

Endothelial Cells Control Pancreatic Cell Fate at Defined Stages through EGFL7 Signaling

Der-I Kao,¹ Laretta A. Lacko,² Bi-Sen Ding,³ Chen Huang,⁴ Kathleen Phung,¹ Guoqiang Gu,⁴ Shahin Rafii,³ Heidi Stuhlmann,² and Shuibing Chen^{1,*}

¹Departments of Surgery and Biochemistry

²Department of Cell and Developmental Biology

³Howard Hughes Medical Institute, Ansary Stem Cell Institute, Department of Genetic Medicine
Weill Cornell Medical College, New York, NY 10065, USA

⁴Vanderbilt University Medical Center, Nashville, TN 37232, USA

*Correspondence: shc2034@med.cornell.edu

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SUMMARY

Although endothelial cells have been shown to affect mouse pancreatic development, their precise function in human development remains unclear. Using a coculture system containing human embryonic stem cell (hESC)-derived progenitors and endothelial cells, we found that endothelial cells play a stage-dependent role in pancreatic development, in which they maintain pancreatic progenitor (PP) self-renewal and impair further differentiation into hormone-expressing cells. The mechanistic studies suggest that the endothelial cells act through the secretion of EGFL7. Consistently, endothelial overexpression of EGFL7 *in vivo* using a transgenic mouse model resulted in an increase of PP proliferation rate and a decrease of differentiation toward endocrine cells. These studies not only identified the role of EGFL7 as the molecular handle involved in the crosstalk between endothelium and pancreatic epithelium, but also provide a paradigm for using hESC stepwise differentiation to dissect the stage-dependent roles of signals controlling organogenesis.

INTRODUCTION

During embryonic development, cell fate is determined by both intrinsic programs and external cell niche. The animal studies suggested that endothelial cell niche provides both supportive and inductive roles throughout pancreas development (Eberhard et al., 2010). Early studies showed that signals from endothelial cells are essential for the induction of pancreatic organogenesis (Lammert et al., 2001). Endothelial cells specifically promote early dorsal pancreas development by inducing Ptf1a⁺ pancreatic progenitors (PPs) by activating FGF10 signaling (Yoshitomi and Zaret, 2004; Jacquemin et al., 2006). Interestingly, some groups recently reported that the endothelial cell niche could restrain epithelium branching and endocrine development. One group shows that blood vessel ablation results in increased pancreatic organ size (Sand et al., 2011). Another group showed that elimination of endothelial cells increases the size of pancreatic buds (Magenheim et al., 2011). Similarly, another group showed that overexpressing vascular endothelial growth factor A increases embryonic endothelial cell populations and perturbs pancreatic endocrine differentiation (Cai et al., 2012). However, a complete understanding of the role of endothelial cells in human pancreatic development is still missing.

Human embryonic stem cells (hESCs) provide an *in vitro* platform to study human development. To better understand the signaling from the endothelial cell niche in pancreatic differentiation, we have developed a coculture

system of endothelial cells with hESC-derived progenitors under serum-free, chemical-defined conditions. By using the coculture system, we found that endothelial cells maintain PP self-renewal and impair further differentiation into hormone-expressing cells by secreting EGFL7.

RESULTS AND DISCUSSION

Endothelial Cells Promote the Proliferation of PDX1⁺ Cells in the Chemically Defined Environment

To systematically probe the role of an endothelial cell niche in human pancreatic development, we set up a coculture system using endothelial cells and hESCs-derived progenitors. The coculture system is established in a chemically defined culture condition to mimic the serum-free environment during embryonic development. The endothelial cells used in this study were AKT-HUVECs (AKT-activated human umbilical vein endothelial cells) (Kobayashi et al., 2010) or MPECs (mouse pancreas islet endothelial cells). BJ cells, which are human skin fibroblasts, were used as a control for cell-type specificity. To explore the stage-dependent effect of endothelial cells, HUES8 cells were differentiated into three different stages: definitive endoderm (DE), foregut endoderm (FE), or PP populations using a previously established strategy (Chen et al., 2009). The hESC-derived populations were cultured together with MPECs or AKT-HUVECs at different ratios and examined for their capacities to self-renew or differentiate (Figure 1A). The

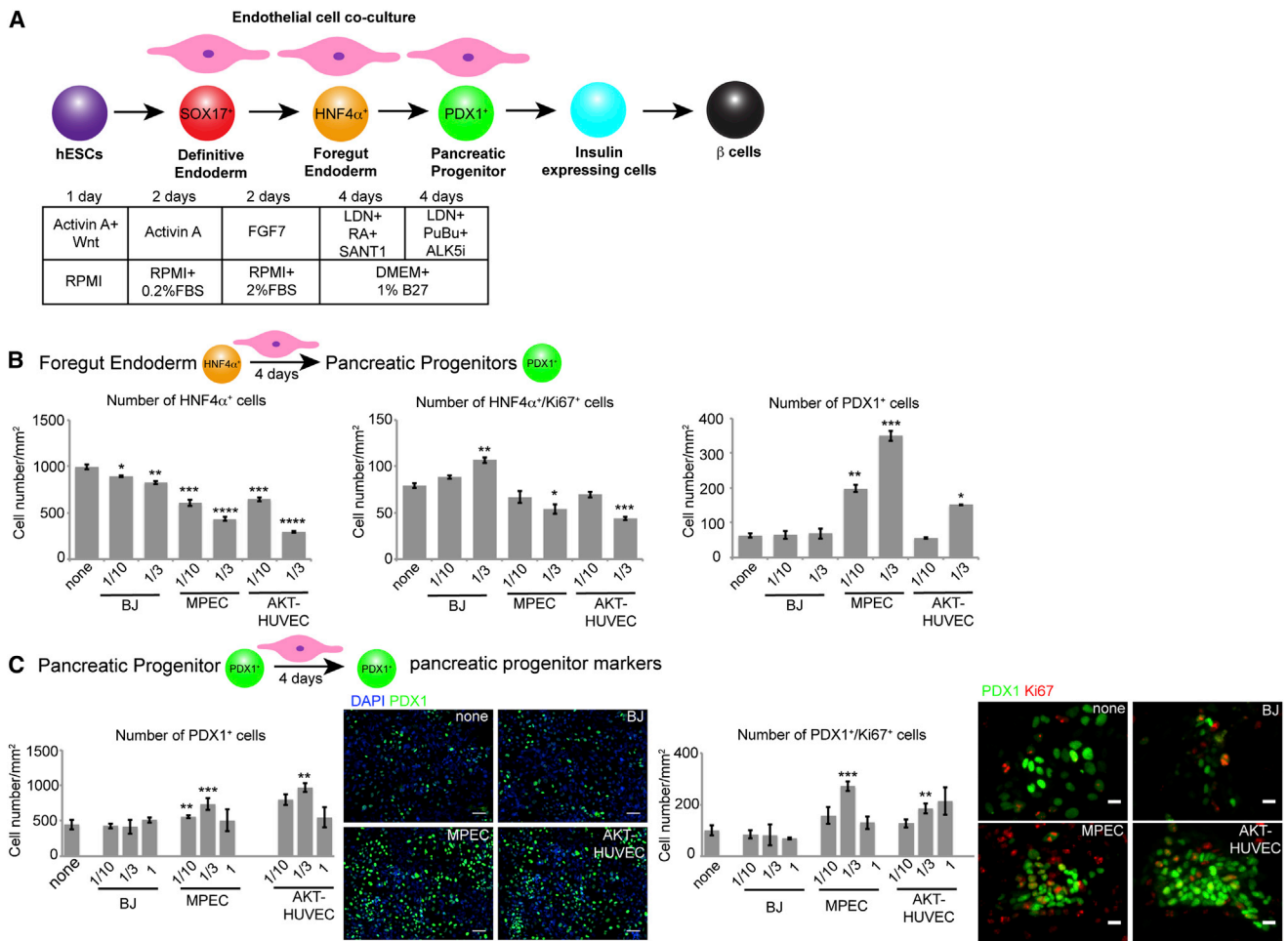


Figure 1. The Role of Endothelial Cells in Human Pancreatic Differentiation

(A) Scheme of coculture between endothelial cells and hESC-derived progenitors.

(B) Cell number per mm² after HUES8-derived FE population were cocultured with BJ cells, MPECs, or AKT-HUVECs at indicated ratios (n = 3).

(C) Cell number per mm² (n = 3) and representative images after HUES8-derived PP population were cocultured with BJ cells, MPECs or AKT-HUVECs. The left scale bar represents 50 μm. The right scale bar represents 10 μm.

Data were presented as mean ± SD.

self-renewal ability was determined by immunostaining with antibodies against a proliferation marker (Ki67) and stage-dependent self-renewal markers, including SOX17 for DE, HNF4α for FE, and PDX1 for PPs. The differentiation ability was determined by immunostaining with antibodies against differentiation markers, including HNF4α for DE, PDX1 for FE, and insulin/glucagon/somatostatin for PPs.

In the coculture condition of MPECs or AKT-HUVECs with the hESCs-derived DE population, neither the number of SOX17⁺/Ki67⁺ cells nor the number of HNF4α⁺ cells changed significantly (Figure S1A available online), suggesting that endothelial cells do not affect either self-renewal or differentiation of DE. In the coculture condition

with the hESC-derived FE population, the number of PDX1⁺ cells was significantly increased in the presence of MPECs and AKT-HUVECs, but not BJ cells (Figure 1B). In addition, when the hESCs-derived PP population was cultured with MPECs and AKT-HUVECs, the number of PDX1⁺ cells was significantly elevated as compared with BJ cells (Figure 1C). The results suggest that endothelial cells, not fibroblasts, promote the generation of PDX1⁺ cells at the FE and PP stages.

Next, we asked whether the generation of PDX1⁺ cells is due to cell proliferation, by examining the proliferation marker Ki67. In cocultures using the HUES8-derived PP population, the number of PDX1⁺/Ki67⁺ cells is significantly higher in the presence of MPECs and AKT-HUVECs



than control conditions (Figure 1C). To further validate that endothelial cells promote PP proliferation, we generated a transgenic *Pdx1*-EGFP HUES8 cell line, which contains the mouse *Pdx1* promoter driving expression of EGFP. The *Pdx1*-EGFP HUES8 cell line was validated by flow cytometry. After 14 days of differentiation, about 45% of cells were EGFP⁺, most of which were positively stained by PDX1 antibody (Figure S1B). *Pdx1*-EGFP⁺ cells were sorted and cocultured with MPECs, AKT-HUVECs, or BJ cells (Figure S1C). The result confirmed that endothelial cells can support the isolated PDX1⁺ cell proliferation. Interestingly, when we cocultured the PP population with endothelial cells, we observed that the differentiation of PPs to hormone-expressing cells was impaired (Figures S1D and S1E). In addition, endothelial cells show similar capacities to increase the number of PDX1⁺ cells and PDX1⁺/Ki67⁺ cells when cocultured with H1-derived PP population (Figure S1F), suggesting that the effect of endothelial cells is not hESCs line dependent. These data together suggest that endothelial cells provide a niche to maintain proliferation and impair differentiation of PDX1⁺ cells toward hormone-expressing cells.

PDX1⁺ Cells after Coculture with Endothelial Cells Retain PP Signature Gene Expression

To determine the cellular identity of PDX1⁺ cells after coculture with endothelial cells, cells were stained with other antibodies against progenitor markers. Immunocytochemistry suggested that most PDX1⁺ cells grew in the presence of endothelial cells expressed PP markers, HNF6, SOX9, and FOXA2 and endocrine marker, NKX6.1 (Figure 2A). The quantified results suggest that the number of PDX1⁺/NKX6.1⁺ cells, PDX1⁺/FOXA2⁺ cells, PDX1⁺/HNF6⁺ cells, and PDX1⁺/SOX9⁺ cells were higher in the endothelial cell coculture conditions than in control cells (Figure 2B). We further tested the mRNA expression of PP markers (*FOXA2*, *HNF6*, and *SOX9*) and endocrine progenitor markers (*NKX6.1*, *NKX2.2*, and *NGN3*) in the purified *Pdx1*-GFP⁺ cells. Quantitative RT-PCR (qRT-PCR) suggested that the expression of PP markers (*FOXA2*, *HNF6*, and *SOX9*; Figures S2B, S2D, and S2E) and endocrine progenitor markers (*NKX6.1*; Figure S2C) in sorted *Pdx1*-EGFP⁺ cells was elevated in MPEC coculture condition, but the expression of endocrine progenitor markers (*NGN3* and *NKX2.2*; Figures S2F and S2G) did not significantly differ between BJ and MPEC coculture conditions (Figure S2F, at day 4, $p = 0.1274$ for none versus MPECs; $p = 0.8968$ for BJ versus MPECs). It suggested that PDX1⁺ cells after coculture with endothelial cells bias to endocrine fate, but have not expressed the endocrine progenitor markers yet. Next, we investigated whether the increased PP population under coculture conditions was due to cell proliferation by measuring the number of PDX1⁺/Ki67 cells that are also


positive for other PP markers (Figure 2C). When cocultured with MPECs and AKT-HUVECs, the number of proliferating PPs increased about 5-fold and 6- to 8-fold, respectively, compared with noncoculture conditions. Sometimes, the number of the proliferating PPs in the presence of BJ cells is lower than noncoculture condition. This might due to the high cell density in coculture condition, which might affect PP proliferation.

Transcriptome profiling provides a way to systematically compare gene expression in different cell populations. PPs before or after coculture with endothelial cells were profiled by RNA-seq. To purify the PDX1⁺ PPs, EGFP⁺ cells were sorted from the *Pdx1*-EGFP HUES8 cell-derived cells before or after coculture with MPECs or AKT-HUVECs. The global gene expression of PPs before and after coculture with endothelial cells showed very high similarity ($R^2 = 0.9611$ in the presence of AKT-HUVECs; Figure 2D left; and $R^2 = 0.9378$ in the presence of MPECs, Figure S2A). In addition, the global gene expression pattern of PPs expanded in the presence of either AKT-HUVECs or BJ cells showed high similarity ($R^2 = 0.9692$; Figure 2D, right). The data confirm that PPs maintain cellular identity after expansion by endothelial cells.

Endothelial Cells Maintain Human PP Self-Renewal by Secreting EGFL7

In the coculture system, endothelial cells might function through a secreted factor(s) or by direct cell-cell contact. To test these possibilities, HUES8 cell-derived PP populations were treated with conditioned medium (Hebrok et al., 2000) collected from MPECs (MPEC-CM) or from AKT-HUVECs (AKT-HUVEC-CM). CM retained similar, but slightly lower, activity compared with intact cells for promoting PP self-renewal (Figure 3A). These data suggest that endothelial cells function, at least in part, through a secreted factor(s). We further analyzed the gene expression profiles of AKT-HUVECs, MPECs, and BJ cells to identify candidate secreted factors (Figures 3B and S3A). Genes encoding secreted proteins, whose expression in endothelial cells is at least 30-fold higher than in the BJ cells, were selected for further analysis. Among the genes encoding secreted molecules (Table S1), we tested the abilities of recombinant proteins ANGPT2, EGFL7, CCL13, IL24, PLAU, DHH, and PDGFB to expand human PPs (Figure 3C). Only EGFL7 was able to promote PP proliferation (Figures 3C–3E). To determine whether EGFL7 was required for the endothelial cell effect, we used shRNA to knockdown EGFL7 expression in AKT-HUVECs. *EGFL7* mRNA expression level was decreased by about 63.5% in AKT-HUVECs infected with *EGFL7* shRNA lentivirus as compared with a scrambled control lentivirus (Figure S3B). When cocultured with *EGFL7*-shRNA-treated AKT-HUVECs, hESC-derived PPs showed significantly lower proliferation activity as



a,b,c. Pancreatic Progenitor (PDX1⁺)  4 days → PDX1⁺ pancreatic progenitor markers (NKX6.1, FOXA2, HNF6 and SOX9)

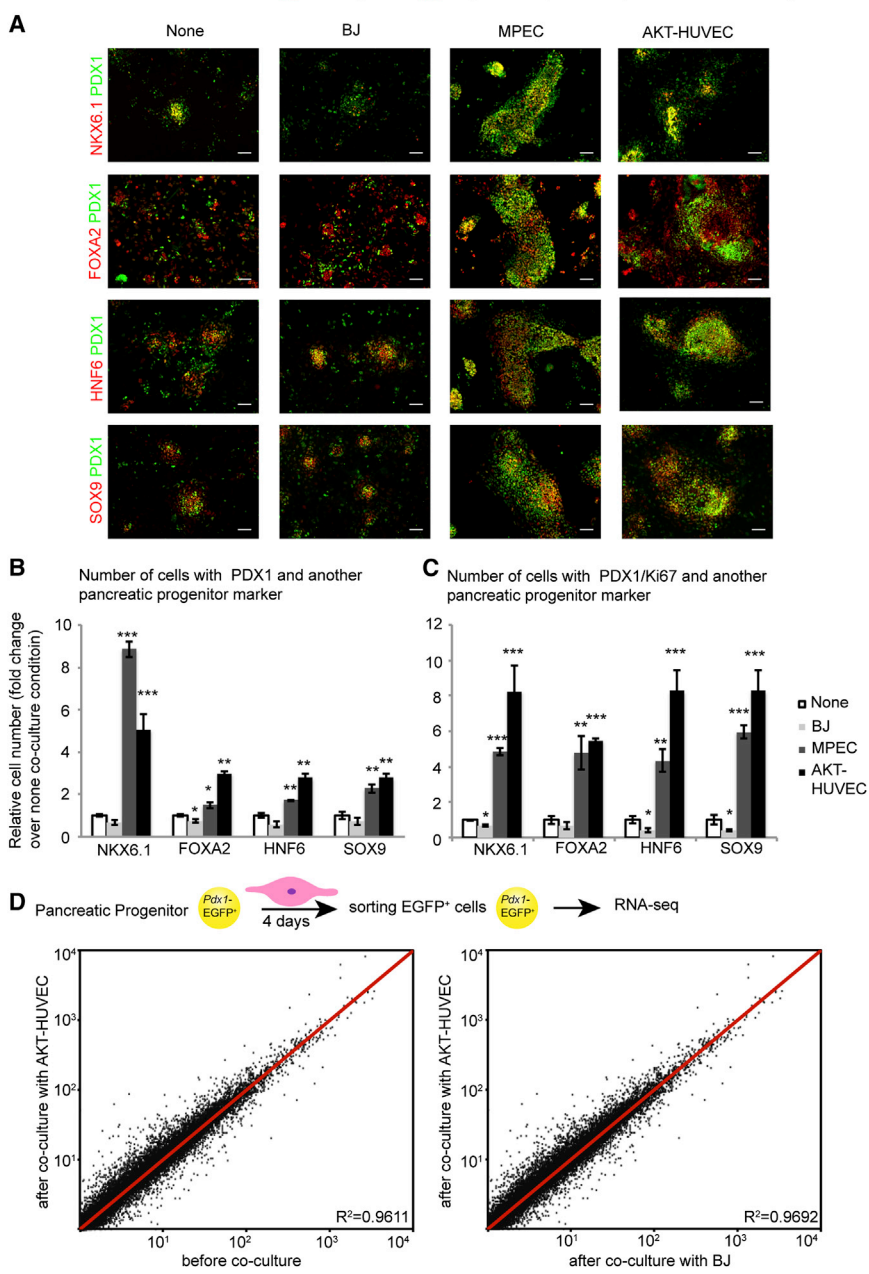


Figure 2. The Cellular Identity of PDX1⁺ PPs Expanded in Endothelial Cell Niche

(A) Representative figures of PDX1⁺ cells costained with another PP marker, FOXA2, HNF6, or SOX9 and endocrine progenitor marker, NKX6.1 under coculture conditions. Scale bar represents 50 μm.
 (B) Relative cell number of PDX1⁺ cells costained with another progenitor marker (n = 3). The relative cell number normalized to control (none) as one.
 (C) Quantified results of the relative cell numbers of PDX1⁺/Ki67⁺ cells costained with another progenitor marker (n = 3).
 (D) Transcriptome analysis by RNA-seq of *Pdx1*-EGFP⁺ cells before and after cocultured with endothelial cells. The scatter plot showed mRNA expression level in sorted *Pdx1*-EGFP⁺ cells before coculture versus after coculture with AKT-HUVECs (left) or after coculture with BJ cells versus after coculture with AKT-HUVECs (right). Data were presented as mean ± SD.

compared with those cocultured with untreated (p = 0.0324) and scrambled-shRNA-treated AKT-HUVECs (p = 0.0387; **Figures 3F and 3G**). These results indicate that EGFL7 is both sufficient and necessary for endothelial cells to maintain PP self-renewal.

Since EGFL7 has been shown to modulate the NOTCH pathway (**Nichol et al., 2010**), we tested whether the NOTCH pathway is involved in endothelial cell-dependent PP expansion. HUES8-derived PP populations were treated with five different NOTCH inhibitors, including DAPT, XX,

compound E, III-31-C, and Sulindac sulfide. None of them mimics EGFL7 effect to promote PP proliferation (**Figure S3C**). In addition, qRT-PCR analysis showed that EGFL7 treatment did not alter the expression of NOTCH target genes, such as *Hes-1* and *Hey-1*, in *Pdx1*-GFP⁺ cells (**Figure S3D**).

Recent studies suggested that EGFL7 might also activate EGF receptor pathway (**Luo et al., 2014; Wu et al., 2009**). Several experiments were performed to determine whether EGF receptor is a downstream signal of EGFL7.

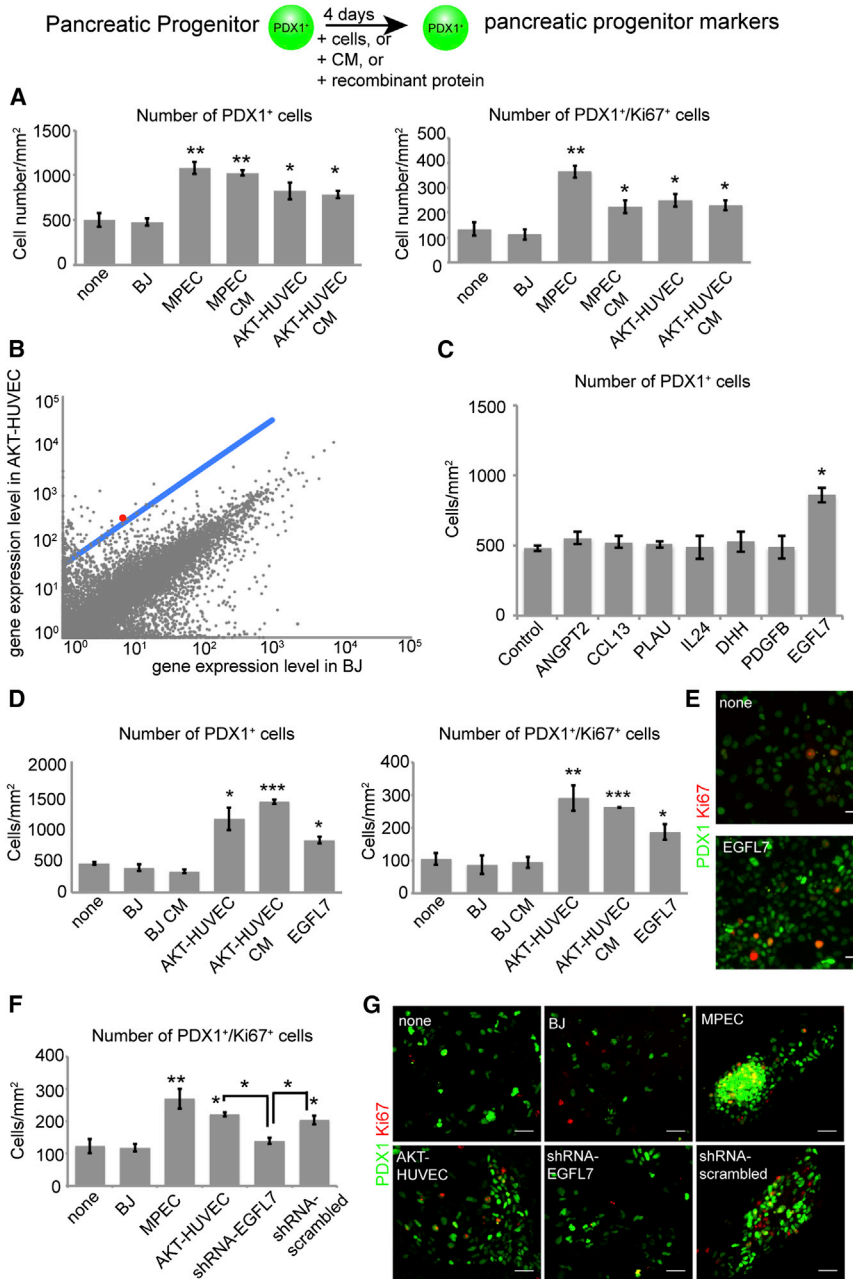


Figure 3. Endothelial Cells Promote PP Proliferation by Secretion of EGFL7

(A) Cell number per mm² 4 days after HUES8-derived PPs were cocultured with endothelial cells or treated with CM (n = 3). (B) Transcriptome analysis of gene expression in BJ cells and AKT-HUVECs by RNA-seq. The blue line indicates the cutoff line of a 30-fold increase in gene expression in AKT-HUVECs as compared with BJ cells. The red dot indicates EGFL7. (C) Number of PDX1⁺ cells per mm² 4 days after hESC-derived PPs were treated with different recombinant proteins (300 ng/ml) (n = 3). (D) PDX1⁺ (left) or PDX1⁺/Ki67⁺ cell number per mm² (right) 4 days after HUES8-derived PPs were treated with CM or 300 ng/ml EGFL7 (n = 3). (E) Representative images of (D). (F) PDX1⁺/Ki67⁺ cell number per mm² 4 days after HUES8-derived PPs were cocultured with AKT-HUVECs carrying shRNA targeting EGFL7 (shRNA-EGFL7) or AKT-HUVECs carrying scrambled control shRNA (scrambled) (n = 3). (G) Representative images of (F). Data were presented as mean ± SD.

Immunocytochemistry suggests that the EGF receptor is expressed in hESC-derived PDX1⁺ PPs (Figure S3E). In addition, microscale thermophoresis binding assay suggested that EGFL7 directly binds to the EGF receptor in vitro ($K_d = 384 \pm 76.5$ nM; Figure S3F). Furthermore, we found that addition of an epidermal growth factor receptor inhibitor (EGFRi) blocks the effect of endothelial cells and EGFL7 to expand PDX1⁺ PPs (Figure S3G). Finally, we found that mRNA expression level for *EGF* is much lower in AKT-HUVECs (0.09 ± 0.02) and MPECs (0.09 ± 0.02) than BJ fibroblasts (0.6 ± 0.1), as determined by RNaseq (Fig-

ure S3H). Since endothelial cells, but not BJ fibroblasts, promote PPs self-renewal, this suggests that endothelial cells do not promote PP proliferation by secreting EGF. Together, these results indicate that endothelial cells promote PP self-renewal through the secretion of EGFL7 and through activation of the EGF receptor signaling pathway.

To determine whether EGFL7 treatment is capable of promoting PP proliferation in long-term cultures, we tested the expansion and differentiation capacity of PDX1⁺ PPs to the endocrine lineage following the pulsed expansion with recombinant EGFL7. HUES8-derived PP



populations were treated with EGFL7 for different periods of time. One set of samples was fixed and stained with PDX1 and Ki67 antibodies to determine cell proliferation rates. The other set of samples were further differentiated in the absence of EGFL7 for additional 7 days and stained with antibodies against endocrine markers to determine the differentiation potential. The percentage of PDX1⁺/Ki67⁺ cells increased during the first week of EGFL7 treatment (Figure S3I). After 1 week, the percentage of PDX1⁺/Ki67⁺ cells started decreasing in all conditions no matter whether EGFL7 was present. However, the percentages of PDX1⁺/Ki67⁺ cells treated with EGFL7 are higher than those of under control condition at all time points (Figure S3I). Consistent with the increase in cell proliferation, the percentage of insulin⁺, C-peptide⁺, and somatostatin⁺ cells increased in PPs cultured under EGFL7 treatment and peaked at 1 week. The percentage of hormone-expressing cells under EGFL7-treated conditions is higher than that under control condition at all time points (Figures S3H–S3L). Together, these results suggest that one week of EGFL7 treatment can expand PDX1⁺ PPs in vitro, resulting in significantly increased differentiation efficiency toward the endocrine cell lineages.

Endothelial Overexpression of *Egfl7* In Vivo Increases Proliferation of PPs

To determine the effect of EGFL7 on the PP cell population in vivo, we analyzed pancreatic development in a transgenic mouse model, Tie2:*Egfl7*. In this strain, the *Egfl7* transgene is under the control of the endothelial-specific *Tie2* promoter, resulting in a 2- to 3-fold overexpression of EGFL7 (Nichol et al., 2010). All animal protocols were approved by the Institutional Animal Care and Use Committee at Weill Cornell Medical College. Whole-mount immunofluorescent staining was performed on E9.5 and E10.5 embryos from C57BL/6 WT mice and Tie2:*Egfl7* transgenic mice (TG) (Figures 4A and 4D). A CD31-positive capillary plexus surrounds the developing pancreatic bud in vivo, demonstrating the proximity of endothelial cells and implicating them as a potential source of EGFL7 to signal to the PDX1⁺ PP population (Figure S4A). Quantification of the vascular coverage at E10.5 demonstrated no significant difference in blood vessel density surrounding the developing pancreatic bud between WT and TG mice (Figure S4B, $p = 0.299$).

At E9.5 and E10.5, the pancreatic buds of TG mice appear larger with less defined edges as compared with WT mice (Figures 4B–4F). To determine whether the larger pancreatic buds in the TG embryos resulted from increased proliferation of PDX1⁺ cells, we performed EdU injections of pregnant E10.5 WT and TG mice (Figures 4G and 4H). We analyzed the percentage of EdU⁺ PP (PDX1⁺) cells in sections of pancreatic buds (Figure 4I). The percentage of

proliferating PPs (EdU⁺/PDX1⁺) in TG mice is significantly higher than WT mice ($*p < 0.05$; Figure 4I). In addition, the PDX1⁺ area in TG mice is significantly increased compared with WT mice ($*p = 0.031$ in Figure S4C and $*p = 0.032$ in Figure S4D). The number of PDX1⁺ cells in TG mice show the same trend toward increase as the PDX1⁺ area, although it does not reach significance ($p = 0.1144$ in Figure S4E, $p = 0.065$ in Figure S4F). The increase of the number of PDX1⁺ cells in TG mice is more pronounced at E15.5 (Figures S4G and S4H, $***p < 0.001$). Together, these data suggest that PP proliferation was stimulated by endothelial-specific overexpression of EGFL7 in vivo.

In addition, WT E11.5 pancreatic buds were isolated from surrounding mesenchyme and cultured in vitro in the presence or absence of recombinant mouse EGFL7 for 2 days. The explants were pulsed for 4 hr with BrdU prior to fixation. The percentage of proliferating PPs (BrdU⁺/PDX1⁺ cells) out of the total number of PPs (PDX1⁺ cells) was significantly increased in the presence of mouse recombinant EGFL7 ($**p < 0.01$, Figures 4J and 4K).

To determine the effect of EGFL7 in vivo on differentiation into hormonal-expressing cells, we analyzed the percentage of insulin⁺ cells and PDX1⁺ cells in E15.5 pancreatic buds (Figure 4L). The percentage of insulin⁺ cells over PDX1⁺ cells in E15.5 pancreatic buds is significantly decreased in TG mice ($*p < 0.05$, Figure 4M). Consistently, the ratio of insulin⁺ area over E-cadherin⁺ area in E15.5 TG pancreatic buds shows a trend toward decrease compared with WT controls (Figures S4G and S4I, $p = 0.396$), although it does not reach significance. We also examined the total number of PDX1⁺ cells and the percentages of PDX1⁺/NKX6.1⁺ cells, PDX1⁺/SOX9⁺ cells and PDX1⁺/NKX6.1⁺/SOX9⁺ cells in total PDX1⁺ cells at E15.5. Consistent with our results for E9.5 and E10.5 embryos, the total number of PDX1⁺ cells in E15.5 TG pancreatic buds is significantly higher than that of WT controls (Figure S4H, $***p < 0.001$). The ratios of PDX1⁺/NKX6.1⁺ cells ($p = 0.385$), PDX1⁺/SOX9⁺ cells ($p = 0.200$), and PDX1⁺/NKX6.1⁺/SOX9⁺ cells ($p = 0.495$) in PDX1⁺ cells do not differ significantly between TG and WT pancreatic buds (Figures S4J and S4K). Importantly, less than 6% of total PDX1⁺ cells coexpress NGN3 in both WT and TG pancreatic buds (Figures S4L and S4M, $p = 0.053$), suggesting that these PPs have not yet fully committed to endocrine progenitors. Together, these data suggest that EGFL7 suppresses PP differentiation into pancreatic endocrine cells, which is consistent with our results using hESC-derived cells (Figures S1D and S1E).

To determine the effect of EGFL7 overexpression in adult beta cell function, we performed a glucose-stimulated insulin secretion (GSIS) test and a glucose tolerance test (GTT) in 8-week-old male TG and WT mice. Strikingly, all four TG mice showed significantly less insulin secretion at

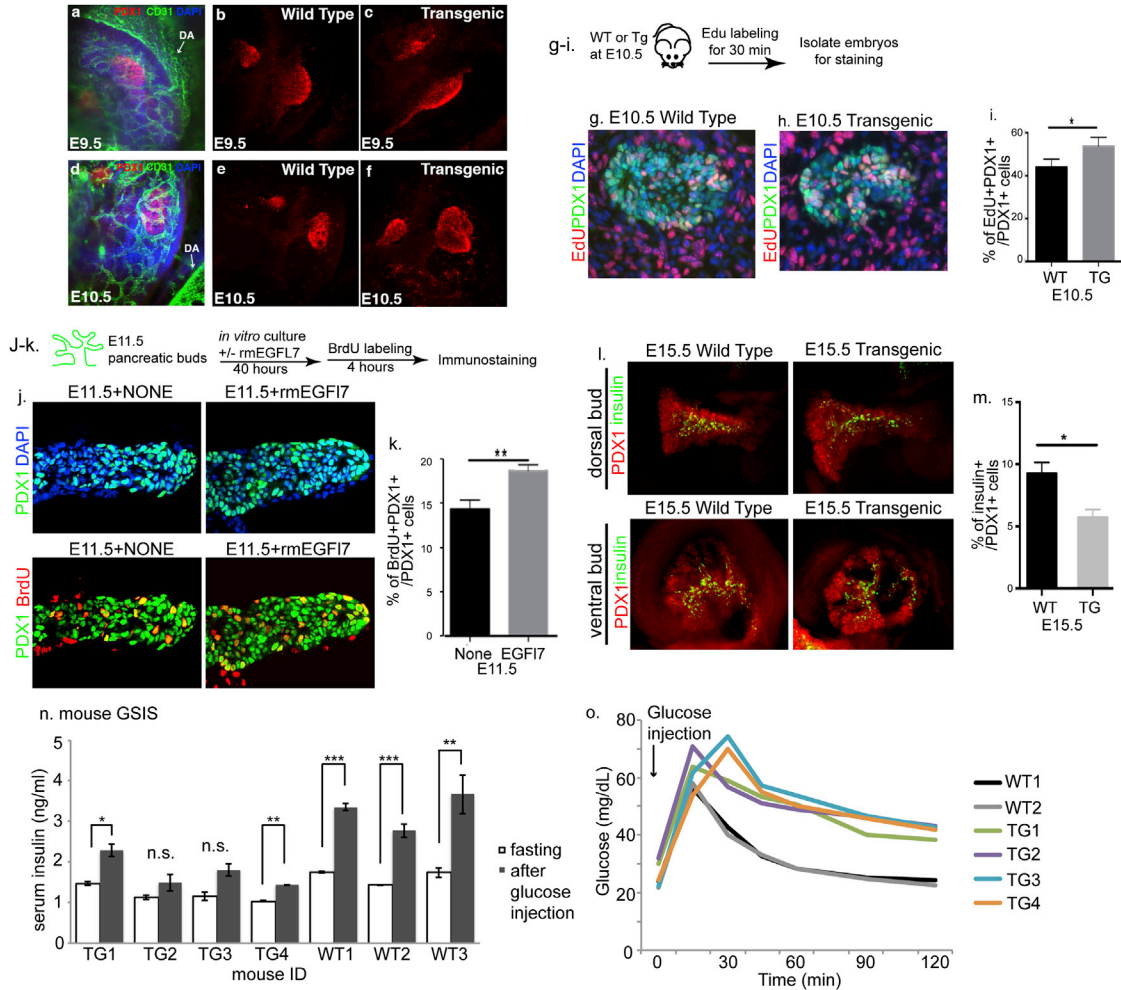


Figure 4. EGFL7 Increases Proliferation of PPs in Both TG and Explant Cultures

(A and D) Representative collapsed z stack confocal image of (A) E9.5 and (D) E10.5 mouse pancreatic buds costained for DAPI, PP marker PDX1, and endothelial cell marker, CD31.

(B, C, E, and F) Representative collapsed z stack confocal images of E9.5 and E10.5 pancreatic buds from WT and TG mice stained for PDX1. (G and H) Representative Images of E10.5 mouse pancreatic bud sections from (G) WT and (H) TG mice, costained for PP marker, PDX1, and proliferation marker, Edu.

(I) Quantification of the percentage of Edu⁺/PDX1⁺ cells of E10.5 WT and TG mice (n = 3).

(J) Representative images of E11.5 WT pancreatic bud explants treated with or without 1 μg/ml recombinant mouse EGFL7 and costained for DAPI, Pdx1, and BrdU.

(K) Quantification of the percentage of BrdU⁺/Pdx1⁺ cells of E11.5 WT pancreatic buds treated with or without recombinant EGFL7 (n = 6).

(L) Representative images of E15.5 dorsal and ventral pancreatic buds from WT and TG mice, costained for PP marker, PDX1, and insulin.

(M) Quantification of the ratio of insulin⁺ cells over PDX1⁺ cells of E15.5 pancreatic buds from WT and TG mice (n = 5).

(N) Glucose-stimulated insulin secretion (GSIS) test of WT control (n = 3) and TG (n = 4) mice.

(O) GTT of WT (n = 2) and TG (n = 4) mice.

Data were presented as mean ± SEM.

both fasting and after glucose stimulation compared with WT controls (fasting, *p = 0.048; after glucose stimulation, *p = 0.027, Figure 4N). Furthermore, the TG mice showed consistently impaired glucose tolerance (Figure 4O). The data suggest that adult TG mice have less functional beta cells than WT control mice.

Stepwise differentiation of hESCs provides a useful platform to study human development. Using a stepwise pancreatic differentiation system, we found that an endothelial cell niche plays a stage-dependent role in human pancreatic development, promoting PP self-renewal and impairing differentiation from PPs toward hormone-expressing cells.



Our finding could explain the results from previous animal studies: removing endothelial cells before the PP stage blocks pancreatic development (Lammert et al., 2001), while forced hypervascularization at the PP stage negatively impacts the later endocrine differentiation (Cai et al., 2012; Magenheimer et al., 2011; Sand et al., 2011). During pancreatic development, the endothelium pattern changes dramatically over time; this might affect the local concentration of paracrine factors, such as EGFL7, which will contribute to the cell fate decision. On the other hand, a recent study proposed that the endothelial cell niche could stimulate PPs to differentiate into insulin-secreting cells (Jaramillo and Banerjee, 2012). In contrast to our coculture conditions, in which MPECs and AKT-HUVECs survive well after 4 days of coculture (Figure S4N), the rat microvascular endothelial cells in that study do not survive well in differentiation conditions and could not be detected after 6 days in culture (Jaramillo and Banerjee, 2012). Although increased insulin expression was detected in the cocultures, we can speculate that the rat microvascular endothelial cells might provide a transient amplifying signal to expand PPs. If the endothelial cells start to die, the block on differentiation of PPs would be relieved. To test this hypothesis, we cultured hESC-derived PPs in endothelial cell CM for 4 days. The medium was then removed to induce spontaneous differentiation. Consistent with our hypothesis, the hESC-derived cells treated with CM show better differentiation toward insulin-expressing cells (Figure S4O).

EGFL7 is a secreted protein that acts as a chemoattractant for endothelial cells, binds to the extracellular matrix, and promotes endothelial cell adhesion (Schmidt et al., 2007). Here, we identified the role of EGFL7 as the molecular handle involved in the crosstalk between endothelium and pancreatic epithelium, which play as a gatekeeper to maintain PP self-renewal. In this context, it is interesting to note that the developmental stage of pancreatic bud formation (E9.0–E11.5) coincides with the peak in EGFL7 expression in the embryonic vasculature (Fitch et al., 2004). Our data also suggested that EGFL7 might function through EGF signaling pathway. Consistently, it has been shown that activation of EGF signaling facilitates the PDX1⁺ cells expansion (Zhang et al., 2009). Although our data strongly support our conclusion that EGFL7 promotes PP proliferation, we cannot exclude the possibility that EGFL7 may also promote foregut epithelium differentiation. In addition, recombinant EGFL7 protein was less efficient for expansion of PDX1⁺ progenitors compared with coculture with endothelial cells. To explain this discrepancy, we considered two possibilities. First, the recombinant EGFL7 protein may lack posttranslational modifications and might not be as functionally active as native EGFL7 secreted by endothelial cells. Second, we cannot exclude the possibility that endothelial cells secrete addi-

tional factors that boost the effect of EGFL7. Finally, since *Egfl7* transgene is overexpressed in all endothelia, we cannot fully rule out the possibility of systemic effects.

The pancreatic development is a highly dynamic process controlling by multiple pathways. Additional work is needed to unravel the dynamic interaction of pancreatic endoderm with endothelial and mesenchymal cell niches. A complete understanding of the underlying mechanism may lead to the development of more efficient strategies to differentiate hESCs/iPSCs into mature glucose-responding cells in vitro.

EXPERIMENTAL PROCEDURES

hESC Culture and Differentiation

HUES8 and H1 cells were routinely cultured on irradiated MEF feeders in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 20% KnockOut Serum Replacement, 2 mM GlutaMAX, 1 mM nonessential amino acids, 1.1 mM β -mercaptoethanol, 10 ng/ml basic fibroblast growth factor, and 50 ng/ml normacin. To differentiate to the PP population, hESCs were cultured on feeders to 80%–90% confluency and then treated with a series of chemicals listed in the supplementary method.

Coculture Experiment

MPECs, AKT-HUVECs, and BJ cells were used in the coculture experiments. hESC-derived populations were trypsinized by 0.25% trypsin (Invitrogen), resuspended in DMEM supplemented with 1 \times B27, and plated at a density of 0.8×10^3 cells/mm² in 96- or 6-well plates. MPECs, AKT-HUVECs, or BJ cells were resuspended in the same medium and plated down in a ratio of 1:1, 1:3, or 1:10 to hESC-derived PPs. One day after plating, medium was changed, and cells were maintained in the incubator for 4 days before fixation or further analysis. Detailed materials and methods are available in the [Supplemental Information](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2014.12.008>.

AUTHOR CONTRIBUTIONS

D.-I.K. and S.C. designed research. D.-I.K., L.A.L., C.H., K.P., and G.G. performed research. D.-I.K., L.A.L., H.S., C.H., G.G., and S.C. analyzed data. B.-S.D. and S.R. provide reagents, and D.K., L.A.L., H.S., and S.C. wrote the paper.

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