Endothelial Cells Mediate Islet-Specific Maturation of Human Embryonic Stem Cell-Derived Pancreatic Progenitor Cells

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It is well recognized that *in vitro* differentiation of embryonic stem cells (ESC) can be best achieved by closely recapitulating the *in vivo* developmental niche. Thus, implementation of directed differentiation strategies has yielded encouraging results in the area of pancreatic islet differentiation. These strategies have concentrated on direct addition of chemical signals, however, other aspect of the developmental niche are yet to be explored. During development, pancreatic progenitor (PP) cells grow as an epithelial sheet, which aggregates with endothelial cells (ECs) during the final stages of maturation. Several findings suggest that the interactions with EC play a role in pancreatic development. In this study, we recapitulated this phenomenon in an *in vitro* environment by maturing the human ESC (hESC)-derived PP cells in close contact with ECs. We find that co-culture with different ECs (but not fibroblast) alone results in pancreatic islet-specific differentiation of hESC-derived PP cells even in the absence of additional chemical induction. The differentiated cells responded to exogenous glucose levels by enhanced C-peptide synthesis. The co-culture system aligned well with endocrine development as determined by comprehensive analysis of involved signaling pathways. By recapitulating cell–cell interaction aspects of the developmental niche we achieved a differentiation model that aligns closely with islet organogenesis.

Introduction

EMBRYONIC STEM CELLS (ESCs) are pluripotent cells that can be propagated in an undifferentiated state indefinitely making them a desirable source of cells for transplantation.¹ These cells can be guided to differentiate into virtually any cell and tissue type by providing appropriate cues in a directed differentiation approach.² In the context of pancreas, directed differentiation consists of stage-wise induction through events known to take place during pancreatic development, beginning with definitive endoderm (DE) formation. This is typically achieved by modulation of the nodal pathway through Activin A³ or more recently, small molecules such as IDE1 and IDE2⁴; Supplementing nodal activity by modulating alternative pathways such as WNT3A⁵ or PI3K inhibition⁶ further enhances DE induction. DE induction is followed by pancreatic progenitor (PP) commitment, marked by the appearance of PDX1, which is the diverging point between pancreatic progression and development of other DE-derived tissues.³ It is well known that appearance of PDX1 is associated with sonic hedgehog (SHH) inhibition during pancreatic development, therefore can be achieved through addition of cyclopamine in an *in vitro* setting.⁷ These PP cells are directed toward en-

docrine progenitors by addition of retinoic acid.⁸ Finally, NEUROG3-expressing endocrine progenitors are matured toward β -cells through different mechanisms including notch inhibition, found during pancreatic development,⁹ and GLP-1 activation, which has been demonstrated to promote regeneration of β -cells through proliferation of already mature β -cells and transdifferentiation of ductal PP cells.¹⁰

Several studies, including previous work in our lab,¹¹ have used this information to develop directed differentiation protocols^{5,6} to yield pancreatic islet-like cells from human ESC (hESC). Many of these existing protocols result in high yield of PP cells. These cells also have the potential for functional maturation upon implantation in diabetic mice models.¹² However, maturing these cells into functional islet-like cells in an *in vitro* setting is yet to be demonstrated.

Organogenesis is a complex and dynamic process involving signals from several parallel inputs including chemical, mechanical, and from contact with neighboring cells. While there is an increasing trend to recapitulate the entire micro-environmental niche, most of the existing protocols use modulation of individual pathways through targeted molecules and growth factors.¹³ In this report, we are presenting an alternate strategy for achieving islet-specific maturation of hESC-derived PP cells. We hypothesize signaling

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from endothelial cells (ECs) during final stages of hESC differentiation will induce islet-specific maturation of the hESC-derived PP cells. This hypothesis is inspired by pancreatic organogenesis, where pancreas and aorta develop in close proximity¹⁴ with considerable crosstalk between these cell types.¹⁵ At several stages of pancreatic development, proximal mesodermal cell types produce signals that play a role in pancreatic differentiation; signaling from blood vessels has been shown to establish the pancreatic bud.¹⁶ ECs have also been implicated in maintenance of PDX1 expression and induction of PTF1 expression in addition to insulin and glucagon expression.^{16,17} In addition to interactions of endothelial and pancreatic cells during development, ECs have also been implicated to increase functionality and survival of β -cells *in vitro*. A recent study¹⁸ showed that rotational culture with ECs increase insulin secretion of human β -cells. Similarly, ECs have been shown to support rat β -cell function hypothesized to be mediated by laminins.¹⁹ Additionally, in a 3D co-culture system rat insulinoma cells demonstrated to have improved insulin secretion when cultured in close proximity of EC.²⁰ These co-culture systems were developed to effectively recapitulate the contributions from diverse cell types constituting native tissues; some of these interactions are not yet understood with sufficient clarity to allow rational reproduction via tissue engineering scaffolds and soluble factors.

In the current study, we recapitulated cell–cell interactions between EC and hESC-derived PP cells in an *in vitro* environment. We find that co-culture with different EC (but not fibroblast) results in pancreatic islet-specific differentiation of hESC-derived PP cells without additional chemical induction. The cells further demonstrated response to exogenous glucose levels by enhanced C-peptide synthesis. Finally, analysis of a comprehensive database of signaling pathways suggests that our co-culture system aligned well with endocrine development and we suggest possible mechanisms involved in the observed phenomenon.

Materials and Methods

hESC maintenance. H1 hESC (WiCell) were maintained in feeder-free conditions as previously described.²¹ EC (VEC Technologies) at passages lower than 10 were maintained using MCDB-131 complete (VEC Technologies). GFP-tagged NIH3T3 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM):F12 supplemented with 10% fetal calf serum.

Differentiation. Once hESC reached an average colony size of 1 mm, DE induction media was added for 4 days. After 4 days media was replaced with PP media for 2 days. After 2 days, all-trans retinoic acid was added to the PP media for 2 additional days. Media was then replaced with maturation media for 2 days, after which ECs were harvested and added to hESC-PP in MCDB-131 media overnight. The same media was added to both N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT) control and co-culture media controls. The next day co-culture media or DAPT control media were added for up to 10 days. Media formulations are included in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/tea).

Quantitative polymerase chain reaction. Quantitative polymerase chain reaction (qPCR) was performed as previously described.²² A list of the primers used can be found in the Supplementary Table S2.

Immunocytochemistry. Cells were fixed, permeabilized, and blocked followed by primary and secondary antibody incubation. Nuclear staining was performed by incubation with Hoechst. Pictures taken using Olympus IX81 inverted microscope and Metamorph imaging software. Antibodies and dilutions found in Supplementary Table S3.

Flow cytometry. Flow cytometry was performed as previously described.²³ As a control for nonspecific staining, cells were incubated in secondary antibody only. Cells analyzed using an Accuri C6 flow cytometry instrument. Antibodies and concentrations can be found in the Supplementary Table 3.

Quantification of intracellular C-peptide. Cells were exposed to low glucose (2.5 mM) or high glucose (25 mM) in Krebs ringer buffer for 4 hours. After glucose stimulation, cells were harvested and lysed using Bio-Plex lysing system according to manufacturer's instructions. C-peptide and glucagon were quantified with Bio-Plex Pro Human Diabetes kit following the product manual and analyzed using the Magpix Luminex system.

Statistical analysis. Every result is reported as an average of five to seven independent experiments. Error bars correspond to standard error and *p*-values were obtained using the Wilcoxon rank-sum test.

Partial least squares regression. We performed partial least squares regression (PLSR) to find correlation between mature markers and the signaling pathways genes from the PCR array. The mature markers were chosen as the output, *Y*, and the signaling pathway genes as the predictors, *X*. The samples chosen contained both co-culture and transwell configurations and the data were normalized with respect to the control sample. From the signaling pathway genes, only those that showed greater than or equal to twofold up- or downregulation were chosen. These data were log2 transformed and the analysis was performed using *plsregress* option from MATLAB (R2010b; Mathworks). The quality of the regression was acceptable for most of the markers with $R^2 > 0.8$.

Ingenuity pathway analysis. Focus genes were analyzed using Ingenuity Pathway Analysis Tool (Version 7.6; Ingenuity® Systems) to identify the biological functions, signaling pathways, and upstream regulators that were differentially regulated under EC co-culture. A threshold of twofold was used for filtering and selection of focus genes. The top networks and the biological functions were analyzed for these focus genes. The networks were assessed for their significance using the score function (*z*) with probability of random placement of the gene given as 10^{-z} . The biological functions were ranked using the significance score obtained from Benjamini-Hochberg (B-H) multiple testing correction method [values presented as $-\log(B-H \text{ } p\text{-value})$]. The upstream regulators that may result in the gene expression

changes observed in the dataset were tested using overlap p -values obtained from Fischer's exact test. Also, the state of activation/inhibition was quantified using an activation z -score. The z -score signifies the consistency in the direction of increase or decrease of all the downstream genes associated with the upstream regulator. z -Score greater than 2 or less than -2 are taken as significant.

Results

Multi-stage directed differentiation protocol

hESCs were differentiated following a multi-stage directed differentiation protocol as described in Figure 1A. The first stage involved DE induction by exposure to Activin A and inhibition of PI3K signaling by Wortmannin. These treatments lead to significant cell death, particularly on the first day. Surviving cells, however, rapidly proliferated with cell mass being recovered by day 4 of treatment.

At the end of DE induction we observed upregulation of DE markers CXCR4, SOX17 and *FOXA2* by qPCR (Fig. 1B). Protein expression was further confirmed by immunostaining, which showed strong nuclear expression of SOX17 (Fig. 1C). Flow cytometry analysis of *FOXA2* showed 90% of cells positive for *FOXA2* stain (Fig. 1D). Taken together, these results confirm DE induction by treatment with Activin A and PI3K inhibition.

The second stage of our protocol consisted of PP induction by SHH inhibition by exposure to Cyclopamine followed by SHH inhibition and retinoid signaling. At the end of this stage, cells were harvested and analyzed for PP markers. qPCR analysis confirmed high upregulation of PP markers, particularly *PDX1*, *HLXB9*, and *ISL1* (Fig. 1E), which showed roughly 21,000-, 800-, and 9,500-fold upregulation over undifferentiated cells respectively. *PDX1* expression was confirmed at the protein level both by immunocytochemistry and flow cytometry. Staining revealed

FIG. 1. Directed differentiation of human embryonic stem cell (hESC) to pancreatic progenitor (PP) cells. (A) Schematic representation of multi-stage differentiation protocol. Definitive endoderm (DE) was induced by modulation of nodal pathway and simultaneously inhibition of PI3K pathway. PP was achieved by sonic hedgehog (SHH) inhibition along with retinoid signaling. Maturation was induced by endothelial cell (EC) co-culture. (B) Quantitative polymerase chain reaction (qPCR) result for DE markers at the end of DE induction. (C) Representative immunocytochemistry for SOX17 and (D) flow cytometry for *FOXA2*. (E) qRT-PCR for PP markers at the end of PP induction. (F) Representative immunocytochemistry and (G) flow cytometry for *PDX1*. Scale bar: 25 μ m. qRT-PCR, quantitative reverse transcriptase polymerase chain reaction. Color images available online at www.liebertpub.com/tea

strong nuclear expression of PDX1 in a large number of the cells (Fig. 1F) and flow cytometry analysis for PDX1 confirmed 78% of the differentiated cells positive for PDX1 (Fig. 1G). These results confirm hESC differentiation into PP cells by exposure to cyclopamine and retinoic acid following DE induction.

ECs mediate islet-specific maturation of hESC-derived PP cells

The PP cells obtained by the protocol mentioned above were next subjected to the maturation protocol. The hESC-derived PP cells were exposed to nicotinamide containing media for 2 days, after which rat heart microvascular endothelial cells (RHMVEC) were directly added to the differentiating population of hESCs to establish a contact co-culture configuration. Since a contact co-culture requires culturing both the differentiating hESCs and the ECs in the same media, it is critical to develop a defined media to support both hESC differentiation and sustain EC survival. RHMVEC are commonly cultured in fetal bovine serum containing media while our differentiation protocol is entirely serum-free. To maintain completely serum-free condition, the differentiation media was supplemented with growth factors that induce RHMVEC survival including epidermal growth factor and crude fibroblast growth factor

(EndoGro) (henceforth referred to as co-culture media). Despite this, RHMVEC survival was limited with considerable cell death observed within 5 days of culture. Figure 2A shows DiI-Ac-LDL-labeled RHMVEC in co-culture with the hESC-derived cells. It is observed that while many of the RHMVEC attach to the empty spots in the plate, a significant portion of the cells are in direct contact with the hESC-derived PP cells. This was further confirmed by immunostaining, which shows Von Willebrand factor (VWF)-positive EC in direct contact with PDX1-positive PP cells (Fig. 2B).

Having established the co-culture system, we next evaluated the effect of co-culture on the differentiating population of hESC-derived PP cells. As described above, the ECs are added to the hESC after the PP stage at a 1:1 ratio and maintained in the co-culture media until RHMVEC viability decreases (around 5 days). At that point the media was switched to regular hESC maturation media, without the EC supplements, and culture was continued for 5 more days to allow for complete depletion of EC. EC depletion was confirmed by absence of DiI-Ac-LDL staining and same media switch was performed on DAPT and control cultures. Interestingly, we found that the RHMVEC survived longer when cultured in the presence of hESC-derived PP than when cultured alone (data not shown). Before complete depletion, RHMVEC were stained for EC-specific markers

FIG. 2. ECs induce maturation of hESC-derived PP into *INS*-expressing cells. (A) Contact co-culture of hESC-derived PP with DiI-Ac-LDL-labeled rat heart microvascular endothelial cells (RHMVEC). EC are observed adjacent (top) or in direct contact (bottom) with hESC-derived PP. (B) Immunocytochemistry (ICC) shows Von Willebrand factor (VWF)-positive EC and PDX1-positive hESC-derived PP cells in direct contact. (C) Maintenance of EC in serum-free co-culture media leads to EC depletion attributed to absence of serum in media. (D) Before EC depletion ICC confirms expression of EC-specific markers. (E) qPCR for *INS* after maturation stage with DAPT, contact co-culture, or co-culture media. Higher expression for cells matured by EC co-culture was observed. DAPT, N-[(3,5-Difluorophenylacetyl-L-alanyl)]-2-phenylglycine-1,1-dimethylethyl ester. Color images available online at www.liebertpub.com/tea

VE Cadherin and VWF to confirm maintenance of EC phenotype (Fig. 2C, D). The cells were harvested after complete EC depletion and analyzed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), which showed over 200,000-fold *INS* upregulation with respect to undifferentiated cells (1500-fold upregulation of with respect to expression at the PP stage [Fig. 2E]). To confirm that the observed effect on differentiation is mediated by the ECs and not from the modified co-culture media, parallel control culture was maintained in the co-culture media without ECs for 5 days and switched to maturation media at the same time as the co-culture group and maintained in this media for the remaining experiment. This control group showed a much lower upregulation of *INS* 68,000 with respect to undifferentiated cells (500-fold with respect to PP stage), hence confirming the positive role of ECs in maturing hESCs to islet-like cells (Fig. 2E). In the next step, we compared the efficiency of differentiation mediated by ECs with that of standard procedures of notch inhibition by DAPT treatment.²⁴ DAPT treatment for 5 days followed by maturation media for 5 additional days resulted in comparable levels of *INS* upregulation (165,000 with respect to undifferentiated cells) as with RHMVEC co-culture, without any significant difference (Fig. 2E). The results suggest that while the basal co-culture media alone is inducing some degree of maturation from the chemical agents known to support islet function, the presence of RHMVEC is further enhancing this effect, resulting in upregulation of insulin comparable to that found using standard maturation methods.

Co-culture-mediated maturation is generalized to ECs but not all cell types

Having confirmed the positive effect of RHMVEC in islet-specific maturation of hESC, we next investigated whether the effect can be reproduced by other ECs as well. Human umbilical vein endothelial cells (HUVECs) were our next choice of ECs since it is a very well studied cell of human origin. HUVECs are more robust in culture and, unlike RHMVEC, survived the entire maturation period. Hence, the HUVECs were labeled with DiO-Ac-LDL and after completion of the differentiation protocol they were sorted out prior to qRT-PCR analysis. hESC-derived PP cells matured in contact with HUVECs showed almost 700,000 upregulation of *INS*, resulting in over 3-fold higher *INS* gene expression compared with RHMVEC co-culture or notch inhibition by DAPT (Fig. 3A) and over 5000-fold insulin upregulation over the PP stage. As a further precaution, the DiO-Ac-LDL-positive sorted populations were also analyzed for *INS* gene expression, which was found to be undetectable (data not shown). Hence, this confirms that the hESCs are maturing into *INS*-expressing cells when co-cultured with ECs.

Our next question was whether the effect of the co-culture was general to all cell types. To test this, we performed a parallel co-culture with a fibroblast cell line (GFP-NIH-3T3). Like HUVECs, the GFP-labeled fibroblast population was sorted out prior to qPCR analysis. The negative hESC-derived population was analyzed for *INS* and *GLUC* expression, which was found to be three and two orders of magnitude lower than cells under DAPT treatment respectively (Fig. 3B). Together, these results confirm that ECs

from different sources have the potential to induce differentiation of hESC-PP cells into insulin-expressing cells and that this effect is not general to all cell types.

EC-mediated maturation of hESCs is augmented by direct contact between cells

Having confirmed that co-culture with ECs specifically induces islet-like maturation of the hESC-derived PP cells, we sought to investigate the effect of density of EC co-culture on differentiation. Since HUVEC was mediating a stronger effect further experiments were performed with HUVEC. ECs were added at a plating ratio of 1:1, 1:2, or 1:10 with respect to hESC-derived PP cells in direct contact and the culture was continued as before. Analysis of the differentiated cells by qPCR after sorting out the ECs confirmed strong *INS* expression under all the co-culture ratios. The effect was, however, strongest with high density of co-culture with *INS* levels being progressively lower for lower co-culture densities (Fig. 3C).

To analyze the mechanism through which the ECs are mediating hESC maturation, we investigated the effect of alternate co-culture configurations on hESC differentiation (Fig. 3D); namely, transwell co-culture in which the ECs and the hESC-derived PP cells were plated on adjacent chambers separated by a semi-permeable membrane that allows diffusion of soluble signals, but prevents direct contact. Additionally, we tested EC-conditioned media where the co-culture media was added to HUVEC (maintaining the 1:1 ratio) for 24 h before being added to the hESC-derived PP with media change in this manner every 24 h. Both conditions were maintained for 7 days, at which point HUVEC viability started to decrease. qRT-PCR analysis for *INS* gene expression under alternate configurations revealed highest upregulation for contact co-culture, followed by two times lower expression in transwell and five times lower in conditioned media culture. This indicates that the maturation is mediated by cell-cell contact along with possibly short-lived soluble factors.

Characterization of cells matured by co-culture with HUVEC

Based on the above analysis, the hESC-derived PP cells were co-cultured in direct contact with high density of HUVEC, and the differentiated cells were further characterized for relevant islet-specific maturation. Analysis by RT-PCR for relevant β -cell markers revealed (Fig. 4A) particularly high upregulation of *GLUT2* reaching 25,000-fold; *NKX2.2*, *NKX6.1*, and *ISL1* also achieved considerable upregulation of 200-, 135-, and 394-fold respectively.

Cells differentiated under this condition showed positive staining for PDX1, Proinsulin, C-peptide, and MAFA (Fig. 4B). We examined whether the resulting cells were polychromal by immunocytochemistry (Fig. 4C). Specifically we co-stained for Glucagon and C-peptide and Somatostatin and Proinsulin. For both cases we found populations of multiple hormone-expressing cells; however, we found a significant number of cells expressing insulin in the absence of other pancreatic hormones, representing fully mature cells. Staining for amylase was found to be negative, suggesting absence of exocrine cells (not shown).

Quantification by flow cytometry showed that about 29% of the cells were positive for C-peptide, compared to 13.8%

FIG. 3. Co-culture-mediated maturation is specific to ECs. Co-culture with **(A)** human umbilical vein endothelial cell (HUVEC) and **(B)** NIH3T3. Cells were sorted to isolate hESC-derived cells and qPCR for *INS* and *GLUC* was performed. qPCR results show that other ECs also mediate insulin upregulation in hESC-derived PP, while non-ECs do not. qPCR results for cells matured by varying EC density **(C)** or co-culture configurations **(D)**. EC number positively correlates with insulin upregulation. *INS* expression is upregulated for all co-culture configurations but to a lower extent than contact co-culture. Transwell co-culture elicits higher upregulation than EC-conditioned media, suggesting some signals may be short lived. Color images available online at www.liebertpub.com/tea

obtained when using notch inhibition as illustrated in Figure 4D. The foremost functionality of β -cells is the ability to respond to external glucose levels. Hence, glucose responsiveness of the hESC-derived cells was analyzed by exposing the cells to 2.5 mM (low) glucose or 25 mM (high) glucose media for 4 h and quantifying intracellular C-peptide levels (Fig. 4F). When stimulated in low glucose, the in-

tracellular C-peptide level was observed to be 2.5 pg/mg of total protein, while at high glucose concentrations it increased to over 16 pg/mg of total protein, representing an almost sevenfold increase over low-glucose condition. These results suggest that the hESC-derived islet-like cells have the capability of modifying insulin biosynthesis in response to external glucose concentration.

FIG. 4. Characterization of hESC-derived cells matured by EC co-culture. **(A)** qRT-PCR analysis of the hESC-derived cells co-cultured with HUVEC for β -cell markers confirms islet-specific maturation of the hESCs. **(B)** Characterization by ICC shows co-expression of C-peptide and Proinsulin. Further, ICC shows high co-expression of Proinsulin and PDX1 and MAFA and C-peptide. Scale bar: 12.5 μ m. **(C)** ICC for glucagon and C-peptide and somatostatin and proinsulin after EC contact co-culture show populations of poly-hormonal cells and populations of cells expressing C-peptide only. **(D)** Flow cytometry results indicate 29% of the cells are positive for C-peptide **(E)** Magpix was used to quantify intracellular C-peptide levels at low (2.5 mM) and high (25 mM) glucose levels. The results show $\sim$ 6-fold increase in C-peptide upon high glucose stimulation. Color images available online at www.liebertpub.com/tea

Analysis of signaling pathways mediating EC co-culture induced pancreatic maturation

Correlation between markers associated with signaling pathways and pancreatic maturation. To identify possible pathways that are differentially modulated by the ECs, the gene expression profile at the end of maturation was analyzed using human signal transduction PCR pathway finder. The signal transduction genes were further supplemented

with relevant pancreatic markers to form our complete dataset, as represented in Figure 5A. Figure 5A presents the heatmap of $-\Delta\Delta C_T$ values of the genes for two co-culture samples and one transwell sample. Since, stem cell cultures show high degree of variability, we analyzed each sample independently without taking mean of the fold-changes. Using a threshold of twofold up- or downregulation, the dataset was filtered to select the genes to be focused for further analysis. These focus genes belonged to the following

FIG. 5. Quantitative analysis of signal transduction pathways during maturation. **(A)** Heatmap of PCR gene expression at the end stage of maturation. Each sample is normalized to the respective control medium. A threshold of twofold upregulation/downregulation is used to filter the dataset and the $-\Delta\Delta C_T$ values of the focus genes are presented here. The Y-axis contains signaling genes and the pancreas-specific markers. Genes in red are upregulated while those in green are downregulated. It is seen that co-culture group shows the highest *INS* levels. **(B)** partial least squares regression (PLSR) coefficients indicating the correlation between the major pancreatic markers and signaling pathway genes. *INS*, *GCG*, *ISL1*, and *NEUROG3* were taken as outputs and the signaling pathway genes from **(A)** are taken as the inputs. The data matrix is log2 transformed before regression analysis. **(C)** Upstream Cyclic AMP signaling and associated target genes. **(D)** Upstream regulators of the focus genes in the dataset associated with deactivation of hypoxia-mediated signaling in co-culture group. Color images available online at www.liebertpub.com/tea

pathways: Hedgehog, JAK/STAT, NF κ B, WNT, TGF β , Hypoxia, PPAR, Notch, and p53. Next, we wanted to identify any possible association between the signaling transcription factors and the pancreatic markers. PLSR analysis was performed with each pancreatic marker as the output and the signaling pathway genes as the input. We observed two groups of output markers based on the type of their relationship with the signaling genes as seen from the sign of the regression coefficient. The outputs *INS*, *GCG*, *ISL1*, and *NEUROG3* formed group 1 and *NKX2-1*, *NKX6-1*,

NEUROD1, and *GLUT2* formed group 2 and these groups showed opposite relationships with the signaling genes. The regression coefficients for group 1 that contained our primary output of interest, *INS*, are presented in Figure 5B. These regression coefficients denote the independent contribution by each signaling transcription factor (TF) to the total output. As seen from the figure, the top five prime contributors were *FCER2*, *ICAM1*, *WISP1*, *EPO*, and *FABP1* while the remaining genes gave very small contributions. *FCER2* (JAK1,3/STAT6), *EPO* (Hypoxia), and *FABP1* (PPAR)

TABLE 1. TOP NETWORKS IN THE DATASET

Key networks	Focus molecules	Score
Endocrine system development and function, tissue morphology, digestive system development and function	<i>FCER2, FOSL1, INS, LFNG, NEUROG3, NKX2-2, NKX6-1, PAX4, PDX1, PTCH1, SLC2A2, SOCS3, TNSFS10</i>	30
Cellular development, cell death and survival, cancer	<i>AXIN2, CCND1, FABP1, HES5, GATA3, TNF, WISP1, WNT1</i>	16
Cardiovascular system development and function, cellular movement, cellular development	<i>BCL2A1, BMP4, CCL5, CEBPD, EMP1, ISL1, OLR1</i>	14

showed a negative correlation while *ICAM1* (NF κ B) and *WISP1* (WNT) elicited a positive correlation. The regression coefficients for group 2 are presented in Supplementary Figure S1.

While this analysis is helpful in identifying independent relationships between each TF in the signaling pathway and the pancreatic markers, it is also informative to analyze the overall activity of pathways representing these genes. Therefore, to infer network level information, an in-depth comparison of focus genes in the dataset to the existing literature was done using ingenuity pathway analysis (IPA) database.

Signaling pathways differentially regulated under co-culture configuration. Network analysis was performed for the most representative co-culture sample that gave the highest *INS* upregulation. Using the IPA database, the top networks associated with the genes in the dataset were

identified and ranked according to their score values. Table 1 lists the identified networks with the corresponding scores and focus genes. The top network is associated with endocrine system development and contains 13 genes from the dataset (estimated probability of random gene placement = 10^{-30} ; i.e., score = 30). The remaining networks were associated with cell death and survival (score = 16) and cardiovascular system development (score = 14). Thus, based on overall gene expression, the hESC co-culture dataset contains gene sets that primarily contribute to endocrine cell development. The IPA generated network of endocrine cell development containing the relevant focus genes is presented in Supplementary Figure S2. A biological functional analysis was also performed for the co-culture sample that further supported the network analysis. The significant functions are presented in Supplementary Figure S3. Among the developmental functions, those associated with digestive system and endocrine system are again identified to be important. The important molecular and cellular functions included cellular development, cell survival, cellular function, and maintenance, and so on.

Using the IPA database, possible upstream regulators resulting in the global gene expression changes were then identified. Table 2 lists the upstream regulators and their predicted activation status. The *z*-scores denote the significance associated with this predicted activation status as compared to a random association and scores with an absolute value equal to and greater than 2 are considered to be significant. Many of the top regulators are significantly activated in the co-culture sample and only one pathway is significantly downregulated. Among the top upstream regulators, Cyclic AMP is predicted to be activated based on the upregulation of *GATA3*, *CDKN1A*, *TNFSF10*, *SOCS3*, and *SERPINE1* and downregulation of *IFNG*, *PTCH1*, *CCND1*, and *TNF*. The influence graph for cyclic AMP is shown in Figure 5C. Hydrogen peroxide (H_2O_2) is also

TABLE 2. TOP UPSTREAM REGULATORS OF GENE EXPRESSION

Upstream regulator	Molecule type	Prediction ^a	Target genes ^b	<i>z</i> -score	<i>p</i> -Value
Cyclic AMP	Endogenous chemical	Up	<i>TNFSF10, TNF, SOCS3, IFNG, GATA3, CEBPD, CDKN1A, CCND1, PTCH1, SERPINE1</i>	2.719	6.86×10^{-12}
H_2O_2	Endogenous chemical	Up	<i>SERPINE1, IFNG, FOSL1, EPO, CDKN1A, CCND1, BCL2A1, TNF, ICAM1</i>	2.697	2.3×10^{-8}
Butyric acid	Endogenous chemical	Up	<i>TNF, ICAM1, GATA3, CDKN1A, CCND1, BCL2A1, SERPINE1, SOCS3</i>	2.335	1.35×10^{-5}
Angiopoietin 2	Growth factor	Up	<i>OLR1, ICAM1, CDKN1A, CCL5</i>	2.0	4.46×10^{-5}
HNF1A	Transcriptional regulator	Down	<i>SERPINE1, SOCS3, SLC2A2, PAX4, CCND1, NKX6-1, NKX2-2, FABP1, EPO</i>	-2.0	3.65×10^{-8}

^aThe prediction denotes the direction of regulation (up or down) of the upstream regulator based on the direction of changes associated with target genes in the dataset. The predictions that pass the *z*-score of 2 are presented here. Cyclic AMP, H_2O_2 , and HNF1A also gave *p*-values $< 10^{-5}$ and are overall good upstream regulators.

^bTop genes predicted from partial least squares regression are colored here. Upregulated genes are colored red and downregulated genes are colored green.

predicted to be activated based upon upregulation of *SERPINE1*, *BCL2A1*, *FOSL1*, *CDKN1A*, and *ICAM1* and down-regulation of *EPO*, *IFNG*, *TNF*, and *CCND1*. This is followed by butyric acid and angiopoietin 2. Among downregulated pathways, only HNF1A upstream regulator was predicted to be significant. HNF1A and H_2O_2 together also form a connected system and interaction between these molecules are shown in Figure 5D by an influence graph with the target genes from the dataset. Overall, the pathway analysis highlights ability of ECs to mediate global gene expression patterns that follow pancreatic organogenesis and identifies the possible signaling pathways that are differentially regulated under the contact co-culture configuration.

Discussion

The goal of tissue engineering is to engineer the cellular microenvironment for enhanced cell and tissue function. This process is often driven by attempts to reproduce the complex *in vivo* organ environment in an *in vitro* culture setting. Such environment manipulation is particularly relevant in stem cell-based regenerative medicine applications, where the goal is to reproduce specific organ function from the uncommitted ESCs. In the context of pancreas development, a key event underlying *in vivo* maturation of the PP epithelium is the interaction with ECs.^{15–17} In this report we are demonstrating a similar role of ECs in inducing pancreatic maturation in a completely *in vitro* setting using hESCs and two different EC types. A detailed pathway analysis further indicates the alignment of this *in vitro* set-up with known *in vivo* signals during pancreas development. These findings can have broad impact on tissue and organ engineering. It underlines the importance of stromal interaction (i.e., ECs) in engineering tissue with high biological fidelity, to achieve an authentic representation of the environmental milieu constituting organ development. Finally, with the advent of whole-organ engineering, diverse cell types are needed to rebuild a complex organ.²⁵ Our study indicates that ECs are an important candidate to consider while reconstructing pancreas.

We performed the co-culture with three different cell-types: two ECs and one non-EC. While both the ECs elicit a strong upregulation of insulin in the differentiating hESCs, the fibroblast cells could not induce any similar maturation effect. Hence, the effect was determined not to be a general response exhibited by all cell types. Of the tested ECs, the effect was stronger in the human EC co-culture compared with the rat heart micro-vascular cells. This could imply that human ECs in general are more supportive of hESC differentiation. However, we also observe that the HUVECs are more robust and survive longer in the co-culture configuration, compared to the RHMVEC cells, and hence they can potentially elicit a stronger effect on the cell maturation.

To investigate the mechanism through which the ECs are mediating the maturation of hESC-derived PP cells, we analyzed two additional co-culture configurations along with the contact co-culture configuration: transwell and conditioned media. The contact co-culture was found to have the maximal effect, followed by transwell, while conditioned media had a positive but reduced effect on maturation. It is important to note that ECs in contact co-culture were found to survive longer in culture, suggesting that their behavior is

also being affected by the hESC-derived cells. This could explain the difference between the transwell, conditioned media, and contact co-culture systems with ECs in the contact co-culture system secreting factors in response to the hESC-derived cells, which modulate differentiation. An additional possibility is deposition of extracellular matrix by ECs. This is in agreement with both developmental and differentiation studies. In terms of development it has been postulated that while pancreatic cells do not produce ECM, they produce vascular endothelial growth factor that attracts ECs. These ECs produce ECM, which aids with pancreatic progression through activation of integrin-mediated pathways.^{26,27} In differentiation studies it has been observed that laminin-1 promotes differentiation of PP cells into insulin-expressing cells.²⁸ Another possible mechanism by which ECs mediate differentiation is restoration of normoxia, which has been suggested to favor β -cell differentiation.²⁹ Related to this, release of nitric oxide from ECs has effects that resemble normoxic conditions such as inhibition of endothelin-1,³⁰ destabilization of hypoxia-inducible factor (HIF), and interference with hypoxia signaling.³¹

To identify specific pathways through which the ECs are mediating hESC maturation, we analyzed the signaling pathways influenced by the co-culture condition. Comparative analysis between the different co-culture configurations revealed the specific effect of contact co-culture. For example, C/EBPD was strongly upregulated under contact co-culture, but not under transwell culture. C/EBPD, which has both anti-apoptotic and anti-inflammatory roles in pancreatic β -cells³² has been found to be upregulated in hepatocytes cultured on laminin-rich extracellular matrix.³³ Upregulation of C/EBPD in our co-culture configuration suggests the possibility of laminin secretion by EC in contact co-culture conditions.

We further investigated possible correlations between the β -cell maturation markers and signaling pathway molecules. PLSR analysis showed several genes that are highly correlated with insulin expression. Among them was WISP1, which is expressed in the adult pancreas³⁴ and has been implicated in increased regenerative ability of β -cells.³⁵ Also, a high negative correlation was found with FABP1, which in the intestine has been found to be suppressed by expression of PDX1.³⁶ Similarly EPO showed a strong negative correlation with insulin expression. EPO is produced and secreted during hypoxic conditions,³⁷ therefore suggesting a negative correlation between hypoxia and PP maturation. Restoration of normoxia by ECs has been suggested to favor β -cell differentiation.²⁹ Related to this, release of nitric oxide from EC has effects that resemble normoxic conditions such as inhibition of endothelin-1,³⁰ destabilization of HIF, and interference with hypoxia signaling.³¹

At this point, most of the analysis considered independent pathways and targets. However, we deemed important to analyze the overall activity of pathways constituting these genes in the IPA database to infer network level information. Detailed pathway analysis revealed several of the differentially regulated genes to be from the signaling pathways belonging to pancreatic organogenesis. Second, biological functions associated with cellular survival were also identified to be important. Among the canonical pathways, those mediated by growth factors were strongly associated with the genes in our dataset. Among them, JAK/STAT has

been recently implicated in the pancreatic maturation process and increased endocrine differentiation.³⁸ From the upstream analysis, IPA inferred many upstream regulators that could possibly be activated by endothelial specific signaling. Among them, cyclic AMP was found to be significantly activated. Cyclic AMP is associated with glucose-induced insulin secretion of mature β -cells and is also important for β -cell differentiation and growth survival.³⁹ The positive influence of cyclic AMP in the maturation of a related cell type, hepatocytes matured from hESC-derived hepatoblasts, was recently demonstrated.⁴⁰ Further, many of the downstream genes identified by IPA also support the close association of H_2O_2 activation and HNF1A downregulation. For example, EPO, which is downregulated in the co-culture sample is inhibited by H_2O_2 ⁴¹ and it is also a downstream target of HNF1A.^{42,43} Also, at later stages of maturation, HNF1A downregulation due to normoxia is found to favor β -cell differentiation.¹⁵ This effect is due to the close association between ECs and β -cells during pancreatic organogenesis.¹⁵

In summary, our pathway analysis along with the correlation analysis identifies a set of signals that could potentially be involved in the crosstalk between ECs and maturing PP. Our network analysis indicates that the gene expression signature under co-culture configuration aligns well with the *in vivo* pancreatic development mechanism. This further emphasizes that establishing the cell-cell interaction by exposing differentiating hESCs to ECs recapitulates the *in vivo* pancreas development niche in an *in vitro* setting.

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Disclosure Statement

No competing financial interests exist.

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