Regulation of multiple signaling pathways promotes the consistent expansion of human pancreatic progenitors in defined conditions

Luka Jarc[1, 2 #], Manuj Bandral[1, 2 #], Elisa Zanfrini[1, 2], Mathias Lesche[3, 4], Vida Kufrin[1], Raquel Sendra[1], Daniela Pezzolla[2, 5], Ioannis Giannios[1, 2], Shahryar Khattak[6, 7], Katrin Neumann[6], Barbara Ludwig[1, 2, 5, 8] and Anthony Gavalas[1, 2, *]

Running title : Expansion of pancreas progenitors under GMP-compliant conditions

1 Paul Langerhans Institute Dresden (PLID) of Helmholtz Center Munich at the University Clinic Carl Gustav Carus of TU Dresden, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany
2 German Centre for Diabetes Research (DZD), Germany
3 (DcGC), TU Dresden, Dresden, Germany
4 Center for Molecular and Cellular Bioengineering (CMCB) Technology Platform, TU Dresden, Dresden, Germany
5 Center for Regenerative Therapies Dresden (CRTD), Faculty of Medicine, TU Dresden, Dresden, Germany
6 Stem Cell Engineering Facility, (SCEF), CRTD, Faculty of Medicine, TU Dresden, Dresden, Germany
7 Current address : KAUST Smart-Health Initiative (KSHI) and Biological & Environmental Science and Engineering (BESE) Division, King Abdullah University of Science & Technology (KAUST), Thuwal, Saudi Arabia
8 Department of Medicine III, University Hospital Carl Gustav Carus and Faculty of Medicine, TU Dresden, Dresden, Germany

# equal contribution
* Correspondence : anthony.gavalas@tu-dresden.de

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ABSTRACT

The unlimited expansion of human progenitor cells in vitro could unlock many prospects for regenerative medicine but it remains an important challenge as it requires the decoupling of the mechanisms supporting progenitor self-renewal and expansion from feed-forward mechanisms promoting their differentiation. The expansion of human pluripotent stem (hPS) cell derived pancreatic progenitors (PP) will accelerate the development of novel therapies for diabetes.

We obtained mechanistic insights into the expansion requirements of PP cells and leveraged them to conduct a hypothesis-driven iterative search to identify conditions for the robust and unlimited expansion of hPS cell derived PP cells under GMP-compliant conditions. We show that the combined stimulation of specific mitogenic pathways, suppression of retinoic acid signaling and inhibition of selected branches of the TGFβ and Wnt signaling pathways are necessary for the effective decoupling of PP proliferation from differentiation. This enabled the selection of $PDX1^+/SOX9^+/NKX6.1^+$ PP cells and their consistent, 2000-fold, expansion over ten passages and 40-45 days. Transcriptome analyses confirmed the stabilisation of PP identity and the effective suppression of differentiation. Using these conditions, $PDX1^+/SOX9^+/NKX6.1^+$ PP cells, derived from different, both XY and XX, hPS cells lines, were enriched to nearly 90% homogeneity and expanded with very similar kinetics and efficiency. Furthermore, non-expanded and expanded PP cells, from different hPS cell lines, were differentiated in micropatterned wells into homogeneous islet-like clusters (SC-islets) with very similar efficiency. These clusters contained abundant β-cells of comparable functionality as assessed by glucose-stimulated insulin secretion assays.

These findings established the signaling requirements to decouple PP proliferation from differentiation and allowed the consistent expansion of hPS cell derived PP cells. They will enable the establishment of large banks of PP cells derived under GMP conditions from diverse hPS cell lines. This will also streamline the generation of SC-islet clusters for further development of the differentiation process, diabetes research, personalized medicine and cell therapies.

INTRODUCTION

Diabetes is a global epidemic affecting > 9% of the global population and its two main forms result from the immune destruction (type 1) or malfunction (type 2) of insulin-producing β cells residing in the pancreatic islets of Langerhans. In severe cases of diabetes, whole pancreas transplantation or pancreatic islet transplantation can restore excellent metabolic control and insulin independence but both approaches are critically limited by the scarcity of tissue donors. Moreover, such transplantations require the use of immunosuppressive drugs that hinder the already limited β-cell self-renewal and are associated with severe side effects and morbidity (Nir et al., 2007).

The remarkable progress over the last decade in the differentiation of human pluripotent stem (hPS) cells into pancreatic islet cells (SC-islet cells) suggests that this approach could provide an unlimited source of β-cells for transplantations and personalized medicine (Amin et al., 2018; Balboa et al., 2022; Du et al., 2022; Hogrebe et al., 2020; Millman et al., 2016; Ramzy et al., 2021; Shapiro et al., 2021). The underlying strategy recapitulates in vitro the stepwise differentiation of epiblast cells in the early embryo initially to definitive endoderm and then to pancreatic progenitors (PP), pancreatic endocrine progenitors and, finally, to pancreatic islet cells (Kroon et al., 2008; Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015; Zeng et al., 2016; Zhu et al., 2016b). Clinical trials that employed either GMP-grade PP cells in a non-immunoprotective macroencapsulation device (Shapiro et al., 2021) (NCT03163511) or GMP-grade SC-islet cells in an immunoprotective macroencapsulation device (NCT02239354) suggested that diabetes cell therapies can become a reality. Obstacles to be overcome include the limited maturation of the resulting β-cells, incomplete conversion of hPS cells into endocrine cells and the large number of SC-islet cells required for a single transplantation. Elucidating the mechanisms that maintain the self-renewal of pure pancreatic progenitors, while inhibiting their differentiation, would allow the establishment of expandable populations of PP cells and address the need for large numbers of pancreatic endocrine cells.

During development, the intersection of several signals induces the pancreatic anlage at the posterior foregut. Repression of posteriorly derived Wnt signaling is initially essential to define the foregut region (McLin et al., 2007). There, the pancreas and liver develop from a common progenitor (Cerda-Esteban et al., 2017; Deutsch et al., 2001). Bipotent progenitors located distant to the cardiac mesoderm, which secretes the pro-hepatic signals FGF10 and BMPs, will be able to adopt the pancreatic fate (Jung et al., 1999; Rossi et al., 2001). The combination of RA signaling, derived from the somitic mesoderm (Martin et al., 2005; Molotkov et al., 2005), and endothelial signals, derived from the dorsal aorta (Lammert et al., 2001), induces the formation of pancreatic progenitors. Complex, stage-specific expression of several transcription factors guides pancreatic cell development (Duvall et al., 2022). The epithelial pancreatic progenitors are characterized by the combined expression of several transcription factors, most notably Pdx1, Nkx6.1 and Sox9, which are essential for progenitor self-renewal and subsequent endocrine differentiation. Pdx1 expression is induced at the boundary between the Sox2-expressing anterior endoderm and the Cdx2-expressing posterior endoderm and it is necessary for the maintenance of the pancreatic identity (Sherwood et al., 2009). Its function is reinforced by Sox9 which cooperates with Pdx1 to repress Cdx2 expression in the pancreatic anlage (Shih et al., 2015). Loss-of-function experiments of Pdx1 (Jonsson et al., 1994; Offield et al., 1996) and Sox9 (Seymour et al., 2007) resulted in pancreatic agenesis, establishing their key role in maintaining pancreatic identity. Sox9 is subsequently essential for the induction of Neurog3, the transcription

factor which is necessary and sufficient for the induction of the pancreatic endocrine lineage (Gradwohl et al., 2000; Grapin-Botton et al., 2001). Nkx6.1 is also a key contributor to the maintenance and self-renewal of pancreatic progenitors (Sander et al., 2000). It is also required for β-cell specification since ectopic Nkx6.1 expression directed endocrine precursors into β-cells, whereas β-cell specific ablation of Nkx6.1 diverted these cells into the other endocrine lineages (Schaffer et al., 2013). Given the importance of these three transcription factors, the identification of conditions to stabilize PDX1$^+$/SOX9$^+$/NKX6.1$^+$ cells will set the stage for unlimited PP cell expansion and their efficient differentiation into SC-islets

PDX1$^+$/SOX9$^+$/NKX6.1$^+$ PP cells undergo self-renewal *in vivo* for a limited amount of time. During development feed-forward loops that steer cells toward differentiation operate in parallel with maintenance and self-renewal mechanisms. Unlimited expansion of PP cells *in vitro* will require disentangling differentiation signals from proliferation/maintenance signals. Several pathways have been implicated in these processes. Notch signaling mediates progenitor self-renewal as well as lineage segregation (Afelik et al., 2012; Murtaugh et al., 2003; Shih et al., 2012) and its downregulation is necessary for endocrine lineage specification (Apelqvist et al., 1999; Jensen et al., 2000). Notch signaling is itself regulated by the extracellular signal sphingosine-1-phosphate (S1p) (Serafimidis et al., 2017). Canonical wnt signaling has also been implicated in PP maintenance (Afelik et al., 2015). Low levels of endogenous RA signaling are involved in subsequent differentiation steps of PP cells (Kobayashi et al., 2002; Lorberbaum et al., 2020; Tulachan et al., 2003; Vinckier et al., 2020). Finally, expression of TGFβ ligands and receptors is widespread during pancreas development (Crisera et al., 1999; Tulachan et al., 2007) and it has been suggested that the TGFβ pathway activity regulates the pancreatic lineage allocation (Miralles et al., 1998; Sanvito et al., 1994).

Culture conditions to expand hPS cell-derived PDX1$^+$/SOX9$^+$ cells were reported but relied either on feeder layers or limiting 3D conditions in an agarose hydrogel matrix and did not maintain the expression of the crucial transcription factor NKX6.1 (Konagaya and Iwata, 2019; Trott et al., 2017). A recent study claimed the expansion of a hPS cell-derived cell population, containing PDX1$^+$/NKX6.1$^+$ cells, on fibronectin and in a defined medium using SB431542, a broad (ALK4/5/7) TGFβ inhibitor (Inman et al., 2002; Nakamura et al., 2022) but, unfortunately, reproducibility of the expansion was not documented. Additionally, the reported percentage of PDX1$^+$/NKX6.1$^+$ PP cells in the expanded cells varied widely, between 65%, 35% and 20%, in the three different hPS cell lines used. Furthermore, RNA Seq data showed that *NKX6.1* expression actually decreased with expansion, in two out of the three samples presented, (E-MTAB-9992) suggesting that NKX6.1 expression was not reliably maintained under those culture conditions. In another study, PP cells, generated through genetic reprogramming of human fibroblasts into endoderm progenitor cells, could be expanded in a chemically defined medium containing epithelial growth factor (EGF), basic fibroblast growth factor (bFGF), and SB431542, another general (ALK4/5/7) TGFβ inhibitor, but the percentage of PDX1$^+$/NKX6.1$^+$ cells was less than 20% (Zhu et al., 2016a). The authors then identified a BET bromodomain inhibitor that could mediate the unlimited expansion of PDX1$^+$/NKX6.1$^+$ PP cells on feeder layers of mouse embryonic fibroblasts (Ma et al., 2022) but the use of feeder layers precluded the use of this method in a clinical setting and did not address the mechanistic requirements for the expansion.

Thus, the elucidation of the mechanistic requirements, for simultaneous PP expansion and decoupling from differentiation, is necessary for reliably expanding hPS-derived PP cells. This will also allow the use of only defined molecules that can be produced under GMP conditions (GMP-

compliant), a necessity for future clinical applications. PP cells express the components of a large number of signaling pathways and we hypothesized that a longitudinal transcriptome analysis of non-expanding and occasionally expanding PP cells would provide candidate signaling pathways reasoning that upregulated pathways in expanding cells would be promoting expansion, whereas downregulated pathways would be blocking expansion and/or favoring differentiation. We leveraged these findings and employed a hypothesis-driven iterative process to define conditions that allowed robust, unlimited expansion, of hPS cell-derived PP cells. We found that the combined stimulation of specific mitogenic pathways, suppression of retinoic acid signaling and inhibition of selected branches of the TGFβ and Wnt signaling pathways enabled the 2000-fold expansion of PP cells over ten passages and 40-45 days. Expansion conditions are GMP-compliant and enable the robust, reproducible expansion, as well as intermediate cryopreservation, of PP cells derived from diverse hPS cell lines with essentially identical growth kinetics. These conditions select PDX1$^+$/SOX9$^+$/NKX6.1$^+$ PP cells, suggesting that they will be advantageous for hPS cell lines that may differentiate less efficiently into PP cells. Expanded PP cells differentiated, with very similar efficiency to non-expanded cells, into SC-islet clusters that contained functional β-cells as shown by glucose-stimulated insulin secretion (GSIS) assays.

These findings will allow the establishment of large banks of PP cells derived under GMP conditions from diverse hPS cell lines. This will streamline the generation of SC-islet clusters for further development of the differentiation procedure, diabetes research, personalized medicine and cell therapies.

RESULTS

Identification of candidate signaling pathways implicated in PP expansion

H1 hPS cells were differentiated into PP cells and PP cell expansion was attempted using the initial conditions (CINI) that relied on EGF and FGF2 and A38-01, a broad TGFβ inhibitor of ALK4/5/7 (Tojo et al., 2005). A similar formulation, using SB431542, another broad TGFβ inhibitor of similar specificity (Inman et al., 2002), was employed in the attempted expansion of PP cells derived through the reprogramming of human fibroblasts into endoderm progenitor cells (Zhu et al., 2016a) (Table S1). PP cells were plated at high density on Matrigel-coated plates and passaged every 4-6 days. In most instances, cell numbers remained either constant or decreased, eventually leading to growth arrest. Only occasionally (one out of five attempts), did PP cells expand and could then be maintained for at least up to ten passages (Figure 1A). Immunofluorescence experiments and qPCR analyses suggested that these expanding PP cells (ePP) maintained their identity since expression of *PDX1*, *NKX6.1* and *SOX9* remained stable at both the protein (compare Figure S1A, B and S1C) and transcript levels (Figure S1D). Immunofluorescence analysis of cryosections showed that these ePP cells could subsequently differentiate into endocrine cells (Figure S1E). These results suggested that EGF, FGF2 and broad TGFβ inhibition do not suffice to reproducibly expand hPS cell-derived PP cells.

PP cells express the components of a large number of signaling pathways (Table S3) and we hypothesized that transcriptome comparison of non-expanding and CINI ePP cells could identify pathways implicated in the expansion. Upregulated pathways in ePP cells would be promoting expansion, whereas downregulated pathways would be blocking expansion and/or favoring differentiation. Thus we compared the RNA Seq profiles of cells directly after their differentiation into pancreatic progenitors (dPP) (p0) (n=4), and CINI ePP cells at passage 5 (p5) (n=3) and p10 (n=3). Analyzed p0 samples included one sample that was subsequently

successfully expanded. Analysis for differentially expressed genes (DEG) identified groups of genes that were continuously up or down-regulated or that were up or down-regulated only within the first five passages (Figure S1F and Table S2). Metric multidimensional scaling (MDS) plot of the Euclidian distances of the samples showed clustering of the samples strictly according to their passage number. The single p0 sample, which subsequently expanded successfully, segregated with the other p0 samples suggesting that it was not fundamentally different from samples that failed to expand. These findings suggested that ePP cells adapted to the culture conditions in a reproducible manner and that this adaptation occurred largely during the first five passages (Figure 1B). GSEA analysis showed that components of the TGFβ signaling pathway were negatively correlated with expansion whereas E2F target genes and DNA replication were positively correlated (Figure 1C). Since E2F transcription factors are indirectly activated by growth signals to regulate multiple cell cycle genes and promote cell proliferation (Ertosun et al., 2016; Rubin et al., 2020), this raised the possibility that expanding cells upregulate their own growth factors engaging themselves in an autocrine growth loop. To gain a better mechanistic insight into additional, possibly involved, signaling pathways, we then examined the expression kinetics of all individual ligands and receptors expressed in dPP and ePP cells.

High expression of TGFβ ligands as well as Type I and Type II receptors suggested that all three branches of this signaling pathway were active at p0 (Table S3). The negative correlation of the TGFβ signaling pathway with expansion appeared to retrospectively justify the use of the broad TGFβ signal inhibitor A38-01 (Tojo et al., 2005). However, this pathway has several branches, which often carry out opposing functions, also depending on the cellular context. Thus we examined the expression kinetics of all expressed TGFβ ligands and receptors. The most highly expressed TGFβ ligands in dPP cells were *TGFb1* and *BMP2* and their expression was repressed nearly four-fold during expansion (Figure 1D, S1G). TGFb1 and BMP2 act through the ALK1/5 and ALK3/6/2 receptors, respectively (Brown and Schneyer, 2010), and, therefore, A38-01 would block TGFB1 but not BMP2 signaling. On the other hand, *ALK4* and its ligand, *GDF11*, were strongly expressed throughout expansion; *GDF11* was even upregulated (Figure 1D, Table S3), suggesting a possible positive role of this TGFβ signaling branch in the expansion of pancreas progenitors. These findings suggested that the use of broad TGFβ inhibitors such as A38-01 or SB431542 may not be optimal because they do not inhibit BMP2 signaling whereas they do block ALK4 signaling, which might promote PP expansion through GDF11. This provided a mechanistic rationale for the subsequent use of more specific TGFβ inhibitors.

To account for the positive correlation of the expression of E2F targets with expansion, we then examined the gene expression kinetics of growth factors during expansion. There was a striking, nearly 30-fold, upregulation of *FGF18* expression (Figure 1E and Table S3) suggesting a strong requirement for the activation of the MAPK pathway. It is noteworthy that FGF18 has a selective affinity for FGFR3 and 4 (Zhang et al., 2006), the two most highly expressed *FGFRs* in dPP and ePP cells. Other *FGFs* were weakly expressed and not upregulated during expansion (Table S3). Another growth factor signaling pathway of possible interest was the PDGF signaling pathway. *PDGF* receptors *A* and *B* were stably and strongly expressed during expansion but the initially strong expression of two of the expressed ligands, PDGFA and B was downregulated by 30- and 15-fold, respectively (Figure 1F, S1H, Table S3). These findings provided a mechanistic rationale to provide FGF18 during expansion and/or inhibit PDGF signaling in subsequent experiments.

Retinoic acid (RA) promotes the differentiation of pancreatic progenitors during development (Lorberbaum et al., 2020; Martin et al., 2005; Ostrom et al., 2008; Vinckier et al., 2020). The retinol dehydrogenase 11 (*RDH11*) and aldehyde dehydrogenase 1a1 (*ALDH1A1*) genes, which encode enzymes that convert vitamin A into retinoic acid, as well as the RA nuclear receptors *RARA*, *RARG*, *RXRA* and *RXRB* were highly expressed in PP cells. Therefore, in the presence of Vitamin A, this pathway could act in an autocrine manner to promote differentiation. Strikingly, during successful expansion, there was a dramatic 25-fold downregulation in the expression of the RA-producing enzyme *ALDH1A1* (Figure 2G, Table S3). Thus, PP cells are poised to initiate RA-mediated differentiation in an autocrine feed-forward loop and, therefore, the PP state might be stabilized by eliminating vitamin A in the medium.

The Notch signaling pathway is implicated in multiple aspects of pancreatic development including the expansion of pancreatic progenitors and lineage selection (Afelik et al., 2012; Apelqvist et al., 1999; Murtaugh et al., 2003; Qu et al., 2013; Seymour et al., 2020; Shih et al., 2012). The expression of Notch receptors, ligands and effectors, such as HES1, was also affected during expansion in a complex pattern (Figure 1H, S1I, Table S3) that suggested an overall attenuation, but not silencing, of the pathway.

In summary, the findings suggested that several mechanisms may contribute to stabilizing the PP state. Alone or in combination, inhibition of selected branches of the TGFβ pathway, attenuation of the Notch pathway, additional mitogenic stimulation with FGF18, and suppression of RA and PDGF signaling may lead to reproducible expansion of the PP cells.

Elimination of RA and selective TGFβ inhibition allow reproducible expansion of PP cells

Several expansion culture conditions (summarized in Table S1) were assessed based on the mechanistic insights discussed above using PP cells generated with an adaptation of published procedures (Balboa et al., 2022; Mahaddalkar et al., 2020; Rezania et al., 2014; Shi et al., 2017) (Table S4). Our approach was to carry out gain-of-function or loss-of-function of specific signaling pathways alone or in combination in order to achieve reproducible, robust PP expansion. We first suppressed RA signaling by substituting the Vitamin A containing B27 supplement with a B27 formulation devoid of vitamin A. Additionally, we substituted A38-01 with the ALK5 II inhibitor (ALK5i II) that targets primarily ALK5, and to a lesser extent ALK3/6, but not ALK4 (Gellibert et al., 2004). This was named ground condition 0 (C0). C0 was further elaborated by substitution of FGF2 with FGF18 (C1) or supplementation with the Notch inhibitor XXI (C2) (Seiffert et al., 2000), or supplementation with the PDGFR inhibitor CP673451 (C3) (Roberts et al., 2005) or with supplementation with both Notch and PDGFR inhibitors (C4). Since FGF2 and FGF18 belong to different FGF subfamilies and have overlapping, but not identical, FGFR specificities (Zhang et al., 2006), we also addressed a possible synergistic effect of these FGFs in PP expansion by combining them in C5. Three of these conditions, (C0, C1 and C5) resulted in the reproducible expansion of PP cells for at least 10 (C0, C1, n=3) or 11 (C5, n=5) passages with doubling times (T_d) of 3.9, 3.6 and 2.3 days, respectively (Figure 2A, Table S1).

Assessment of the expression of key PP genes by qPCR at p5 and p10 suggested that, in all three conditions, initial high levels of *PDX1, NKX6.1* and *SOX9* expression were maintained (*NKX6.1*, *SOX9*) or even transiently increased at p5 (C5, *PDX1*) (Figure 2B-D). Expression of *PTF1A* was dramatically decreased in all three conditions suggesting a restriction to a bipotent endocrine/duct progenitor identity (Figure S2A). Expression of *FOXA2*, which helps maintain pancreatic progenitor cells and is important for the development of the endocrine lineage (Gao et

al., 2010; Gao et al., 2008; Lee et al., 2005; Lee et al., 2019), was upregulated in C5 indicating that cells expanded in this condition might be more amenable to terminal endocrine differentiation (Figure S2B). On the other hand, there was a statistically significant increase in the expression of the liver and gut markers *AFP* and *CDX2* in C0 and C1 and a similar, but less strong, trend in C5 (Figure S2C, D). This suggested that these conditions (C0, C1, C5) allowed cells to express aspects of liver and gut programs. Since C5 had a significantly lower T_d and a less pronounced increase in *AFP* and *CDX2* expression, we concentrated on the analysis and further improvement of C5. Notably, C5 ePP cells could also be frozen and recovered after long storage with high survival rates (>85%) and no apparent loss of proliferative capacity.

To first confirm the C5 qPCR results at the protein level, we assessed the expression of several markers by immunofluorescence for C5 ePP cells. Immunofluorescence suggested that PDX1 and SOX9 were uniformly expressed at p0, p5 as well as p10 and that a large number of PP cells were NKX6.1$^+$ at all three different time points even though expansion appeared to somewhat reduce the number of NKX6.1$^+$ cells. (Figure 2E-G). Similarly, FOXA2 remained widely expressed at p0, p5 and p10 (Figure S2E-G). Expression of both *AFP* and *CDX2* increased transiently upon expansion, at p5 (Figure S2H-J). We then quantitated the expression of the key PP markers PDX1, SOX9 and NKX6.1 by flow cytometry at p0, p5 and p10. These experiments confirmed the immunofluorescence experiments showing that 90%, or more, of all cells were PDX1$^+$/SOX9$^+$ at all passages examined (Figure S2K-N), whereas nearly 50% of the cells at p0 and p5 and nearly 40% at p10 were PDX1$^+$/SOX9$^+$/NKX6.1$^+$. There was a small, apparent progressive drop in the number of PDX1$^+$/SOX9$^+$ as well as the number of PDX1$^+$/SOX9$^+$/NKX6.1$^+$ cells which did not reach statistical significance (Figure 2H-K, S2K-N).

These experiments established that C5 allowed reproducible and robust PP expansion. The basis for this was the elimination of RA signaling and selective TGFβ inhibition. Additionally, EGF, FGF2 and FGF18 synergized to promote a more robust expansion.

Expansion conditions promote primarily the proliferation of PP cells

To understand the mechanism of the successful, reproducible expansion, we asked whether it was due to enhanced survival, proliferation, or a combination of both. *De novo* generated PP cells were expanded in either C5 or CINI and assayed at p3 for EdU incorporation and apoptosis. The EdU incorporation analysis revealed that C5 cultures contained significantly more EdU$^+$ expanding cells (10.4 ± 1.2%) than CINI (4.2 ± 0.6%) cultures (*n=4*) (Figure 3A-C, S3A). The apoptosis assays showed that even though the percentage of 7-AAD$^+$/Annexin V$^+$ cells appeared higher in CINI-expanded cells (22.1 ± 5.0%) than in C5-expanded cells (17.2 ± 4.7%) this difference did not reach statistical significance (*n=6*) (Figure 3C and Figure S3B, C). Therefore, C5, as compared to CINI, promotes primarily proliferation rather than survival of PP cells.

Canonical Wnt inhibition restricts upregulation of hepatic fate and promotes PP identity

The upregulation of *AFP* and *CDX2* in C5 ePP cells suggested a drift towards hepatic and intestinal fates that would hinder the efficiency of differentiation into endocrine cells and subsequent maturation (Nair et al., 2019). During pancreas development, non-canonical Wnt signaling specifies bipotent liver/pancreas progenitors to pancreatic fates whereas canonical Wnt signaling leads to liver specification and the emergence of gastrointestinal identity (Ober et al., 2006; So et al., 2013; Muñoz-Bravo et al., 2016; Rodríguez-Seguel et al., 2013). PP cells, at p0

as well as subsequent passages, strongly and stably expressed several *WNT* receptors, co-receptors as well as canonical and non-canonical signals (Table S3). Thus, to suppress the upregulation of hepatic and/or intestinal fates, we supplemented C5 with the canonical Wnt inhibitor IWR-1 to selectively inactivate the canonical Wnt signaling (Chen et al., 2009) (condition 6; C6).

The growth curve of C6 ePP cells, over ten passages, showed that IWR-1 supplementation did not significantly affect the growth rate since the T_d was 2.5 days as opposed to 2.3 for cells expanded in C5 (Figure 4A). Expansions in C6 were also undertaken using vitronectin-N (VTN-N) as a substrate instead of Matrigel without any changes in expansion efficiency and promotion of the PP identity (*n=3*). VTN-N is a defined peptide that can be produced under GMP-conditions and, since all other media components can be produced under similar conditions, this finding established that this approach is also GMP-compliant (Figure 5A).

C6 ePP cells retained strong expression of *PDX1, NKX6.1* and *SOX9* as well as similar expression levels, to C5 ePP cells of the same passage no, of *PTF1A* and *FOXA2* (Figure S4A). Importantly, the expression of the liver markers examined, *AFP, HHEX* and *TTR* were all significantly lower by p10 in the C6 ePP cells, as compared to C5 ePP cells (Figure S4B), but expression of *CDX2* was only marginally reduced (Figure S4A). The expression of *PDX1, NKX6.1, SOX9* and *FOXA2* was also examined at the protein level by immunofluorescence which suggested that their expression was stable and persisted at high levels (Figure 4B-D, S4C-E). Similarly to C5 ePP cells, expression of AFP and CDX2 in C6 ePP cells was detectable by immunofluorescence at p5 but significantly reduced at p10 (Figure S4F-H). We then quantitated and compared the expression of the key PP markers by flow cytometry at p0, p5 and p10 (Figure 4E-I, S4I-L). Strikingly, the number of $PDX1^+/SOX9^+/NKX6.1^+$ C6 ePP cells increased significantly from 48%±11% (n=5) at p0 to 90%±10% (n=3) at p5 and 95%±5% (n=5) at p10 (Figure 4H). This, together the reduction in the expression of liver markers, was an additional significant improvement over C5 ePP cells at either p5 or p10 (Figure 4I). Similarly to C5 ePP cells, C6 ePP cells could be cryopreserved and recovered with high survival rates (>85%) and no apparent loss of proliferative capacity. Chromosomal stability was also assessed after 16 passages analyzing the G-banding of at least 20 metaphases and no alterations were found (Figure 4J).

To further repress the expression of liver and gut markers, we reconsidered BMP inhibition. ALK5i II is considered a relatively weak inhibitor of ALK3 (Gellibert et al., 2004). However, BMP2, a ligand of ALK3, was significantly downregulated in the CINI ePP cells (Figure S1G, Table S3). Attempting to effectively inhibit ALK3, we substituted ALK5i II with LDN-193189, which inhibits ALK3 with higher potency (Sanvitale et al., 2013). LDN-193189 was used alone (C7) or in combination with IWR-1 (C8) but none of these conditions was efficient in PP cell expansion (Table S1).

In summary, in C6, inhibition of the canonical Wnt signaling by IWR-1, promoted the selection of $PDX1^+/SOX9^+/NKX6.1^+$ in the expanding cell population and mitigated the upregulation of liver markers at both the gene and protein expression levels. C6 is a robust, highly reproducible, GMP-compliant procedure suitable for application in cell therapies.

Expansion stabilizes PP cell identity by repressing differentiation and alternative cell fates

To understand the effects of the C6 expansion procedure on the transcriptome of PP cells we performed RNA-Seq analyses on dPP cells (p0) derived from independent differentiations and

the corresponding p5 and p10 ePP cells. Principal component analyses (PCA) showed that the main component, PC1, represented most (81%) of the variance among samples and clearly separated p0 PP cells from either p5 and p10 ePP cells. All expanded cells were clustered remarkably close together on the PC1 axis (Figure S5A), suggesting that the ePP transcriptome stabilized quickly, after just five passages. This was confirmed by correlation analyses of the transcriptome profiles showing that all major changes occurred between p0 and p5 (Figure 5A, B, S5B). Results from GO and KEGG analyses of DEGs between p0 and p10 were consistent with a cell adaptation to culture conditions, the signaling molecules used for the expansion and an effect on the differentiation process (Figure 5C, S5C). We then compared the transcriptome of our ePP cells, expanded on VTN-N, with that of ePP cells expanded on feeders (Ma et al., 2022) or on fibronectin (FN) (Nakamura et al., 2022) as well as with FACSorted pancreatic progenitors from human fetuses (Ramond et al., 2018). Initial PCA analyses suggested that all *in vitro* derived PP cells clustered away from fetal cells (Figure S5D) and thus we restricted subsequent comparisons only among *in vitro* derived PP cells.

Comparative PCA analysis, with the other *in vitro* derived ePP cells (Ma et al., 2022; Nakamura et al., 2022), clustered our ePP cells separately and showed very high similarity among our p5 and p10 ePP cells (Figure 5D). To identify the molecular basis of this difference, we used a variance stabilizing transformation (vst) function (Love et al., 2014) and hierarchical clustering to identify genes that are differentially regulated in our ePP cells in comparison to the feeder (Ma et al., 2022) or FN ePP cells (Nakamura et al., 2022) (Figure S5E). Interestingly, these consisted only of upregulated genes and GO analyses showed that affected GO categories referred to epithelial maintenance and differentiation, cellular signaling response and metabolic processes (GO BP), membrane components (GO CC) and metabolism (GO MF and KEGG) (Figure S5F). An interesting upregulated gene in our ePP cells was *GP2*, a gene encoding a zymogen granule membrane glycoprotein that has been identified as a unique marker of human fetal pancreatic progenitors (Cogger et al., 2017; Ramond et al., 2017). Importantly, $GP2^{+}$ enriched hPS cell-derived PP cells are more efficiently differentiating into pancreatic endocrine cells (Aghazadeh et al., 2022; Ameri et al., 2017). Direct comparison of *GP2* expression among ePP cells showed that our method resulted in a progressive strong *GP2* upregulation and expression. This expression was nearly 50-fold higher than expression in the FN ePP cells at p10 whereas feeder ePP cells did not express this marker to any appreciable extent (Figure 5E, Table S4). Robust GP2 expression in our ePP cells was confirmed by immunofluorescence (Figure 5F).

To further understand the differences among ePP cells we compared the expression of several additional pancreatic gene markers. *PDX1* expression was generally reduced after expansion but remained at levels comparable among the three procedures (Figure 5G, Table S5). *SOX9* expression followed the same pattern in our ePP cells whereas it remained stable or even increased in feeder and FN ePP cells, respectively (Figure 6G, Table S5). SOX9 is also a major driver of the ductal pancreatic program and, to assess whether higher SOX9 levels might be associated with higher levels of this program, we examined transcription of duct program transcription factors (TFs), such as *PROX1*, *HES1*, *GLIS3* and *ONECUT1*. During development, expression of these genes segregates from bipotent progenitors to duct progenitors and differentiated duct cells and is excluded from the endocrine compartment (Bastidas-Ponce et al., 2017). Overall levels of these transcription factors were higher in FN ePP cells suggesting that the ductal program was more active in these cells (Figure S5G, Table S5).

During pancreas development, both *NKX6-1* and *NKX6-2* are expressed in progenitor cells, acting in concert to define bipotent progenitors and to subsequently specify endocrine cells (Binot et al., 2010; Henseleit et al., 2005; Nelson et al., 2007; Pedersen et al., 2005; Schaffer et al., 2010). The antibody used here most likely recognizes both proteins, given the very high similarity of NKX6-1 and NKX6-2 at the region of the antigen (Figure S5H). Consistent with the flow cytometry experiments, our expanded cells showed upregulated and high combined *NKX6-1/2* expression, similar to that of feeder ePP cells (Figure 5G, Table S5). Interestingly, that was due to a strong, expansion-dependent upregulation of *NKX6-2* (Figure S5I, Table S5). Here it is important to note that whereas *NKX6-1/2* expression in feeder and our ePP cells is strong in all samples analyzed, it is very low in all FN ePP samples with the exception of a single sample (Figure 5G, Table S5). This suggests a lack of reproducibility in the expansion of PDX1$^+$/NKX6-1$^+$ FN ePP cells. The expression of other progenitor TF genes such as *FOXA2* and *RBJ* remained comparable in all expanded cells, although *FOXA2* retained higher levels in the FN ePP cells and *RBPJ* retained higher levels in our ePP cells (Figure S5I, Table S5). Similarly, the expression of acinar TFs such as *BHLHA15* and *RBPJL* was virtually undetectable in all expanded cells. Of note, expression of *PTF1A* was also undetectable in all ePP cells (Table S5).

Specification of NEUROG3$^+$ pancreatic endocrine progenitors depends on the lengthening of G1 phase and these progenitors, once specified, divide very rarely, if at all, employing a feed-forward mechanism for their differentiation (Azzarelli et al., 2017; Krentz et al., 2017; Wang et al., 2008). Therefore, it is expected that an efficient PP expansion procedure would efficiently repress the endocrine program. Indeed, a common feature of all expansion procedures was the repression of the endocrine differentiation program. Key transcription factors such as *NEUROG3* and its downstream effectors *NEUROD1*, *NKX2-2*, and *INSM1* were essentially switched off with very similar efficiency during expansion, although it is worth noting that expression of these genes was already very low in the Ma et al protocol (Ma et al., 2022) in p0 cells (Figure 5G, S5I, Table S5). Notable exceptions were the substantial expression levels of *RFX3* and *RFX6*, particularly the latter, expression which was also retained in the FN ePP cells (Figure 5G, S5I, Table S5). Expression of terminal endocrine differentiation markers in all ePP cells was also negligible, particularly at late passages (Table S5).

We finally compared the expression of liver and gut markers. Feeder ePP cells retained only negligible expression of the liver markers *AFP* and *HHEX*. However, expression of these markers was nearly 7-fold and 10-fold higher, respectively, in the FN ePP cells, as compared to our ePP cells, suggesting an overall decreased propensity to endocrine differentiation (Figure 5G, S5I, Table S5). Finally, expression of the gut marker *CDX2* was slightly higher in the feeder ePP cells but generally comparable in all three methods (Figure S5I, Table S5).

In summary, the comparative transcriptome analyses suggested that our C6 expansion procedure is more efficient at strengthening the PP identity and efficiently repressing the initiation of endocrine differentiation and alternative liver fate.

C6 expansion selects PDX1$^+$/SOX9$^+$/NKX6.1$^+$ cells derived from either XX or XY hPS cells

Different hPS cell lines may vary in their differentiation efficiency and this complicates the clinical implementation of this technology. C6 expansion resulted in the selection of PDX1$^+$/SOX9$^+$/NKX6.1$^+$ cells and this would be advantageous for iPS cell lines that may not differentiate as efficiently. Thus, we asked whether the C6 expansion condition, established using the male H1 human embryonic stem (hES) cell line, was similarly applicable to other hPS cell

lines of either sex. To address this, we used the female H9 hES cell line, a line that has a preference for neural rather than endoderm differentiation, and a male iPS cell line derived in the CRTD (CRTD1, hPSCreg: CRTDi004-A) with unknown lineage preference. Cells were differentiated into PP cells in monolayer culture (Table S4) and PP cells were then expanded in C6 for at least ten passages. Expansion efficiency was comparable as assessed by the growth curves with a T_d of 2.3 and 2.2 days for H9- (H9-PP) and CRTD1- (CRTD1-PP) derived ePP cells, respectively. Expansion in VTN-N coated cell culture surface was at least equally efficient as an expansion on Matrigel for H9- and CRTD1- PP cells(Figure 6A, B).

We then compared the maintenance of the PP identity during the expansion of H9-PP and CRTD1-PP cells to that of H1-PP cells, first by qPCR for gene expression levels at p0, p5 and p10. Expression of *PDX1*, *NKX6.1* and *SOX9* in H9-derived PP cells was strikingly similar to that of H1-derived PP cells at p0 and also following expansion. Expression of these markers in CRTD1-PP cells diverged with decreased *PDX1* expression, at p0 and p10, but increased *NKX6.1* expression at p5 and p10 (Figure S6A-C). Expression of *FOXA2* and *PTF1A* in H9-PP and CRTD1-PP p0 and expanded cells was also very similar to that in H1-PP cells with the exception of a transient increase of *FOXA2* expression at p5 (Figure S6D, E). Expression of *AFP* and *CDX2* were also very similar in H9-PP and CRTD1-PP cells as compared to H1-PP cells except for a transient *CDX2* upregulation in CRTD1-PP cells at p5 (Figure S6F, G).

We then evaluated the presence of $PDX1^+/SOX9^+$ and $PDX1^+/SOX9^+/NKX6.1^+$ cells in H9-PP and CRTD1-PP cells at p0, p5 and p10 using flow cytometry. H9 cells were less efficiently differentiated into $PDX1^+/SOX9^+$ cells in comparison to either H1 or CRTD1 cells giving rise to 77%±8% (n=6) as opposed to 98%±2% (n=5) and 89%±5% (n=3) $PDX1^+/SOX9^+$ PP cells for H1- and CRTD1-PP cells, respectively. However, this percentage was 91% (n=3) by p10, similar to that for H1- (98%±1%) (n=5) and CRTD1- (95%±6%) (n=4) p10 ePP cells (Figure 4H, 6C, D). Importantly, the % of $PDX1^+/SOX9^+/NKX6.1^+$ PP cells increased from 45%±29% and 39%±6% at p0 to 90% and 85%±6% at p10 for H9- and CRTD1-derived PP cells respectively. This was a very similar selection to that for H1-derived PP cells, which was from 48%±11% at p0 to 89%±13% at p10 (Figure 4H, 6C, D). Chromosomal stability, following expansion, was confirmed also for these lines, since no alterations were found after analysis of the G-banding of at least 20 metaphases for each line at p12 (H9-PP cells) or p13 (CRTD1-PP cells) (Figure S6H, I).

All ePP cells differentiate into SC-islets containing functional β-cells

Having established that our PP expansion procedure can be applied with very similar efficiency in PP cells derived from different hPS cell lines, we asked whether ePP cells can be differentiated equally efficiently into SC-islets. H1 dPP cells as well as H1, H9 and CRTD1 ePP cells, expanded for at least ten passages, were clustered in micropatterned wells and differentiated using an adaptation of published media (Mahaddalkar et al., 2020; Rezania et al., 2014; Shi et al., 2017) (Table S4) to generate SC-islets.

Both H1 dPP and ePP cells gave rise to similar clusters containing $INS^+/NKX6.1^+$, $INS^+/MAFA^+$, GCG^+ and SST^+ endocrine cells (Figure 7A, B and S7A, B) as well as similar percentages of INS^+, INS^+/GCG^+ and GCG^+ cells as determined by flow cytometry (Figure 7C, S7C, D). The total number of INS^+ and GCG^+ cells was between 50% and 55% in both H1 dPP- and ePP- derived SC-islets (Figure 7C).

Gene expression levels for differentiated endocrine markers including β-cell markers such as *PDX1*, *NKX6.1*, *SLC30A8* (Figure 7D), *INS*, *MAFA* (Figure S7E) as well as *GCG* and *SST*

(Figure S7E) were very similar between H1 dPP derived SC-islets and SC-islets derived from either H1, H9 or CRTD1 ePP cells. Expression of *PDX1*, *NKX6.1*, *SLC30A8* and *SST* was comparable between human islets and dPP or ePP SC-islets (Figure 7D, S7E) but expression of *INS*, *GCG* and *MAFA* was substantially higher in human islets presumably reflecting the less advanced maturation of SC-islets (Figure S7N).

Finally, we assessed the functionality of the β-cells in H1 dPP- as well as H1, H9 and CRTD1 ePP- derived SC-islets in static GSIS assays where SC-islets were sequentially incubated in Kreb's buffer containing low glucose levels (2.8mM), Kreb's buffer containing high glucose levels (16.7 mM) and finally a depolarizing Kreb's buffer containing high glucose levels (16.7 mM) and KCl (30 mM). As a comparison, human islets were processed in a similar manner. Human C-pep levels were measured from the supernatant of successive incubations and used to calculate the fold stimulation. These experiments showed that dPP- and ePP- derived SC-islets contained β-cells of very similar functionality (Figure 7E) and, as expected, close, but not similar, functionality to human islets.

In summary, both dPP and ePP cells, derived from different hPS cell lines, differentiate into SC-islets with essentially the same efficiency and contain β-cells of similar functionality.

DISCUSSION

The unlimited expansion of progenitor cells can offer many therapeutic advantages but remains an important challenge for regenerative medicine. Progenitor cells are dynamic entities and the key objective in these efforts is to uncouple survival and proliferation from widely employed feed-forward mechanisms that promote their differentiation. The latter are not exclusively regulated from extrinsic signals but rely to a large extent on internal regulators as well as autocrine signals. Thus, the hallmarks of efficient progenitor expansion would be the maintenance of key progenitor features, the efficient suppression of differentiation programs and alternative lineages and the capacity to efficiently differentiate under appropriate conditions. To be suitable for therapeutic applications, such expansion should be efficient, reproducible, applicable across different cell lines and compatible with chemically defined culture media.

Regarding pancreatic development in particular, several feed-forward networks have been documented with exquisite detail (Arda et al., 2013). In the early pancreatic progenitors, a Sox9/Fgf feed-forward loop is essential to escape liver fate and promote pancreas identity and expansion (Seymour et al., 2012). Fgf10 was identified as a possible extrinsic signal but it was shown that it also promotes liver identity (Hart et al., 2003; Jung et al., 1999; Norgaard et al., 2003; Rossi et al., 2001). At a later stage, Ptf1a initially forms heterodimers with Rbpj to promote expansion of pancreatic progenitors and activate transcription of Rbpjl which eventually replaces Rbpj in its complexes with Ptf1a. The Ptf1a·Rbpjl complexes then promote the specification of acinar progenitors (Masui et al., 2010). S1p signaling in pancreas progenitors promotes progenitor survival but also their differentiation into the acinar and endocrine lineages through the attenuation of Notch signaling (Serafimidis et al., 2017). Subsequently, the transcription factors Myt1 and Neurog3 form a feed-forward loop to promote the final commitment into the endocrine lineage (Wang et al., 2008).

However, the identity of signalling pathways that promote progenitor proliferation and of those initiating differentiation is unclear precluding a rational approach to expanding PP cells in vitro while blocking differentiation. To address this, we first undertook longitudinal transcriptome analyses and comparisons of non-expanding and occasionally expanding PP cells. The analyses

focused on components of signaling pathways and several candidates were identified providing a rational, mechanistic approach to address the expansion of PP cells. We followed a hypothesis-driven, iterative, approach to identify conditions enabling the robust unlimited expansion of hPS cell derived PP cells under chemically defined conditions that are applicable for different hPS cell lines of both sexes.

We found that a combination of EGF, FGF2 and FGF18 promoted robust expansion of hPS cell derived PP cells. EGF promotes *NKX6.1* activation (Nostro et al., 2015) but on its own it could not support consistent PP cell expansion (CINI, Table S1). FGF2 is a widely used mitogen and whereas it enhanced PP proliferation (C1) it was less efficient than FGF18 (C5, Table S1). There was a clear synergy of the two mitogens in promoting expansion and possibly the enrichment in NKX6.1$^+$ cells (C6, Table S1). NKX6.1 regulates multiple cell cycle genes (Taylor et al., 2015) and thus some of the effects of these mitogens might be indirectly reinforced through NKX6.1. Pancreas progenitor cells express the enzymes necessary to convert vitamin A into retinoic acid (RA) (Table S3) which promotes differentiation of the pancreas progenitors (Lorberbaum et al., 2020; Martin et al., 2005; Ostrom et al., 2008; Vinckier et al., 2020). Vitamin A is one of the B-27 components in its most common formulation and to suppress the RA driven differentiation of PP cells we used, in all new conditions tested, the vitamin A free variant of B-27 (Table S1). The TGFβ signaling pathway plays a complex role in the induction, maintenance and endocrine differentiation of pancreas progenitors (Guo et al., 2013; Sanvito et al., 1994; Spagnoli and Brivanlou, 2008; Tulachan et al., 2003) and the diverging gene expression kinetics of receptors and ligands during expansion in CINI reflected this complexity. Accordingly, our experiments suggested that the highly specific ALK5i II inhibitor (Sanvitale et al., 2013) (C5, C6) was more efficient than the widely specific A83-01 inhibitor (Tojo et al., 2005) (CINI) in promoting PP expansion. Further inhibition of the pathway with the addition of LDN193198 which targets ALK3 more efficiently than ALK5i II (Gellibert et al., 2004) dramatically reduced PP expansion (C7, Table S3). In line with the effects of these interventions affecting primarily the proliferation of PP cells, we documented that preferential expansion of progenitors rather than cell death was the main mechanism. Finally, the addition of the canonical Wnt inhibitor IWR-1 (Chen et al., 2009) significantly reduced AFP expression and promoted an efficient selection of NKX6.1$^+$ cells without affecting proliferation, presumably by reducing the divergence of expanding cells towards the hepatic fate.

We report here that this is a robust procedure resulting in a 2000-fold expansion and selecting up to 90% PDX1$^+$/SOX9$^+$/NKX6.1$^+$ PP cells after ten passages over 40-45 days. High levels of NKX6-1 expression suggested that the expanded cells may resemble more bipotent progenitors as in mice (Schaffer et al., 2010) and it has also been proposed that high levels of NKX6-1 are necessary to produce β cells in humans (Russ et al., 2015). The same procedure applied to H9- and CRTD1-PP cells gave essentially identical results, even though the initial differentiation of these hPS cells into PP cells was less efficient. Thus this procedure will be particularly useful for hPS cell lines with reduced initial capacity to differentiate into PP cells. Of note, for all cell lines a high initial density was necessary in achieving robust early expansion.

Culture conditions to expand hPS cell-derived PP cells have recently been reported. The first relied on feeder layers of transformed cells and a BET bromodomain inhibitor (Ma et al., 2022) to expand an, up to 90% PDX1$^+$/SOX9$^+$/NKX6.1$^+$, population of PP cells and the second relied on a simple expansion medium and fibronectin as a cell culture substrate (Nakamura et al., 2022). While the first is limited by the use of feeder layers, which preclude its use in a clinical

setting, the second did not document reproducibility of the expansion procedure and the percentage of NKX6.1$^+$ cells reached an average of only 40% with large variability among different cell lines. The comparison of the transcriptome profiles showed that our procedure is unique in effecting a strong upregulation of *GP2*, a unique marker of human fetal pancreas progenitors (Cogger et al., 2017; Ramond et al., 2017) with a high preference for endocrine differentiation (Aghazadeh et al., 2022; Ameri et al., 2017) and strong upregulation of *NKX6.2*, another marker of PP cells which complements *NKX6.1* function but is not retained in differentiated endocrine cells (Binot et al., 2010; Henseleit et al., 2005; Nelson et al., 2007; Pedersen et al., 2005; Schaffer et al., 2010). Expression of other progenitor markers such as *FOXA2* and *RBPJ* were retained at similar but variable levels. Levels of *TFs* that are engaged in the duct program appeared higher in the FN expansion procedure (Nakamura et al., 2022) whereas expression of acinar *TFs* was very low in all three procedures. As expected, the expression of *TFs* driving the endocrine program is strongly reduced in all three procedures albeit our procedure is more efficient in this respect, particularly with regard to *RFX3* and *RFX6* expression. Expression of *AFP* and *HHEX*, markers of the liver lineage, were significantly higher in the FN-expansion procedure (Nakamura et al., 2022) whereas all three procedures resulted in noticeable upregulation of CDX2, a gut marker.

Importantly, ePP cells from H1, H9 and CRTD1 hPS cells all differentiated with similar efficiency to dPP cells into SC-islets containing similar numbers of β-cells of comparable functionality. This may appear surprising because ePP cells contained a much higher percentage of PDX1$^+$/SOX9$^+$/NKX6.1$^+$ cells. It should be noted, however, that the liver marker AFP and, particularly, the gut marker CDX2 were upregulated, whereas expression of *PTF1A*, recently shown to promote endocrine differentiation of hPS cells (Miguel-Escalada et al., 2022), was essentially lost. The same pattern, and even higher upregulation of gut and liver markers, was also seen in the other expansion procedures (Ma et al., 2022; Nakamura et al., 2022). Additionally, the strong upregulation of *NKX6.2* in our procedure suggested that our ePP cells may have retracted to an earlier PP stage. Thus, means to suppress *AFP* and *CDX2* expression during expansion and restore *PTF1A* expression during the resumption of the differentiation are expected to result in a very high percentage of endocrine cells in the SC-islets derived from expanded cells.

The unlimited expansion of PP cells reported here is applicable to different hPS cell lines and presents several advantages in the efforts to scale-up the generation of islet cells, including β-cells, for the cell therapy of diabetes. It reduces the number of differentiation procedures to be carried out starting at the hPS cell stage, thus eliminating a source of variability and allows the selection of the most optimally differentiated PP cell population for subsequent expansion and storage. Since it is currently acknowledged that current differentiation procedures do not produce fully functional β-cells, these ePP cells will provide a convenient springboard to refine downstream differentiation procedures. Suitable surface markers for the selection of endocrine cells at the end of the differentiation procedure have been reported (Li et al., 2020; Veres et al., 2019) but such procedures may prove too expensive in a clinical setting. Alternatively, expanded PP cells could be directly used for transplantations as it has been shown that they can mature in vivo (Kroon et al., 2008; Shapiro et al., 2021). Whether PP cells or terminally differentiated SC-islet cells would be the best approach in future tansplantations is still discussed but, in any of these cases, the availability of a highly pure GMP-grade, hPS cell derived PP cell population has several advantages as discussed above. Therefore, expansion of PP cells will facilitate the generation of

unlimited number of endocrine cells, initially for studying diabetes, drug screening for personalized medicine and eventually cell therapies. The chemically defined expansion procedure we report here will be an important step toward generating large numbers of human pancreatic endocrine cells that are of great interest for biomedical research and regenerative medicine.

Author contributions

A.G. conceptualized the study, L.J., M.B., E.Z, V.K., R.S., D.P and I.G. performed experiments, A.G., L.J., and M.B. analysed the data, A.G. and M.L. analysed the transcriptome data, S.K. and K.N. generated and expanded the CRTD1 line, B.L. provided the human islets, A.G. wrote the manuscript with input and editing from L.J. and M.B., A.G. supervised and acquired funding.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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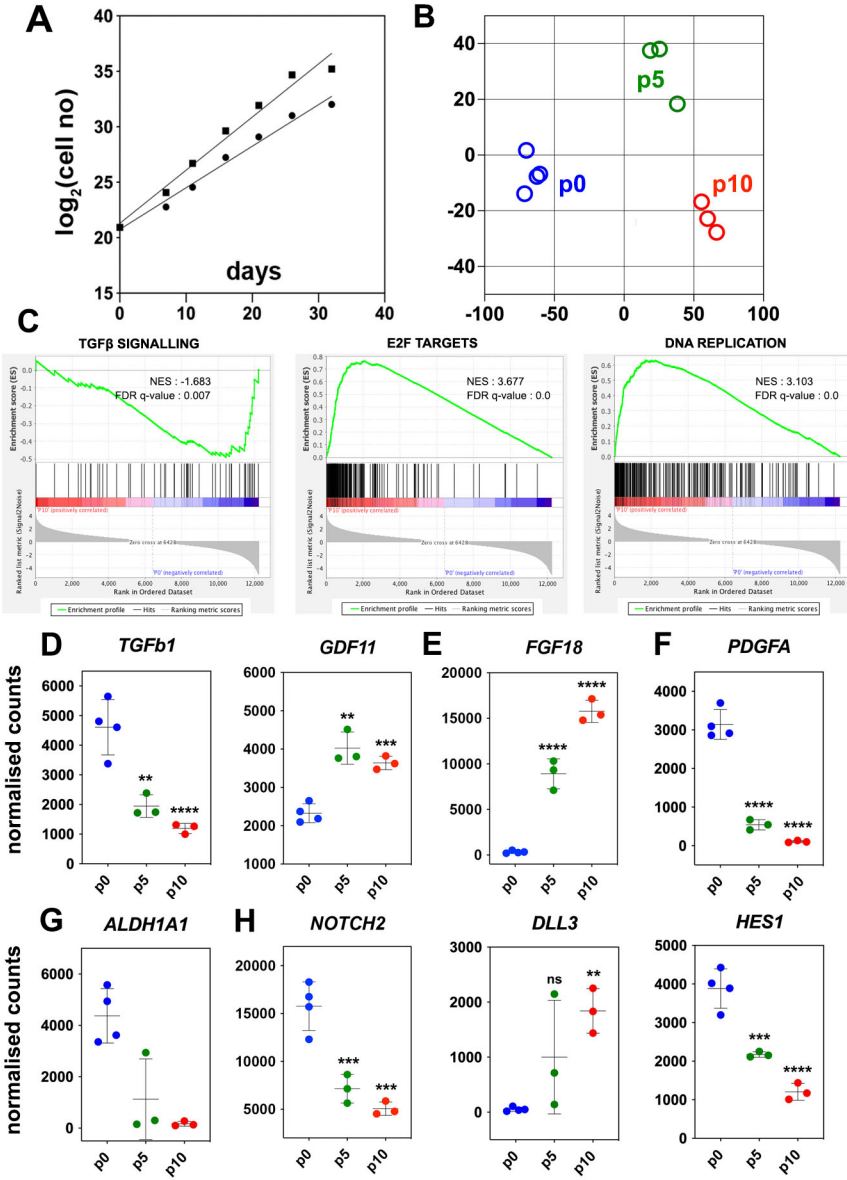
A
log2(cell no)
days
B
p5
p0
p10
C
TGFβ SIGNALLING
NES : -1.683
FDR q-value : 0.007
E2F TARGETS
NES : 3.677
FDR q-value : 0.0
DNA REPLICATION
NES : 3.103
FDR q-value : 0.0
Enrichment score (ES)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
D
TGFb1
GDF11
E
FGF18
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DLL3
HES1
normalised counts
p0
p5
p10
ns

Figure 1

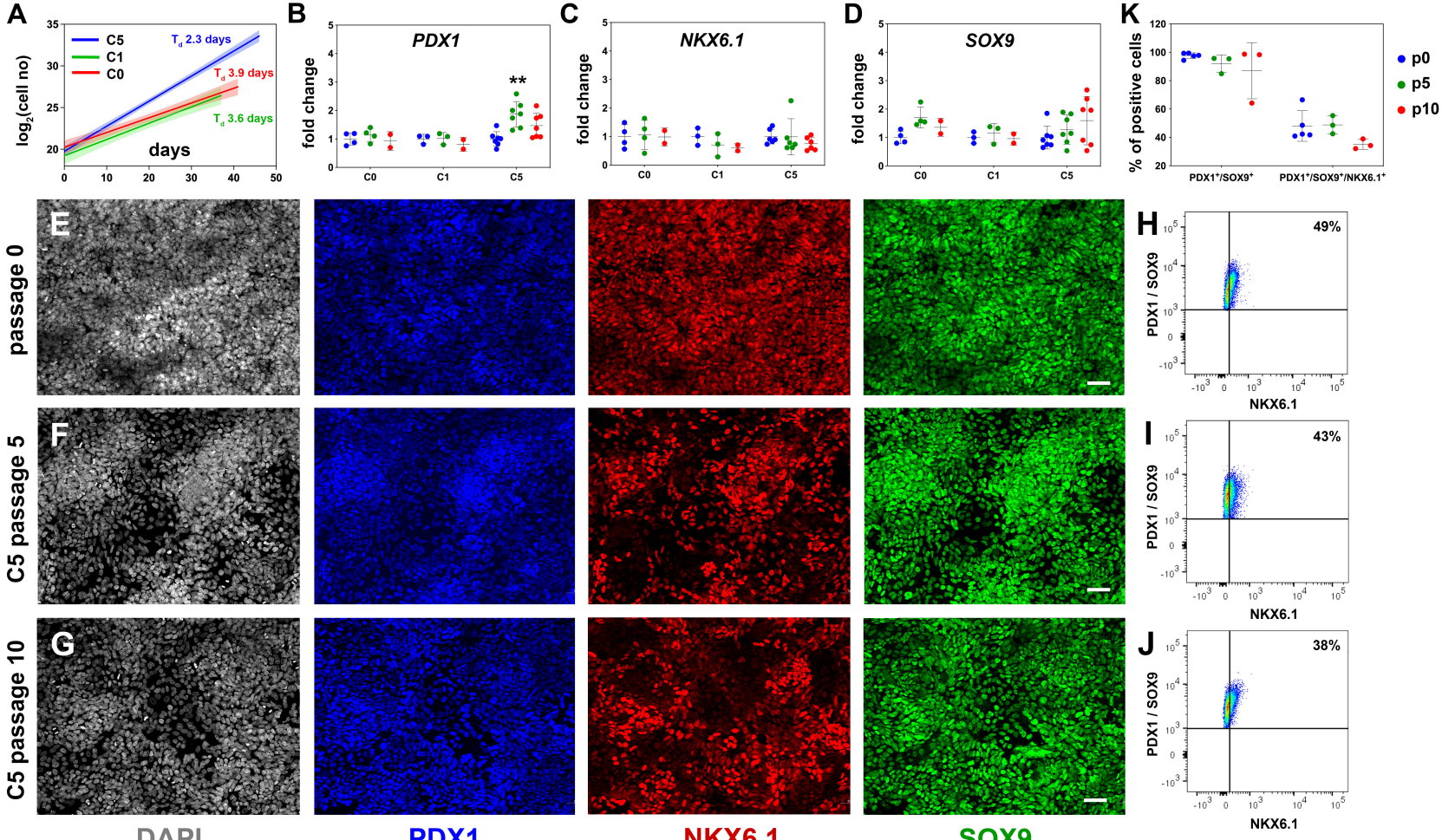

A
C5
C1
C0
T_d 2.3 days
T_d 3.9 days
T_d 3.6 days
log_2(cell no)
days
B
PDX1
fold change
**
C0
C1
C5
C
NKX6.1
D
SOX9
K
% of positive cells
p0
p5
p10
PDX1+/SOX9+
PDX1+/SOX9+/NKX6.1+
E
F
G
passage 0
C5 passage 5
C5 passage 10
H
I
J
49%
43%
38%
PDX1 / SOX9
NKX6.1
DAPI
PDX1
NKX6.1
SOX9

Figure 2

Lorem Ipsum

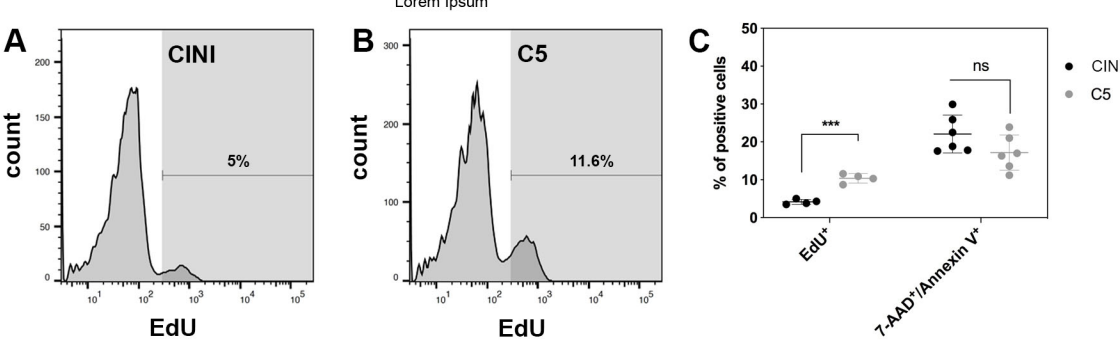


Figure 3

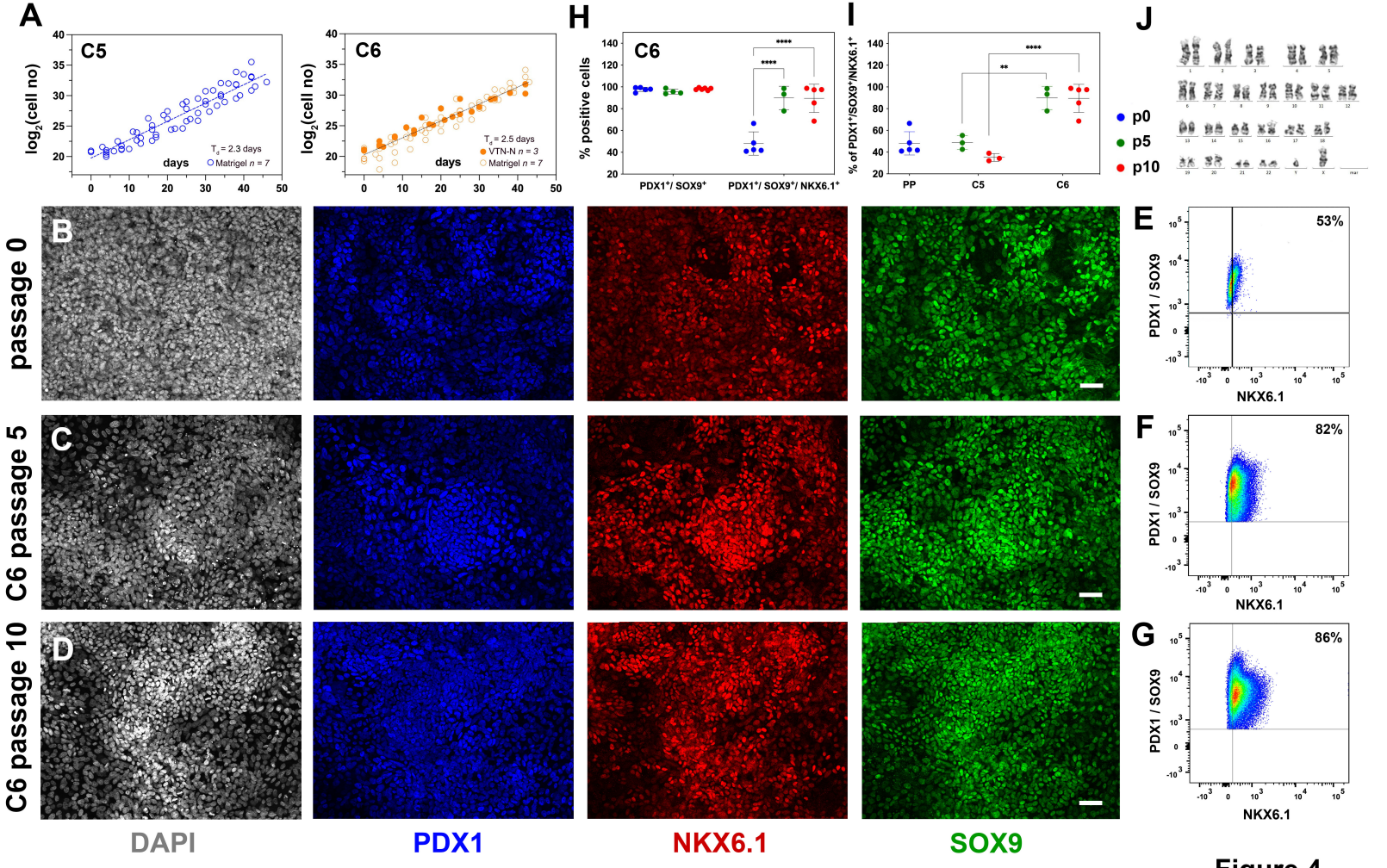
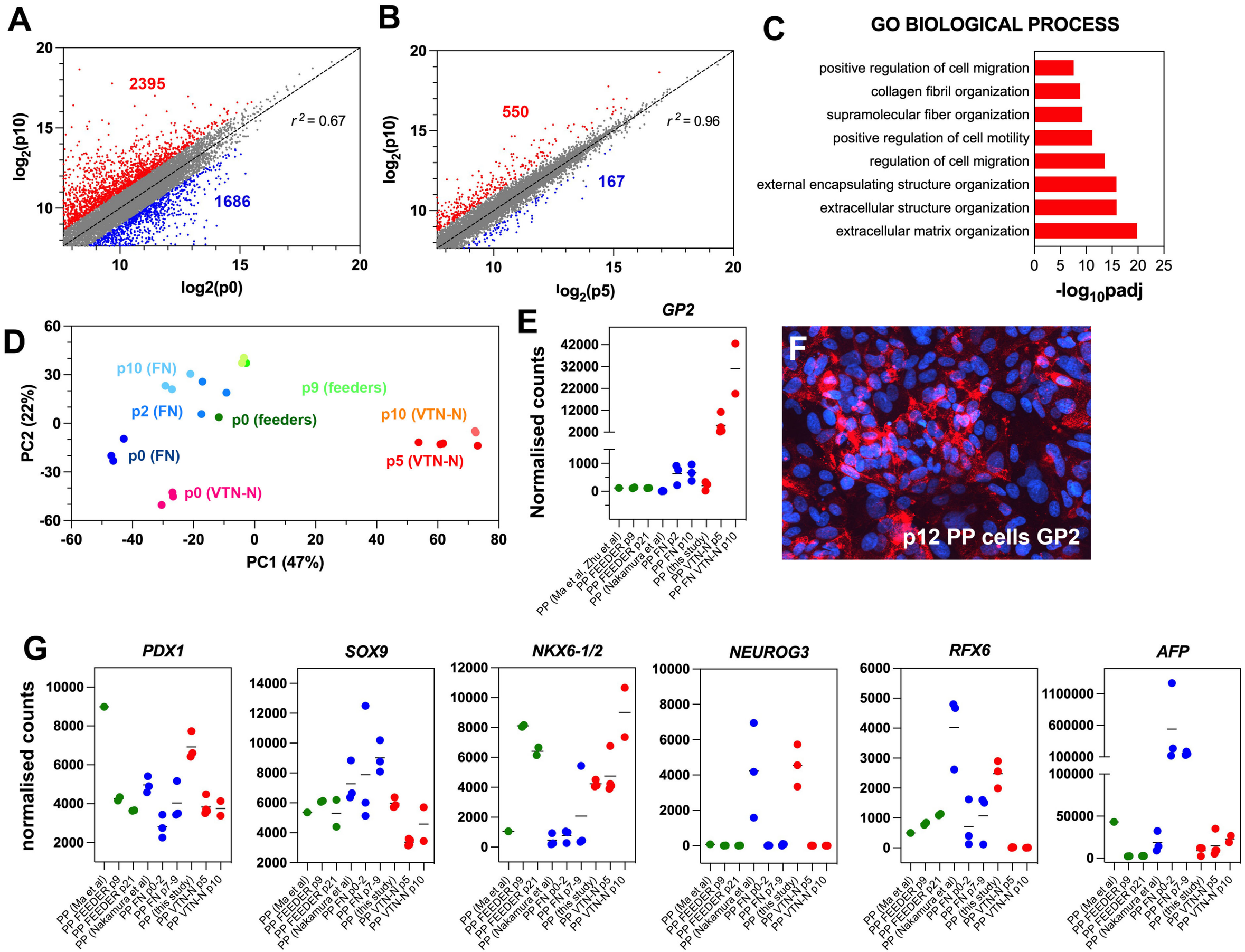

A
C5
log2(cell no)
days
Td = 2.3 days
Matrigel n = 7
C6
Td = 2.5 days
VTN-N n = 3
Matrigel n = 7
H
C6
% positive cells
PDX1+/ SOX9+
PDX1+/ SOX9+/ NKX6.1+
I
% of PDX1+/SOX9+/NKX6.1+
PP
C5
C6
J
p0
p5
p10
B
C
D
passage 0
C6 passage 5
C6 passage 10
E
53%
F
82%
G
86%
PDX1 / SOX9
NKX6.1
DAPI
PDX1
NKX6.1
SOX9

Figure 4


A
2395
1686
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log2(p0)
log_2(p10)
B
550
167
$r^2 = 0.96$
log_2(p5)
log_2(p10)
C
GO BIOLOGICAL PROCESS
positive regulation of cell migration
collagen fibril organization
supramolecular fiber organization
positive regulation of cell motility
regulation of cell migration
external encapsulating structure organization
extracellular structure organization
extracellular matrix organization
$-log_{10}$padj
D
p10 (FN)
p9 (feeders)
p2 (FN)
p0 (feeders)
p10 (VTN-N)
p0 (FN)
p5 (VTN-N)
p0 (VTN-N)
PC1 (47%)
PC2 (22%)
E
GP2
Normalised counts
PP (Ma et al, Zhu et al)
PP FEEDER p9
PP FEEDER p21
PP (Nakamura et al)
PP FN p2
PP FN p10
PP (this study)
PP VTN-N p5
PP FN VTN-N p10
F
p12 PP cells GP2
G
PDX1
SOX9
NKX6-1/2
NEUROG3
RFX6
AFP
normalised counts
PP (Ma et al)
PP FEEDER p9
PP FEEDER p21
PP (Nakamura et al)
PP FN p0-2
PP FN p7-9
PP (this study)
PP VTN-N p5
PP VTN-N p10

Figure 5

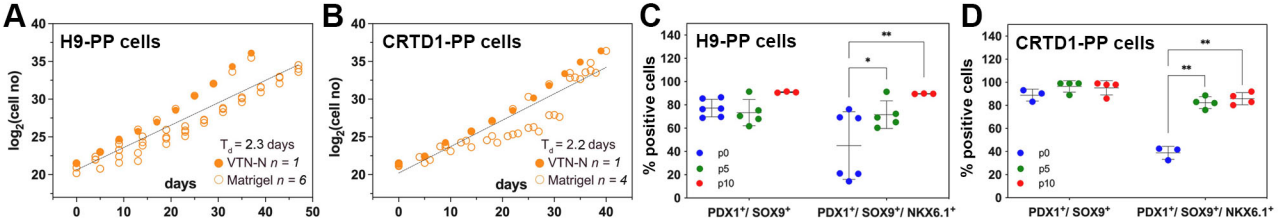
A
H9-PP cells
log2(cell no)
days
Td = 2.3 days
VTN-N n = 1
Matrigel n = 6
B
CRTD1-PP cells
log2(cell no)
days
Td = 2.2 days
VTN-N n = 1
Matrigel n = 4
C
H9-PP cells
% positive cells
p0
p5
p10
PDX1+/ SOX9+
PDX1+/ SOX9+/ NKX6.1+
D
CRTD1-PP cells
% positive cells
p0
p5
p10
PDX1+/ SOX9+
PDX1+/ SOX9+/ NKX6.1+

Figure 6

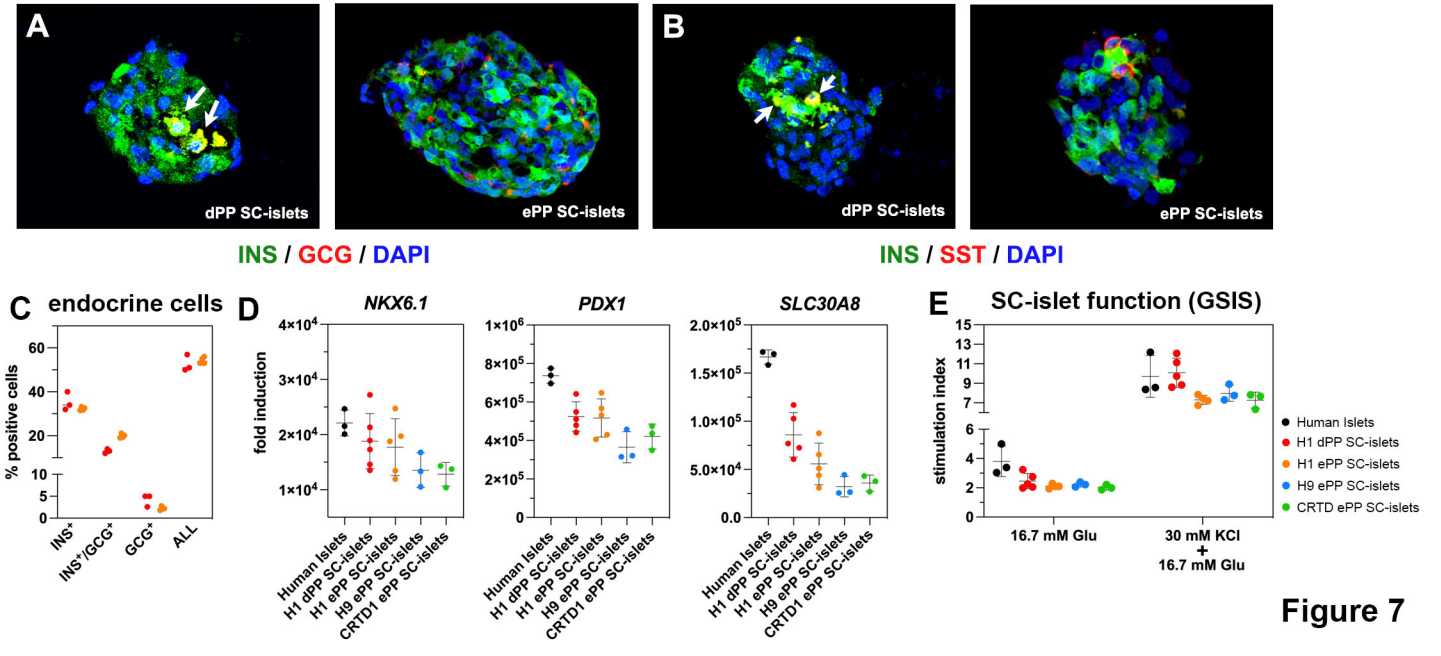
A
dPP SC-islets
ePP SC-islets
INS / GCG / DAPI
B
dPP SC-islets
ePP SC-islets
INS / SST / DAPI
C endocrine cells
% positive cells
INS+
INS+/GCG+
GCG+
ALL
D
NKX6.1
PDX1
SLC30A8
fold induction
Human Islets
H1 dPP SC-islets
H1 ePP SC-islets
H9 ePP SC-islets
CRTD1 ePP SC-islets
E SC-islet function (GSIS)
stimulation index
16.7 mM Glu
30 mM KCl
+
16.7 mM Glu
Human Islets
H1 dPP SC-islets
H1 ePP SC-islets
H9 ePP SC-islets
CRTD ePP SC-islets

Figure 7

Figure 1 Regulated genes and signaling pathways during PP expansion under initial conditions

(A) Regression lines of two samples showing exponential expansion of PP cells for 32 d using initial conditions (CINI).

(B) MDS plot representing the Euclidian distance of the samples p0 (n=4), p5 (n=3) and p10 (n=3).

(C) Enrichment plots for regulated genes (p0 vs P10) of the TGF-ß signaling pathway, E2F target genes and DNA replication showing a negative correlation of the TGF-ß pathway with the expansion but a positive correlation of E2F target genes and DNA replication with the expansion.

(D-H) Average transcript levels in normalized RNA-Seq counts of p0 (n=4), p5 (n=3) and p10 (n=3) cell populations for genes encoding signals or receptors of the TGFβ (D), FGF (E) and PDGF (F) signaling, the RA producing enzyme ALDH1A1 (G) as well as components of NOTCH (H) signaling.

Horizontal lines represent the mean ± SD. Statistical tests were one-way ANOVA using p0 as the control condition for the comparison with $p \leq 0.033$ (*), $p \leq 0.002$ (**), ≤ 0.0002 (***) and ≤ 0.0001 (****).

Figure 2 Reproducible expansion of PP cells in condition 5

(A) Growth curves and regression analysis for PP cells expanded in C0, C1 and C5 for at least 10 passages. The doubling time (T_d) of C5-expanded cells (n=7) was 2.3 days with a 95% confidence interval (CI) of 2.13-2.51 days. This was clearly increased compared to C0- (n=2, Td=3.92 days, 95% CI = 3.22-4.98 days) and C1-expanded cells (n=2, Td=3.55 days, 95% CI = 2.88-4.62 days). The translucent shading represents the 95% CI of the growth rate of the different conditions.

(B-D) Gene expression profile of C0-, C1- and C5-expanded cells as shown by qPCR for expression of the key pancreas progenitor markers *PDX1* (B), *NKX6.1* (C) and *SOX9* (D) during the expansion. Expression is normalized against expression of each marker at p0.

(E-G) Representative images of immunofluorescent staining of p0 PP cells (E) as well as C5-expanded cells at p5 (F) and p10 (G) for the PP transcription factors PDX1, NKX6.1 and SOX9.

(H-J) Flow cytometry analysis of p0 PP cells (H), as C5-expanded cells at p5 (I) and P10 (J) for PDX1, NKX6.1 and SOX9.

(K) Cumulative results of the flow cytometry analyses for $PDX1^+/SOX9^+$ and $PDX1^+/SOX9^+/NKX6.1^+$ C5-expanded cells at p0, p5 and p10.

Horizontal lines represent the mean ± SD. Statistical tests were two-way ANOVA with Tukey's test, using p0 as the control condition for the comparison with $p \leq 0.033$ (*), $p \leq 0.002$ (**), ≤ 0.0002 (***) and ≤ 0.0001 (****). Scale bar corresponds to 50 um.

Figure 3 PP expansion conditions promote primarily proliferation rather than survival of PP cells.

(A, B) Histogram plots showing the % of cells that had incorporated EdU-Alexa488 in the CINI-expanded PP cells (A) in comparison to the C5-expanded PP cells (B).

(C) Summary of flow cytometry data comparing proliferation using EdU-Alexa488 incorporation (n=4) as well as cell death by Annexin V/7-AAD staining in INI- and C5-expanded PP cells (n=6).

Horizontal lines represent mean ± SD. Means were compared with multiple t-tests and significance is $p \leq 0.033$ (*), $p \leq 0.0021$ (**), $p \leq 0.0002$ (***) or $p \leq 0.0001$ (****).

Figure 4 Reproducible expansion in condition 6 promotes PP identity
(A) Growth curves and regression analysis for PP cells expanded in C5- and C6 for ten passages. The regression line for C5 showed a doubling time of 2.3 d (n=7) compared to C6 with 2.5 d (n=10).
(B-D) Representative images of immunofluorescent staining of p0 PP cells (B) as well as C6-expanded cells at p5 (C) and p10 (D) for the PP transcription factors PDX1, NKX6.1 and SOX9.
(E-G) Flow cytometry analysis of non-expanded p0 PP cells (E) and C6-expanded cells at p5 (F) and p10 for $PDX1^+/SOX9^+/NKX6.1^+$ cells (G).
(H, I) Cumulative results of the flow cytometry analyses for $PDX1^+/SOX9^+$ and $PDX1^+/SOX9^+/NKX6.1^+$ C6-expanded cells at p0, p5 and p10 (H) and comparison of the % of C5- and C6- expanded $PDX1^+/SOX9^+/NKX6.1^+$ cells at p5 and p10 (I).
(J) Karyotyping of C6-expanded PP cells after sixteen passages showed no chromosomal abnormalities.

Horizontal lines represent the mean ± SD. Statistical tests were two-way ANOVA with Tukey's test, using p0 as the control condition for the comparison with $p \leq 0.033$ (*), $p \leq 0.002$ (**), ≤ 0.0002 (***) and ≤ 0.0001 (****). Scale bar corresponds to 50 um.

Figure 5 Expansion stabilizes PP cell identity by repressing endocrine differentiation.
(A, B) Correlation analyses of the transcriptome profiles of non-expanded (p0) and p10 expanded PP cells (A) and the transcriptome profiles of p5 and p10 expanded cells (B). The numbers of upregulated and downregulated genes (normalized counts ≥ 200 and 0.5 ≥ FC ≥ 2) are shown in red and blue, respectively and r is the correlation coefficient.
(C) Most affected biological processes between p0 and p10.
(D) PCA of feeder expanded cells and corresponding p0 cells (shades of green), fibronectin (FN) expanded cells and corresponding p0 cells (shades of blue) as well as vitronectin-N (VTN-N) expanded cells and corresponding p0 cells (shades of red). Darker shades correspond to earlier passages.
(E) Expression levels of *GP2* in normalized RNA-Seq counts.
(F) GP2 immunofluorescence of p12 expanded PP cells.
(G) Expression levels of progenitor and endocrine markers in normalized RNA-Seq counts.

Figure 6 Expansion of H9-derived and CRTD1-derived PP cells
(A, B) Growth curve and regression analysis of the expansion of H9-derived PP cells (A) and CRTD1-derived PPs cells (B).
(C, D) Flow cytometry analysis for $PDX1^+/SOX9^+$ and $PDX1^+/SOX9^+/NKX6.1^+$ cells during the expansion in C6 of H9-derived PP cells (C) and CRTD1-derived PP cells (D) at p0 and during their expansion in C6 at p5 and p10.

Figure 7 Differentiation of ePP cells into SC-islets containing functional β-cells.
(A, B) Immunofluorescence analysis of SC-islets derived from p0 PP cells (dPP) or expanded PP cells for at least ten passages (ePP) for INS and GCG expression (A) or INS and SST expression (B).
(C) Percentages of INS^+, INS^+/GCG^+ as well as GCG^+ cells in SC-islets derived from dPP or ePP cells as determined by flow cytometry.
(D) Expression levels of NKX6.1, PDX1 and SLC30A8 in relation to expression levels in hPS cells (fold induction) as determined by qPCR.
(E) Secretion of C-peptide following sequential stimulation by 16.7 mM glucose and 16.7 mM glucose / 30 mM KCl after exposure in basal conditions with 2.8 mM glucose. Stimulation index is determined by the ratio of secretion under these conditions to secretion in basal conditions.

Horizontal lines represent the mean ± SD. Statistical tests were two-way ANOVA with Tukey's test, using p0 as the control condition for the comparison with $p \leq 0.033$ (*), $p \leq 0.002$ (**), ≤ 0.0002 (***) and ≤ 0.0001 (****). Scale bar corresponds to 50 um. Scale bar corresponds to 100 µm (E, F)

MATERIALS AND METHODS

Derivation of the CRTD1 human iPS cell line

The CRTD1 human iPS cell line (hPSCreg: CRTDi004-A) was generated from previously published foreskin fibroblasts (termed Theo) of a consenting healthy donor (Wolf et al., 2016). Isolation of cells and reprogramming to hiPS cells was approved by the ethics council of TU Dresden (EK169052010 und EK386102017). Theo fibroblasts were reprogrammed at the CRTD Stem Cell Engineering Facility at the Technical University of Dresden using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fischer Scientific A16517) according to the supplier's recommendations for transduction. Following transduction with the Sendai virus, cells were cultured on irradiated CF1 Mouse Embryonic Fibroblasts (Thermo Fisher Scientific 15943412) in KOSR-based medium containing 80% DMEM/F12 (Thermo Fisher Scientific 31330095), 20% KnockOut Serum Replacement (Thermo Fisher Scientific 10828028), 2 mM L-glutamine (Thermo Fisher Scientific 25030024), 1% Nonessential Amino Acids (Thermo Fisher Scientific 11140035) and 0,1 mM 2-mercaptoethanol (Thermo Fisher Scientific 21985023) supplemented with 10 ng/ml human FGF2 (Stem Cell Technologies 78003). Individual iPSC colonies were mechanically picked, expanded as clonal lines and adapted to Matrigel (Corning 354277), mTeSR1 (Stem Cell Technologies 85850) and ReLeSR (Stem Cell Technologies 05873) conditions after several passages. Master and working hiPS cell banks were established from clones with the best morphology.

To characterize the newly generated CRTD1 hiPS cell line, several tests were performed, accessible at https://hpscreg.eu/cell-line/CRTDi004-A. Pluripotency was initially analyzed by Alexa Fluor 488 conjugated anti-Oct3/4 (BD Pharmingen 560253), PE conjugated anti-Sox2 (BD Pharmingen 560291), V450 conjugated anti-SSEA4 (BD Pharmingen 561156), and Alexa Fluor 647 conjugated anti-Tra-1-60 (BD Pharmingen 560122) used according to the manufacturer's recommendations and analyzed on a BD LSRII Flow Cytometer. Then, the three germ layer differentiation assay was performed as described previously (Cheung et al., 2011) and resulting cells were stained using the 3-germ layer Immunocytochemistry Kit (Thermo Fisher Scientific A25538) according to the instruction manual. For endoderm differentiation, a SOX17 primary antibody (Abcam ab84990) followed by Alexa Fluor 488 goat anti-mouse IgG (Thermo Fisher Scientific A11001) was used. Quantitative RT-PCR for pluripotency and trilineage spontaneous differentiation was performed according to the instruction manual of the human ES cell Primer Array (Takara Clontech).

Cells were analyzed for chromosomal abnormalities using standard G banding karyotyping. Cells were treated with 100 ng/ml KaryoMAX Colcemid solution (Thermo Fisher Scientific 15212012) for 4 h at 37°C, harvested and enlarged with 0.075 M KCl solution (Thermo Fisher Scientific 10575090) for 20 min at 37°C. After fixing with 3:1 methanol (VWR 20846.326) : glacial acetic acid (VWR 20102.292), cells were spread onto glass slides and stained with Giemsa at the Institute of Human Genetics, Jena University, Germany. G-bandings of at least 20 metaphases were analyzed.

Isolation of human pancreatic islets

Human islets were obtained through the XXX Islet Transplantation Program approved by the XXX Institutional Review Board (EK 255062022) with written informed consent obtained from each islet donor participant. Islets were isolated and purified from resected pancreas tissue according to a modified Ricordi method. Briefly, Collagenase, neutral protease (Serva Electrophoresis, Heidelberg, Germany), and Pulmozyme (Roche, Grenzach, Germany,) were infused into the main pancreatic duct. Islets were separated from exocrine tissue by centrifugation on a continuous Biocoll gradient (Biochrom AG, Berlin, Germany) in a COBE 2991 cell processor (Lakewood, CO, USA).

Maintenance and karyotyping of human pluripotent stem cell lines

The H1 and H9 hES cell lines were purchased from WiCell (Wisconsin, USA). H1 and H9 hES cells as well as CRTD1 iPS cells were maintained on cell culture dishes coated with hES cell qualified Corning Matrigel (BD Bioscience, 354277) diluted 1:50 with DMEM/F-12 (Gibco, 21331-020) and daily changes of mTeSR1 medium (STEMCELL Technologies, 85850) supplemented with 1x penicillin/streptomycin (Gibco, 15140-122). The cells were passaged at around 70% confluency, approximately every 4 days at a ratio of 1:6 to 1:9, as small aggregates using ReLeSR (STEMCELL Technologies, 05872). Karyotyping for H1 and H9 cells was as described above for the CRTD1 iPS cell line and cells were routinely tested for mycoplasma contamination by PCR as published previously (Young et al., 2010).

Differentiation of hPS cells to PP cells

Initially, the H1 ES cell line was differentiated to PP cells using the STEMdiff™ Pancreatic Progenitor Kit (STEMCELL Technologies, 05120) according to the manufacturer's instructions. In short, the hES cell colonies were dissociated into single cells using TrypLE Express (Gibco, 12604-013) and seeded on Matrigel coated plates as described above at a concentration of 95,000 cells/cm^2 in mTeSR supplemented with 20 µM ROCKi. The medium was replaced the next day by mTeSR and differentiation was initiated by replacing it with the S1d1 differentiation medium when cells were 60-70% confluent, typically two days after the initial seeding. Daily washes with DPBS (Gibco, 14190250) and media changes were done until S4d5 when the cells reached the end of PP stage. The monolayer of PP cells was then dissociated using Accumax™ (STEMCELL Technologies, 07921) and cells were used for expansion under INI conditions.

Later, H1, H9 and CRTD1 iPS cells were differentiated into PP cells using an adaptation of published procedures (Mahaddalkar et al., 2020; Rezania et al., 2014; Shi et al., 2017) (Table S4). The monolayer of PP cells was then dissociated using TrypLE Express (Gibco, 12604-013) for 2 minutes and cells were used for expansion under C0 to C8 conditions.

Expansion and cryopreservation of PP cells

The monolayer of PP cells was dissociated using TrypLE Express (Gibco, 12604-013) and cells were used for expansion. Expansion cultures were maintained on polystyrene cell culture plates (Corning, CLS3516) coated with Matrigel (Corning, 354277) or Cultrex (R&D systems, 3434-005-02), diluted 1:50 in DMEM/F-12 or with 20 ug/mL recombinant truncated vitronectin (VTN-N) (Thermo Fischer Scientific, A31804) diluted in DMEM/F-12 (Gibco, 21331-020) for 1 hr at room temperature. PP cells were resuspended in PP expansion media CINI-C8 (Table S1) and seeded at a density of 2.1×10^5/cm^2. Expansion media were supplemented, during the first day, with 10

μM ROCKi. The expansion medium was then changed daily, and cells were typically passaged every 4th day using TrypLE Express dissociation into single cells. Karyotyping for expanded PP cells and mycoplasma testing was as described above for the hPS cell lines.

Expanding PP cells were routinely frozen at later passages using mFreSR (Stem Cell technologies, 05854), supplemented with 20 μM ROCKi, at a density of 10 million cells/ml. To ensure proper controlled freezing (− 1 °C/min), cryotubes were placed in Mr. Frosty™ Freezing Container (Thermo Fischer Scientific, 5100-0001) at − 80 °C. After 24 hours, cryotubes were transferred to a liquid nitrogen chamber. For thawing, frozen cells were placed at 37°C and then transferred to 6ml DMEM/F-12 at room temperature for centrifugation. After spinning down the cells at 600xg, the pellet was resuspended using PP expansion media and cells were counted using the Countess II Automated Cell Counter (Thermo Fischer Scientific) and Trypan blue. Typical recovery rates were above 85%. Cells were then seeded as described above for expansion.

Differentiation of CINI ePP cells into pancreatic endocrine cells using ALI culture

PP cells generated using the STEMdiff™ Pancreatic Progenitor Kit and expanded under CINI conditions were dissociated into single cells using Accutase™ (STEMCELL Technologies, 07920) at 37 °C and then resuspended in PEP medium (Table S4) supplemented with 10 μM ROCKi at concentration of 50,000 cells/μl. The Falcon® Cell Culture Inserts (Corning, 353493) were placed in their companion plate wells (Corning, 353502) containing 1.5 ml of complete PEP media supplemented with 10 μM ROCKi. Ten droplets of 5 μl of the cell suspension were dropped on the insert to create ten 3D clusters per well, each containing 250,000 cells. Daily media changes were conducted according to Table S4 with the following changes : (a) during S5, the XX Notch inhibitor (Millipore, 565789) was added at a concentration of 10 nM (b) final glucose concentration at S6 and S7 was 20 mM and (c) no sodium pyruvate was used at S7.

Differentiation of PP cells into SC-islets using micropatterned wells

PP cells were dissociated using TrypLE Express at 37°C for 2-3 mins and seeded in microwells of AggreWell 800 plates (STEMCELL Technologies, 34825), using the recommended procedure by the supplier. Directly differentiated and ePP cells were seeded at a density of 5000 or 2000 cells per microwell, respectively. Media were an adaptation of published procedures (Balboa et al., 2022; Mahaddalkar et al., 2020; Rezania et al., 2014; Shi et al., 2017) (Table S4). Expanded PP cells in micropatterned wells were kept in expansion media for 24 hours and, during the first day of S5, S5 medium was supplemented with one-fourth of the concentration of C6 additional factors. Media of S5-S7 were as described in Table S4 and the medium was changed daily.

Immunofluorescence analyses

For immunofluorescence (IF), cells were cultured on 12 mm diameter Matrigel-coated coverslips (Carl Roth, P231.1) placed in 12-well wells (Corning, CLS3513) and differentiated or expanded as described above. Cells were then fixed in 4% paraformaldehyde (PFA) for 20 min at 4˚ C and washed with PBS. Cells were blocked and permeabilized for 1 h at RT using a 5% serum / 0.3% Triton X-100 PBS solution. Samples were then incubated at 4°C in 2.5% serum / 0.3% Triton X-100 in PBS containing the primary antibodies in the appropriate concentration (Table S6). The following day, samples were incubated for 1 h at RT in 2.5% serum / 0.3% Triton X-100 in PBS

containing the appropriate secondary antibodies, conjugated with either Alexa 488, 568 or 647, at a 1:500 dilution (Table S7). The coverslip with the stained cells was then placed on a microscope slide, covered with ProLong™ Gold Antifade mounting medium with DAPI (Invitrogen, P36931) and overlayed with a rectangular coverslip.

IF images were acquired using a Zeiss Axio Observer Z1 microscope coupled with the Apotome 2.0 imaging system and with consistent exposure times for the Alexa 488, 568 and 647 channels in between passages and conditions to allow for direct comparison of the signal intensities.

Fluorescence-activated cell sorting (FACS) of hPS cell derived PP and endocrine cells

Cells were first washed with 2% BSA in DMEM-F12 and then dissociated into single cells using TrypLE Express (Gibco, 12604-013). Following dissociation, the cells were counted using the Countess II Automated Cell Counter (Thermo Fischer Scientific) and Trypan blue. Then, cells were washed with PBS and fixed at a concentration of 4 million/ml in in 4% PFA for 10 min at 4°C. For staining with transcription factor antibodies, cells were washed with PBS and permeabilized using the Foxp3 Transcription Factor Staining Buffer Set (Invitrogen, 00-5523-00) for 1 h in dark at 4°C. Cells were then washed again using the 1x permeabilization buffer. Thereafter, $2x10^6$ cells/sample were blocked using 100 µl of 5% serum in 1x permeabilization buffer and then incubated with primary antibodies at the appropriate concentration (Table S5) in the same buffer overnight at 4°C. Then, they were washed twice with 1x permeabilization buffer and incubated with secondary antibodies at the appropriate concentration (Table S7) in the same buffer at room temperature for 1 hour in dark. For cytoplasmic factors, $2x10^6$ cells/sample were blocked for 30 mins at RT in 100 µl PBS containing 2% serum and 0.3% Triton X-100. Then cells were washed with 0.1% Triton X-100 in PBS solution and incubated with primary antibodies at the appropriate concentrations (Table S5) in the same buffer overnight. Thereafter, cells were washed twice with 0.1% Triton X-100 in PBS solution and incubated with secondary antibodies at the appropriate concentration (Table S7) at room temperature in the dark for 1 hour. For conjugated antibodies, the appropriate number of cells was used, as directed by the manufacturer, for both the isotype control and staining sample. Samples were stained overnight with conjugated antibodies and then washed with 0.1% Triton X-100 in PBS solution. FACS data were acquired using BD FACSCanto™ II and analysed using the FlowJo software.

Proliferation and cell death assays

The EdU proliferation assay was performed with the Click-iT™ Plus EdU Alexa Flour™ 488 Flow Cytometry Assay Kit (Invitrogen, C10632), that contains all the necessary reagent except PBS and BSA, and according to the kit protocol. In short, on the day of passaging the expanding cells, the expansion media was supplemented with 10 µM EdU for 2 h and cells were then dissociated and washed in 3 ml of 1% BSA in PBS. A pellet of 1 million cells was resuspended in 100 µl of Click-iT™ fixative for 15 min and washed again with 3 ml of 1% BSA in PBS. The pelleted cells were resuspended in 100 µl of permeabilization and wash reagent for 15 min. Following the incubation, 0.5 ml of the Click-iT™ Plus reaction cocktail containing 1X buffer additive, the Alexa Fluor™ 488 picolyl azide and a copper protectant in PBS, was added to the sample and incubated in the dark at RT for 30 min. The cells were then washed with 3 ml of 1X permeabilization and wash reagent. The pelleted cells were resuspended in 0.5 ml of permeabilization and wash

reagent, passed through a 40 µm strainer (PluriSelect, 43-10040) and analysed on the BD FASCanto™ II.

The flow cytometry staining for necrosis and apoptosis was was performed with the FITC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend, 640922). Once the cells were incubated in 10 µM of EdU, they were detached and washed in 1% BSA in PBS and an aliquot of 250,000 cells was taken and pelleted in a 1.5 ml microcentrifugation tube. The cell pellet was resuspended in 100 µl of Annexin V Binding Buffer containing 5 µl of FITC Annexin V and 5 µl of 7-AAD Viability Staining Solution and cells were incubated for 15 min at RT in the dark. After the incubation, an additional 400 µl of Annexin V Binding Buffer was added to the sample and passed through a 40 µm strainer (PluriSelect, 43-10040) before the live cells were analysed on the BD FACSCanto™ II.

Static glucose-stimulated insulin secretion (GSIS) assay

At the end of S7 (S7d10 – S7d14) 150 clusters were collected, washed with PBS and incubated for 1 hour in 700 µl of fresh Kreb's buffer (2.5mM $CaCl_2$, 129mM NaCl, 4.8mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 1mM Na_2HPO_4, 5mM $NaHCO_3$, 10mM HEPES, 0.1% BS, pH adjusted to 7.4 with 5M NaOH). After first incubation, clusters were incubated with Kreb's buffer containing low glucose (2.8mM) for 1 hr, then high glucose (16.7mM) for 1 hr and finally KCl/high glucose (30 mM/16.7 mM) for 1 hr. Incubations were for exactly one hour and then supernatant was collected. Collected supernatants and pelleted cells were frozen at -80 ^{o}C until the analysis. C-peptide detection was performed using the human C-peptide ELISA kit (Mercodia, 10-1141-01), readings were taken using an ELISA plate reader and the standard curve was generated. Cell pellets were used to isolate genomic DNA with the DNeasy Blood & Tissue kit (Qiagen, 69504) and quantification was done using a Nanodrop spectrophotometer.

RNA isolation and qRT-PCR (qPCR)

Total RNA was prepared using the RNeasy kit with on-column genomic DNA digestion (Qiagen, 74004) following the manufacturer's instructions. First strand cDNA was prepared using the TAKARA PrimeScript RT Master Mix (TAKARA RR036A). Real-time PCR primers (Table S8) were designed using the Primer 3 software (SimGene), their specificity was ensured by *in silico* PCR and they were further evaluated by inspection of the dissociation curve. Reactions were performed with the FastStart Essential DNA Green Master mix (Roche 06924204001) using the Roche LightCycler 480 and primary results were analyzed using the on-board software. Reactions were carried out in technical triplicates from at least three independent biological samples. Relative expression values were calculated using the ΔΔCt method by normalizing to H1 undifferentiated expression levels and the *TBP* housekeeping gene.

RNA sequencing and bioinformatics analysis

Cells were differentiated and from hPS cells as described above. Three independent samples from distinct differentiations and independent expansions were used as biological replicates. Total RNA prepared as above with an integrity number of ≥ 9 was used and subsequent steps were performed at the Biotec Sequencing Core of TU Dresden. mRNA was isolated from 1 ug of total RNA by poly-dT enrichment using the NEBNext Poly(A) mRNA Magnetic Isolation Module according to the manufacturer's instructions. Final elution was done in 15ul 2x first strand cDNA

synthesis buffer (NEBnext, NEB). After chemical fragmentation by incubating for 15 min at 94°C the sample was directly subjected to the workflow for strand specific RNA-Seq library preparation (Ultra Directional RNA Library Prep, NEB). For ligation custom adaptors were used 1: (Adaptor-Oligo 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT-3', Adaptor-Oligo 2: 5'-P-GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC-3'). After ligation, adapters were depleted by an XP bead purification (Beckman Coulter) adding bead in a ratio of 1:1. Indexing was done during the following PCR enrichment (15 cycles) using custom amplification primers carring the index sequence indicated with 'NNNNNN'. (Primer1: Oligo_Seq AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T, primer2: GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T, primer3: CAA GCA GAA GAC GGC ATA CGA GAT NNNNNN GTG ACT GGA GTT. After two more XP beads purifications (1:1) libraries were quantified using Qubit dsDNA HS Assay Kit (Invitrogen). For Illumina flowcell production, samples were equimolarly pooled and distributed on all lanes used for 75bp single read sequencing on Illumina HiSeq 2500.

After sequencing, FastQC (http://www.bioinformatics.babraham.ac.uk/) was used to perform a basic quality control of the resulting sequencing data. Fragments were aligned to the human reference genome hg38 with support of the Ensembl 104 splice sites using the aligner gsnap (v2020-12-16) (Wu and Nacu, 2010). Counts per gene and sample were obtained based on the overlap of the uniquely mapped reads with the same Ensembl annotation using featureCounts (v2.0.1) (Liao et al., 2014). Normalization of raw fragments based on library size and testing for differential expression between the different cell types/treatments was done with the DESeq2 R package (v1.30.1) (Love et al., 2014). Sample to sample Euclidean distance, Pearson' and Spearman correlation coefficient (r) and PCA based upon the top 500 genes showing highest variance were computed to explore correlation between biological replicates and different libraries. To identify differential expressed genes, counts were fitted to the negative binomial distribution and genes were tested between conditions using the Wald test of DESeq2. Resulting p-values were corrected for multiple testing with the using Independent Hypothesis Weighting (v1.18.0) (Ignatiadis et al., 2016). Genes with a maximum of 5% false discovery rate (padj ≤ 0.05), 0.5>fold regulation>2.0 and counts above 200 were considered as significantly differentially expressed.

To directly compare the transcriptome profile of our expanded PP cells with previously published data sets, raw sequencing data of the GEO archives GSE156712 (Ma et al., 2022) were downloaded. The array express, E-MTAB-9992 archive from EBI (Nakamura et al., 2022) and EGAS00001003127 from EGA (Ramond et al., 2018) offered bam files. Here, the fastq files were extracted with picard tools (v2.25.6). All these data sets underwent the same processing procedure. Fragments/Reads were aligned to the human reference genome hg38 with support of the Ensembl 104 splice sites using the aligner gsnap (v2020-12-16). Counts per gene and sample were obtained based on the overlap of the uniquely mapped reads with the same Ensembl annotation using featureCounts (v2.0.1) (Liao et al., 2014). The various strand-specificity of the several projectes was taken into account for the gene counting.

Normalization of raw fragments based on library size and scaling the count on a log2 scale was done with the DESeq2 R package (v1.30.1) (Love et al., 2014) and the Variance Stabilizing Transformation (vst) function. These values were used for plotting.

Original RNA Seq data have been deposited in GEO under the GSE216266 accession number and accessible with the token sfwbsqyslfirbcj (C6 ePP transcriptome) and under the GSE216179 accession number and accessible with the token enyjwwycfjsjdub (C6 ePP transcriptome). GO and KEGG analyses have been carried out using the Enrichr suite https://maayanlab.cloud/Enrichr/ (Kuleshov et al., 2016) and GSEA analyses using the UCSD Broad Institute suite (https://www.gsea-msigdb.org/gsea/index.jsp) (Kuleshov et al., 2016).

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