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"Christa Caneda is a fourth-year Regents Scholar from Sacramento, California. She is a Biological Sciences major, with her studies emphasizing on Microbiology and Immunology. She avidly looks forward to graduating with UC Merced's Class of 2013, and hopes that her future in research will provide a suitable lifelong environment for her passion towards biology. Christa will be taking a working year after graduation, and has currently accepted a full-time research position at The Scripps Research Institute in La Jolla, California. In addition, she currently enjoys sharing her experiences in biology through her position as an Upper Division Biology Tutor with the Bright E. Success Center. When Christa is not sporting her lab coat to perform cell culture, she enjoys the pastimes of drawing, baking, and theater. She also loves to end long workdays with pineapple milk tea, and hopes that her friends reading this will get the hint."



**Embryoid Body Formation is Required for Differentiation of  
Insulin-Producing Cell Clusters from Mouse Embryonic Stem Cells**

## Abstract

In Type 1 diabetes, insulin-producing pancreatic cells, or beta cells, are destroyed by an autoimmune response. Current clinical treatments are indefinite insulin replacement therapy or transplantation of the pancreas or beta islets. The latter two treatments are limited in available donors; a potential alternative is the use of insulin-producing cell clusters (IPCCs) differentiated from embryonic stem cells (ESCs). We hypothesize that IPCCs will reproduce the insulin-producing capacity of healthy beta cells of an adult mouse, and we are testing the efficiency of distinct IPCC culture methods to achieve this goal. Among several existing ESC-IPCC differentiation protocols, Blyszczuk et al. developed the most successful method to date in producing IPCCs that showed similarities to pancreatic beta cells. However, this method is time-intensive, requiring approximately 41 days. We attempted to streamline the protocol by bypassing the formation of embryoid bodies (EBs), reducing the differentiation timeline to 27 days. At several time points during this protocol, IPCC cultures were analyzed by RT-PCR and immunofluorescence for genes and proteins expressed in pancreatic beta islet cells. The mRNA and protein expression of insulin was not observed. Furthermore, ELISA analysis detected low intracellular insulin response after challenging IPCCs with different glucose concentrations. These results reject the hypothesis that EB formation is not required for ESC-IPCC differentiation in vitro. However, it is possible that alpha cells can be differentiated, as glucagon was detected.

## Introduction

Type 1 diabetes is an autoimmune disease that involves the destruction of insulin-producing beta cells when autoreactive CD8<sup>+</sup> cytotoxic T cells are activated by CD4<sup>+</sup> helper T cells. Current treatments are limited to indefinite insulin therapy, pancreas transplantation, and beta islet transplantation. The latter two treatments are afflicted by a lack of suitable donors, low transplantation success rates, and transiently restored glucose regulation. Thus, stem cell therapy has been proposed as an alternative treatment<sup>1,9,10</sup>. This can potentially be achieved through beta islet replacement by embryonic stem cell (ESC) differentiation to insulin-producing cell clusters (IPCCs).

Currently, several laboratories have developed and published ESC-IPCC differentiation protocols with

varying levels of similarities to pancreatic beta cells. To date, one of the most successful protocols in recreating IPCCs with beta islet resemblance is the method established by Blyszczuk et al. However, its methodology is demanding in time and labor, as it incorporates embryoid body (EB) formation<sup>1,9</sup>.

There are multiple ways that embryonic stem cells can be differentiated. One way is by the formation of embryoid bodies, which are compact clusters of loosely organized tissue that resemble a gastrulating embryo; another method is a two-dimensional approach, where embryonic stem cells are induced as a monolayer on a tissue culture plate in specific differentiation media<sup>8</sup>. In the protocol by Blyszczuk et al., the intermediate step of EB formation causes the method to require about 41 days for ESC-IPCC differentiation. Eliminating this step would reduce the differentiation timeline to 27 days, potentially providing a more efficient and less labor intensive production of IPCCs from mouse ESCs, as has been demonstrated with the generation of human IPCCs without EB formation<sup>3,4</sup>.

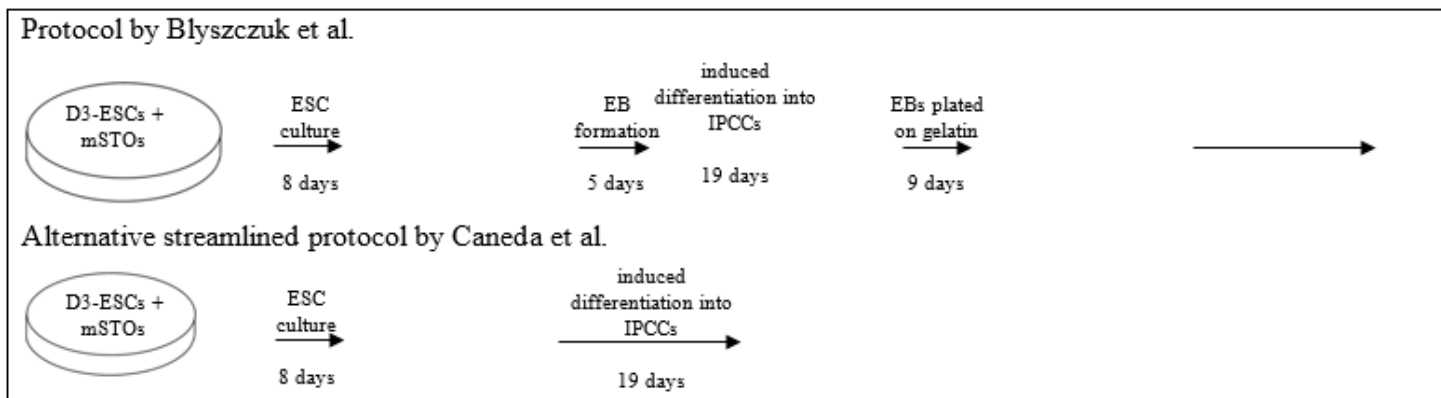


Figure 1. Project Goals

Figure 1. Differentiation timeline outlining differences between original protocol by Blyszczuk et al. and the alternative protocol with goals to optimize the Blyszczuk protocol to become less time-intensive, namely by bypassing embryoid body formation.

## Methods and Materials

### Cell Culture

ES-D3 mouse embryonic stem cells were cultured on a feeder layer of murine STO fibroblasts cells, which were mitotically inactivated by treatment with 10  $\mu\text{g}/\text{mL}$  Mitomycin C<sup>5</sup>. These cells were incubated at 37°C with 5% CO<sub>2</sub> in a medium consisting of 15% ES-qualified fetal calf serum (FCS), 0.15 mM monothio-

glycerol (MTG), 1000 U/mL leukemia inhibitory factor (LIF), 1X penicillin/streptomycin, and Dulbecco's Modified Eagle Medium (DMEM) for two days. These cells were then expanded by trypsin subculture for an additional three days at identical conditions. After this time-point, ES cells were detached by trypsinization and then replated onto 60-mm plates coated with 0.1% gelatin in differentiation media (DM1) consisting of ES-qualified FCS, L-glutamine, non-essential amino acids, MTG, penicillin/streptomycin, and Iscove's Modified Dulbecco's Medium (IMDM)<sup>9</sup>. ES cells were allowed to culture in this environment for two days.

Afterward, ES cells were then replated onto 10  $\mu\text{g}/\text{cm}^2$  collagen I-coated plates with specific differentiation media that was more selective for IPCC differentiation. The differentiation media contained progesterone, putrescine, laminin, nicotinamide, insulin,  $\text{Na}_2\text{SeO}_3$ , transferrin, B27 supplement, penicillin/streptomycin, 10% FCS, and DMEM: Nutrient Mixture F-12 (DMEM/F12)<sup>9</sup>. After incubating in media with 10% FCS for one day, cells were moved to specific differentiation media with 5% FCS, with other reagents being otherwise identical. Cells were cultured on collagen I coated wells for a total of 19 days, with samples collected on days 7 and 19 for further analysis.

### *Immunofluorescence Analysis*

On Days 7 and 19 of differentiation, ESC-IPCC cells were collected for immunofluorescence analysis (IFA). These cells were fixed onto microscope slides at  $1.0 \times 10^5$  cells per slide with 4% paraformaldehyde for 15 minutes at room temperature. Cell clusters were incubated for 30 minutes at room temperature with blocking buffer consisting of 10% normal goat serum, 0.1% Triton-X, and phosphate buffered saline. In addition, cultures of the INS-1 832/13 insulinoma cell line (cells derived from an insulin-secreting mouse pancreas tumor) were also fixed with identical conditions to be utilized as a control against ESC-IPCCs<sup>2</sup>.

After blocking, samples and controls were incubated at 4°C with the following primary antibodies: guinea pig anti-insulin, rabbit anti-c-peptide, and rabbit anti-glucagon. Several samples of INS 832/13 cells were not stained with primary antibodies, keeping them as negative controls. Samples were then rinsed and incubated for 45 minutes at 37°C with secondary antibodies against guinea pig IgG FITC and rabbit IgG Atto 633. Finally, slides were stained with DAPI for nuclei visualization and then sealed with mounting medium

before performing analysis by fluorescence microscopy, using the Olympus BX51 Microscope and Image-Pro Plus 5.0 software.

### *Gene Expression*

Gene expression in ESC-IPCCs was analyzed by polymerase chain reaction (PCR) for comparison with genes expressed by pancreatic cells in adult mice. To prepare ESC-IPCCs for PCR analysis, cell culture samples were first suspended in RNeasy Lysis Buffer (Qiagen). Then, RNA extraction was performed on cells with the Qiagen RNeasy Mini Kit according to manufacturer instructions.

PCR testing was conducted for the following genes: *insulin-1*, *insulin-2*, *glucagon*, and  *$\beta$ 5-tubulin*. First-strand cDNA was synthesized from total RNA using reverse transcriptase. 5  $\mu$ M oligo-dT and 1 mM dNTPs were added to 500 ng of total RNA from the following samples: ESCs cultured on gelatin as an undifferentiated negative control, adult mouse pancreatic cells as a positive control, and RNase-free water as a template-free negative control. In addition, oligo-dTs and dNTPs were also added to 33.6 ng of RNA from ESC-IPCCs on Day 7, and 50.4 ng of RNA from ESC-IPCCs on Day 19. Due to low cell numbers, these were the maximum attainable amounts of RNA from these samples.

These samples were incubated at 65°C for 5 minutes, using the Eppendorf Mastercycler EP Gradient S Thermal Cycler. Afterward, cDNA synthesis was performed by incubating samples in 1X RT buffer, 5 mM MgCl<sub>2</sub>, 0.1 M DTT, 2 units/ $\mu$ L RNase OUT, and 10 units/ $\mu$ L SuperScript III RT at 50°C for 50 minutes. The synthesis reaction was terminated by keeping samples at 85°C for 5 minutes. Finally, RNA was removed from each sample by adding 1  $\mu$ L of RNase H, then incubating at 37°C for 20 minutes.

PCR amplification was then performed on the resultant cDNA. This PCR master mix was composed of the following components: 1X *Taq* buffer, 0.2  $\mu$ M forward/reverse primers for the specific gene of interest (Table 1), 0.2 mM dNTPs, and 0.06 units/ $\mu$ L *Taq* polymerase. This mix was then added to 10  $\mu$ L of each synthesized cDNA sample. These samples were then amplified in a thermal cycler at the following conditions: denaturation at 95°C for 2 minutes, followed by 45 cycles of 95°C for 1 minutes, 60°C for 1 minute, and 72°C for 1 minute. The cycle ended at 70° C for 10 minutes, and samples were then held in the cycler at 4°C prior to gel

electrophoresis.

Gel electrophoresis was conducted at 80-V for approximately 45 minutes with a 2% agarose gel stained with ethidium bromide. Separated PCR products were then photographed with the BioRad ChemiDoc imaging system. The sizes of amplified PCR products were confirmed by comparison with a 100 bp Low Scale DNA ladder (Fisher Scientific).

Table 1. PCR genes and primers

Gene	Primer Sequence (forward/reverse)	Size (base pairs)
<i>insulin-1</i>	5'-TAG TGA CCA GCT ATA ATC AGA GAC-3' 5'-CGC CAA GGT CTG AAG GTC-3'	288
<i>insulin-2</i>	5'-CCC TGC TGG CCC TGC TCT T-3' 5'-AGG TCT GAA GGT CAC CTG CT-3'	213
<i>glucagon</i>	5'-CAT TCA CAG GGC ACA TTC ACC-3' 5'-CCA GCC CAA GCA ATG AAT TCC-3'	207
<i>β5-tubulin</i>	5'-TCA CTG TGC CTG AAC TTA CC-3' 5'-GGA ACA TAG CCG TAA ACT GC-3'	318

### Challenge With Glucose

On Day 19 of the streamlined ESC-IPCC differentiation protocol, ESC-IPCCs were cultured in insulin-free media for 48 hours prior to challenging with glucose. Media was changed again 3 hours before glucose challenge. Cells were washed five times with sterile 1X PBS, then incubated at 37°C in 2.5 mM glucose and Krebs Ringer bicarbonate HEPES (KRBH) buffer (118 mM NaCl, 4.7 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 3.4 mM CaCl<sub>2</sub>, 2.5 MgSO<sub>4</sub>, 10 mM HEPES, and 2 mg/ml BSA at 7.4 pH) for 90 minutes. The KRBH buffer was replaced with KRBH containing either 2.5 mM glucose, 5.5 mM glucose and tolbutamide, or 27.7 mM glucose for glucose challenge. Cells were incubated in these conditions for one hour, after which they were dissociated by trypsinization and resuspended in acid ethanol.

Upon resuspension, cells were sonicated three times for thirty seconds each, with 1 minute cooling intervals. Proteins were extracted with acid ethanol overnight at 4°C; the acid ethanol was then neutralized with 1 M Tris Buffer, pH 7.5. Intracellular insulin levels were analyzed by the Mercodia Ultrasensitive Mouse In-

sulin Enzyme-Linked Immunosorbent Assay (ELISA) according to manufacturer instructions.

**Results:**

ESCs were differentiated as depicted in Figure 1. ESC-IPCC were analyzed for morphology (Figure 2), expression of specific proteins and genes (Figures 3 and 4), and for insulin-production after glucose challenge (Figure 4).

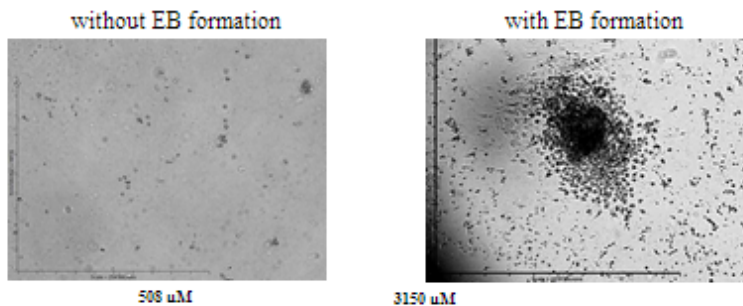


Figure 2. ESC-IPCC Morphology

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Figure 2. Cell morphology during ESC-IPCC differentiation was analyzed by light microscopy. Cells without EB formation displayed low levels of proliferation in comparison to IPCCs differentiated through the Blyszczuk et al. protocol (with EB formation) after 41 days of differentiation.

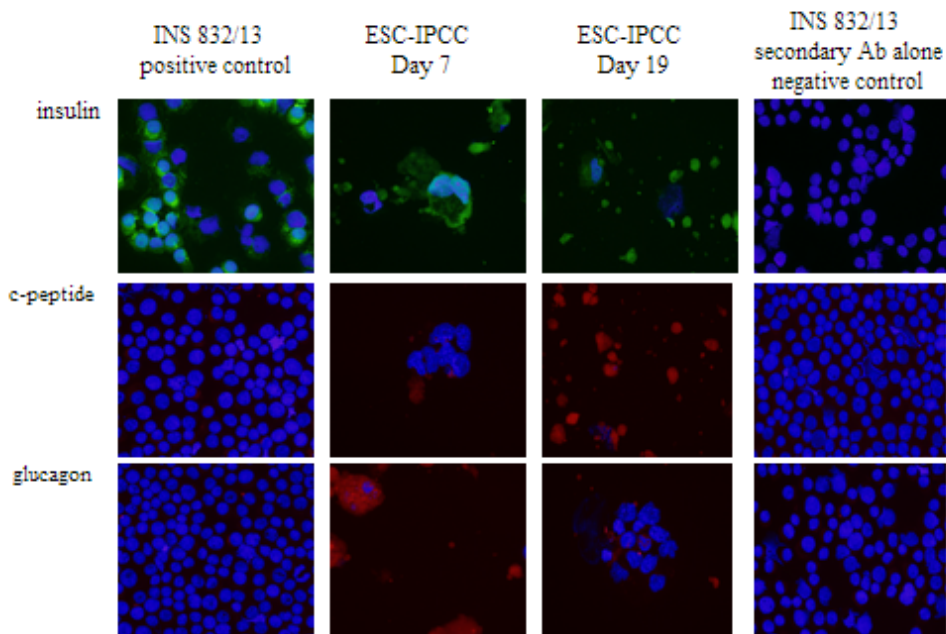


Figure 3. Immunofluorescence Analysis

Figure 3. Immunofluorescence analysis of ESC-IPCCs under 400X magnification. Cells were stained for insulin in green, and DAPI in blue (marker for cell nuclei).

As expected of healthy insulinoma cells, staining of insulinoma cells demonstrated colocalization of DAPI and insulin in the same cell. In contrast, ESC-IPCC staining showed DAPI-only and insulin-only staining, indicative of debris.

The insulinoma positive control for c-peptide did not demonstrate a substantial signal when compared to the negative control. Due to a lack of signal in the positive control, antibody visualization produced by ESC-IPCCs was negligible. However, the IFA for c-peptide in ESC-IPCCs continued to demonstrate staining without abundant DAPI colocalization, supporting the presence of debris.



Figure 4. Gene Expression Analysis

Figure 4. Analysis for gene expression was performed for pancreatic endocrine-specific genes. The number of genes analyzed by RT-PCR was limited by low RNA concentrations during sample collection, with  $\leq 10$  ng/ $\mu$ L for ESC-IPCCs collected on Days 7 and 19 due to a low number of cells.

Products were amplified by polymerase chain reaction and separated for visualization by gel electrophoresis. Analysis was performed on the following samples from left to right: embryonic stem cells (undifferentiated negative control), ESC-IPCCs Day 7, ESC-IPCCs Day 19, adult mouse pancreas (positive control), and RNase-free water (template-free negative control). Conserved *β5-tubulin* was utilized as a housekeeping gene, and was visualized in all samples. *Insulin-1* and *insulin-2* were not detected, while *glucagon* signal was present in ESC-IPCCs Day 7.

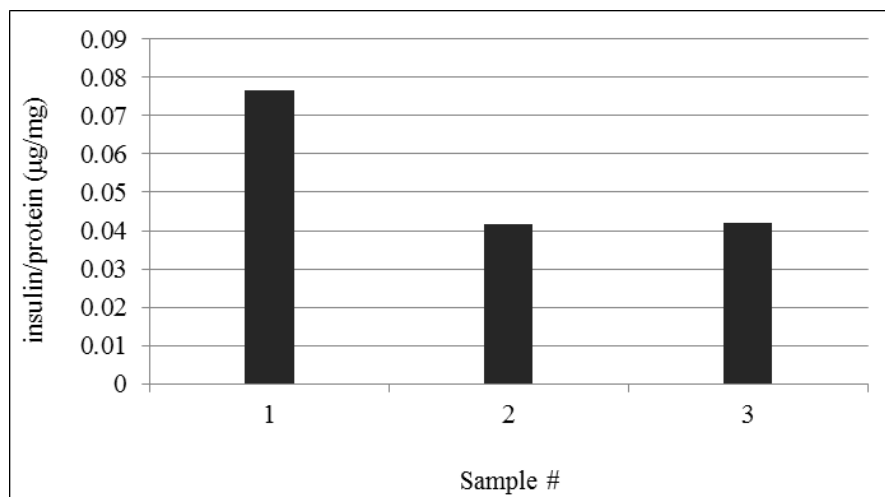


Figure 5. Challenge with Glucose

Figure 5. Intracellular response of ESC-IPCCs after glucose challenge, as measured by ELISA. Samples were challenged after Day 19 with: 1) 2.5 mM glucose, 2) 5.5 mM glucose and tolbutamide, and 3) 27.7 mM glucose. The observed insulin response was low ( $< 0.08 \mu\text{g}/\text{mg}$ ), whereas the Blyszczuk et al. protocol produced an intracellular response of  $\approx 0.31 \mu\text{g}/\text{mg}$ .

### Discussion:

Results throughout the streamlined differentiation protocol demonstrated that embryoid body formation is necessary for healthy ESC-IPCC differentiation, as measured by the presence of minimal cell numbers with low expression for pancreas-specific genes and proteins.

Visualization of c-peptide expression by IFA was not reliable due to limited expression in the insulinoma positive control, where c-peptide signals were below detection level. This may be due to an incompatibility of the c-peptide antibody with the IFA conditions that were applied, or the quality of the antibody itself may be limited, which would require further investigation.

There are several possibilities that could contribute to the underperformance of the ESC-IPCCs produced by the streamlined protocol. For example, the lack of EB formation could hinder effective cell signaling and/or formation of the endoderm germ layer prior to ESC differentiation, which is essential to proper pancreatic beta cell development<sup>9</sup>. However, the *glucagon* expression observed in our cultures suggests that alpha cells, in contrast to beta cells, can be differentiated without EB formation. This suggests that the streamlined

ESC-IPCC protocol may be applicable to other afflictions to glucose homeostasis, such as hypoglycemia due to glucagon deficiency. The secretion of glucagon by pancreatic alpha cells is critical in opposing insulin actions and regulating physiological glucose levels<sup>7</sup>.

Awareness of EB formation requirements for ESC-IPCC differentiation furthers the understanding of effectively optimizing the protocol of Blyszczuk et al., and enables focus to be placed towards tailoring alternative variables of the protocol. Future directions for optimizing the ESC-IPCC differentiation protocol of Blyszczuk et al. would incorporate EB formation prior to induction into IPCCs, and instead consist of alterations in cell concentrations, changes to differentiation media reagents, and measure of insulin release. Alternatively, endoderm formation without EB formation could be induced prior to IPCC differentiation as described<sup>3,4</sup>.

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