Video Article

Endothelial Cell Co-culture Mediates Maturation of Human Embryonic Stem Cell to Pancreatic Insulin Producing Cells in a Directed Differentiation Approach

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Abstract

Embryonic stem cells (ESC) have two main characteristics: they can be indefinitely propagated *in vitro* in an undifferentiated state and they are pluripotent, thus having the potential to differentiate into multiple lineages. Such properties make ESCs extremely attractive for cell based therapy and regenerative treatment applications¹. However for its full potential to be realized the cells have to be differentiated into mature and functional phenotypes, which is a daunting task. A promising approach in inducing cellular differentiation is to closely mimic the path of organogenesis in the *in vitro* setting. Pancreatic development is known to occur in specific stages², starting with endoderm, which can develop into several organs, including liver and pancreas. Endoderm induction can be achieved by modulation of the nodal pathway through addition of Activin A³ in combination with several growth factors⁴⁻⁷. Definitive endoderm cells then undergo pancreatic commitment by inhibition of sonic hedgehog inhibition, which can be achieved *in vitro* by addition of cyclopamine⁸. Pancreatic maturation is mediated by several parallel events including inhibition of notch signaling; aggregation of pancreatic progenitors into 3-dimensional clusters; induction of vascularization; to name a few. By far the most successful *in vitro* maturation of ESC derived pancreatic progenitor cells have been achieved through inhibition of notch signaling by DAPT supplementation⁹. Although successful, this results in low yield of the mature phenotype with reduced functionality. A less studied area is the effect of endothelial cell signaling in pancreatic maturation, which is increasingly being appreciated as an important contributing factor in in-vivo pancreatic islet maturation^{10,11}.

The current study explores such effect of endothelial cell signaling in maturation of human ESC derived pancreatic progenitor cells into insulin producing islet-like cells. We report a multi-stage directed differentiation protocol where the human ESCs are first induced towards endoderm by Activin A along with inhibition of PI3K pathway. Pancreatic specification of endoderm cells is achieved by inhibition of sonic hedgehog signaling by Cyclopamine along with retinoid induction by addition of Retinoic Acid. The final stage of maturation is induced by endothelial cell signaling achieved by a co-culture configuration. While several endothelial cells have been tested in the co-culture, herein we present our data with rat heart microvascular endothelial Cells (RHMVEC), primarily for the ease of analysis.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3759/>

Protocol

1. Cell Maintenance

1. H1 hESC (WiCell) were maintained on hESC qualified matrigel coated wells with mTeSR1 media, with media change every day. Wells were coated by dilute matrigel solution, prepared by adding 300 μ l of hESC matrigel in 25 ml of DMEM:F12. 1 ml of this matrigel solution was added to each well of a six well plate, or 400 μ l added to each well of a 12 well plate and allowed to coat for 1 hour at room temperature. Cells were mechanically passaged at a split ratio of 1:4 by scraping colonies once they reached between 1 and 1.5mm in diameter.
2. RHMVEC (VEC technologies) was maintained in MCDB-131 media with media change every other day. Cells were split by trypsinization once 80% confluence was reached at a split ratio of 1:3. Endothelial cells between passages 3 and 7 were used for this study.

2. Preparation of Stock Solutions

1. Activin A was reconstituted at 50 μ g/mL in sterile PBS containing 0.1% bovine serum albumin. The solution was aliquoted and stored at -20 °C.
2. EGF was reconstituted at 500 μ g/mL in sterile 10 mM Acetic Acid. The solution was aliquoted and stored at -20 °C.
3. Insulin was reconstituted at 10 mg/ml by adding acidified H₂O (pH $\leq$ 2), prepared by addition of 0.1 ml glacial acetic acid to 10 ml of water.
4. DAPT was reconstituted in DMSO at 18 mg/ml. It was further diluted to 300 μ M concentration in DMEM:F12, aliquoted and stored at -20 °C.
5. KAAD-Cyclopamine was reconstituted in DMSO at 5mg/ml. It was further diluted to 2 μ M concentration in DMEM:F12, aliquoted and stored at -20 °C.

- All-trans retinoic acid was reconstituted at 2.7 mg/ml in 95% ethanol. It was further diluted to 2mM concentration in DMEM:F12, aliquoted and stored at -80 °C.

3. Definitive Endoderm (DE) and Pancreatic Progenitor (PP) Induction

- Once hESC colonies reached between 1 and 1.5mm in diameter, DE induction was performed by adding DMEM:F12 supplemented with B27, 0.2% BSA, 100 ng/ml Activin A and 1 μ M Wortmannin for 4 days (Day 0-Day 4) with media change every day.
- After DE induction was complete, pancreatic progenitor induction was induced by changing the media to DMEM:F12 supplemented with B27, 0.2% BSA and KAAD-Cyclopamine at 0.2 μ M concentration for 24 hours (Day 4-Day 5).
- After 24 hours media was changed to DMEM:F12 supplemented with B27, 0.2% BSA, KAAD-Cyclopamine at 0.2 μ M concentration and all-trans retinoic acid at 2 μ M concentration for 3 days with media change every day (Day 5-Day 8).

4. Pancreatic Maturation

- After PP induction, all groups were changed to maturation media composed of DMEM:F12 supplemented with B27, 0.2% BSA, 10 mM Nicotinamide, 25 μ g/ml insulin, 30 nM Na₂SeO₃ and 50 μ g/ml transferrin for 2 days with media change every day (Day 8-Day 10).
- After 2 days in maturation media, the final maturation step is carried out in parallel using several different conditions for comparison. Differentiation was induced under the following conditions: (i) using notch inhibitor DAPT (ii) using co-culture media only, without endothelial cells as control and (iii) using contact co-culture with RHMVEC cells. Cells were maintained in DAPT or co-culture media for a week (Day 10-Day 17) with media change every day.
 - DAPT media was prepared by supplementing the maturation media with 30 μ M DAPT. Cells in this groups were first exposed to complete MCDB131 for 24 hours and then exposed to DAPT media for 6 days (Day 11-Day 17).
 - Co-culture media was prepared by supplementing MCDB-131 media with B27, 0.2% BSA, 10 mM Nicotinamide, 10 ng/ml EGF, 1 μ g/ml hydrocortisone, 10 mg EndoGro, 90 μ g/ml heparin. For media control condition the differentiating cells were exposed to MCDB-131 complete medium for 24 hours (Day 10-Day 11) after which the media was changed to co-culture media for 6 days (Day 11-Day 17) (no endothelial cells).
 - For contact co-culture condition, one million RHMVEC was added to the differentiating cells in complete MCDB-131 media (VEC technologies) for 24 hours (Day 10-Day 11) to allow attachment. After 24 hours media was changed to co-culture media for 6 days (Day 11-Day 17) with media change every day.

5. qRT-PCR Analysis

- At the end of each stage of differentiation (endoderm; pancreatic progenitor; mature islet) were lysed and RNA was extracted using NucleoSpin RNA II extraction kit according to manufacturer's instructions.
- RNA quality was analyzed by checking RNA absorbance at 260 nm and 280 nm, followed by RNA quantification using a SmartSpec Plus spectrophotometer.
- Reverse transcription was performed using ImProm II reverse transcription kit according to manufacturer's instructions. All reactions were performed with 100 ng of RNA.
- qRT-PCR was performed using the Mx3005P QPCR system (Agilent) and Brilliant II SYBR Green QPCR master mix according to manufacturer's instructions. The primers used are listed on table 1. The initialization step was performed at 50 °C for 2 minutes followed by 95 °C for 10 minutes. 50 amplification cycles were performed as follows: 95 °C for 30 seconds, 54 °C for 30 seconds and 72 °C for 1 minute.
- Fold change of target mRNA expression over undifferentiated cells was calculated from CT values using the following formulas:

$$\Delta CT(\text{Marker}_i) = CT(\text{Marker}_i) - CT(\text{GAPDH})$$

$$\Delta \Delta CT(\text{Marker}_i) = \Delta CT(\text{Marker}_i)(\text{Sample}) - \Delta CT(\text{Marker}_i)(\text{Undifferentiated cells})$$

$$\text{Relative expression}(\text{Marker}_i) = 2^{-\Delta \Delta CT(\text{marker}_i)}$$

6. Immunocytochemistry

- At the end of each stage of differentiation (endoderm; pancreatic progenitor; mature islet) cells were fixed in 4% formaldehyde for 15 minutes at room temperature.
- Fixed cells were permeabilized using 0.25% Triton X-100 (TX) for 15 minutes.
- Non-specific staining was blocked by incubation in 10% donkey serum in 0.05% TX for 30 minutes.
- Primary antibody incubation was performed overnight at 4 °C in blocking buffer at the recommended antibody dilution (see table 1).
- Cells were washed with 0.05% TX three times for 5 minutes.
- Secondary antibody incubation was performed for one hour at room temperature in the dark with appropriate antibodies diluted in blocking buffer.
- Cells were washed with 0.05% TX three times for 5 minutes.
- Nuclear staining was performed by incubation with Hoechst stain at 1:1000 dilution in PBS for 5 minutes followed by 3 washes with PBS.
- Fixed and stained cells were imaged using Olympus IX81 inverted microscope and Metamorph imaging software.

7. Representative Results

Addition of Activin A and Wortmannin to undifferentiated hESC for 4 days induces definitive endoderm as confirmed by qRT-PCR analysis for DE markers Sox17, Cxcr4, and Foxa2 and immunostaining for Sox17 as illustrated in **Figure 2**. Differentiation to pancreatic progenitor cells after addition of cyclopamine and retinoic acid was confirmed by qRT-PCR of pancreatic progenitor markers and Immunostaining for Pdx1 as illustrated in **Figure 3**.

At the final stage of differentiation, contact co-culture with RHMVEC cells strongly induced upregulation of insulin expression in the hESC derived pancreatic progenitor cells. The efficiency of co-culture mediated differentiation was analyzed by comparing with control condition using DAPT. DAPT was used as a positive control since it is currently one of the most widely used methods to achieve pancreatic maturation. Since the

co-culture was performed in a modified media supplemented by factors supportive of endothelial cells, additional control was performed with the media in absence of endothelial cells to verify the effect of media on differentiation. Details of this analysis are presented in **Figure 4**.

* Rat Heart Microvessel Endothelial Cells (RHMVEC)

Figure 1. Multi-stage protocol for differentiation of hESC into insulin expressing cells.

Figure 2. Definitive endoderm induction of human embryonic stem cells. A) qRT-PCR analysis of representative DE markers in hESC cells exposed to Activin A and Wortmannin for 4 days. Results are normalized with respect to undifferentiated hESC. B) Sox17 staining (Green) and DAPI (blue) show nuclear expression of Sox17. Scale bar: 50 μ m.

Figure 3. Pancreatic progenitor induction of the embryonic stem cell derived endoderm cells. A) qRT-PCR analysis of early pancreatic markers in hESC derived endoderm cells exposed to cyclopamine and retinoic acid for 4 days after DE induction. Results normalized with respect to undifferentiated hESC. B) Pdx1 staining (violet) and DAPI (blue) show nuclear expression of Pdx1. Scale bar: 50 μ m.

Figure 4. Pancreatic maturation stage A) qRT-PCR analysis of pancreatic hormones in hESC after pancreatic maturation using DAPT, contact co-culture or co-culture media (control). Results normalized with respect to undifferentiated hESC. p values obtained by student t-test. B) Co-culture with DiI-Ac-LDL labeled RHMVEC (Red) show regions where ECs attach to the plate if there are empty spaces (top), other RHMVEC can be found in direct contact with the differentiating ESC(bottom). C) C-peptide (Green) staining for cells differentiated using co-culture method. Scale bar: 12.5 μ m (Top) and 50 μ m (Bottom) D) Immunostaining of RHMVEC demonstrates no insulin expression in the RHMVEC.

Marker	Primer1 (5' TO 3')	Primer2 (5' TO 3')	Ref
SOX17	CTCTGCCTCCTCCACGAA	CAGAATCCAGACCTGCACAA	12
CXCR4	CACCGCATCTGGAGAACCA	GCCCATTTCTCGGTGTAGTT	4
FOXA2	GGAGCGGTGAAGATGGAA	TACGTGTTTCATGCCGTTTCAT	12
PDX1	AAGTCTACCAAAGCTCACGCG	GTAGGCGCCGCCTGC	4
PTF1	CATAGAGAACGAAACCACCCTTTGAG	GCACGGAGTTTCTGGACAGAGTTC	12
HLXB9	CACCGCGGGCATGATC	ACTTCCCCAGGAGGTTTCGA	4
HNF6	TGTGGAAGTGGCTGCAGGA	TGTGAAGACCAACCTGGGCT	5
PAX6	CGAATTCTGCAGGTGTCCAA	ACAGACCCCTCGGACAGTAAT	5
NKX6.1	AGACCCACTTTTTCCGGACA	CCAACGAATAGGCCAAACGA	5
ISL1	GATCTATGTCACCTCGCAAGG	TACAACCACCATTTCACTG	12
GLUCAGON	AGGCAGACCCACTCAGTGA	AACAATGGCGACCTCTTCTG	4
INSULIN	AAGAGGCCATCAAGCAGATCA	CAGGAGGCGCATCCACA	12

Table 1. Primers used for qPCR reactions.

Discussion

During pancreatic development, differentiating pancreatic cells are in close proximity with endothelial cells from the aorta; furthermore, pancreatic islets are densely vascularized to promote rapid exchange of blood glucose and islet hormones. Given these facts, it is not surprising that endothelial cells play an important role in the process of pancreatic organogenesis. While the importance of endothelial cells during pancreatic development is increasingly being appreciated, its role in *in vitro* differentiation of embryonic cells is less investigated. In our earlier report we established the positive role of endothelial cells on pancreatic maturation of mouse embryonic stem cells¹³. In the current study we investigate the effect of endothelial cell signaling on differentiating human embryonic stem cells.

Multiple endothelial cells were used in this protocol, all of which resulted in similar overall effect although with variations in efficiency of differentiation. In the current report we present the effect of co-culturing hESC derived pancreatic progenitor cells with rat heart microvascular endothelial cells (RHMVEC). Endothelial cells can be conveniently visualized by AC-LDL staining, which is specific to only endothelial cells. Co-culture of the differentiating ESCs with pre-stained endothelial cells showed that while most of the endothelial cells were viable in culture for extended period of time, the RHMVEC gradually disappear after 4 days and could no longer be detected after 6 days of maturation. Absence of the RHMVEC simplifies analysis by eliminating the need for sorting, hence was chosen for optimization of the co-culture protocol.

While the present study clearly indicates the positive effect of endothelial cells in inducing pancreatic maturation, the exact mechanism of such induction is still unknown and being currently investigated in our laboratory. A few plausible mechanisms could be (i) the effect of cell-secreted molecules (ii) endothelial cell mediated inhibition of notch signaling (iii) the role of extracellular matrix secreted by the endothelial cells. While the first category does not require cell-cell contact, such contact is mandatory for the second and third category. Hence we performed some preliminary analysis using different co-culture configurations: (i) contact co-culture (ii) transwell co-culture and (iii) conditioned media culture. It was observed that contact co-culture resulted in maximum differentiation, as judged by insulin expression; followed by transwell co-culture; while

conditioned media culture resulted in minimal effect on insulin expression (data not shown). These analyses indicate that while cell-cell contact is important, there might be some short lived secreted molecules which are important as well.

Disclosures

We have nothing to disclose.

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