Stem Cell Research & Therapy

RESEARCH

Open Access

Generation of vascularized pancreatic progenitors through co-differentiation of endoderm and mesoderm from human pluripotent stem cells

Xiaopu Sang[1,2,3†], Junming Xu[4†], Yihang Wang[1], Jingyi Li[5], Jiasen Xu[1], Xiaoni Chen[1], Xianjie Shi[4*] and Fenfang Wu[1,5*]

Abstract

Background The simultaneous differentiation of human pluripotent stem cells (hPSCs) into both endodermal and mesodermal lineages is crucial for developing complex, vascularized tissues, yet poses significant challenges. This study explores a method for co-differentiation of mesoderm and endoderm, and their subsequent differentiation into pancreatic progenitors (PP) with endothelial cells (EC).

Methods Two hPSC lines were utilized. By manipulating WNT signaling, we optimized co-differentiation protocols of mesoderm and endoderm through adjusting the concentrations of CHIR99021 and mTeSR1. Subsequently, mesoderm and endoderm were differentiated into vascularized pancreatic progenitors (vPP) by adding VEGFA. The differentiation characteristics and potential of vPPs were analyzed via transcriptome sequencing and functional assays.

Results A low-dose CHIR99021 in combination with mTeSR1 yielded approximately 30% mesodermal and 70% endodermal cells. Introduction of VEGFA significantly enhanced EC differentiation without compromising PP formation, increasing the EC proportion to 13.9%. Transcriptomic analyses confirmed the effectiveness of our protocol, showing up-regulation of mesodermal and endothelial markers, alongside enhanced metabolic pathways. Functional assays demonstrated that vPPs could efficiently differentiate into insulin-producing β-cells, as evidenced by increased expression of β-cell markers and insulin secretion.

Conclusion Our findings provide a robust method for generating vPPs, which holds significant promise for regenerative medicine applications, particularly in diabetes treatment.

Keywords Human pluripotent stem cells, Endoderm differentiation, Mesoderm differentiation, Multi-lineage co-differentiation, Vascularized pancreatic progenitors

[†]Xiaopu Sang and Junming Xu contributed equally to this work.

*Correspondence:
Xianjie Shi
shixj7@mail.sysu.edu.cn
Fenfang Wu
wufenfang19@126.com
[1]Department of Central Laboratory, Shenzhen Hospital, Beijing University of Chinese Medicine, Shenzhen, Guangdong, China
[2]School of Traditional Chinese Medicine, Hubei University of Chinese Medicine, Wuhan, Hubei, China
[3]Hubei Shizhen Laboratory, Wuhan, Hubei, China
[4]Department of Hepatobiliary and Pancreatic Surgery, The Eighth Affiliated Hospital of Sun Yat-sen University, Shenzhen, Guangdong, China
[5]Biotherapy Center, Shenzhen Third People's Hospital (The Second Affiliated Hospital of Southern University of Science and Technology), Shenzhen, Guangdong, China

Introduction

Human pluripotent stem cells (hPSCs) possess unlimited self-renewal capabilities and can differentiate into various cell lineages derived from the three embryonic germ layers under different in vitro cultivation conditions [1–3]. These properties render hPSCs an attractive source for cell therapies targeting a variety of diseases. Induced functional stem/progenitor cells, in particular, show great potential for treating intractable diseases and metabolic injuries. Recent clinical trials have demonstrated that hPSC-derived pancreatic progenitor cells or islet tissues hold promise for treating diabetic patients with impaired islet function [4, 5]. Vascularization is vital for proper islet development and function [6, 7]. However, there is currently no satisfactory protocol to achieve vascularization of hPSC-derived pancreatic progenitor cells. A critical challenge in this field is the simultaneous induction of multiple lineages from a single culture of hPSCs, which is essential for creating functional vascularized tissues. The ability to co-differentiate endodermal and mesodermal lineages is particularly important for generating tissues such as the pancreas, which requires both pancreatic progenitors and a supporting vascular network [8].

Over the past two decades, several protocols have been established for the stepwise differentiation of hPSCs into definitive endoderm (DE), pancreatic progenitors (PP) and pancreatic β-cells [9–11]. Improvements in the efficiency and purity of PP differentiation have been achieved more recently [12–16]. However, these strategies often focus on acquiring highly purified DE cells, which may result in a single cell type that does not accurately replicate the cellular diversity observed during embryonic development. Achieving the complexity and maturation representative of human organogenesis remains a significant challenge. Recent studies have highlighted the crucial role of endothelial cells (EC) in orchestrating the development and maturation of organs [17–19]. ECs, which arise from mesodermal lineages, and PPs, which differentiate from endodermal lineages; both originate from the same epiblast. The initial interactions between the mesoderm and endoderm are critical for early pancreatic development [17–19]. However, the developmental interplay between PP and EC in humans remains poorly understood, underscoring the necessity to establish a co-differentiation system for generating both PP and EC from hPSCs [20].

The establishment of a co-differentiation system for PPs and ECs relies on the simultaneous induction of endoderm and mesoderm. However, the precise methodology for achieving this remains unclear. The differentiation of DE from hPSCs using Activin A (ActA) is well-documented [21]. CHIR99021 (CHIR), a small-molecule inhibitor of glycogen synthase kinase-3β (GSK3β), activates the canonical WNT pathway and enhances the purity of DE differentiation when used in combination with ActA in *vitro* [11, 22, 23]. It has been identified that manipulating WNT signaling in a dose-dependent manner can lead to mesodermal and/or endodermal specification [15, 24]. Additionally, incorporating a specific proportion of mTeSR1 in the early differentiation medium can promote endoderm formation alongside a small portion of mesoderm [25]. These studies suggest that mesoderm and endoderm can be inducted simultaneously by regulating signaling pathways, although this approach has not been reported in pancreatic differentiation. Furthermore, several multi-lineage co-differentiation organoids, such as hepatobiliary organoids, cardio-pulmonary micro-tissues, and vascularized lung/intestinal organoids, provide valuable references for developing multi-lineage differentiation systems for the pancreas [24–26]. Nevertheless, attaining a precise equilibrium between these lineages within a single culture remains a substantial challenge.

In our study, we described a stepwise protocol for the co-differentiation of pancreatic progenitors and endothelial cells, where endoderm and mesoderm are simultaneously induced from a single culture of hPSCs. Notably, the vascularized pancreatic progenitors demonstrated the capacity to further differentiate into β-cells capable of secreting insulin. Our findings provide a robust and reproducible method for generating vascularized pancreatic progenitors, holding significant promise for developing effective therapies for diabetes and other conditions requiring complex tissue regeneration.

Methods

Cell culture and maintenance

The human-induced pluripotent stem cell line UC [25] was obtained from the Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Science. The human embryonic stem cell line H1 was acquired from the Wisconsin Cell Research Institute. Passages of the cell lines ranged from 40 to 50. Cell cultures were maintained on Matrigel-coated (Corning, cat.#356231) plates in mTeSR™1 medium (STEMCELL Technologies, cat.#85850) at 37 °C in a humidified atmosphere with 5% CO_2. The medium was refreshed daily, and cells were passaged upon reaching approximately 80% confluency. The recommended cell passage ratio was between 1:6 and 1:8.

Cell cryopreservation

When the cells reach approximately 80% confluency, aspirate the medium and wash the cells once with PBS (Solarbio, cat.#P1032). Add Accutase solution (Gibco, cat.#A1110501) and place the cells in an incubator to facilitate digestion for approximately five minutes. DMEM/F12 (Gibco, cat.#10565018) should then be used

to halt the digestion process. After centrifugation, resuspended the cells in CS10 (Sigma, cat.#C2874-100ML), transfer them to freezing tubes, and document the process. Place the cells in a freezing container at -80 °C overnight before transferring them to liquid nitrogen for long-term storage.

Vascularized pancreatic progenitor cells differentiation

Prior to the commencement of pancreatic differentiation, iPSCs underwent were dissociated into clumps with Accutase (Gibco, cat.#A1110501), and subsequently plated onto Matrigel Basement Membrane (Corning, cat.#356231). Once the cells reached 90% confluence, the medium was replaced with MCDB131 (Solarbio, cat.#C3231) containing 1.5 g/L NaHCO3 (Sigma, cat.#S6297), 0.5% FAF-BSA (Proliant, cat.#69700), 10mM glucose, 100ng/mL activin A (Peprotech, cat.#120−14), 3μM CHIR99021 (APExBIO, cat.#A3011-25), and 2.5% mTeSR™1 (STEMCELL Technologies, cat.#85850) for 3 days. Following this, the last three components were replaced with 0.25 mM Vitamin C (Sigma, cat.#A4403), 50 ng/ml KGF (NovoProtein, cat.#CH73-10), and 300 ng/ml VEGF-A (Gibco, cat.#AF-100-20-1MG) for 2 days. Then, the medium was supplemented with ITS-X (procell, cat.#PB180431), 1μM Retinoic acid (Sigma, cat.#R2625), 200 nM PdBU/PKC Activator II (Sigma, cat.#524390), 0.25 μM SANT-1(Sigma, cat.#S4572), and 100 nM LDN193189 (MCE, cat.#HY-12071) and maintained in culture for an additional 5 days. The differentiation protocol and reagents are detailed in Table S1 and Table S4.

Pure pancreatic progenitor cells differentiation

Following the protocols of Chen et al., differentiation into pancreatic progenitor cells was initiated with decreasing concentrations of CHIR99021 over the first three days, without the addition of mTeSR™1 or VEGFA during subsequent progenitor cell differentiation.

Differentiation into β-Cells

To assess the potential of vPP to differentiate into β-cells, late-stage differentiation medium from Chen's protocol was applied. Cells were cultured until day 27. The expression of β-cell markers (NKX6.1, INS, MAFB) and endothelial marker (CD34) was analyzed using qPCR, immunofluorescence, and flow cytometry. Insulin secretion was measured in culture supernatants using an enzyme-linked immunosorbent assay (ELISA).

RNA extraction and qPCR

Total RNA was extracted from the cells using TRIzol™ Reagent (Invitrogen). The PrimeScript RT Reagent Kit with gDNA Eraser (Takara) was used to synthesize cDNA from 500 ng of total RNA. Quantitative PCR was conducted using TB Green Premix Ex Taq II (Takara) on a LightCycler 480 II (Roche). Data were quantified relative to a standard curve and normalized to the expression of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used are listed in Table S2.

Immunofluorescence and quantification

The procedure was conducted following Wu's protocol [25]. Cultures were fixed with 4% paraformaldehyde (Solarbio, cat.#P1110) for 15 min at room temperature (RT, 20–25℃). Subsequently, they were blocked and permeabilized using a solution of 5% donkey serum (The Jackson Laboratory, cat.#JAC-017-000-121) and 0.3% Triton X-100 (Sigma-Aldrich, cat.#T9284) in PBS for 1 h. After each step, cultures were washed three times with PBS for 5 min at RT. Then, the cultures were incubated overnight at 4℃ with primary antibodies diluted in a solution containing 3% donkey serum and 0.3% Triton X-100. This was followed by incubation with secondary antibodies diluted in 1% donkey serum in PBS for 30 min at RT. Subsequently, nuclei were stained with DAPI (Sigma-Aldrich, cat.#D9542). After antibodies and DAPI staining, cells were rinsed four times for 5 min with PBS. Immunofluorescence images were acquired utilizing a Leica DMI8 inverted microscope, and processed with LAS X software (Leica). At least three samples per group were stained, with three non-overlapping fields of view captured per sample. The positive area was quantified using ImageJ (v.ij154) and expressed as a ratio to the total DAPI-stained area. The primary and secondary antibodies used are provided (Table S3).

Flow cytometry

This experiment followed the methodology outlined by Wu [25]. Digestion and separation of cultures into single cell suspensions using Accutase/Trypsin-EDTA solution (Sigma-Aldrich, cat.#T4174). Cells were then subjected to centrifugation, followed by resuspension and fixation in 4% paraformaldehyde at RT for 15 min. After an additional round of centrifugation, cells were resuspended in Intracellular Staining Permeabilization Wash Buffer (BD, cat.#554714) for 5 min, a process repeated four times. Subsequently, cells were incubated with the fluorescent antibody for 15 min at RT, protected from light. Results were analyzed using the FACS Calibur flow cytometer (BD Biosciences) and processed with FlowJo VX software.

RNA-sequencing

Cells were harvested for RNA sequencing on day 3 and day 10 of vPP differentiation, as well as from the control cells (Chen's protocol) and undifferentiated hPSCs. Total RNA was isolated and purified using TRIzol™ reagent

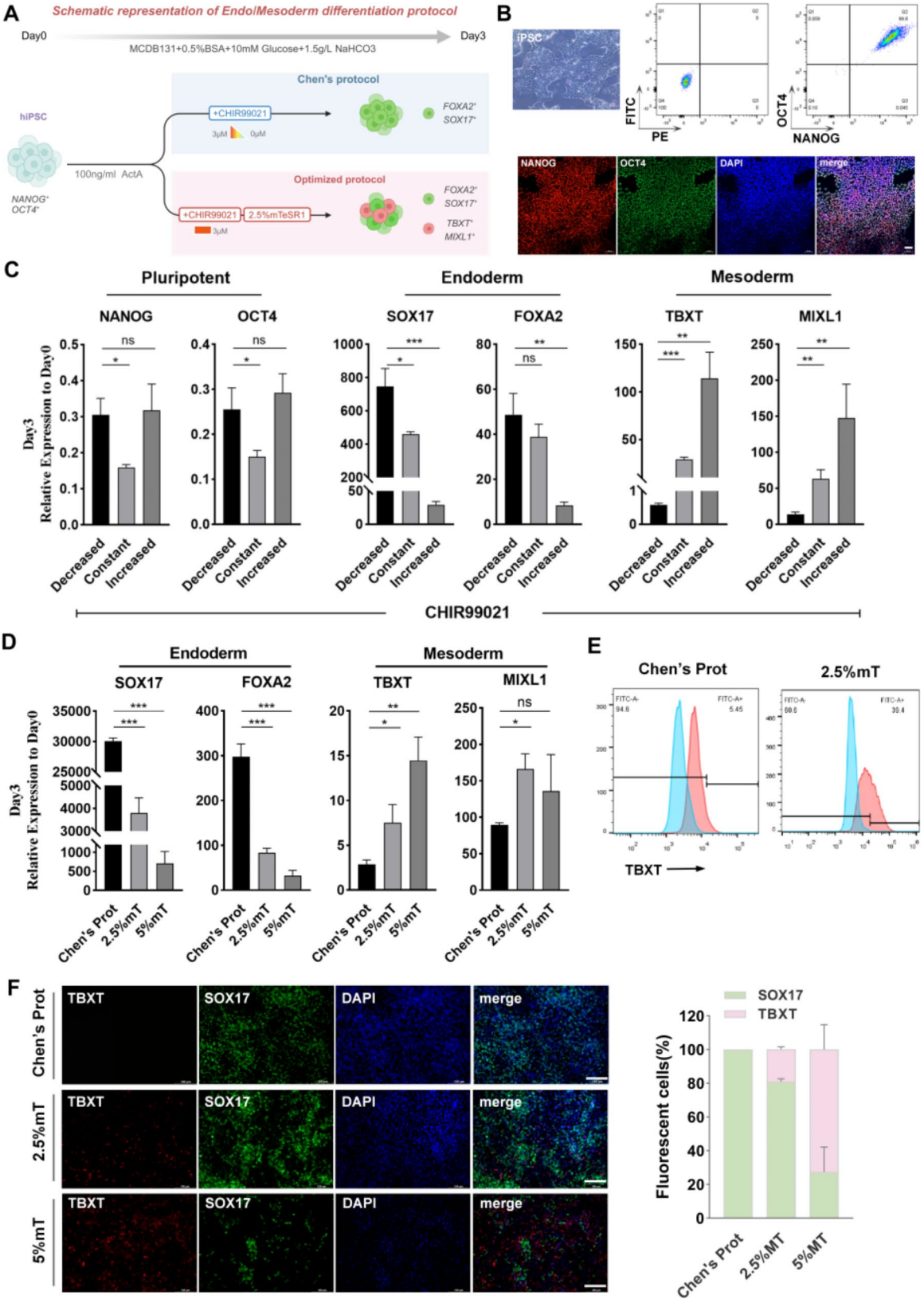

Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 Optimization of co-differentiation of endoderm and mesoderm from hPSC. (**A**) A schematic representation of the differentiation protocol from Day 0 to Day 3. The endoderm differentiation scheme was adapted from Chen et al. Our optimized protocol facilitates the simultaneous induction of both endoderm and mesoderm. (Created in BioRender: https://BioRender.com/j57p699). (**B**) Immunofluorescence and flow cytometry analysis confirmed that most Day 0 hPSCs expressed NANOG and OCT4. Scale bar, 50 μm. (**C**) Quantitative PCR analysis. High levels of CHIR99021 promote the optimal proportion of mesodermal and endodermal co-differentiation compared to protocols with increased or decreased CHIR99021 concentrations. (**D**) Quantitative PCR analysis, (**E**) flow cytometry, and (**F**) immunofluorescence showed that 2.5% mTeSR1 induces an appropriate proportion of mesoderm and endoderm. ($n=3$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ns: no statistical differences)

(Invitrogen), following the manufacturer's instructions. The NanoDrop ND-1000 (NanoDrop) was used to determine the quantity and purity of RNA in each sample. The 2×150 bp paired-end sequencing (PE150) was conducted on an Illumina NovaSeq™ 6000 (LC-Bio Technology CO., Ltd.) in accordance with the vendor's recommended protocol.

Bioinformatics analysis

Bioinformatics analysis was performed utilizing the OmicStudio suite of tools, which is accessible via the following link: https://www.omicstudio.cn/tool. Principal components analysis (PCA) and heatmaps were generated to visualize the distribution of samples. Differentially expressed genes (DEGs) were identified, and Gene Ontology (GO) and KEGG pathway enrichment analyses were performed. Additionally, gene set enrichment analysis (GSEA) was conducted to further elucidate the biological processes influenced by the differentiation protocols.

Human INS (insulin) ELISA assay

Insulin concentration in the supernatant was quantified using a human insulin ELISA kit (Elabscience, cat.#E-EL-H2665c) according to the manufacturer's instructions. Supernatant samples (20 μL) were stored at -20℃ until measurement.

Transmission electron microscope

Samples were initially fixed with 2.5% glutaraldehyde in PBS at 4℃ for 2 h. This was followed by a post-fixation with 1% osmium tetroxide at RT for 1 h. The fixed samples were dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%), then embedded in Epon resin, and polymerized at 60℃ for 24 h. Ultra-thin sections with a thickness of 50–70 nm, were prepared by cutting with an ultramicrotome and mounted on copper grids. The sections were then stained with uranyl acetate for 10 min, followed by lead citrate for 5 min. A transmission electron microscope (EOL, JEM1400) operated at 80 kV was used to examine the sections, and images were captured for subsequent analysis using SIS Montega (2k×4k) and Cantega (2k×2k).

Statistical analyses

Statistical analyses were conducted using GraphPad Prism 9.5.1. For inter-group comparisons, a two-tailed unpaired Student's t-test was employed. A p-value of <0.05 was considered significant, with significance levels indicated as follows: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Results

Simultaneous induction of endoderm and mesoderm via WNT signaling manipulation

Human-induced pluripotent stem cell line UC was assessed for pluripotency prior to differentiation. Immunofluorescence and flow cytometry analysis showed that more than 99% of the cells co-expressed the pluripotency markers NANOG and OCT4 (Fig. 1B). It has been reported that a high dose of CHIR induces mesoderm, whereas a low dose induces endoderm [15]. To achieve co-differentiation of both endoderm and mesoderm from hPSCs in a single dish, we tested different combinations of ActA and CHIR (Fig. 1A) to precisely manipulate WNT signaling over three days (day 1–3).

Initially, we evaluated various concentration of CHIR within a three-day treatment protocol. Consistent with previous studies, the expression of mesoderm markers (*TBXT*$^{+}$, *MIXL1*$^{+}$) increased with higher concentrations of CHIR, while the expression of endoderm markers (*SOX17*$^{+}$, *FOXA2*$^{+}$) decreased (Figure S1A). Consequently, we choose the low dose of CHIR for further testing. Both the dosage and duration of CHIR treatment are critical for determining the fate of cells towards mesoderm or endoderm. The combination of ActA with decreasing concentrations of CHIR has been shown to generate highly purified DE [23]. We then sought to identify the optimal processing time for CHIR application. Compared to decreasing concentration, an incremental or constant concentration of CHIR favored mesoderm induction and suppressed endoderm differentiation. However, the constant concentration had a reduced effect on endoderm suppression (Fig. 1C). Therefore, maintaining a constant concentration of CHIR for three days appears to be an effective strategy for the simultaneous induction of both endoderm and mesoderm.

In our previous study, we demonstrated mTeSR1 (mT) contributes to the co-differentiation of endoderm and mesoderm for generating vascularized hepato-biliary organoids [25, 27]. To access the effect of mT on the co-induction of endoderm and mesoderm, various ratios of mT were added into the basic medium for three days. The expression of endoderm makers decreased with increasing ratios of mT, while the expression of mesoderm

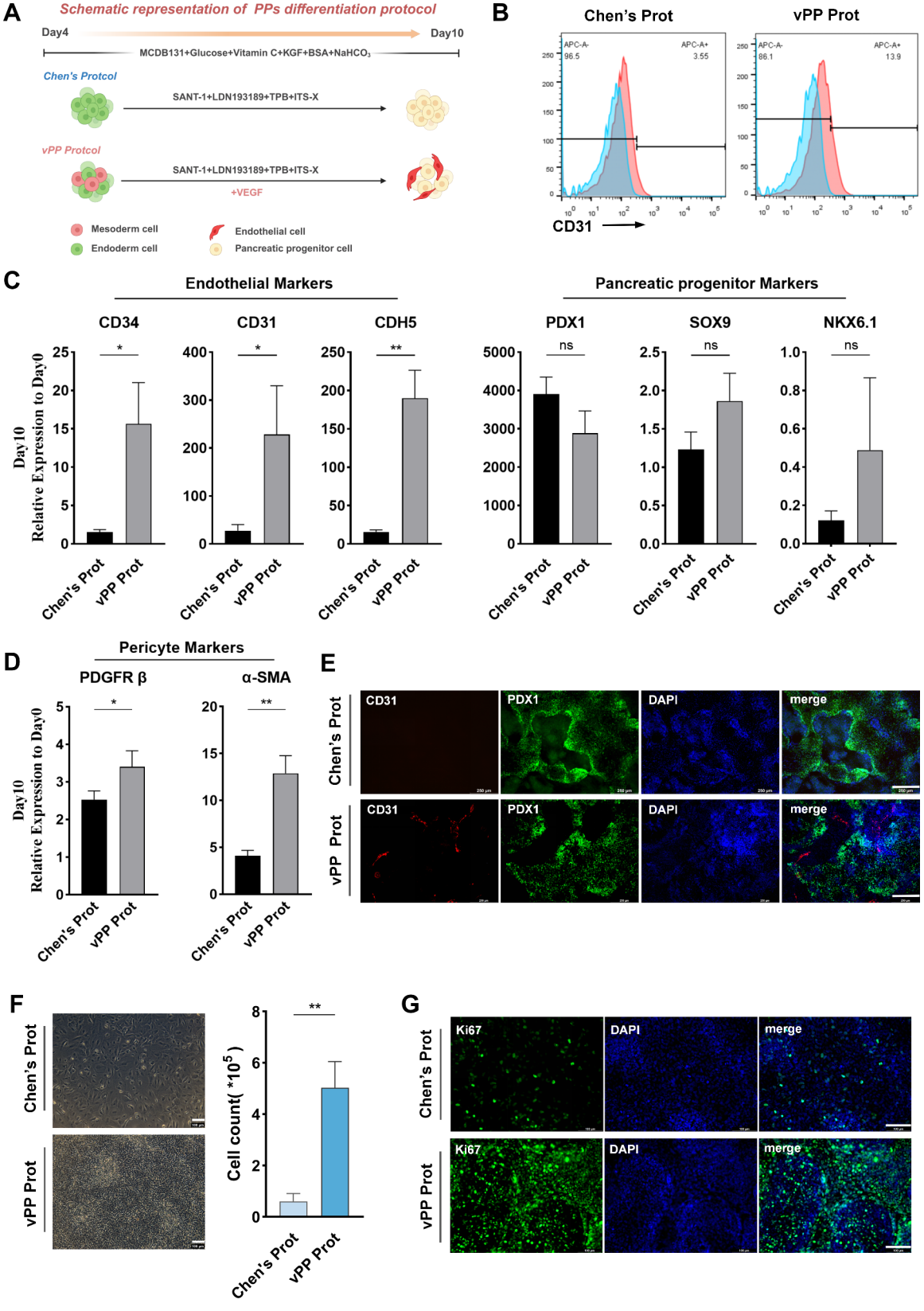

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Generation of Vascularized Pancreatic Progenitors (vPP). (**A**) A schematic representation of the PP and vPP differentiation protocol. The PP differentiation scheme was adapted from Chen et al. Our vPP protocol facilitates the simultaneous induction of both EC and PP from Day 4 to Day 10. (Created in BioRender: https://BioRender.com/d21p796). (**B**) Flow cytometry analysis of EC marker CD34. (**C** and **D**) Quantitative PCR analysis of EC markers (*CD34*, *CD31*, *CDH5*), PP markers (*PDX1*, *SOX9*, *NKX6.1*), and pericyte markers (*PDGFRβ*, *α-SMA*). (**E**) Immunostaining of generated PP (day 10) against PDX1 and CD31, comparing the vPP and Chen's protocols. Scale bar, 250 μm. (**F**) Cell counting analysis. Statistical analysis of cell counts after re-inoculation for 3 days with an equal number of cells on Day 10 under both protocols. (**G**) Immunostaining of generated PP (day 10) against Ki67, comparing the vPP and Chen's protocol. Scale bar, 100 μm. ($n=3$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: no statistical differences)

markers increased (Fig. 1D-F). Immunoflurescence and flow cytometry further confirmed that a 2.5% mT treatment achieved an optimal proportion of mesodermal (~30%) and endodermal (~70%) cells (Fig. 1F).

Bone morphogenetic protein 4 (BMP4) has been reported to induce mesoderm differentiation from hPSCs. Therefore, we tested the effect of supplementing BMP4 during the first three days of differentiation. Unexpectedly, BMP4 did not show a positive effect in our protocol (Figure S1B). The initial cell density of hPSCs can significantly affect differentiation efficiency. We tested different initial cell densities, applying the same ActA and CHIR treatment for three days. Immunofluorescence analysis of the mesoderm marker TBXT revealed that starting differentiation at cell densities between 80% and 100% resulted in stable mesodermal cell populations by day 3 (Figure S1C).

In summary, by manipulating WNT signaling and supplementing with mTeSR1, we achieved the co-induction of both endoderm and mesoderm from a single culture of hPSCs. This approach effectively prepares the cells for subsequent co-differentiation into pancreatic progenitors and endothelial cells.

Promotion of endothelial cells differentiation by VEGFA in pancreatic progenitors specialization

We used Chen's protocol as a control, which effectively differentiates DE into pancreatic progenitors (PP) [23]. To generate vascularized pancreatic progenitors (vPP) from mesoderm and endoderm, we initially applied the same supplement as in Chen's protocol from day 4 to day 10 (Figure S2A). By day 10, compared to Chen's protocol, there was a significant increase in the expression of endothelial cell makers (*CD34*$^+$, *CDH5*$^+$) (Figure S2C). However, expression of the key pancreatic progenitor marker *PDX1* was lower, even though *NKX6.1* and *SOX9* levels were higher than those in the control group (Figure S2C). The proportion of ECs was approximately 7% (Figure S2B). Therefore, we need to identify a strategy to promote EC differentiation without hindering PP specialization.

VEGFA and FGF2 are key differentiation factors that promote angiogenesis [28].We investigated the effectiveness of supplementing these angiogenic factors to facilitate EC differentiation during PP specialization (day 4–7). A high dose of VEGFA increased mRNA expression of CD31, while FGF2 did not have the same effect (Figure S2F). We then combined VEGFA and with the PP differentiation protocol to generate both ECs and PPs, referred to as the vPP protocol (Fig. 2A).

Compared to Chen's protocol, the expression of endothelial cells makers (*CD31*$^+$, *CD34*$^+$, and *CDH5*$^+$) significantly increased, with no adverse impact on PP differentiation markers (*PDX1*$^+$, *SOX9*$^+$, and *NKX6.1*$^+$) (Fig. 2C). The proportion of ECs increased to 13.9% (Figure S2B). Additionally, pericytes, identified by the molecular markers PDGFRβ and α-smooth muscle actin (SMA), were more prevalent in vPPs (Fig. 2D, Figure S2D).

In contrast to prior methods that primarily focused on producing pure PPs, our approach successfully controlled the co-development of DE and mesodermal lineages within the same culture, leading to the formation of vascularized pancreatic progenitors (Fig. 2E, Figure S2E). The robustness of vPP differentiation was maintained, with both cell proliferation ability and cell numbers significantly improved compared to the original protocol (Fig. 2F-G).

To verify these results, we repeatedly induced vPP using H1 human embryonic stem cell lines, consistently achieving the same outcomes (Figure S5).

Transcriptome analysis of vascularized pancreatic progenitors

To evaluate the transcriptomic profile of vPPs, we performed RNA sequencing on cells at day 3 and day 10 of vPP differentiation. Cells from Chen's protocol and hPSCs were used as controls. Principal components analysis (PCA) and heatmaps demonstrated a clear distribution of all samples (Figure S3A and B).

Our optimized protocol, applied from day 1 to day 3, enhanced the expression of several makers involved in mesoderm development, including *SFRP2*, *SMAD3*, *TBXT*, *LEF1*, and *DKK1* (Fig. 3A and C, Table S5), while it suppressed the expression of makers associated with endoderm development, such as *APELA*, *BMP4*, *GATA4*, *GATA6*, and *HNF1B* (Fig. 3A and D, Table S5). Gene ontology (GO) enrichment analysis revealed that differentially expressed genes (DEGs) were significantly involved in the biological process of mesoderm and endoderm development (Fig. 3B). This was further supported by gene set enrichment analysis (GSEA). Compared to controls, the gene set related to mesoderm formation was significantly up-regulated (P value=0.024, FDR value=0.1142, ES=0.557), while the set related

to endodermal cell differentiation was down-regulated (*P* value=0, FDR value=0.0026, ES=-0.792) (Fig. 3E-F). To further understand the underlying mechanisms, KEGG enrichment analysis of DEGs showed that pathways involved in oxidative phosphorylation (*P* value=0, FDR value=0, ES=0.574) and linoleic acid metabolism (*P* value=0.0050, FDR value=0.0289, ES=0.626) were significantly up-regulated in our optimized protocol in comparison to the previous method. Conversely, the PI3k-AKT signaling pathway (*P* value=0, FDR value=0.0305, ES=-0.4954) and FOXO signaling pathway (*P* value=0.0028, FDR value=0.0408, ES=-0.5061) were significantly down-regulated (Fig. 3G).

We further analyzed DEGs in vPPs, compare to PPs from Chen's protocol [23]. Most genes related to endothelium development were up-regulated (Fig. 4A and C, Table S5). GO enrichment analysis of up-regulated DEGs in vPPs highlighted functions involved in angiogenesis (GO: 0001525), heart development (GO: 0007507), and positive regulation of angiogenesis (GO: 0045766) (Fig. 4B). GSEA suggested that the vPP protocol may promote processes related to hematopoietic cell lineage, vasculature development, and angiogenesis. Importantly, we did not find any down-regulated DEGs impacting pancreatic development.

Key transcription factors in vascularized pancreatic progenitor differentiation

Transcription factors (TFs) are specialized proteins that bind to specific regions of DNA and regulate gene transcription. The roles of TFs in gene regulation during vPP differentiation were further elucidated through bioinformatics analysis. On day 3, 154 TF genes were significantly differentially expressed, with 71 TF genes up-regulated in our optimized protocol compare to the control (Fig. 5A, Table S5). The biological processes associated with these up-regulated TF genes were linked to mesoderm development and vasculogenesis (Fig. 5C). By day 10, 59 TF genes were significantly differentially expressed, with 33 TF genes up-regulated in our optimized protocol compared to the control (Fig. 5B, Table S5). These upregulated genes continued to be associated with mesoderm development and vasculogenesis (Fig. 5D).

Potency of vascularized pancreatic progenitors to differentiate into β-cells

To demonstrate the potential of vascularized pancreatic progenitors to differentiate into β-cells, we applied the late-stage differentiation medium from Chen's protocol and compared these cells with those directly differentiated using the same protocol. We examined markers of mature β-cells (NKX6.1, INS, and MAFB), immature β-cells (PDX1 and SOX9), and ECs (CD34). The expression levels of *NKX6.1* and *INS* were significantly up-regulated in cells at day 27 that differentiated from vPP compared to the control, while other markers showed no significant differences (Fig. 6A). Immunofluorescence demonstrated co-expression of INS and MAFA in cells at day 27 (Fig. 6B). Flow cytometry analysis revealed that approximately 60% of vPP differentiated into insulin-producing β-cells by day 27, with the ratio of C-peptide$^+$ cells being higher than in the control group (Fig. 6C). Insulin secretion in the culture supernatant at the end of the culture period was higher in cells differentiated from vPP compared to the control (Figure S4A). Transmission electron microscopy and immunofluorescence confirmed the presence of insulin-secreting granules in the β-cells derived from vPP (Figure S4B and C). These observations suggest that vPP can differentiate into insulin-producing β-cells and may do so more efficiently than direct differentiation.

Discussion

This study successfully establishes a robust protocol for the co-induction of endoderm and mesoderm from hPSCs via WNT signaling manipulation (Fig. 1A). To achieve an optimal ratio of approximately 30% mesoderm and 70% endoderm, our novel protocol employs a constant low dose of CHIR in combination with mTeSR1 (Fig. 1C-F). This ratio is particularly advantageous for subsequent differentiation steps aimed at producing vascularized tissues. Contrary to our expectations, the addition of BMP4 did not enhance mesoderm differentiation in our protocol, diverging from its recognized role in mesoderm induction (Figure S1B). This discrepancy might be attributed to context-specific responses of hPSCs, illustrating the necessity for tailored protocols depending on the specific cell line and differentiation target.

Our novel protocol, with the addition of VEGFA, significantly enhanced endothelial cell (EC) differentiation without compromising the efficiency of pancreatic progenitor (PP) formation (Fig. 2). The proportion of ECs reached 13.9%, representing a notable improvement over previous methodologies that primarily focused on pure PP populations. The increased expression of endothelial markers CD31, CD34, and CDH5 in our vPPs underscores the efficacy of VEGFA in promoting angiogenesis [28]. Additionally, the presence of pericytes highlights the formation of more complex and mature vascular structures within the PP context, further validating the robustness of our differentiation protocol. We also repeatedly induced vPP using H1 human embryonic stem cell lines, consistently achieving the same outcomes (Figure S5). This dual differentiation approach holds promise for creating more functional and vascularized tissues, which are essential for successful transplantations.

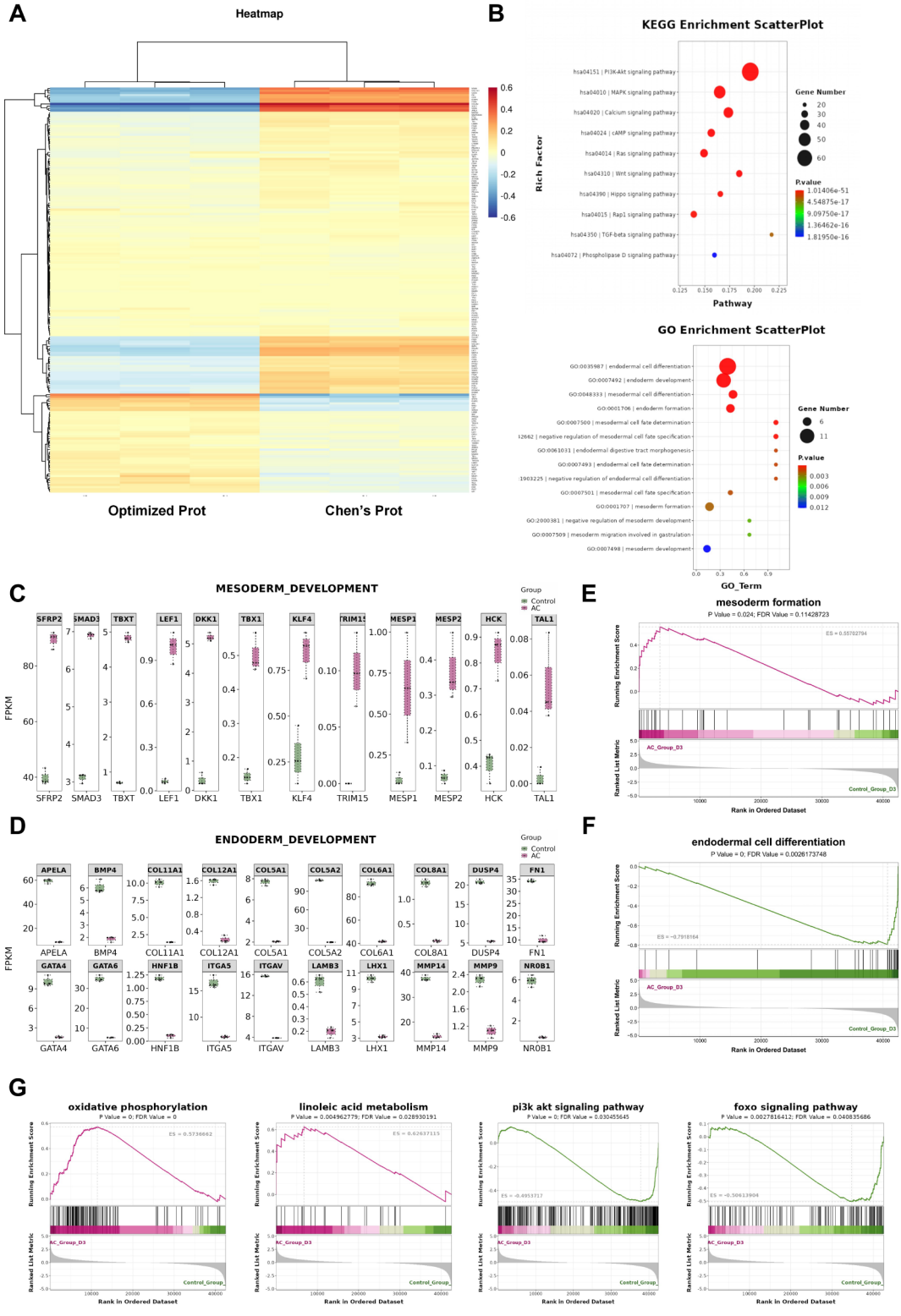

Fig. 3 Characterization of endoderm and mesoderm co-differentiation via RNA sequencing. (**A**) Heatmap representation of DEGs of endoderm and mesoderm co-differentiation from Optimized Protocol compared to control protocol (Chen's Protocol). (**B**) Gene Ontology (GO) enrichment analysis of DEGs involved in mesoderm and endoderm development. (**C** and **D**) Up-regulation of mesoderm-associated genes and down-regulation of endoderm markers in the optimized protocol. (**E** and **F**) Gene set enrichment analysis (GSEA) indicating significant up-regulation of mesoderm formation and down-regulation of endodermal cell differentiation. (**G**) GSEA for KEGG pathway analysis

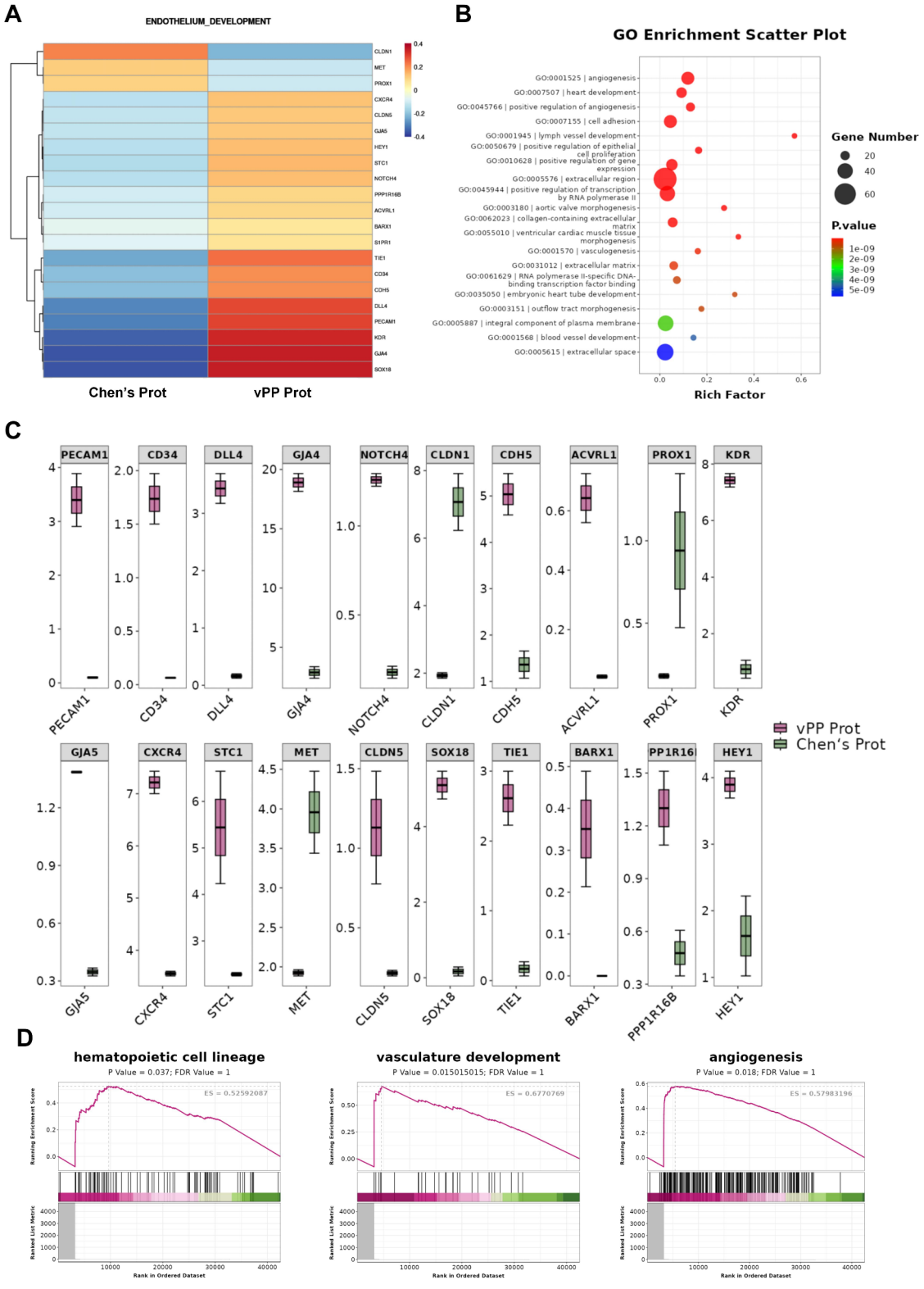

Fig. 4 Characterization of vascularized pancreatic progenitors (vPP) via RNA sequencing. (**A**) Heatmap showing upregulated endothelium development-related genes in vPP from vPP Protocol compared to PP from Chen's protocol. (**B**) GO enrichment analysis of up-regulated DEGs in vPP. (**C**) Up-regulation and down-regulation of endothelium development-related genes in vPP protocol, compared to Chen's protocol. (**D**) GSEA results suggesting vPP protocol promotes processes of hematopoietic cell lineage, vasculature development, and angiogenesis

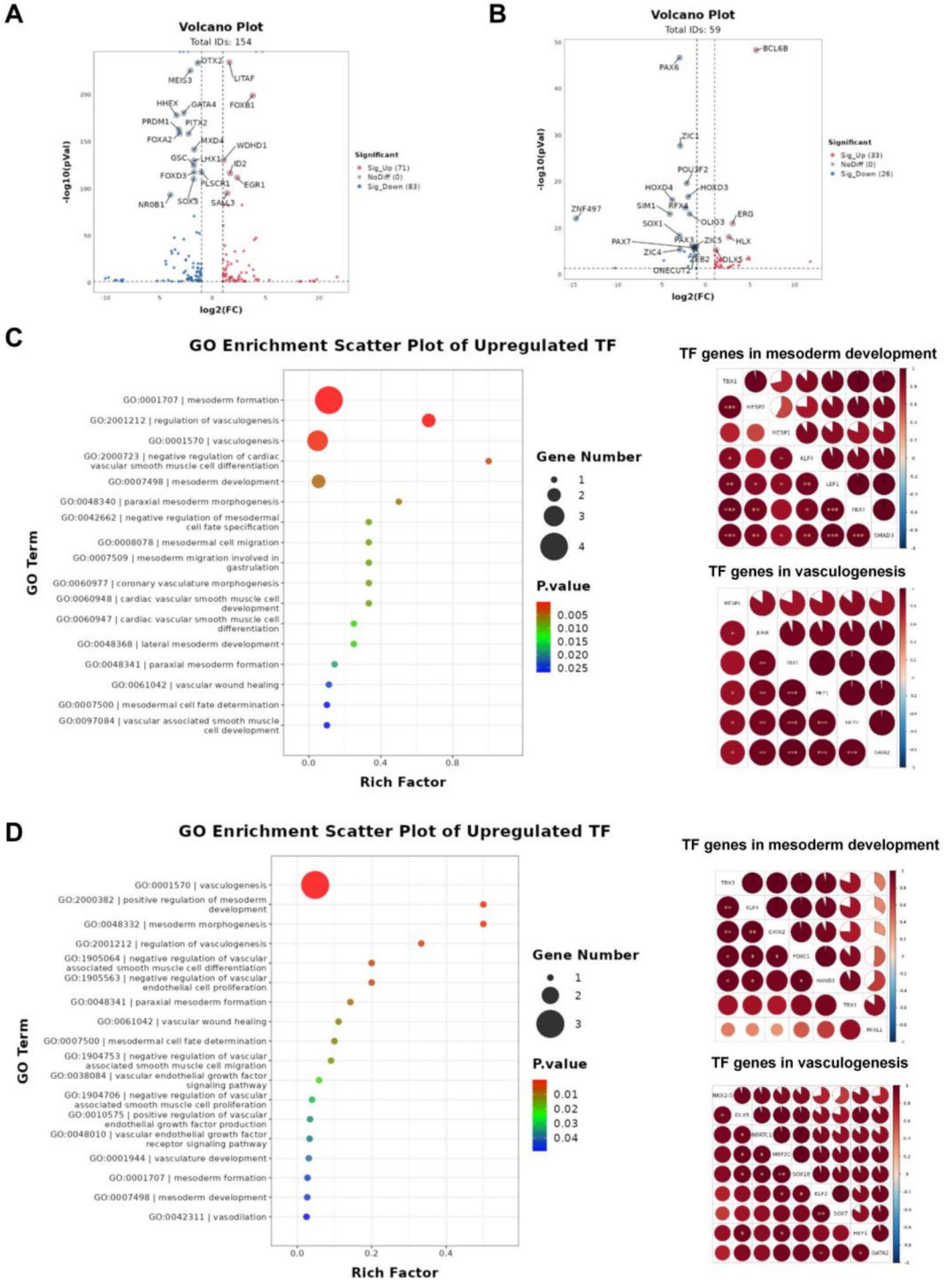

Fig. 5 Transcription factor analysis for vascularized pancreatic progenitors (vPP). (**A**) Volcano plot for differentially expressed TF genes of endoderm and mesoderm co-differentiation from Optimized Protocol compared to control protocol (Chen's Protocol) on day 3. (**B**) Volcano plot for differentially expressed TF genes in vPP Protocol compared to PP from Chen's protocol on day 10. (**C**) GO enrichment and gene correlation analysis of up-regulated differentially expressed TF genes associated with mesoderm development and vasculogenesis on day 3. (**D**) GO enrichment and gene correlation analysis of up-regulated differentially expressed TF genes associated with mesoderm development and vasculogenesis on day 10

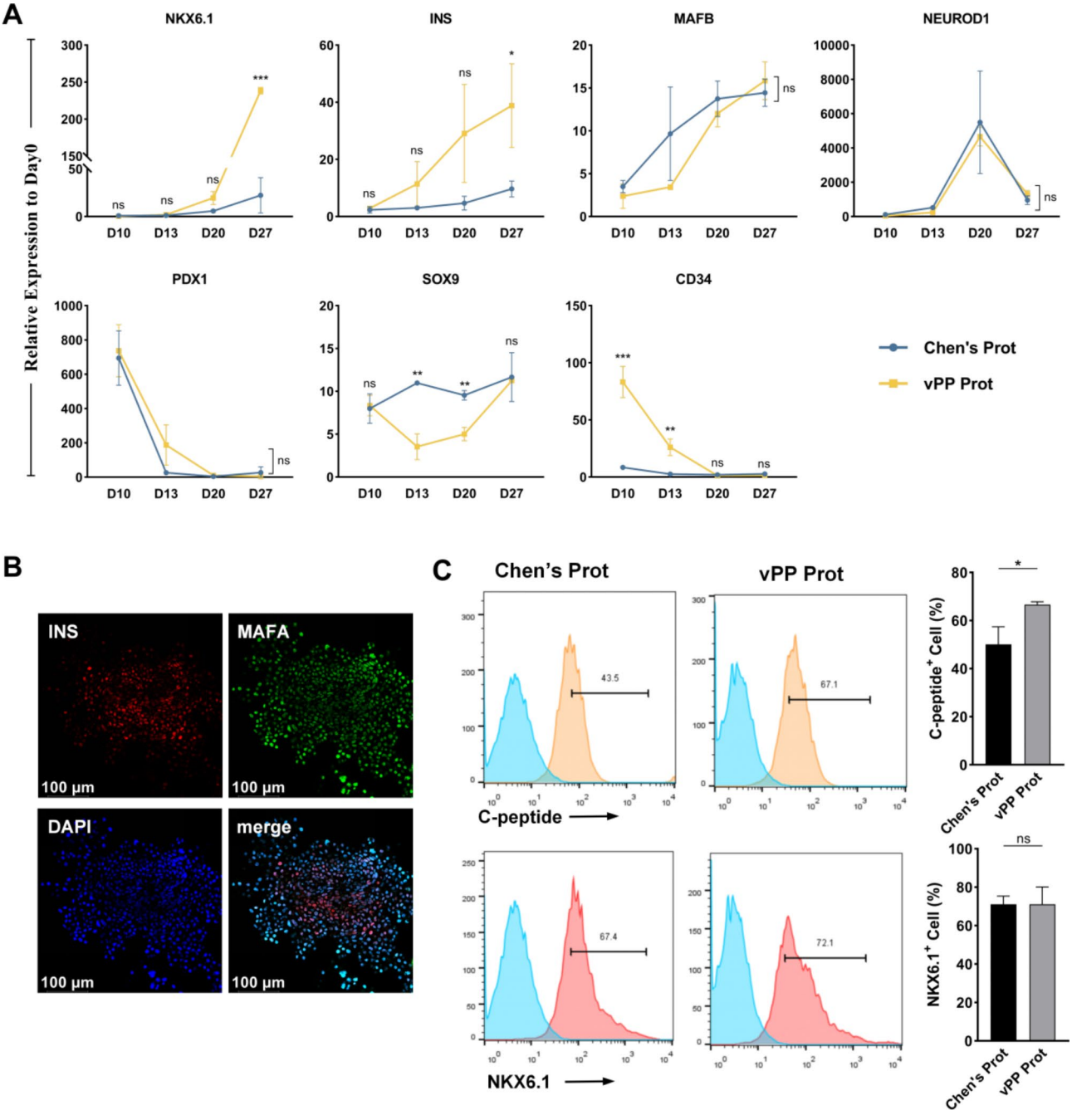

Fig. 6 Differentiation of vPP into β-Cells. (**A**) Quantitative PCR analysis of mature β-cells (*NKX6.1*, *INS* and *MAFB*), immature β-cells (*PDX1* and *SOX9*), and ECs (*CD34*) at day 27. (**B**) Immunofluorescence images showing co-expression of INS and MAFA in vPP-derived β-cells at day 27. (**C**) Flow cytometry analysis of insulin-producing β-cells (C-peptide$^+$ cells), indicating a higher ratio in vPP-derived β-cells compared to the control. ($n = 3$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: no statistical differences)

RNA-sequencing data corroborated the biological efficacy of our protocol. The up-regulation of mesoderm-associated genes (e.g., *SFRP2*, *SMAD3*, *TBXT*) and the suppression of endoderm markers (e.g., *APELA*, *BMP4*) align with the observed phenotypic outcomes (Fig. 3). GO and KEGG pathway analyses further supported these findings, illustrating significant involvement in mesoderm and endothelial development processes. Importantly, pathways related to oxidative phosphorylation and linoleic acid metabolism were up-regulated, indicating enhanced metabolic activity in the differentiated cells, which may contribute to their robustness and functionality. Recent studies have shown that cellular metabolism can influence stem cell differentiation,

including the development of mesodermal tissues [29–31]. The emerging field of metabolic regulation in stem cell biology continues to uncover new insights into how energy production and cellular metabolism influence development at the molecular level.

The final phase of our study demonstrated the functional potential of vPP to differentiate into insulin-producing β-cells. The enhanced expression of mature β-cell markers *NKX6.1* and *INS*, along with increased insulin secretion, signifies efficient maturation of these cells (Figure S4). Co-expression of INS and MAFA, detected via immunofluorescence, reconfirms the successful differentiation into functional β-cells. The functional maturation of islet cells, particularly insulin-producing β-cells, is crucial for maintaining glucose homeostasis. Vascularization plays a critical role in the development, maturation, and function of these cells, as it ensures an adequate supply of nutrient and oxygen, facilitates the removal of metabolic waste, and enables proper signaling interactions [32, 33].

Conclusions

Our study successfully establishes a robust protocol for the co-induction of endoderm and mesoderm from hPSCs by optimizing WNT signaling manipulation and integrating essential growth factors. This approach facilitates the generation of vascularized pancreatic progenitors, addressing a critical need in regenerative medicine. Future research should aim to further refine these protocols and explore the long-term functionality and stability of the differentiated cells in vivo. This work represents a substantial step toward developing effective therapies for diabetes and other diseases requiring complex tissue regeneration.

Abbreviations

hPSCs	Human pluripotent stem cells
PP	Pancreatic progenitors
EC	Endothelial cells
CHIR	CHIR99021
VEGFA	Vascular endothelial growth factor A
vPP	Vascularized pancreatic progenitors
DE	Definitive endoderm
ActA	Activin A
GSK3β	Glycogen synthase kinase-3β

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-024-04120-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Acknowledgements

Not applicable.

Author contributions

F.W. led and supervised the project. F.W. and Xianjie Shi designed the experimental strategy. Xiaopu Sang and Junming Xu interpreted the results and wrote the manuscript. Xiaopu Sang, Y.W., Junming Xu, Jiasen Xu, J.L., and X.C. performed the experiments. Xiaopu Sang performed the bioinformatics analysis. Junming Xu and Y.W. drew the figures. All authors reviewed and approved the manuscript.

Funding

This work was supported by grants from the Shenzhen Science and Technology Program (JCYJ20220818103407016), the Shenzhen Medical Research Fund (SMRF.D2301015), the National Natural Science Foundation of China (82172107), the Guangdong Basic and Applied Basic Research Foundation (2024A1515011222), and the Special Funds for Strategic Emerging Industry of Shenzhen (Grant No. F-2022-Z99-502266), and the Shenzhen Longgang District Science and Technology Innovation Special Fund (LGKCYLWS2022007).

Data availability

The RNA sequencing dataset supporting the conclusions of this article is included within the article (Supplementary materials). The data underlying this article will be shared on reasonable request to the corresponding author.

Declarations

Ethical approval and consent to participate

This study does not involve human participants or animals. Use of human pluripotent cell lines (UC and H1) was approved by the ethics committee of Shenzhen Hospital, Beijing University of Chinese Medicine in March 2021 (SZLDH2021LSYA-010).

Consent for publication

Not applicable.

Artificial intelligence

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

Competing interests

The authors have declared no competing interests.

Received: 30 August 2024 / Accepted: 18 December 2024
Published online: 24 December 2024

References

1. Yamanaka S. Pluripotent stem cell-based Cell Therapy-Promise and challenges. Cell Stem Cell. 2020;27:523–31.
2. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663–76.
3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145–7.
4. Wu J, Li T, Guo M, Ji J, Meng X, Fu T, et al. Treating a type 2 diabetic patient with impaired pancreatic islet function by personalized endoderm stem cell-derived islet tissue. Cell Discov. 2024;10:45.
5. Wang S, Du Y, Zhang B, Meng G, Liu Z, Liew SY, et al. Transplantation of chemically induced pluripotent stem-cell-derived islets under abdominal anterior rectus sheath in a type 1 diabetes patient. Cell. 2024;187:6152–64.
6. Narayanan S, Loganathan G, Dhanasekaran M, Tucker W, Patel A, Subhashree V, et al. Intra-islet endothelial cell and β-cell crosstalk: implication for islet cell transplantation. World J Transpl. 2017;7:117–28.
7. Lammert E, Cleaver O, Melton D. Induction of pancreatic differentiation by signals from blood vessels. Science. 2001;294:564–7.
8. Ranjan AK, Joglekar MV, Hardikar AA. Endothelial cells in pancreatic islet development and function. Islets. 2009;1:2–9.
9. Jin W, Jiang W. Stepwise differentiation of functional pancreatic β cells from human pluripotent stem cells. Cell Regen. 2022;11:24.

10. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol. 2014;32:1121–33.
11. Pagliuca FW, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, et al. Generation of functional human pancreatic β cells in vitro. Cell. 2014;159:428–39.
12. Cuesta-Gomez N, Verhoeff K, Dadheech N, Pawlick R, Marfil-Garza B, Razavy H, et al. AT7867 promotes pancreatic progenitor differentiation of human iPSCs. Stem Cell Rep. 2023;18:2084–95.
13. Arkenberg MR, Ueda Y, Hashino E, Lin CC. Photo-click hydrogels for 3D in situ differentiation of pancreatic progenitors from induced pluripotent stem cells. Stem Cell Res Ther. 2023;14:223.
14. Nakamura A, Wong YF, Venturato A, Michaut M, Venkateswaran S, Santra M, et al. Long-term feeder-free culture of human pancreatic progenitors on fibronectin or matrix-free polymer potentiates β cell differentiation. Stem Cell Rep. 2022;17:1215–28.
15. Jiang Y, Chen C, Randolph LN, Ye S, Zhang X, Bao X, et al. Generation of pancreatic progenitors from human pluripotent stem cells by small molecules. Stem Cell Rep. 2021;16:2395–409.
16. Wesolowska-Andersen A, Jensen RR, Alcántara MP, Beer NL, Duff C, Nylander V, et al. Analysis of differentiation protocols defines a common pancreatic progenitor molecular signature and Guides refinement of endocrine differentiation. Stem Cell Rep. 2020;14:138–53.
17. Jones RC, Karkanias J, Krasnow MA, Pisco AO, Quake SR, Salzman J, et al. The Tabula Sapiens: a multiple-organ, single-cell transcriptomic atlas of humans. Science. 2022;376:l4896.
18. Cao J, O'Day DR, Pliner HA, Kingsley PD, Deng M, Daza RM et al. A human cell atlas of fetal gene expression. Science. 2020;370.
19. Vargas-Valderrama A, Messina A, Mitjavila-Garcia MT, Guenou H. The endothelium, a key actor in organ development and hPSC-derived organoid vascularization. J Biomed Sci. 2020;27:67.
20. Pierreux CE, Cordi S, Hick AC, Achouri Y, Ruiz DAC, Prévot PP, et al. Epithelial: endothelial cross-talk regulates exocrine differentiation in developing pancreas. Dev Biol. 2010;347:216–27.
21. D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol. 2005;23:1534–41.
22. Velazco-Cruz L, Song J, Maxwell KG, Goedegebuure MM, Augsornworawat P, Hogrebe NJ, et al. Acquisition of dynamic function in human stem cell-derived β cells. Stem Cell Rep. 2019;12:351–65.
23. Ghazizadeh Z, Kao DI, Amin S, Cook B, Rao S, Zhou T, et al. ROCKII inhibition promotes the maturation of human pancreatic beta-like cells. Nat Commun. 2017;8:298.
24. Ng WH, Johnston EK, Tan JJ, Bliley JM, Feinberg AW, Stolz DB et al. Recapitulating human cardio-pulmonary co-development using simultaneous multilineage differentiation of pluripotent stem cells. Elife. 2022;11.
25. Wu F, Wu D, Ren Y, Huang Y, Feng B, Zhao N, et al. Generation of hepatobiliary organoids from human induced pluripotent stem cells. J Hepatol. 2019;70:1145–58.
26. Miao Y, Tan C, Pek NM, Yu Z, Iwasawa K, Kechele DO et al. Deciphering Endothelial and Mesenchymal Organ Specification in Vascularized Lung and Intestinal Organoids. bioRxiv. 2024.
27. Wu D, Chen X, Sheng Q, Chen W, Zhang Y, Wu F. Production of functional hepatobiliary organoids from human pluripotent stem cells. Int J Stem Cells. 2021;14:119–26.
28. Wimmer RA, Leopoldi A, Aichinger M, Wick N, Hantusch B, Novatchkova M, et al. Human blood vessel organoids as a model of diabetic vasculopathy. Nature. 2019;565:505–10.
29. Tyurin-Kuzmin PA, Molchanov AY, Chechekhin VI, Ivanova AM, Kulebyakin KY. Metabolic regulation of mammalian stem cell differentiation. Biochem (Mosc). 2020;85:264–78.
30. Zhao J, Yao K, Yu H, Zhang L, Xu Y, Chen L, et al. Metabolic remodelling during early mouse embryo development. Nat Metab. 2021;3:1372–84.
31. Mahmoud AI. Metabolic switches during development and regeneration. Development. 2023;150.
32. Eberhard D, Kragl M, Lammert E. Giving and taking': endothelial and beta-cells in the islets of Langerhans. Trends Endocrinol Metab. 2010;21:457–63.
33. Cao Z, Wang X. The endocrine role between β cells and intra-islet endothelial cells. Endocr J. 2014;61:647–54.

Publisher's note