Enhanced Differentiation of Human Pluripotent Stem Cells into Pancreatic Endocrine Cells in 3D Culture by Inhibition of Focal Adhesion Kinase

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Abstract

Background The generation insulin-producing cells from human pluripotent stem cells (hPSC) in vitro would be useful for drug discovery and cell therapy in diabetes. Three-dimensional (3D) culture is important for the acquisition of mature insulin-producing cells from hPSC, but the mechanism by which 3D culture promoted β cell maturation is poorly understood.

Methods We established a stepwise differentiation method to induce human embryonic stem cells (hESC) into mature monohormonal pancreatic endocrine cells (PEC) at high efficiency, with the last maturation stage in 3D culture. To make a comprehensive comparison between 2D culture and 3D culture, we examined gene expression, pancreatic-specific markers and functional characteristics of 2D culture-induced PEC and 3D culture-induced PEC. And the mechanisms was discussed from the perspective of cell-cell and cell-extracellular matrix interactions which are fundamentally different between 2D and 3D culture.

Results Expression of pancreatic endocrine-specific transcription factors PDX1, NKX6.1, NGN3, ISL1, PAX6 and hormones INS, GCG, SST were significantly increased in 3D culture-induced PEC. 3D culture gave rise to monohormonal endocrine cells, while 2D culture-induced PEC co-expressed INS and GCG or INS and SST, and some cells even expressed all three hormones. We found that FAK phosphorylation level was significantly downregulated in 3D culture-induced PEC, and treatment with a selective FAK inhibitor PF-537228 could improve the expression of β cell-specific transcription factors. We further demonstrated that 3D culture may promote endocrine commitment by limiting FAK-dependent activation of the Smad2/3 pathway. Moreover, the expression of gap junction protein Connexin 36 is much higher in 3D culture-induce PEC than 2D culture-induced PEC, and inhibition of FAK pathway in 2D culture increased Connexin 36 expression.

Conclusion We developed a differentiation strategy to induce monohormonal mature PEC from hPSC and found limited FAK-dependent activation of the Smad2/3 pathway and unregulated expression of Connexin 36 in 3D culture-induced PEC. This study has important implications for the generation of mature, functional β-cells for drug discovery and cell transplantation therapy in diabetes, and sheds new light on the signaling events that regulate endocrine specification.

Backgroud

Diabetes a globally widespread disease characterized by hyperglycemia due to autoimmune destruction of insulin-producing β cells (type 1 diabetes; T1D) or to extensive β cell exhaustion and depletion after hypersecretion of insulin to overcome insulin resistance (type 2 diabetes; T2D). All T1D and many T2D patients require exogenous insulin delivery, but the challenges of managing insulin dosing may lead to poor overall glycemic control. Whole pancreas or pancreatic islet (~ $6–10 \times 10^5$ islets or ~ 10^9 β cells) transplantation is considered to be one of the most effective therapies to treat patients with severe diabetes without the use of exogenous insulin [1–3]. However, it is severely limited by the shortage of

donor organ and the life-long use of immunosuppressive drugs to prevent rejection of the transplanted islets. Human pluripotent stem cells (hPSC), including human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC) can serve as a renewable source of β cells due to their capacity for extensive expansion and commitment to various somatic cell fates.

Step-wise protocols have been reported to differentiate hPSC into insulin-secreting cells, which mimic pancreatic development along the stages of definitive endoderm (DE), primitive gut tube (PGT), pancreatic progenitors (PP), and endocrine precursors (EP) that finally mature into pancreatic endocrine cells (PEC)[4–9]. Most of the studies were focused on promoting the differentiation of ESC into islet cells by using various growth factors or pharmacological molecules that regulate specific signaling pathways [4–9]. But cell-cell and cell-extracellular matrix (ECM) interactions which play vital roles in cell proliferation, differentiation and functional maintenance were often underestimated.

Pancreatic islets are three-dimensional arrangements of cells with intricate cell-cell and cell-ECM interactions. It is important that the culture environment takes into account the spatial organization of the cell. Three-dimensional (3D) cell culture more accurately imitates the in vivo conditions compared to traditional 2D culture, which allows biological cells to grow or interact with their surroundings in all three dimensions[10, 11]. Cell-cell and cell-ECM interactions have been confirmed to be essentially different between 2D and 3D cultures. These differences then resulted in alterations of the molecular pathways that regulate cell behaviors, leading to distinct biological outcomes, such as cell phenotypes and functions[12]. A typical and clinically relevant example of a dimensionality-mediated cell response was reported in 1990[13]. When growing as monolayers, the murine mammary tumor cells did not display the drug-resistant phenotypes that previously had been seen only in vivo. However, cells cultured under 3D conditions recapitulated the drug resistance properties. Recently, 3D cell culture has been increasingly used for stem cell research, in which cell phenotypes needs to be strictly controlled [14, 15].

Studies on islet function have found that intact islets isolated from the body had better insulin release function than dispersed islet cells, and when the dispersed islet cells re-aggregated, the insulin-secreting activity could be restored [16]. Bergsten et al reported that aggregated mouse insulinoma-derived MIN6 cells, which display characteristics of pancreatic β cells, secrete insulin in response to glucose stimulation [17]. These findings suggested that the spherical structure of islets may be associated with the differentiation and maturation of islet cells. It was previously reported that when stem cells were differentiated to insulin-secreting cells, the cells spontaneously aggregated into clusters, and three-dimensional aggregate formation was necessary to generate insulin-producing cells.[18]. Moreover, Suemori et al found that 3D culture plays an important role in the induction of functional INS-expressing cells with insulin secretion from hPSC [19]. Although many studies reported that 3D culture is important for the acquisition of mature insulin-producing cells from hPSC [19–21], none of them made a thorough comparison between 2D and 3D cultures, and the mechanism by which 3D culture promoted β cell maturation is poorly understood.

In this study, we developed a stepwise strategy to differentiate hESC into mature monohormonal PEC using 3D culture in the maturation stage. To make a comprehensive comparison between 2D culture and 3D culture, we examined gene expression, pancreatic-specific markers and functional characteristics of 2D culture-induced PEC and 3D culture-induced PEC. 3D culture significantly increased the pancreatic specification efficiency and enhanced functional maturation of PEC. Furthermore, the mechanisms was discussed from the perspective of cell-cell and cell-extracellular matrix which are fundamentally different between 2D and 3D culture. We showed that 3D culture promoted endocrine commitment by limiting FAK-dependent activation of the Smad2/3 pathway, and enhanced functional maturation of insulin-producing cells by upregulating Cx36 expression.

Methods

Cell Culture. The (hESC line H9 was grown in feeder-free conditions in six-well Nunclon surface plates (Nunc) coated with Matrigel (BD Biosciences) and maintained in mTESR1 media (Stem Cell Technologies). Cells were passaged at a 1:3 ~ 4 ratio using dispase (Invitrogen). All Matrigel plates were coated with a 1:80 dilution in Advanced DMEM-F12 (Life Technologies) and incubated at room temperature for at least 1 hr before use.

Generation of pancreas endocrine cells (PEC) from ESCs. Human ESCs were passaged with Accutase (Sigma) and plated at a density of 100,000 cells/cm^2 in mTeSR with 10 μM Y27632 (Selleck) on RPMI1640 (Gibco), Matrigel (BD) and collagen IV (BD) (5:2:1) mixed gel coated-plate (Corning). In the restriction of definitive endoderm (DE) stage (S1), cells were cultured for 24 hrs in RPMI1640 with B27 supplement (1:50, Gibco), N2 supplement (1:50, Gibco), 100 ng/ml Activin A (R&D) and 50 ng/ml Wnt3a (R&D), and then treated with 100 ng/ml Activin A and 0.2% FBS for 2 days. In the stage (S2) to get primitive gut tube (PGT), the culture medium was replaced with RPMI1640 supplemented with B27 supplement (1:50), N2 supplement (1:50), 30 ng/ml FGF7, 5 ng/ml Wnt3a, 0.75 μM Dosomophine, 2% FBS for 3 days. And in the stage (S3) of pancreatic progenitors (PP), cells were cultured in Advanced DMEM-F12 supplemented with B27 supplement (1:100), 2 μM RA, 0.25 μM Cyclop, 30 ng/ml FGF7, 50 ng/ml Noggin, 0.3 μM IL-5, and 6 μM SB431542 for 6 days. At the end of stage3, media were changed to DMEM (Gibco) supplemented with B27 supplement (1:100), 50 ng/ml Exendin-4, 6 μM SB431542, 50 ng/ml Noggin, and 10 mM Nicotinamide. For 3D culture, cells at stage 3 were digested with Accumax and replated at a density of 3×10^5/ml in ultra-low attachment 6-well plates (Corning), and the plates were placed on a 3D orbital shaker set at rotation rate of 80 rpm in a 37°C incubator, 5% CO_2. Cells were photographed during differentiation using a Nikon phase contrast microscope (Nikon Microscopes).

Quantitative real-time PCR analyses. Total RNA was isolated using an RNeasy extraction kit. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed with SYBR Green real-time PCR master mix (TOYOBO) on a Bio-Rad iQ5 Real-Time PCR detection system (Bio-Rad). The data were analyzed using the delta-delta Ct method. The primers are listed in supplementary **Table S1**.

Immunofluorescent Staining. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature and blocked with 10% goat or donkey serum for 1 h, followed by incubation with primary antibodies at 4 ºC overnight. Labeled isotype-specific secondary antibodies were added and incubated 1 h at room temperature. Cells were counterstained with 4´,6-diamidino-2-phenylindole (DAPI) for visualization of cell nuclei and observed using a confocal microscopy (PerkinElmer) and Volocity Software (PerkinElmer). Antibodies used in this study were summarized in supplementary **Table S2**.

Flow cytometry. Single cell suspensions were obtained by dissociation with Accutase for 3–5 min. Cell surface antigen staining was performed in PBS at 4 ℃. Intracellular staining was performed with the BD Cytofix/Cytoperm™ Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, cells were fixed and permeabilized with BD Cytofix/Cytoperm solution for 20 min at 4 °C. Intracellular antigen staining was performed in BD Perm/Wash solution.

The stained cells were analyzed or sorted with BD FACSCalibur (BD Biosciences), and the data was analyzed using the Flowjo software (TreeStar). The sources and concentrations of primary, secondary antibodies and isotype controls are listed in supplementary **Table S2**.

C-peptide release assay. The pancreatic endocrine cells were used for the C-peptide release assay as previously described. Briefly, after a 1 hour-wash in KRBH medium, 300 µl of basal media that contains 2 mM D-glucose (Sigma) were added to each well of 12-well dishes. After 1 hour incubation, the basal media were changed into 300 µl of stimulation media (20 mM D-glucose or 30 mM KCl). The cultures were incubated at 37 °C in a 5% CO_2 environment for 30 min. For each experiment, 6 wells of supernatants were pooled together and stored at -20 °C until assay, meanwhile the cells were harvested for protein determination using the Bio-Rad Protein Assay K (Bio-Rad) according to the Bradford method. Ultra-sensitive human C-peptide ELISA kit (Mercodia) has been used and the assays done according to manufacturer's instructions.

Transmission electron microscopy. The cell samples were rinsed with PBS and fixed in 3% glutaraldehyde/0.1 M sodium cacodylate, pH 7.4 overnight. Following three rinses with sodium cacodylate buffer, the samples were postfixed for 1 hour in 1% osmium tetroxide/0.1 sodium cacodylate buffer. After rinsing in deionized water, samples were dehydrated and embedded in Polybed 812 epoxy resin (Polysciences, Inc., Warrington, PA). The samples were sectioned perpendicular to the substrate at 70 nm using a diamond knife. Ultrathin sections were collected on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 15 min, followed by Reynolds' lead citrate for 7 min. Samples and stained sections were observed using a H7650 transmission electron microscope (HITACHI) operating at 80 kV (H7650 Electron Microscopy) and photographed using an AMT XR16M CCD Digital Camera and AMT Capture Engine Software Version 600.259 (AMT).

Western blotting. Cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.25 mM sodium-deoxycholate, 150 mM NaCl, 2 mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100) containing protease and phosphatase inhibitors (Roche). Lysates were sonicated for 30 s, maintained on ice for

30 min, and then spun at 15,000 rpm for 15 min at 4 ˚C. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with antibodies listed in supplementary **Table S2**. Proteins were detected by enhanced chemiluminescence HRP substrate (Millipore).

Statistics. Data are shown as means and SEMs. For most statistic evaluation, 2-tailed Student's t test was applied for calculating statistical probability in this study. *P* values less than 0.05 were considered to be statistically significant. For all statistics, data from at least three independent samples or repeated experiments were used.

Results

Generation of pancreatic endocrine cells from human embryonic stem cells

Our strategy to PEC from hESC in vitro is outlined in Fig. 1A. A stepwise four-stage protocol modified from previous studies[4, 6] was used to induce hESCs differentiation through the stages of DE, PGT, PP and EP to yield PEC, with the first three stages in monolayer culture and the last stage in 3D culture (Fig. 1B). SRY (sex determining region Y)-box 17 (SOX17)- and forkhead box protein A2 (FOXA2)-positive definitive endoderm (DE) was efficiently induced in the first three days during stage 1, with high expression levels of EpCAM and CXCR4 (Fig. 1C and Figure S1). Pancreatic-specific transcriptional factors pancreatic and duodenal homeobox 1 (PDX1), NK6 homeobox transcription factor-related locus 1 (NKX6.1) and Neurogenin 3 (NGN3) were significantly increased in PP, over 95% of which co-expressed PDX1 and NKX6.1 (Fig. 1C and Figure S1). In addition, flow cytometry analysis showed that more than 68% PP expressed CD142 —— a surface marker used for enrichment of pancreatic endoderm cells, which was much higher than previously reported[21, 22] (Figure S2). In stage 4, when continuing 2D culture, large numbers of cell clusters emerged from the underline monolayer cells (Fig. 1B), resembling normal pancreatic islet topologically. In order to mimic pancreatic islet development, we dissociated PP from stage 3 into single cells and replated them in ultra-low attachment cell culture plates for 3D culture. The cells in suspension self-assembled to form three-dimensional clusters with diameters ranging from 100 to 400 μm (Fig. 1B). Endocrine cell-specific transcription factors paired box 6 (PAX6) and ISL LIM homeobox 1 (ISL1) and hormones Insulin (INS), C-peptide (C-pep), Glucagon (GCG) and Somatostatin (SST) were induced in EP and PEC from both 2D and 3D culture (Fig. 1C and Figure S1). Overall, human pluripotent stem cells could be differentiated into pancreatic endocrine cells stepwise following our four-stage protocol.

3D culture promoted maturation of pancreatic endocrine cells

To investigate the effect of 3D culture on pancreatic differentiation, PEC induced under 2D and 3D culture were compared at different levels. qRT-PCR analysis showed that the mRNA expression level of transcription factors *PDX1*, *NKX6.1*, *NGN3*, *ISL1*, *PAX6*, as well as hormones *INS*, *GCG*, *SST* were significantly higher in 3D culture-induced PEC (Fig. 2A). Immunostaining of pancreatic hormones illustrated that many 2D culture-induced PEC co-expressed INS and GCG or INS and SST, and some cells even expressed all three hormones, resembling the expression pattern in primary fetal islets (Fig. 2B). Nevertheless, the three hormones were mostly expressed in different endocrine cells induced under 3D culture, with a large proportion of monohormonal INS-expressing β cells and moderate percentage of monohormonal GCG-expressing α cells. Although the percentage of SST-positive cells was slightly high, the hormone expression pattern in 3D culture-induced PEC was more like that in primary adult islets (Fig. 2B). Moreover, the percentage of INS-expressing cells was much higher in 3D culture-induced PEC than that of 2D culture-induced PEC (Fig. 2C). Taken together, these data highlighted the difference between PEC derived from 2D culture and 3D culture and suggested that 3D culture promoted endocrine cells maturation.

Enhanced β cell function in 3D culture-induced PEC

The β cells in the islet can be specifically labeled with the zinc-chelating dye dithizone (DTZ) owing to the presence of zinc in insulin-containing secretory granules[23], therefore, DTZ staining can efficiently stain INS-expressing regions of pancreatic endocrine cells in the cultures. We observed discrete areas of DTZ staining in 2D culture and much darker DTZ staining in 3D culture-induced PEC clusters (Fig. 3A). Analysis of ultrastructure by transmission electron microscopy (TEM) revealed that PEC induced under 3D culture contained a high density of endocrine granules (Figure S3). As previously reported, three types of insulin granules were generally observed in mature human beta cells by TEM: (i) light gray, diffuse core, (ii) dense, round core, and (iii) dense, rod-shaped core with a crystalline appearance[8]. Notably, we observed examples of each type of insulin granule in hESC-derived PEC under 3D culture (Figure S3).

To confirm the de novo synthesis and release of insulin by hESC cell–derived β cells, we monitored the release of C-peptide into the culture medium in response to high glucose and stimulus (Fig. 3B). Under basal glucose conditions, PEC induced under 3D culture released over four times more C-peptide than 2D culture-induced PEC. Moreover, we observed a 2-fold induction of human C-peptide from 3D culture-induced PEC exposed to high glucose over the course of a 1-h incubation, while 2D culture-induced PEC did not response sensitively to high glucose. Direct depolarization of the cells by addition of potassium chloride (KCl) resulted in increased C-peptide secretion in 3D culture-induced PEC during 1-h incubation. Together, these data suggested that PEC induced under 3D culture were capable of producing appropriately packaged insulin granules. Moreover, 3D culture-induced PEC could respond more sensitively to glucose and stimuli than 2D culture-induced PEC, which indicated that 3D culture enhanced functional maturation of hESC-derived β cells.

3D culture might promote endocrine specification by inhibiting FAK-dependent activation of the Smad2/3 pathway

As opposed to conventional 2D monolayer culture, 3D culture is thought to mimic the natural environment found in vivo, allowing cells to interact with each other, the extracellular matrix (ECM), and their microenvironment. Integrins constitute the primary set of receptors for ECM components, communicating with the intracellular cytoskeleton and transmission of growth factor signals[24]. A central regulator of integrin signaling is focal adhesion kinase (FAK), a ubiquitously expressed nonreceptor tyrosine protein kinase, which gets activated by integrin ligation and clustering[25]. FAK signaling alters the association between cells and the underlying extracellular matrix, which in turn can have profound consequences for anchorage-dependent growth and differentiation[26, 27]. We noticed that phosphorylated FAK was nearly undetectable in 3D culture-induced PEC, in contrast to high FAK phosphorylation level in PEC under 2D culture (Fig. 4A). Therefore, we further determined whether pharmacological inhibition of FAK would promote endocrine specification under 2D culture. PF-228 is a small compound inhibitor that selectively inhibits FAK catalytic activity by blocking phosphorylation at Tyr-397[28]. PEC induced under 2D culture were treated with or without PF-228 for 48 hours on day 9, and its impact was evaluated 3 days later. Western blot showed that the FAK inhibitor PF-228 abolished the phosphorylation of FAK, as expected. Importantly, the mRNA expression of proendocrine transcription factor *NGN3*, β cell-specific transcription factors *NKX6.1* and *ISL1*, and *INS* were elevated in the presence of PF-228 (Fig. 4B). Together these data suggested that 3D culture promoted endocrine specification by inhibiting FAK activation.

Inhibitors of TGFβ-dependent Smad2/3 activation have been shown to induce the endocrine commitment of hESCs derivatives[6]. And it was confirmed that inhibition of SFK/FAK signaling promoted endocrine specification of human embryonic stem cell derivatives by limiting activation of the TGFβR/Smad2/3 pathway[29]. We observed sharp decrease of SMAD2 expression in 3D culture-induced PEC, and confirmed that the presence of PF-228 in 2D culture significantly downregulated SMAD2 expression (Fig. 4C). Based on these observations, we concluded that 3D culture may promote endocrine commitment by limiting FAK-dependent activation of the Smad2/3 pathway.

3D culture might enhance β cell function by regulating connexin 36

As described above, 3D culture not only improved the pancreatic differentiation efficiency, but also enhanced insulin secretory response to glucose compared with 2D monolayer culture. Cell–cell coupling mediated by gap junctions formed from connexin contributes to the control of insulin secretion in the endocrine pancreas. Insulin-secreting β cells within the pancreatic islet are exclusively coupled by Cx36 gap junctions in mice, and strongly coupled by Cx36 gap junctions in humans[30]. It was reported that

adult β cells, which respond to glucose, expressed significantly higher levels of Cx36 than fetal β cells, which respond poorly to the sugar[31, 32]. We confirmed that INS-expressing cells in human adult β cells expressed much higher levels of Cx36 protein than those in human fetal β cells by immunostaining (Fig. 5A). And we observed that Cx36 was expressed in INS-expressing cells in late maturation stage (day 27), but not expressed in the early stage of differentiation, though some cells already expressed INS (Fig. 5A). Moreover, Cx36 expression is much higher in 3D culture-induce PEC than 2D culture-induced PEC as determined by qRT-PCR and westernblot, suggesting that 3D culture might enhance glucose responsiveness through promoting Cx36 expression (Fig. 5B and 5C). Furthermore, treatment of PF-228 in 2D culture increased Cx36 expression at both mRNA level and protein level, indicating that FAK signaling pathway was involved in Cx36 regulation (Fig. 5B and 5C). Taken together, our data suggested that 3D culture might regulate Cx36 expression by inhibiting FAK pathway, thus promoting β cell maturation.

Discussion

Cells naturally grow, differentiate and mature in a three dimensional environment. 3D cell culture model can almost perfectly mimic in vivo cells behaviors and organization, therefore using 3D culture is an accurate way to reproduce this process in vitro. We established a four-stage differentiation method that induced PEC from hESC at high efficiency, with the first three stages in monolayer culture and the last maturation stage in 3D culture. Comparison between 2D culture-induced PEC and 3D culture-induced PEC showed that expression of pancreatic-specific transcription factors *PDX1*, *NKX6.1*, *NGN3*, *ISL1*, *PAX6* and endocrine hormones *INS*, *GCG*, *SST* were significantly increased in 3D culture-induced PEC. Importantly, 3D culture gave rise to monohormonal endocrine cells, while 2D culture-induced PEC co-expressed INS and GCG or INS and SST, and some cells even expressed all three hormones. Bruin et al. demonstrated that hESC-derived polyhormonal insulin-expressing cells lacking the mechanism to transport glucose into the cell, because the glucose transporter was transcribed but not translated[33]. Also, hESC-derived polyhormonal insulin-expressing cells only displayed mild K^+ channel activity, which did not appear to be mediated by functional K_{ATP} channels[33–35]. Additionally, the processing of proinsulin to form mature insulin hormone was hindered in polyhormonal insulin-expressing cells as a result of lack of prohormone convertases expression[33, 36, 37]. The defects in glucose transporter expression, K_{ATP} channel function and prohormone processing enzymes may contribute to the lack of glucose-responsiveness in hESC-derived polyhormonal insulin-producing cells. Consistent with these studies, our 3D culture-induced PEC could respond more sensitively to glucose and stimuli than 2D culture-induced PEC. These data suggested that 3D culture promoted functional maturation of pancreatic endocrine cells.

3D cell cultures mimic the specificity of native tissue with greater physiological relevance than conventional 2D, because they establish physiological cell-cell and cell-ECM interactions. The integrin receptors play a major role in tissue morphogenesis and homeostasis by regulating cell interactions with ECM proteins. The expression of integrin receptors in the human fetal pancreas play multiple roles in islet cell biology, including adhesion, function, and survival[38]. FAK represents a crosstalk point for integrin signaling. It was reported that inhibition of SFK/FAK signaling potentiates endocrine differentiation by

inhibiting the TGFβR/Smad2/3 pathway[29]. Previous studies have shown that pharmacological inhibitors that target the TGFβ type I receptor ALK5 (ALK5 inhibitor II) or ALK5 and its relatives ALK4 and ALK7 (SB431542) promoted the endocrine specification of hESC derivatives[6] and the subsequent derivation of insulin producing β-cells [39]. We observed lower FAK phosphorylation level and decreased SMAD2 expression in 3D culture-induced PEC, compared to PEC under 2D culture. In the presence of FAK inhibitor PF-228 in 2D culture, FAK phosphorylation was abolished and SMAD2 expression was downregulated. Furthermore, inhibition of FAK by PF-228 in 2D culture increased the expression of *NGN3*, *NKX6.1*, *ISL1* and *INS*, suggesting 3D culture may promote endocrine commitment by limiting FAK-dependent activation of the Smad2/3 pathway.

Insulin secretion and most other functions of pancreatic islets are multicellular processes, which allow for a rapid regulation of hormonal secretion in order to match the changing levels of circulating glucose. The insulin-producing β-cells of pancreatic islets are connected by a large number of small gap junction plaques, which ensure cell-to-cell coupling via Cx36 gap junctions[40]. Glucose stimulation induces much stronger secretory and metabolic responses from either intact pancreatic islets or clusters of islet cells, than those of single β-cells[41, 42]. In our study, Cx36 expression is much higher in 3D culture-induce PEC than 2D culture-induced PEC, which, in turn, leading to better coupling between pancreatic β-cells to synchronize the activity of individual cells. In addition, previous studies demonstrated that changes in Cx36 resulted in altered expression of specific β-cell genes that played key roles in the glucose-induced insulin secretion[43, 44]. And we also found that treatment of PF-228 in 2D culture increased Cx36 expression. Collectively, our result indicated that 3D culture might enhance glucose responsiveness through promoting Cx36 expression, and FAK signaling pathway was involved in Cx36 regulation.

Conclusion

In conclusion, we developed a differentiation strategy to induce monohormonal mature PEC from hPSC with the last maturation step in 3D culture. In particular, 3D culture increased the differentiation efficiency and promoted the functional maturation of hESC-derived PEC. Moreover, we discussed the mechanism and found limited FAK-dependent activation of the Smad2/3 pathway and unregulated expression of Cx36 in 3D culture-induced PEC, indicating that 3D culture promoted endocrine specification of hESC through comprehensive modulation of cell-cell and cell-ECM interactions. Our method might provide a new platform for in vitro anti-diabetic drug discovery and characterization for human metabolism and diabetes. On the other hand, small compound inhibitors which can enhance the derivation of β-cells prior to transplantation should help to bring us closer to a universal cell-based therapy for diabetes.

Abbreviations

hPSC: human pluripotent stem cells; 2D: two-dimensional; 3D: three-dimensional; hESC: human embryonic stem cells; PEC: pancreatic endocrine cells; DE: definitive endoderm; PGT: primitive gut tube; PP: pancreatic progenitors; EP: endocrine precursors; ECM: cell-extracellular matrix; Cx36: connexin 36;

T1D: type 1 diabetes; T2D: type 2 diabetes; DTZ: dithizone; TEM: transmission electron microscopy; FAK: focal adhesion kinase.

Declarations

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Authors' contributions

XL was responsible for conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. JQ was responsible for collection and/or assembly of data, data analysis and interpretation, manuscript writing. MC and SW was responsible for technical support, ESC culture and differentiation. YL reviewed the manuscript. XP reviewed the manuscript. YW was responsible for conception and design, manuscript writing and final approval of manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Ethical approval and consent to participate

Human pancreata were obtained from patients in the Chinese PLA General Hospital with patient consent. The study was approved by the academic committee of the Institute of Health Service and Transfusion Medicine and the ethics committee of the PLA General Hospital.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Figures

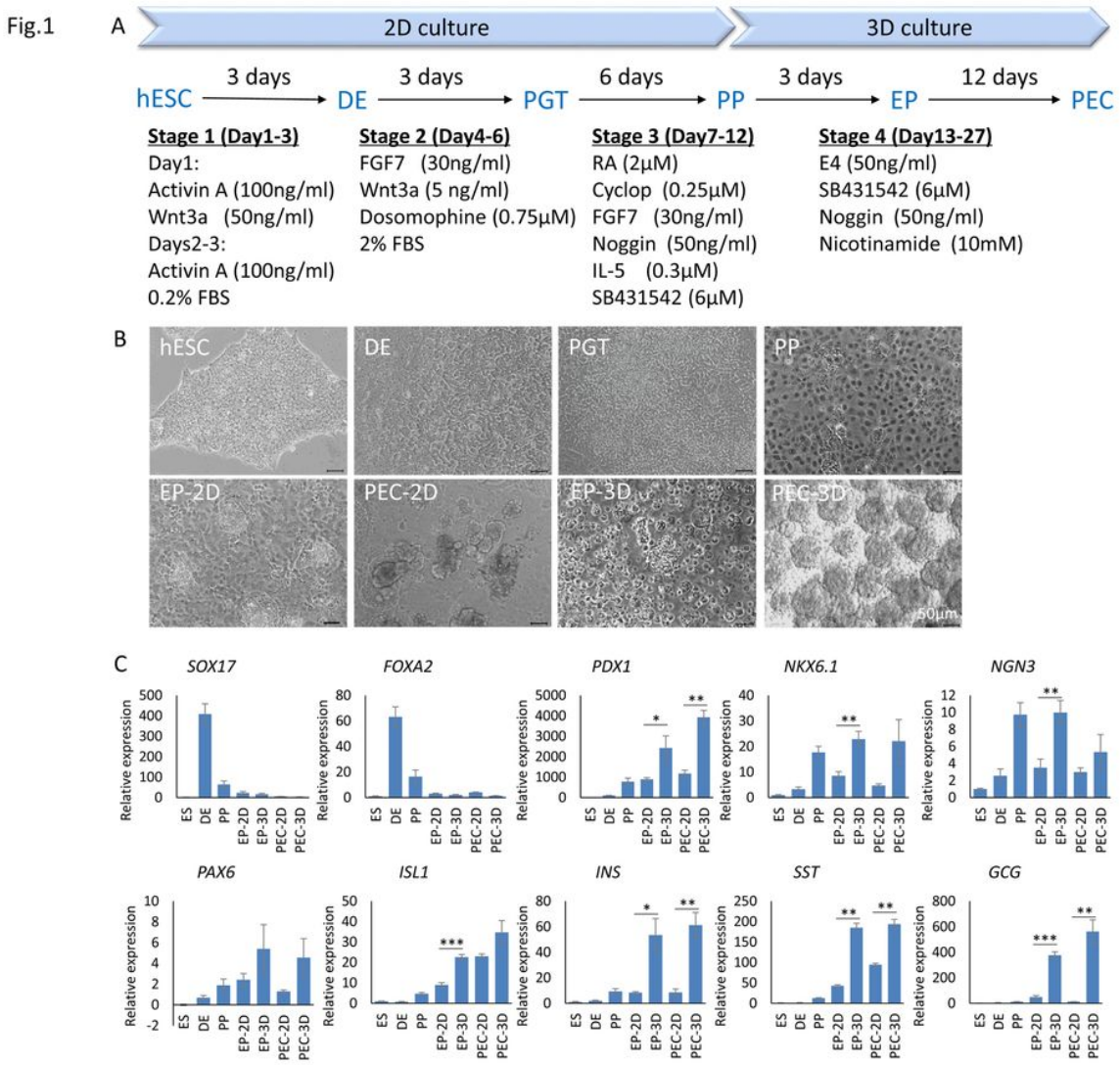

Figure 1

Differentiation of human embryonic stem cells to pancreatic endocrine cells. (A) Schematic overview of the protocols used for the differentiation of human embryonic stem cells (hESC) into pancreatic endocrine cells (PEC). hESC are differentiated through the stages of definitive endoderm (DE), primitive gut tube (PGT), pancreatic progenitors (PP) and endocrine precursors (EP) to yield PEC using a 4-stage protocol. (B) Representative morphology of cells at different stages. Scale bars, 50μm. (C) qRT-PCR analysis of cells at different stages. Data represent mean ± SEM (n=3), *p < 0.05, **p < 0.01,***p < 0.001.

Fig.2

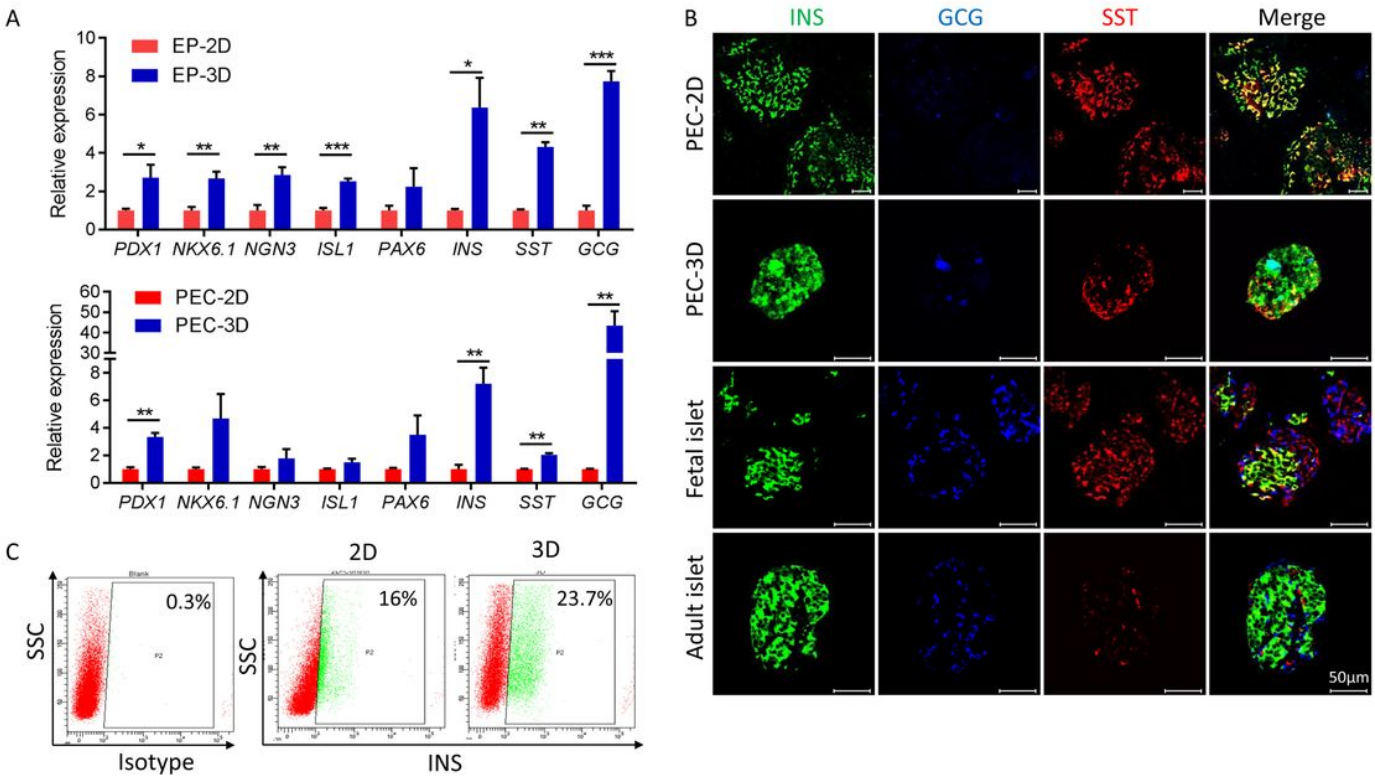

Figure 2

Comparison of pancreatic endocrine cells derived from 2D and 3D culture. (A) qRT-PCR analysis of PEC induced under 2D or 3D culture conditions. (B) Flow cytometry analysis of INS in PEC induced under 2D or 3D culture conditions. (C) Immunostaining of Insulin (INS), Glucagon (GCG) and Somatostatin (SST) in PEC induced under 2D or 3D culture conditions and fetal or adult islets. Data represent mean ± SEM (n=3), *p < 0.05, **p < 0.01,***p < 0.001.

Fig.3

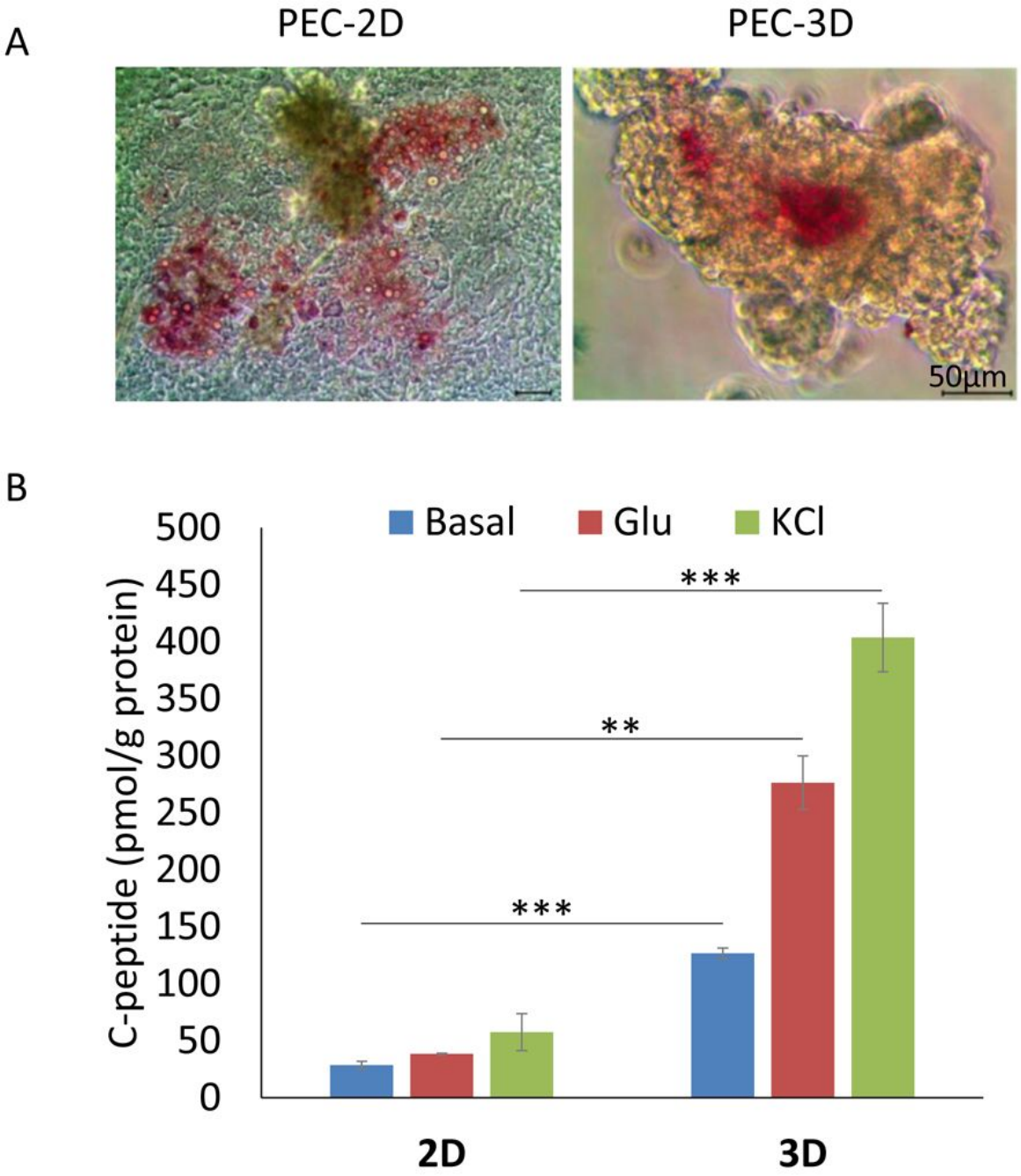

Figure 3

3D culture enhanced function maturation of insulin-producing cells. (A) Representative images of endocrine cells stained with dithizone. Scale bars, 50μm. (B) Secreted C-peptide in response to high (20 mM) concentrations of D-glucose or 30mM potassium chloride (KCl) was measured with a C-peptide ELISA kit. Data represent mean ± SEM (n=3), **p < 0.01,***p < 0.001.

Fig.4

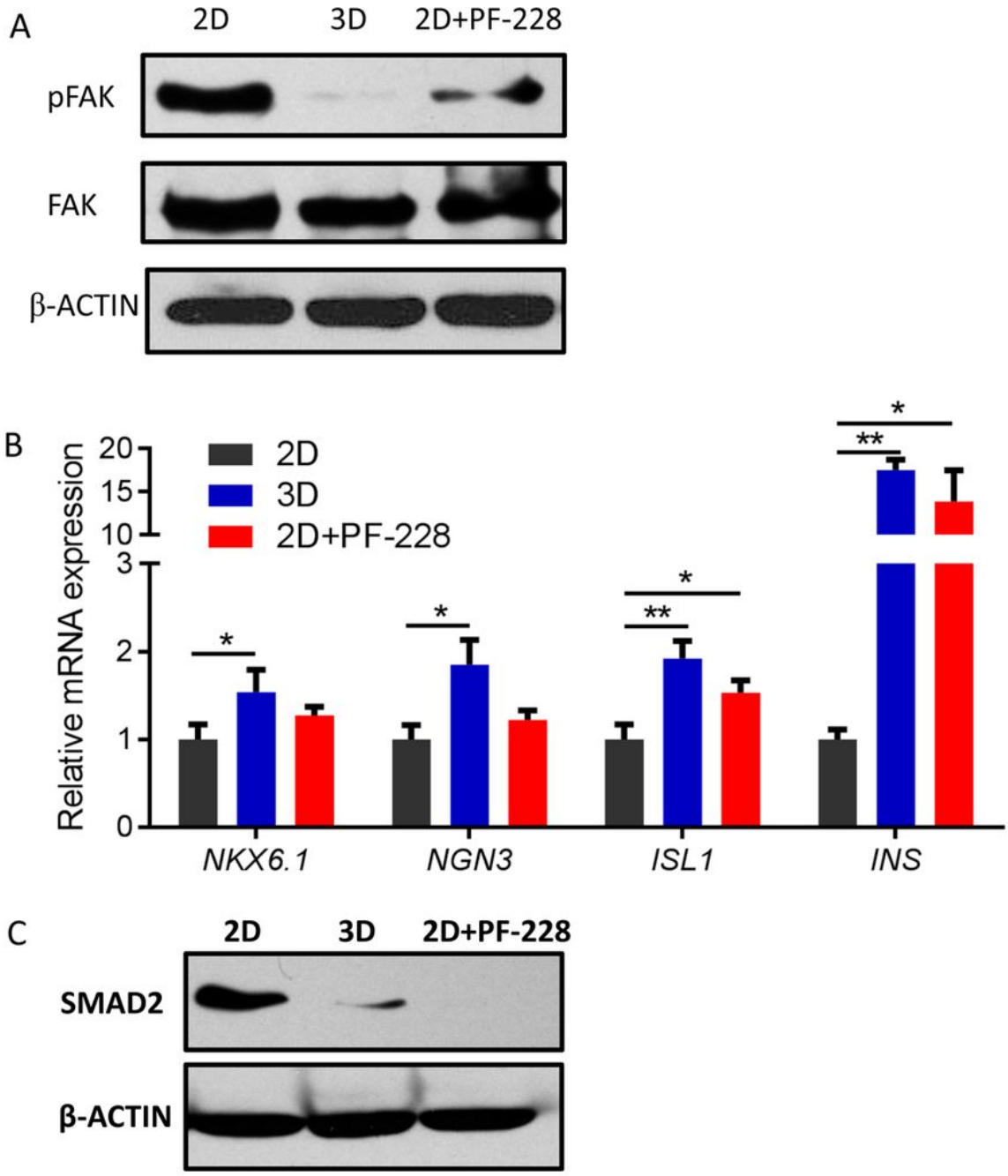

Figure 4

Inhibition of FAK signaling and TGFβ signaling was involved in 3D culture. (A) Western blot showing pFAK levels in PEC induced under 2D culture, 3D culture and 2D culture treated with PF-288. PF-228 was added on day 9, and the lysates were generated 48 h later. The membranes were probed with pAbs specific for pFAK (Tyr-397) or total FAK. (B) qRT-PCR analysis of PEC induced under 2D in the presence or

absence of PF-228. (C) Western blot showing SMAD expression levels in PEC induced under 2D culture, 3D culture and 2D culture treated with PF-288. Data represent mean ± SEM (n=3), *p < 0.05, **p < 0.01.

Fig.5

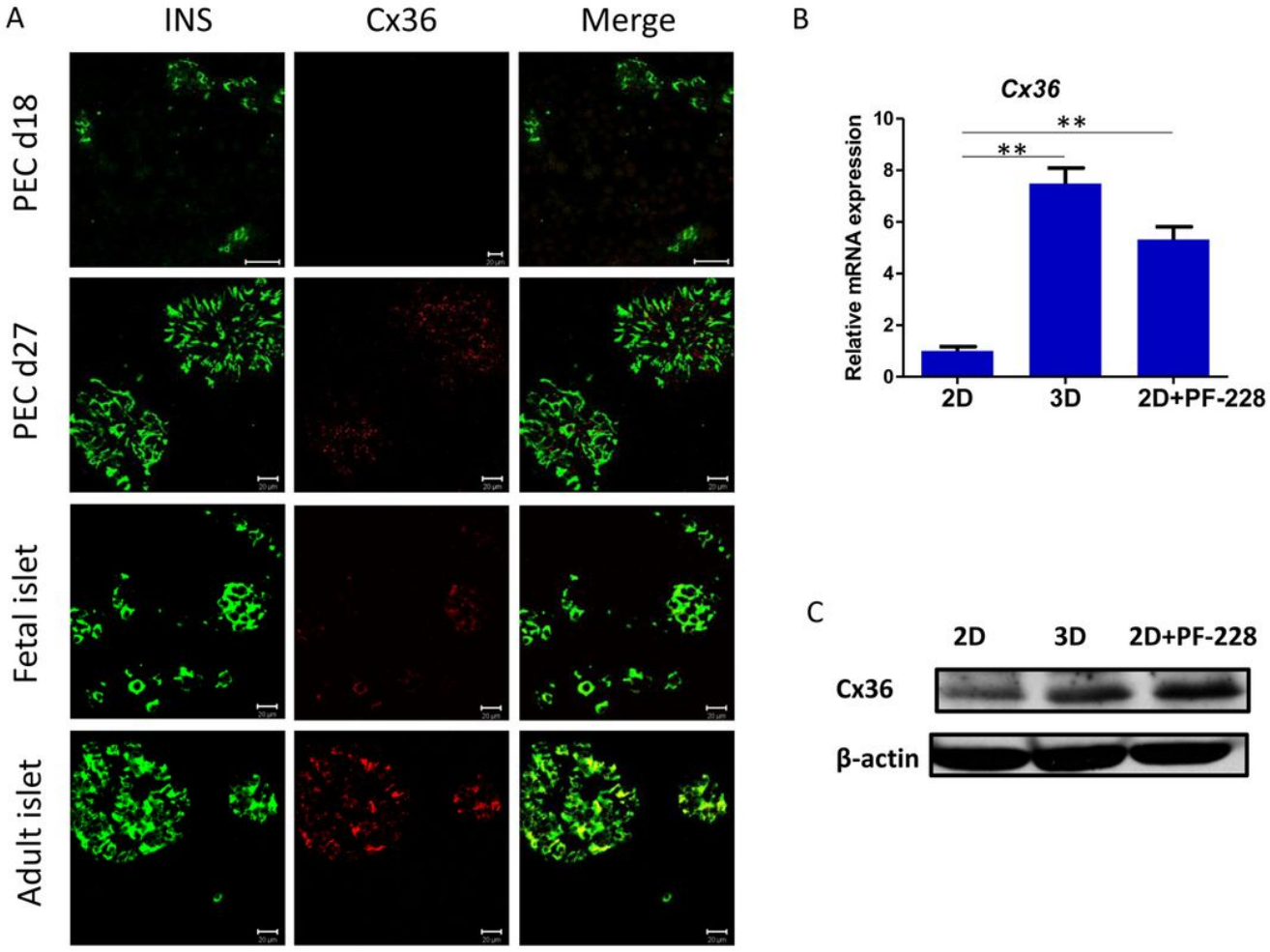

Figure 5

Upregulation of Cx36 in 3D culture. (A) Immunostaining of INS and Cx36 in ESC-derived PEC, fetal and adult islets. Scale bar, 20 μm. (B) qRT-PCR analysis of Cx36 in PEC induced under 2D culture, 3D culture and 2D culture treated with PF-288. (C) Western blot showing Cx36 expression levels in PEC induced under 2D culture, 3D culture and 2D culture treated with PF-288. Data represent mean ± SEM (n=3), **p < 0.01.

Supplementary Files

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