicillin-streptomycin (EuroClone) during the differentiation process. The differentiation protocol is shown in Fig. 1. RNA samples were collected after the end of each differentiation step.

**Real-time PCR**

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions and quantified using Nanodrop 2000 spectrophotometer (ThermoFisher Scientific). RNA was reverse-transcribed into cDNA using the High-Capacity RNA-to-cDNA kit (Applied Biosystem, ThermoFisher Scientific). The real-time PCR protocol consists of an initial denaturation for 30 s at 95 °C, followed by 40 cycles of heating at 95 °C (5 s), 60 °C (1 min) and then, a final extension for 5 s at 65 °C. Real-time PCR was performed using pre-designed primers (Table 1) and the KiCqStart SYBR Green qPCR Ready Mix (Sigma-Aldrich) in a Thermal Cycle Dice Real Time System (BIO-RAD). Relative quantification is represented as 1/2<sup>-ΔCt</sup> with respect to the housekeeping gene beta-actin (*ACTB*). All experiments were carried out in duplicate and the reproducibility of the observations was confirmed in two or three independent experiments.

**Immunofluorescence assays**

To confirm the successful differentiation of human iPS cells, immunofluorescence analysis was performed analyzing PDX1 expression, a marker specific to pancreatic progenitor cells, and α-amylase expression, a marker characteristic of pancreatic exocrine cells.



**Fig. 1** Protocol for the differentiation of human iPS cells into pancreatic exocrine cells

**Table 1** Primers (Sigma-Aldrich) for real-time PCR analysis of differentiation markers. *ACTB* (beta-actin), *AMY2A* and *AMY2B* (pancreatic isoforms of *α-amylase*), *FOXA2* (Forkhead Box A2), *HNF* (hepatocyte nuclear factors), *OCT4* (*POU class 5 homeobox 1*), *MYC proto-oncogene*, *PDX1* (pancreatic and duodenal homeobox 1), *SOX17* (SRY-box 17)

Gene	Primer	Sequence 5'→3'	T melt-ing (°C)	Product size (bp)
<i>OCT4</i>	Forward	CCTCACTTCACTGCACTGTA	82.5	164
	Reverse	CAGGTTTTCTTCCCTAGCT		
<i>NANOG</i>	Forward	CCAGAACCAGAGAATGAAATC	58.0	120
	Reverse	TGGTGGTAGGAAGAGTAAAG		
<i>SOX17</i>	Forward	GGCGCAGCAGAATCCAGA	80.5	60
	Reverse	CCACGACTTGCCAGCAT		
<i>FOXA2</i>	Forward	GGGAGCGGTGAAGATGGA	82.5	89
	Reverse	TCATGTTGCTCACGGAGGAGTA		
<i>PDX1</i>	Forward	AAAACGTAGTGATTGGAGG	86.0	122
	Reverse	CCAGACCTTAAAAGAAGAC		
<i>AMY2A</i>	Forward	ACTTTTCATTTACCAGGAGG	79.0	148
	Reverse	GTAAGACATCTTCTCTCCATTC		
<i>AMY2B</i>	Forward	CTACAATGATGCTACTCAGG	79.5	181
	Reverse	AATTGCCTTTATGTCTCCAG		
<i>ACTB</i>	Forward	CGCCGCCAGCTCACCATG	86.5	120
	Reverse	CACGATGGAGGGGAAGACGC		

Undifferentiated human iPS cells and pancreatic exocrine cells (stage IV) were analyzed. Cells were cultured and differentiated in 24-well plates on Matrigel-coated glasses. Cells were washed with 500  $\mu$ L of PBS, fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min, permeabilized with 0.1% Triton X-100 in PBS, and then treated with 1.5% donkey serum to block non-specific staining. After 30 min, the following diluted primary antibodies were added and incubated overnight with the fixed cells at 4 °C using the goat anti-PDX1 (1:200; R&D Systems) and rabbit anti- $\alpha$ -amylase (1:500; Sigma-Aldrich) antibodies. After overnight incubation, cells were washed three times with PBS and incubated with a donkey anti-goat-PerCP 678 (1:1000 in PBS; Santa Cruz Biotechnology) or a donkey anti-rabbit 520 (1:1000 in PBS; Sigma-Aldrich) secondary antibody together with 4,6-diamidino-2-phenylindole dihydrochloride for nuclei staining (DAPI; 1:1000 in PBS). The specimens were observed by a ZEISS Axio Observer Z1 or the Nikon Eclipse E800 and images were processed using the Fiji software.

#### Freezing protocol of differentiated cells

The possibility of freezing pancreatic progenitors and exocrine cells was tested. The differentiation medium was removed and cells were gently washed with 2 mL of PBS. Then, cells were incubated at 37 °C with Versene for 6–9 min or with 0.05% Trypsin-EDTA 0.02% in PBS

(Sigma-Aldrich) for 5 min for detaching pancreatic progenitors or exocrine cells, respectively. After the incubation time, the dissociation reagent was removed with floating cells and kept in a falcon tube. Pancreatic progenitors still attached to the well were gently detached with 2 mL of media composed by RPMI 1690 with penicillin 10,000 UI/mL (EuroClone, Milan, Italy), streptomycin 10 mg/mL (EuroClone, Milan, Italy), 1% L-glutamine 200 mM (EuroClone, Milan, Italy) and 1X B27 Supplement (Gibco) while exocrine cells with RPMI 1690 with penicillin 10,000 UI/mL (EuroClone, Milan, Italy), streptomycin 10 mg/mL (EuroClone, Milan, Italy) + 1% L-glutamine 200 mM (EuroClone, Milan, Italy), and fetal bovine serum. The dissociation reagents with floating cells were mixed with detached cells. Cells were centrifuged at 400 xg for 5 min and resuspended in 1 mL of CryoStor<sup>®</sup> cryopreservation media (Sigma-Aldrich). Cells in the CryoStor<sup>®</sup> solution were stored in appropriate cryogenic vials in a cryostep container for the first 24 h at -80 °C allowing gradual freezing. The day after, cells were moved in liquid nitrogen for long-term storage.

#### Thawing protocol

The process of thawing pancreatic progenitors and exocrine cells has to be quick but gentle. Before starting the process, 9 mL of stage III or stage IV differentiation media were pre-warmed in a 37 °C water bath. Then, the vial of interest containing cells was quickly thawed in a 37 °C water bath until only a small ice crystal was observable. Cells were gently transferred to the warmed medium and centrifuged for 5 min at 400 xg. The medium was removed and cells were gently resuspended in an adequate volume of fresh differentiation medium containing 10  $\mu$ M of Y-27,632. The day after the media was changed with fresh differentiation media of interest. RNA samples were collected before and 4 days after thawing.

#### Re-plating of differentiated cells

Pancreatic progenitors and exocrine cells were detached incubating them with Versene solution for 6–9 min. Versene was collected together with the floating cells and the cells remained attached to the well with 2 mL of media composed by RPMI with penicillin 10,000 UI/mL (EuroClone, Milan, Italy), streptomycin 10 mg/mL (EuroClone, Milan, Italy), 1% L-glutamine 200 mM (EuroClone, Milan, Italy) and 1X B27 Supplement (Gibco). Cells were centrifuged at 400 xg for 5 min, the supernatant removed and the pellet resuspended in stage III medium composed of RPMI 1640 medium, cyclopamine (0.25  $\mu$ M, Sigma-Aldrich), noggin (50 ng/mL, Invitrogen) and all-trans retinoic acid (2  $\mu$ M, Sigma-Aldrich). Cells were seeded in a Matrigel pre-coated plate and exposed to 10  $\mu$ M of Y-27,632 (Rock inhibitor, Miltenyi Biotec) for

24 h to facilitate cell adhesion. RNA samples were collected before and 3/4 days after re-plating cells.

### Cell cycle

The cell cycle of human iPS cells was analyzed using the flow-cytometry propidium iodide cellular uptake assay [9]. For human iPS, cells were cultured for 72 h and harvested before reaching the confluence. Definitive endoderm and pancreatic progenitor cells were collected at the end of the days necessary to differentiate them as indicated in the differentiation protocol.

Cells ( $2.0 \times 10^6$ ) were fixed in 70% ethanol on ice, washed twice with PBS, and allowed to stay in PBS for 1 h at 4 °C. Cells were stained overnight with 2 mL of a PBS/EDTA 0.5 mM solution containing 200  $\mu$ L of propidium iodide (0.1 mg/mL) and 25  $\mu$ L of 1 mg/mL RNase (Sigma-Aldrich). Samples were analyzed by the flow cytometer CYTOMICSTM FC500, Beckman Coulter Inc. Fullerton, CA. All flow cytometric measurements were analyzed with the FCS Express V3.

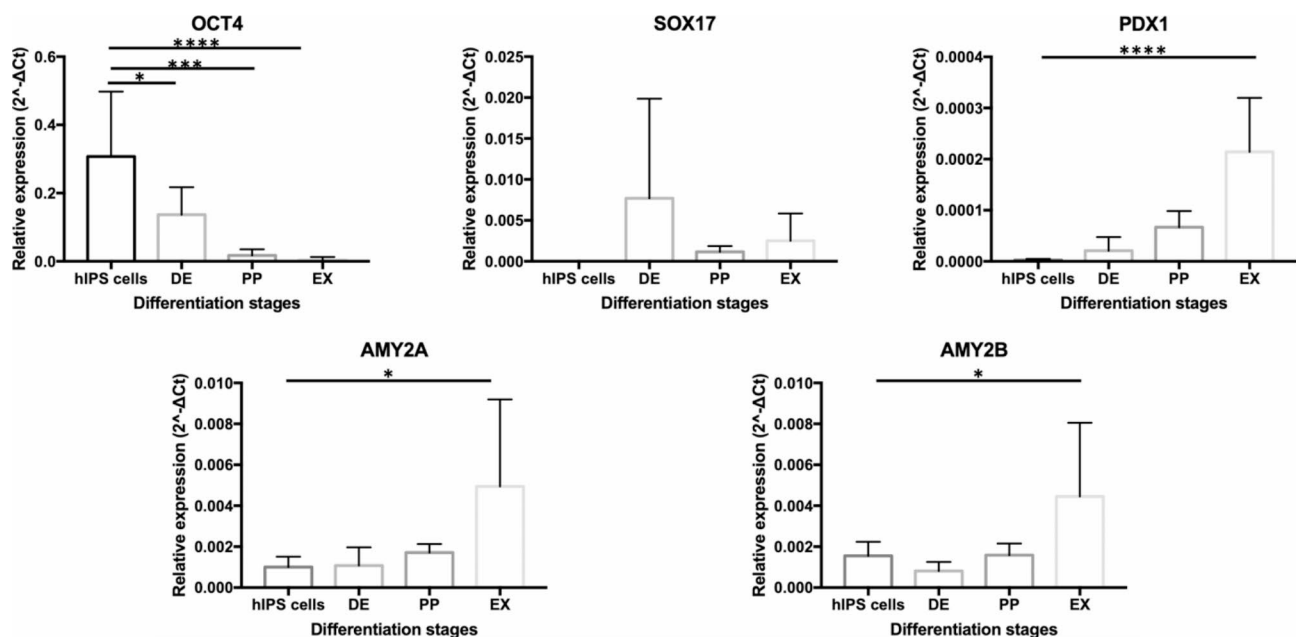
### Statistical analysis

Data were determined as means  $\pm$  SD obtained from at least three independent experiments. The results were processed using GraphPad Prism 7, and analyzed with Student's t-test or one-way ANOVA setting  $p < 0.05$  as the significant level.

## Results

### Differentiation of human iPS cells into pancreatic exocrine cells

Differentiation experiments using the protocol developed for differentiating human embryonic stem cells [6] were performed on two Crohn's disease patient-derived human iPS cell lines, studying one clone per human iPS cell line [8] (Fig. 1). Differentiation markers were analyzed by real-time PCR. The mRNA levels of *OCT4*, *SOX17*, *PDX1*, and the isoforms *AMY2A* and *AMY2B* of  $\alpha$ -amylase in undifferentiated cells, definitive endoderm cells (stage I of differentiation), pancreatic progenitors (stage III of differentiation), and exocrine cells were analyzed to evaluate human iPS cell differentiation efficiency. As shown in Fig. 2, *OCT4* mRNA was significantly higher in undifferentiated human iPS cells compared to the other stages (one-way ANOVA,  $p < 0.05$ ,  $p < 0.001$ ,  $p < 0.0001$ , respectively for endoderm cells, pancreatic progenitors, and exocrine cells). The mRNA expression of the other key markers analyzed indicates that human iPS cells were successfully differentiated into pancreatic exocrine cells. In particular, *SOX17* resulted more expressed in definitive endoderm cells, despite this difference was not statistically significant probably due to the high standard deviations noticed. Moreover, *PDX1* expression was higher in pancreatic progenitors, despite not significantly; on the other hand, its expression was significantly higher in exocrine cells (one-way ANOVA,  $p < 0.0001$ ) compared to undifferentiated iPS cells. The



**Fig. 2** Real-time PCR analysis showing the dynamics of mRNA expression for several key markers of human iPS cells differentiated to pancreatic exocrine cells applying the protocol reported in Fig. 1. Comparison between human iPS cells and differentiated counterpart (one-way ANOVA *OCT4*  $p < 0.0001$ ; one-way ANOVA *PDX1*  $p < 0.0001$ ; one-way ANOVA *AMY2A*  $p < 0.05$ ; one-way ANOVA *AMY2B*  $p < 0.05$ ; Dunnett's multiple comparisons test, \*:  $p < 0.05$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ ). DE, definitive endoderm; PP pancreatic progenitors, EX exocrine cells

two isoforms of amylase were also significantly higher in exocrine cells compared to iPS cells (one-way ANOVA,  $p < 0.05$ ), as expected.

The differentiation efficiency of human iPS cells was also analyzed by immunofluorescence in terms of PDX1 and alpha-amylase protein expression. Almost all pancreatic exocrine cells obtained from human iPS cell differentiation resulted PDX1 and amylase positive, showing respectively a strong nuclear and cytoplasmic signal as expected (Fig. 3).

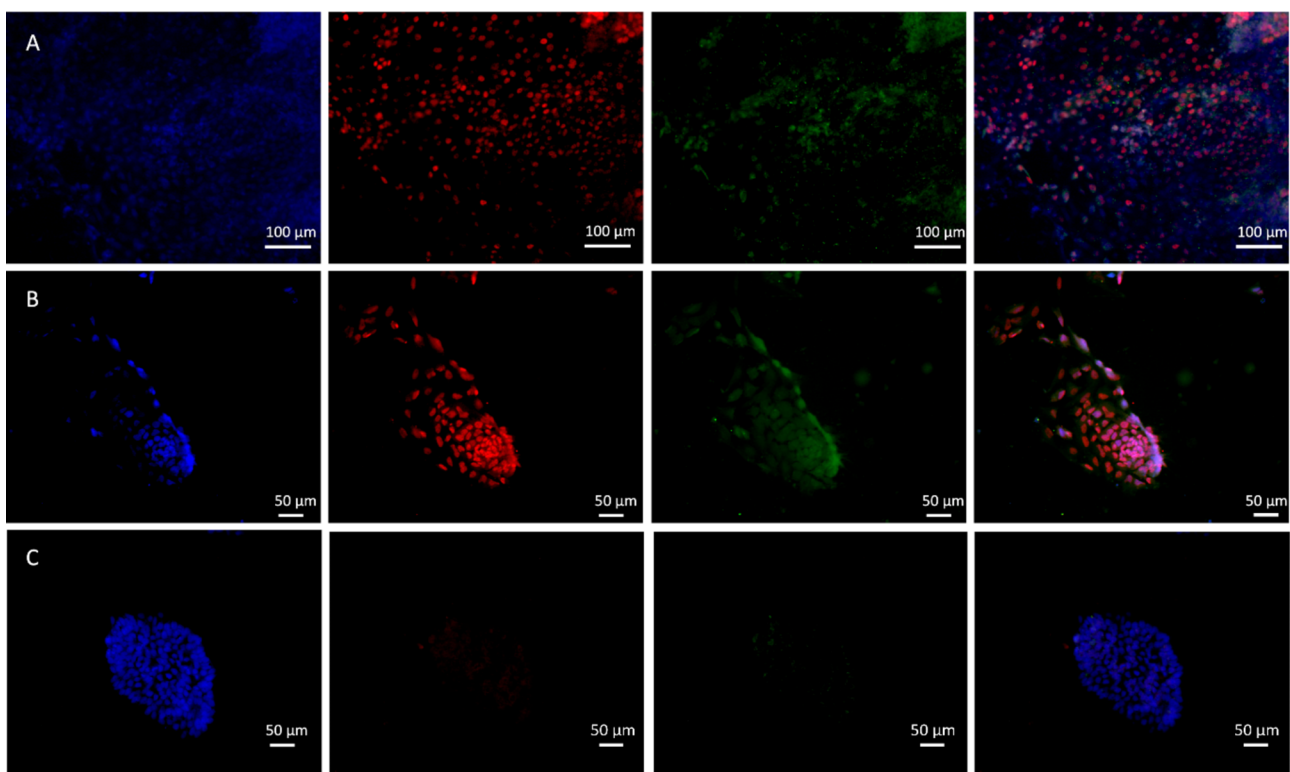
To optimize the time necessary for obtaining differentiated cells, we focused on mRNA levels of exocrine cells after 4 and 20 days of stimuli exposure. Results showed no differences in terms of key marker expression (Fig. 4) between the two differentiation times. We compared pancreatic exocrine cells differentiated for 4 or 20 days to the immortalized healthy ductal pancreatic line H6C7; no differences were identified except for PDX1 levels, which resulted significantly higher ( $p < 0.05$ , one-way ANOVA) in exocrine cells differentiated for 20 days in comparison to H6C7 line. To corroborate our result, we also analyzed by immunofluorescence the protein expression of PDX1 and amylase after 4 and 20 days of stimuli exposure, respectively (Fig. 5). From this analysis, the expression of PDX1 in exocrine cells differentiated for 20 days resulted lower, indicating a reduction in terms of pancreatic

progenitor cells. However, there were no changes in the expression of amylase. These results, along with those of real-time PCR analyses, suggested that it is possible to reduce the number of days of differentiation in the last step without affecting the percentage of exocrine cells obtained. Precisely, the quantification of immunofluorescence images indicated that exocrine cells differentiated for 4 days express about 40% of amylase-positive cells, while cells differentiated for 20 days present about 45% of amylase-positive cells. On the other hand, about 37% and 27% of PDX1-positive cells were found in the population differentiated for 4 and 20 days, respectively.

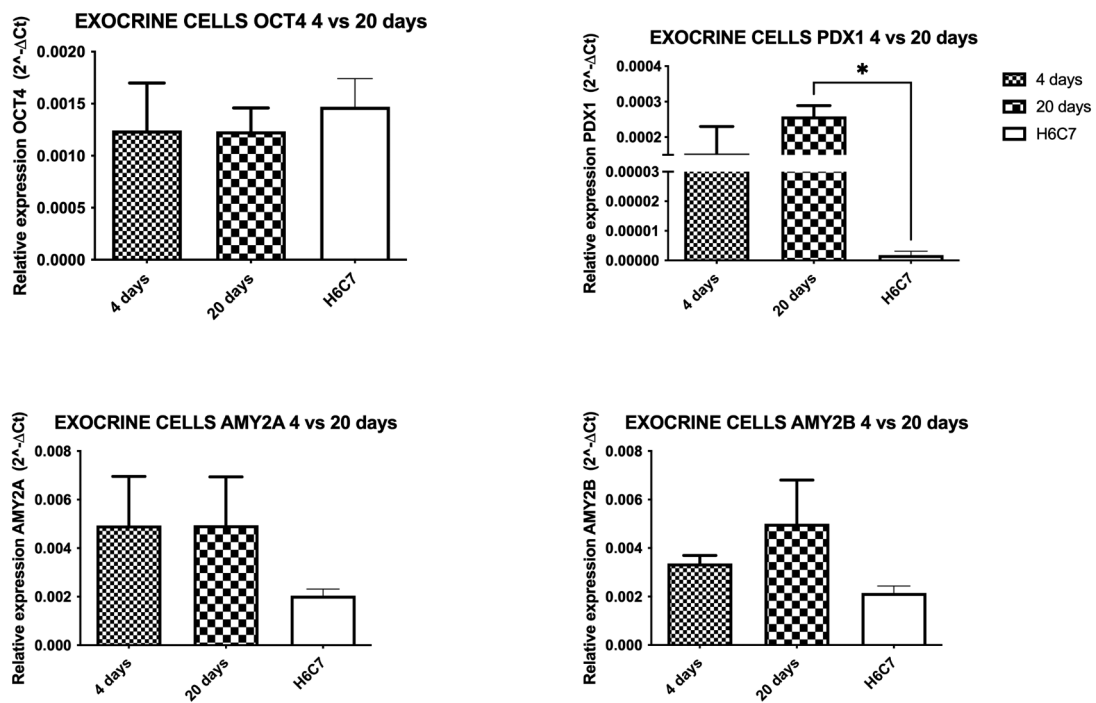
No differences were identified between the two patient cell lines in terms of mRNA or protein expression (supplementary figure).

### Re-plating of differentiated cells

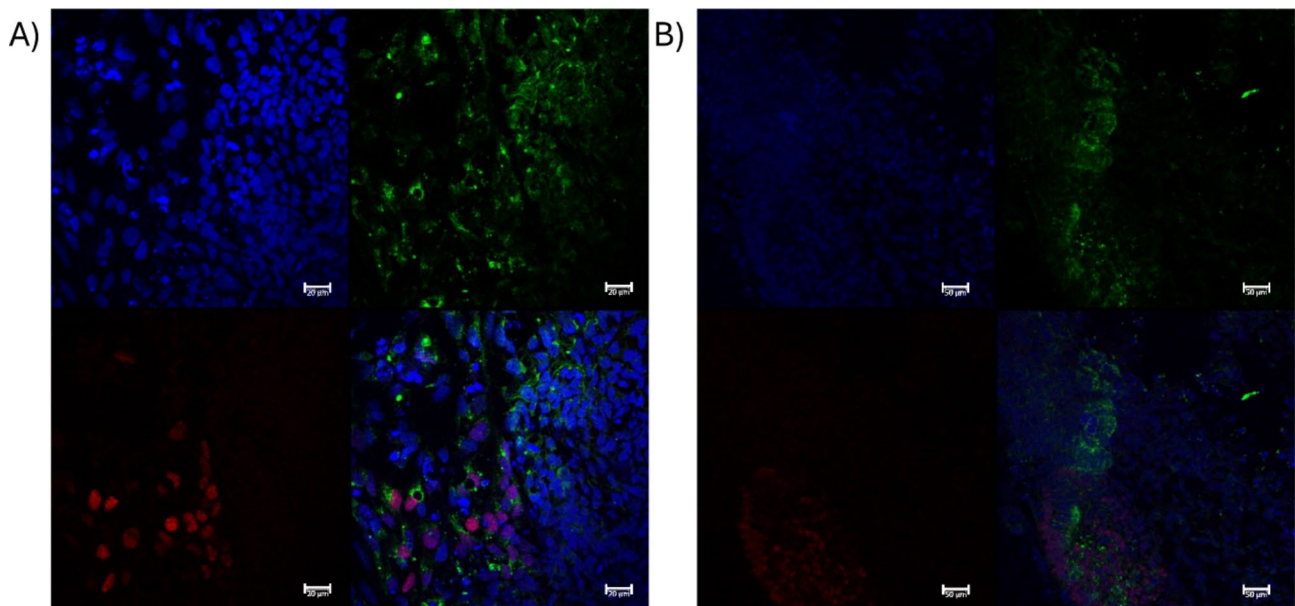
We explored the possibility of maintaining in culture pancreatic progenitors, re-plating them twice. The mRNA levels of differentiation key genes were assessed by real-time PCR. The expression levels of all the markers considered (*SOX2*, *NANOG*, *SOX17*, *PDX1*, *AMY2A*, and *AMY2B*) were not statistically different respecting the not re-plated control (one-way ANOVA,  $p > 0.05$ ) (Fig. 6), except for *OCT4* level, which resulted significantly lower after re-plating cells one and two times (one-way



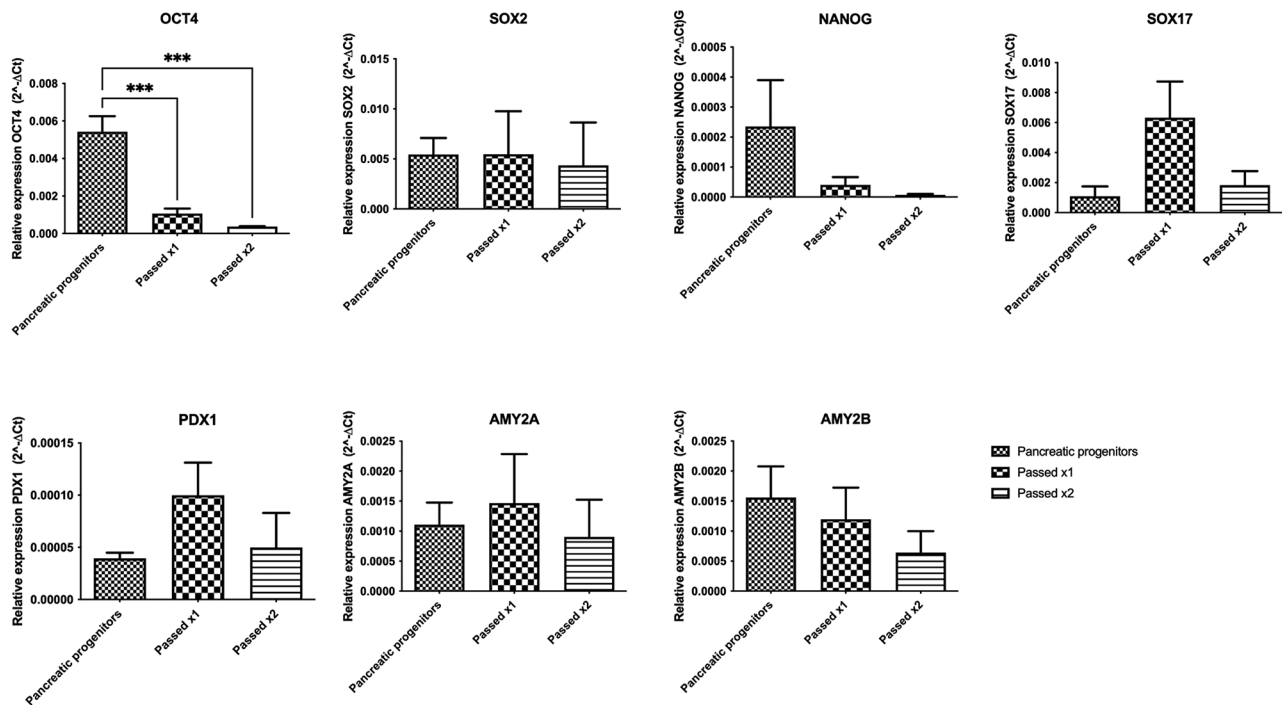
**Fig. 3** Double immunostaining shows that positive cells for PDX1 (red) and amylase (green) are present in pancreatic exocrine cells obtained after human iPS cell differentiation (A, B). Negative control, represented by undifferentiated human iPS cells (C). In blue the nuclear staining (DAPI) is represented



**Fig. 4** Real-time PCR analysis showing the dynamics of mRNA expression for several key markers of pancreatic differentiation. Comparison between pancreatic exocrine cells differentiated for 4 and 20 days and the healthy pancreatic ductal cell line H6C7 (one-way ANOVA PDX1  $p < 0.05$ ; Tukey's multiple comparisons test \*:  $p < 0.05$ )



**Fig. 5** Double immunostaining showing positive cells for PDX1 (red) and amylase (green) pancreatic exocrine cells obtained after human iPS cell differentiation for 4 (A) or 20 days (B). In blue the nuclear staining (DAPI) is represented. Scale bar: 20  $\mu\text{m}$  (A), 50  $\mu\text{m}$  (B)



**Fig. 6** Real-time PCR analysis of mRNA expression for key markers of differentiation. Analysis of mRNA level of pancreatic progenitors maintained in culture (pancreatic progenitors) and re-plated at half density two times (passed x1, passed x2). One-way ANOVA OCT4  $p < 0.001$ ; Dunnett's multiple comparison test \*\*\*:  $p < 0.001$

ANOVA,  $p < 0.001$ ). Also, the stemness marker *NANOG* was reduced after subculturing, despite this difference was not statistically significant. In addition, an increment in *PDX1* and *AMY2A* mRNAs expression was noticed after one passage of re-plating, even if not significant.

**Freezing, thawing, and re-plating of differentiated cells**

We explored the possibility of freezing and thawing differentiated cells for keeping thawed cells in culture. For pancreatic progenitors, no statistically significant differences in terms of mRNA levels of all marker genes (one-way ANOVA,  $p > 0.05$ ) were identified with respect to control (pancreatic progenitors not frozen and thawed), except for *OCT4* level which resulted significantly reduced after freezing, thawing, and re-plating steps (one way ANOVA,  $p < 0.05$ , Dunnett's multiple comparison test vs. pancreatic progenitors \*  $p < 0.05$ ) (Fig. 7). In addition, expression levels of *PDX1*, *AMY2A*, and *AMY2B* were higher after thawing, despite not significantly.

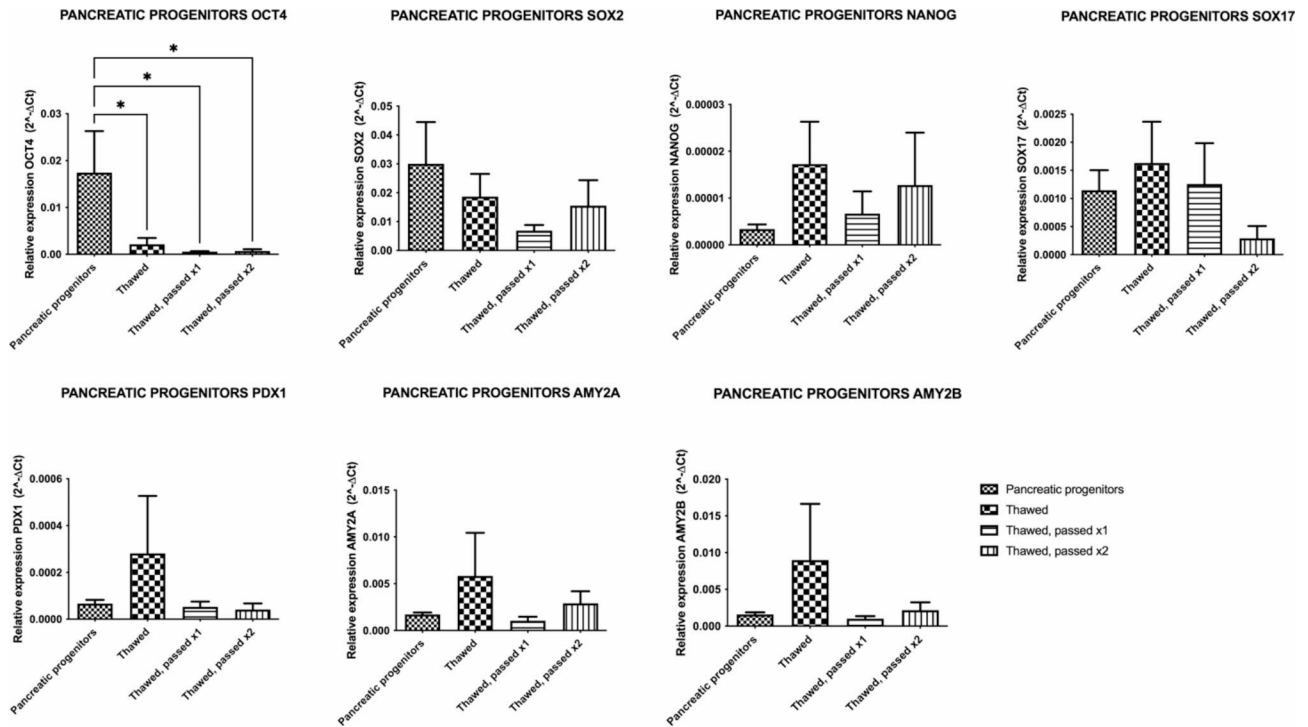
Exocrine cells showed no significant differences in terms of all the mRNA levels after freezing, thawing, and re-plating steps compared to control (one-way ANOVA,  $p > 0.05$  vs. control exocrine cells not frozen and thawed) (Fig. 8). Also in this case, expression levels of *PDX1*, *AMY2A*, and *AMY2B* resulted higher after thawing, even though not significantly.

For both pancreatic progenitors and exocrine pancreatic cells, no differences were identified between the two patient cell lines.

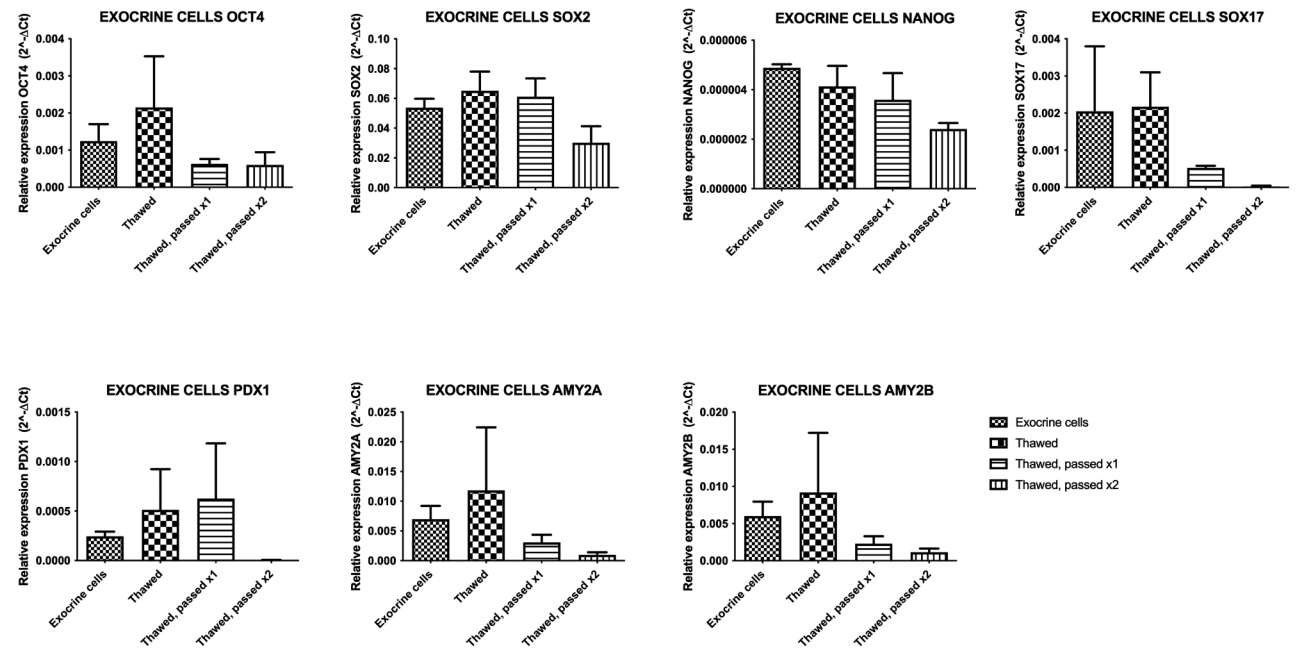
**Effect of differentiation on cell cycle**

The cell cycle was analyzed for human iPS cells, definitive endoderm, and pancreatic progenitors. Higher G0 percentages were shown in definitive endoderm (one-way ANOVA,  $p < 0.05$ ) and progenitor cells in comparison to undifferentiated human iPS cells. No differences were highlighted in the S phase, while statistically lower levels of G2/M were identified in definitive endoderm compared to iPS (one-way ANOVA,  $p < 0.01$ ) (Fig. 9). No differences were identified between the two patient cell lines.

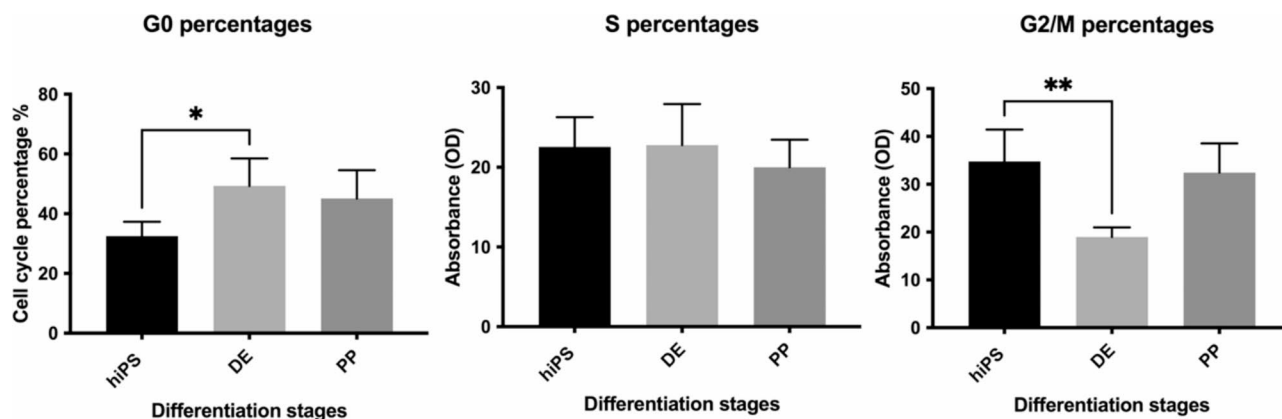
Human iPS cell stage: the definitive endoderm was obtained after 4 days of stimulation with activin A and CHIR99021. Stage I: during the next 3 days, the medium was changed and fibroblast growth factor 7 (FGF7) was added. Stage II: the culture medium was changed and cyclopamine, noggin, and retinoic acid were added for 3 days. Stage III: the media was changed and supplemented with FGF7, glucagon-like peptide 1, and nicotinamide for 4 days. Stage IV: exocrine pancreatic cells. Cell markers: endodermal cell markers (*SOX17*), pancreatic progenitor cell marker (*PDX1*) pancreatic exocrine cell marker (alpha-amylase). The differentiation stimuli and



**Fig. 7** Real-time PCR analysis shows mRNA expression dynamics for several key markers of pancreatic differentiation. Comparison of mRNA level of pancreatic progenitors after thawing and passaging. One-way ANOVA OCT4  $p < 0.05$ ; Dunnett's multiple comparison test \*:  $p < 0.05$



**Fig. 8** Real-time PCR analysis shows mRNA expression dynamics for several key markers of pancreatic differentiation. Comparison of mRNA levels of pancreatic exocrine cells in culture after thawing



**Fig. 9** Analysis of cell cycle measured by propidium iodide uptake of human iPS cells, definitive endoderm, and pancreatic progenitor cells (G0 one-way ANOVA  $p < 0.05$ , G2/M one-way ANOVA  $p < 0.01$ , Dunnett's multiple comparison test \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ )

procedure is a slightly modified version of a previously published protocol [6].

Supplementary figure. Real-time PCR analysis expression of key marker gene for each step of differentiation (human iPS – definitive endoderm DE, pancreatic progenitors PP, exocrine cells EX) compared between the two patients.

## Discussion

Since their discovery in 2006 [1], human iPS cells have proven to be a useful tool for disease modeling, regenerative medicine, drug discovery, and personalized medicine [10–12]. Over the years, there have been many advances in human iPS cell culture and differentiation technologies, and today, different protocols are available for differentiating them in almost every cellular type of the human body [13]. Nonetheless, available protocols often have some issues, such as the long time needed for the differentiation, the heterogeneity of differentiated cells obtained, and a lack of studies regarding the culture of cells after differentiation [14, 15].

In this work, we successfully differentiated patient-specific iPS cells into pancreatic exocrine cells, applying a protocol previously developed for ESCs [6], indicating that the protocol is versatile for both types of stem cells. This also suggests that iPS cells differentiate similarly to ESCs [16, 17], also in the context of exocrine pancreatic differentiation.

Furthermore, given that the majority of human iPS cell differentiation protocols are time-consuming, for the first time, we successfully optimized the protocol for exocrine pancreatic differentiation shortening its duration by reducing the time needed for obtaining exocrine cells. To shorten the time required for differentiation, we first examined whether shortening the pancreatic progenitors' exposure to the final differentiation step's stimuli would affect the expression of important marker genes. To produce mature exocrine cells, Takizawa-Shirasawa

and colleagues [6] suggested subjecting pancreatic progenitors to particular stimuli for 15 days to obtain mature exocrine cells. Since 10 days are needed to obtain pancreatic progenitors from human iPS cells, the complete differentiation into exocrine pancreatic cells requires 25 days in total. Hence, there is a need to optimize the protocol as much as possible, reducing the time for obtaining exocrine pancreatic cells. Our findings suggest that it is possible to reduce from 15 to only 4 days the time necessary for differentiating pancreatic progenitors in amylase-producing cells without losing differentiation efficiency, and from 25 to 14 days the total time required. Indeed, our immunofluorescence assays indicated similar percentages of amylase-positive cells in the populations obtained after 4 (40%) and 20 days (45%) of differentiation, respectively. In this regard, the differentiation of pancreatic progenitors into acinar cells physiologically occurs from embryonic day 12 to 15 in mice [18, 19], suggesting that also in vitro a short time could be needed for obtaining exocrine pancreatic cells from iPS-derived pancreatic progenitors. Therefore, our findings are in line with this observation, suggesting that it is possible to get an efficient exocrine differentiation in only 4 days. Although human pancreatic embryogenesis is less well understood [20], the overall process is thought to be comparable to that of mice [21]. Moreover, the reduction in terms of PDX1 positive cells from about 37% after 4 days of differentiation to 27% after 20 days, as shown in our immunostaining assay, indicates a depletion of pancreatic progenitors leading to a purer culture of pancreatic exocrine cells. This finding is in line with the current literature, reporting that PDX1 is down-regulated after late embryonic development, with only a restricted subset of exocrine pancreatic cells expressing it in the post-natal pancreas [22, 23]. Even if there is a lack of studies regarding the improvement of differentiation protocols for obtaining exocrine cells, some evidence is available for other cell types [24–26].

Another important issue related to human iPS cell differentiation is the difficulty of keeping in culture differentiated cells, mainly due to biological and technical factors [27]. In this context, differentiated cells generally exhibit a reduced DNA synthesis and a low replication capacity compared to their undifferentiated counterpart [28]. Nonetheless, some differentiated cells, among which pancreatic progenitors, maintain the ability to proliferate when cultured under optimal conditions [29, 30], being these cells multipotent [31]. Therefore, due to the challenges in culturing differentiated cells, we explored the possibility of keeping in culture pancreatic progenitors and exocrine pancreatic cells and freezing and thawing them after differentiation.

For the purpose of reducing the time needed for obtaining differentiated cells, we successfully maintained in culture fresh human iPS-derived pancreatic progenitors performing cell passages, without recording any reduction in *PDX1* and amylase mRNAs over time, rather observing an increment of *PDX1* mRNA after the first passage. In this regard, studies reported in the literature indicate that subculturing pancreatic progenitors leads to a notable increase in the proportion of *PDX1*+ cells. One example in this field was provided by Nakamura and co-authors in 2022 [30], who demonstrated that pancreatic progenitors' long-term expansion leads to enrichment in *PDX1*+ cells. In particular, the increment over time of *PDX1* in pancreatic progenitors positively affects pancreatic differentiation efficiency, being *PDX1* an indispensable precursor of pancreatic development [32]. In this way, the possibility of keeping in culture pancreatic progenitors allows us to avoid starting each time from human iPS cells to obtain differentiated cells. Furthermore, *PDX1*-positive pancreatic progenitors are versatile cells that can be further differentiated into both exocrine and endocrine pancreatic cells [33]. Therefore, the possibility of maintaining this cellular population in culture constitutes an advantage and allows to reduce time consumption. Moreover, the stemness marker *OCT4* mRNA resulted significantly lower after subculturing, suggesting a strong reduction of undifferentiated iPS cells leading to a purer population of pancreatic progenitors. Also, the stemness marker *NANOG* mRNA resulted lower after passaging, corroborating this result. It is known that the regulation of *OCT4* expression is influenced by epigenetic changes that occur during differentiation; these modifications can silence the genes responsible for maintaining pluripotency, thus contributing to their decreased expression after passaging [34]. In summary, the decrease of *OCT4* mRNA after passaging pancreatic progenitors may be primarily driven by differentiation processes [35], regulatory network changes [34], and the effect of passaging. These factors collectively contribute to the transition from a pluripotent state toward a purer

differentiated pancreatic lineage. Altogether, these findings suggest that to obtain purer progenitor cultures, at least one passage should be done. This is in line with the findings reported by Konagaya and Iwata in 2019 [29], who noticed an increase in pancreatic progenitors' markers, such as *PDX1*, after several passages of pancreatic progenitors.

Then, we focused on freezing, thawing, and keeping in culture thawed differentiated cells, exploring a field poorly discussed in the literature in the context of pancreatic cells. Interestingly, in our analysis we noticed a significant decrease in *OCT4* mRNA expression in iPS-derived pancreatic progenitors after freezing and thawing procedures, and also in thawed pancreatic progenitors maintained in culture, in comparison to unfrozen cells, suggesting a probable reduction of undifferentiated cells. In this regard, different studies reported in the literature [36–38] indicate that the cryo-preserved agent DMSO reduces *OCT4*-positive cells in embryoid bodies and stimulates differentiation into definitive endoderm cells, and their differentiated counterpart (i.e. pancreatic progenitors or hepatic cells). In this regard, as shown by Sousa and colleagues in 2020 [39], DMSO improves responsiveness for differentiation into multiple lineages by promoting a higher percentage of cells in the G1 phase of the cell cycle, which is crucial for effective differentiation [39]. According to these findings, even if we did not assess the cell cycle in thawed cells, we can speculate that the decrease of *OCT4* could be related to this mechanism. Indeed, the solution that we used for freezing differentiated cells, the CryoStor® reagent, contains 10% DMSO, a high concentration that can be responsible for the increase of the differentiation capability. Moreover, *SOX17*, *PDX1*, and amylase mRNA levels were also increased after thawing in both pancreatic progenitors and exocrine pancreatic cells indicating an increase of differentiation. Furthermore, although the expansion of thawed pancreatic progenitors reduced *PDX1* mRNA levels compared to thawed cells, it did not alter it compared to the control (not thawed), suggesting that cells can be further expanded. To the best of our knowledge, only few previous works report on the possibility of freezing, thawing, and culturing iPS-derived cells, and specifically pancreatic progenitors. For example, recently Trott and co-workers [40] and Konagaya and Iwata [29] identified different stimuli to maintain in culture pancreatic progenitors from human iPS cell lines, which were able to effectively proliferate, even after thawing, in a chemically defined medium without losing differentiation efficiency, as in our case. Although we did not notice any reduction in pancreatic progenitor proliferation, we could further implement our protocol with other combinations of stimuli [29, 40] to investigate the possibility of keeping cells in culture for a longer time and restoring the higher *PDX1*,

*AMY2A*, and *AMY2B* mRNA levels reported after thawing. In addition, despite Trott et al. [40] and Konagaya and Iwata [29] used stimuli different from those used in our work, in 2017 other authors [41] used noggin, and B27, which are also present in our culture conditions, indicating that our mix of stimuli is suitable for maintaining proliferative pancreatic progenitor cells, strengthening the results of our study.

Despite the encouraging dynamic of mRNA marker levels described above, our analysis showed an overall decrease of all mRNA markers after the second seeding of mature exocrine cells. To date, no works are available regarding the culture of differentiated exocrine cells. Although this field is poorly discussed in the literature, we can speculate that the decrease in mRNA markers could be related to culturing conditions. Particularly, achieving high purity and functionality of iPS-derived exocrine pancreatic cells remains a challenge, mainly due to incomplete differentiation protocols, which constitutes the first issue. Moreover, it is important to optimize as much as possible the culture conditions. Indeed, exocrine pancreatic cells require a medium rich in nutrients and growth factors to support the metabolic needs of the cells. In this regard, the medium that we used is rich in growth factors necessary for pancreatic cell survival, e.g. FGF7, and GLP1 [6, 42], but it could be further enriched in nutrients. Therefore, the optimization of the culture medium is a pivotal point to address in the future. In support of this, in 2023, Inui et al. [43] developed a method for culturing, cryopreserving, and thawing human iPS cell-derived hepatocytes, overcoming issues due to the loss of hepatic functions after reseeded by applying different culture conditions and dissociation agents. Finding the optimal culture conditions for improving cell expansion and maintaining appropriate levels of specific markers could also be resolute in our case, and our study could be optimized for long-term and stable maintenance of human iPS cell-derived pancreatic progenitors, and exocrine cells. Nonetheless, it is important to highlight that, differently from exocrine pancreatic cells, hepatocytes still retain the ability to proliferate, due to the maintenance of stemness features (e.g. high telomerase activity) [44]. Instead, as already mentioned, terminally differentiated cells, such as exocrine cells, generally exhibit a reduced DNA synthesis and a low replication capacity compared to their undifferentiated counterpart [28]. Therefore, we assume that contrary to pancreatic progenitors, which still maintain multipotent stemness characteristics, pancreatic exocrine cells cannot be extensively expanded given their terminally differentiated nature, but can probably be maintained in culture for the time necessary for performing long-term experiments, such as toxicological assays.

In the present study, we also analyzed the cell cycle of human iPS cells, definitive endoderm cells, and pancreatic progenitors using the flow-cytometry propidium iodide cellular uptake assay. Our results indicated a lower percentage of cells in the G0 phase in undifferentiated iPS cells compared to pancreatic progenitors and definitive endoderm cells. This result is in line with the well-known proliferation ability of stem cells [45] and suggests that differentiated cells are in a higher percentage in a quiescent state, as expected [28]. Then, the analyses showed similar percentages of cells in the S phase between undifferentiated iPS cells, definitive endoderm, and pancreatic progenitor cells, suggesting that, similarly to undifferentiated cells, differentiated cells can replicate DNA in culture. Lastly, a significantly larger proportion of iPS cells were in G2/M phases compared to definitive endoderm. These results are consistent with the available literature, indicating that G2-specific pathways actively promote the pluripotent state [46]. Altogether, little evidence about cell cycle progression and regulation in human iPS-derived differentiated cells is reported. A better understanding of the cell cycle in iPS and differentiated cells can be useful for example to study more accurately the cytotoxicity of cell cycle-specific drugs [47]. Therefore, despite some evidence already existing in the literature, there is a strong need for a better knowledge of the cell cycle in human iPS cells, especially in differentiated cells, which constitute a poorly investigated field.

Overall, there are still major limits in the field of iPS differentiation, such as the purity of the culture obtained, the long time needed to differentiate iPS, and the set-up of the appropriate culture conditions important for maintaining unaltered the genetic background of the patient. In addition, our study has some limitations, such as the need for a deeper characterization of differentiated cells by omics approaches. Moreover, despite the decrease of stemness markers after passaging, our population of differentiated cells is still not completely pure, due to the presence of some undifferentiated iPS cells, as indicated by other studies [48, 49]. Indeed, in order to use iPS cells or their differentiated counterpart for clinical applications, technology must be standardized to ensure safety and efficacy. Despite this aspect being currently inadequately investigated [50], in recent years many studies, among which our work, have been focusing on these challenges, making great progress in this field.

## Conclusions

The results highlighted in this study are a promising starting point for optimizing existing technologies in patient-specific iPS-derived pancreatic cell differentiation and culture. In this work, we successfully differentiated patient-specific iPS cells into pancreatic exocrine cells, reduced the time needed for exocrine cell generation, and

kept in culture patient-specific pancreatic cells. Our work advances the tailored therapy field, leading to relevant clinical research-related applications laying the foundation for creating a bank of patient-specific pancreatic cell lines exploitable for tailored pharmacological assays.

#### Abbreviations

DMSO	Dimethyl sulfoxide
ESCs	Embryonic stem cells
FGF7	Fibroblast growth factor 7
GLP	Glucagon-like peptide
iPS cells	Induced pluripotent stem cells
HNF	Hepatocyte nuclear factor
OCT4	Octamer-binding transcription factor 4
PBMCs	Peripheral blood mononuclear cells
PDX1	Pancreatic and duodenal homeobox 1
SOX17	SRY-box transcription factor 17
SOX2	SRY-box transcription factor 2

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13287-024-04068-6>.

Supplementary Material 1: Supplementary figure. Real-time PCR analysis expression of key marker gene for each step of differentiation (human iPS – definitive endoderm DE, pancreatic progenitors PP, exocrine cells EX) compared between the two patients.

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The authors declare that they have not use AI-generated work in this manuscript.

#### Author contributions

EG and PR performed, analyzed, and interpreted the data and were the major contributors in writing the manuscript. RB performed cytofluorimetry experiments. FY, ML, GD, and GS interpreted the data. All authors read and approved the final manuscript.

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#### Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Declarations

##### Ethics approval and consent to participate

The study was approved by the Ethical Committee of the Institute of Maternal and Child Health IRCCS Burlo Garofolo, with approval number 1556 (internal ID of the study RC 07/14 and RC 44/22). Title of the approved project: "Patient-derived induced pluripotent stem cells for personalizing therapy: the paradigm of thiopurine pancreatitis in Crohn's disease" and "Comparison of 2D and 3D patient-derived pancreatic exocrine models for the study of thiopurine induced pancreatitis in pediatric IBD patients: an innovative approach therapy personalization"; Name of the institutional approval committee: Ethical Committee of the Institute of Maternal and Child Health IRCCS Burlo Garofolo (Trieste, Italy); Approval number: 1556; Date of approval: 14.07.2014.

##### Informed consent

Obtained from all subjects involved in the study.

#### Competing interests

The authors declare that they have no competing interests.

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