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Cerberus1 Regulates the Differentiation of Human Embryonic Stem Cells into Definitive Endoderm

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Cerberus1 Regulates the Differentiation of Human Embryonic Stem Cells into  
Definitive Endoderm**

A thesis submitted in partial satisfaction of the requirements for the degree Master of  
Science

in

Biology

by

Sahar Zargar

Committee in charge:

Professor Ulupi Jhala, Chair  
Professor Dong-Er Zhang, Co-Chair  
Professor Michael David

2017



The Thesis of Sahar Zargar is approved and it is accepted in quality and form for  
publication on microfilm and electronically:

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University of California, San Diego

2017

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## **ABSTRACT OF THE THESIS**

Cerberus1 Regulates the Differentiation of Human Embryonic Stem Cells into  
Definitive Endoderm

by

Sahar Zargar

Master of Science in Biology

University of California, San Diego, 2017

Professor Ulupi Jhala, Chair  
Professor Dong-Er Zhang, Co-Chair

Cerberus1 is a secreted protein that has emerged as a critical regulator of embryogenesis in mouse, frog, and more recently human embryos. Despite the wealth of information about its roles in embryogenesis, there has yet to be a complete understanding of its role in human embryonic stem cell (hESC) pluripotency and differentiation into cells of the Definitive Endoderm (DE) and eventually insulin-producing, glucose responsive cells. These insulin-producing, glucose responsive cells regulate blood glucose levels and are the focus of regenerative therapies for the treatment of Type 1

Diabetes. Understanding how biochemistry and signal transduction regulate this transition out of pluripotency is not only critical to understand how cells communicate with the extracellular environment but also to develop safe and effective protocols to generate tissues from select cell lines. In this report, we created a model system to modulate DE formation and look at the role of secreted proteins on differentiation. We show that changes in the media from directed differentiation protocols signal the cells, which then actively respond by upregulating secreted proteins such as Cer1. We used conditioned media, Cer1 antibodies, and exogenous Cer1 to show that Cer1 modulates cell fate when it's secreted by hESCs. Taken together, these results show that Cer1 is not simply a marker of DE but it also plays an active role in regulating differentiation by signaling to the cells that DE formation is complete and therefore halting differentiation. These findings provide insight into how cells communicate with the extracellular aid in enhancement of the production of insulin-secreting, glucose responsive cells across different hESC lines.

## INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which the insulin-producing beta ( $\beta$ ) cells of the pancreas are destroyed, resulting in absolute insulin deficiency and subsequent inability to regulate blood glucose levels<sup>1,2</sup>. According to the Centers for Disease Control, the prevalence of T1D is increasing, with approximately 40,000 people newly diagnosed each year in the U.S. alone<sup>1,3</sup>. Current treatments include daily insulin injections, complete pancreas replacement, or islet transplantation.<sup>4</sup> Each of these approaches have their own limitations<sup>3</sup>. For the typical T1D patient, regular insulin injections are required along with constant monitoring of blood glucose levels to prevent potentially life threatening spikes or drops in blood glucose levels<sup>1,3,5,6</sup>. Although patients on this strict regimen are expected to live a normal life span, serious complications including neuropathy, retinopathy, and increased heart disease are common<sup>1</sup>. In the past 30 years, there have been attempts to cure T1D through both whole pancreas and islet transplants<sup>5,6</sup>. These procedures have been met with moderate success, are costly, and are limited by the number of organs available<sup>1,3,6</sup>. Patients who opt for these approaches have a risk of organ rejection, and must take immunosuppression drugs<sup>5,6</sup>. The most successful treatment has been islet transplants based on the Edmonton protocol<sup>4</sup>. Although initially promising, this approach has shown to be an inefficient solution because the transplant fails after 2 years and patients again become reliant on insulin injections<sup>4,6,7</sup>. These obstacles highlight the need to develop more effective treatments.

Stem cell research and regenerative medicine show great promise for the treatment of T1D, with current research focusing on ways to improve  $\beta$  cell replacement

strategies<sup>6</sup>. One such method to generate insulin secreting  $\beta$  cells requires a complete understanding how human embryonic stem cells (hESCs) differentiate into insulin-producing, glucose responsive cells.

Human embryonic stem cells are isolated and cultured from the inner cell mass (ICM) of the preimplantation embryo.<sup>8</sup> The ICM is a cluster of cells that will develop into the embryo before implantation into the uterine wall<sup>8</sup>. The cells of the ICM exhibit characteristics of stem cells; they have the capacity for unlimited self-renewal,<sup>5</sup> proliferation, and give rise to cells from all three germ layers: endoderm, ectoderm, and mesoderm<sup>9</sup>. The ability of pluripotent hESCs to reproduce indefinitely while retaining the ability to differentiate into any somatic cell makes them ideal for cell based T1D treatment options and disease modeling *in vitro*<sup>5</sup>. However, for hESCs to be used therapeutically, the biochemistry and signal transduction pathways that regulate the differentiation process must be fully understood to avoid teratomas – tumors that arise when a small population of stem cells do not exit from pluripotency and contain tissues of organs from the multiple germ layers<sup>5,10</sup>. Teratomas pose the greatest therapeutic hurdle because pluripotent cells still possess the large replicative potential<sup>10</sup>.

As hESCs differentiate into a specific cell type *in vivo*, they pass through developmental milestones that are categorized by the up and down regulation of specific transcription factors and other proteins<sup>11</sup>. Specifically, the pluripotent cells of the ICM express the transcription factors: Oct4, Sox2, and Nanog, key markers for pluripotency<sup>12,13</sup>. While there are many other markers for pluripotency as determined by Wang et al., via computational analysis of the hESC transcriptional network, Oct4, Sox2, and Nanog are classical markers and will be used for the scope of this study<sup>14</sup>. As this

unique transcriptional profile shifts and the expression of these transcription factors decreases (**Figure 1**), this signals the cells have begun to exit pluripotency and started to differentiate<sup>13</sup>. As seen in Figure 1, to recreate the pancreatic progenitor differentiation process *in vitro*, specific growth media must be added to push the cells toward endodermal and eventually pancreatic cell fate<sup>12</sup>. In addition to downregulation of signaling through the lipid kinase phosphatidylinositol-3-kinase (PI3K)<sup>16</sup>, the growth factors Activin A and Wnt3a, must be added to drive the cells out of pluripotency and initiate mesendoderm (ME) formation<sup>15,17</sup>. The molecular details of this process are well understood. Serum is removed which decreases signaling through PI3K to Akt which regulates survival of GSK3<sup>16</sup>. Low levels of Activin A mimic Nodal signaling, determines the anterior-posterior axis of the embryo,<sup>18</sup> and induces the formation of ME cells, cells that are bipotent for mesoderm and definitive endoderm<sup>19,20</sup>. Addition of Noggin and BMP (bone morphogen protein) push ME cells into mesoderm<sup>15,17</sup>. However, to avoid mesodermal fate, serum and high levels of Activin A must be added at day 2 of differentiation<sup>15,17</sup>. Wnt3a has been shown to optimize the development of pancreatic cell fate by mimicking the natural process of development<sup>17,21</sup> and interacts with anterior-posterior axis determination<sup>22</sup>. By day 4 of embryonic development, the key markers of DE are expressed: *SOX17*, *Cerberus (CER)*, *FOXA2*, and *CXCR4*<sup>15,17,23–25</sup>. After DE formation, the cells progress through the four other stages of development until they express the hormone insulin (**Figure 2**)<sup>15</sup>. This differentiation process is not linear; the characteristic transcription factors are expressed at more than one time and can have different roles<sup>11</sup>. Because of this, the complete understanding of the differentiation process and the interaction between the proteins is not fully understood<sup>11</sup>. This project

focuses on the 4 day time period from pluripotency to DE because it is the crucial step where the stem cells become one of the three germ layers<sup>26</sup>. While the protocol for *in vitro* generation of DE cells is well-established<sup>17</sup>, different hESC cell lines have intrinsically different capacities to form DE<sup>5,11,27</sup>. These variances in differentiation between cell lines suggest that factors secreted into the media are helping drive hESC cell fate independently of growth factors or proteins supplemented in the media. Therefore, in this study we aim to identify the roles that these proteins play in facilitating differentiation.

To understand the biochemistry that regulates DE formation, an expanded understanding of Activin signaling is required. Activins are members of the TGF $\beta$  superfamily, which consists of 3 main ligand subfamilies: BMPs, Activins, and TGF $\beta$ <sup>28,29</sup>. These ligands bind their cognate type I and type II receptors and stimulate differentiation and growth responses<sup>29</sup>. The type II receptor phosphorylates type I receptor which subsequently recruits and phosphorylates SMADS (a homology of the *Drosophillia mothers against decapentaplegic*) which moves into the nucleus and acts as transcriptional activators<sup>29</sup>. It is important to note that signal transduction through this pathway is regulated at multiple points<sup>29,30</sup>.

At the highest level, ligands for these receptors are bound and sequestered in the cytosol by regulatory proteins<sup>30</sup>. One family of secreted proteins, the DAN (differential screening-selected gene aberrative in neuroblastoma) family has been previously demonstrated to bind and sequester TGF $\beta$  family ligands<sup>30</sup>. All DAN family members are cysteine-knot proteins, are secreted into the extracellular media, antagonize Nodal, Wnt, and BMP signaling pathways, and have been demonstrated to be required for

development<sup>30,31</sup>. This family of proteins contains 7 members: Nbl1 (DAN1), CER1, GREM1 (Gremlin 1), PRDC (Gremlin 2), COCO, SOST (Sclerostin), and USAG1 (uterine sensitization-associated gene-1)<sup>30</sup>. All are differentially expressed during development and involved in limb formation, and left-right axis deliniation<sup>24,30–32</sup>. Although all DAN family members antagonize TGF $\beta$ /Wnt signaling, each acts and is expressed differentially during development<sup>30</sup>. In addition to their antagonistic effects on TGF $\beta$  signaling, certain DAN family members can interact with other signaling cascades. For example, GREM1 has recently been shown to interact with the VEGFR (vascular endothelial growth factor receptor), playing a role in tumorigenesis and angiogenesis<sup>33</sup>. Furthermore, it was previously shown that the CER1 from *Xenopus* interferes with Nodal signaling and reduces the metastasis of breast cancer cells<sup>34,35</sup>. Recently, Aykul (2015), found that the human CER1 has an inhibitory role in Activin/Nodal signaling and suppresses migration of aggressive breast cancer cells<sup>34</sup>. CER1 is upregulated as hESCs transition from pluripotency to DE and is primarily used as a marker to identify this change<sup>15,36</sup>. No studies have explored the specific role of CER1 in differentiation<sup>15,36</sup>. These findings not only demonstrate the unknown function of DAN proteins in human embryonic stem cell development, but also highlight that extensive research is necessary to further understand their role in disease and hESC differentiation.

While the specific role and molecular action of these proteins in the differentiation process has not been elucidated, our preliminary research suggests that these proteins may regulate differentiation<sup>36</sup>. Our understanding of how extracellular secreted proteins, such as those of the DAN family, interact with stem cells and drive cell fate decisions remain to be fully understood. Of particular interest is CER1. CER1 is currently used as a marker

for DE formation but its role in DE formation is unknown. Our preliminary, unpublished data have shown that CER1 is highly upregulated as hESCs differentiate into DE, suggesting that it either signals the cells to differentiate or to remain pluripotent. We also hypothesize that once this protein is secreted into the extracellular stem cell environment, it might then interact with the TGF $\beta$  signaling pathway and influence differentiation by driving or modulating DE formation. The aims of this project are to create a model system in hESCs that will allow us to modulate DE formation to investigate the role of secreted proteins on hESC differentiation. We hypothesize that hESCs respond to changes in their environment by upregulating receptors and secreted proteins which then influence their own differentiation (**Figure 3**). Furthermore, we will specifically explore the expression profile of CER1 with DE formation and to look at its possible role on differentiation. We will measure gene and protein levels along with localization of these proteins within the stem cells and their extracellular environment. By studying the role of CER1, GREM 1, and the other DAN family proteins in the formation of DE and primitive pancreatic tissue, we will gain a better understanding of differentiation and enhance the production of insulin-secreting, glucose-responsive cells to therapeutically treat T1D.

## MATERIALS AND METHODS

### Materials:

The following antibodies were used for Western Blots: Nanog antibody at 1:1000 dilution (Cell Signaling, Cat #4893). Heat Shock Protein (Hsp90) antibody at 1:1000 dilution (BD Biosciences, Cat #610419); Extracellular signal-regulated kinase (Erk) antibody at 1:1000 dilution (BD Biosciences, Cat# 610030); Oct4a antibody at 1:1000 dilution (Cell Signaling, #28905). Gremlin1 antibody at 1:500 dilution (Santa Cruz, Cat#18274). Cerberus1 antibody at 1:500 dilution (Santa Cruz, Cat#515324). The following Antibodies were used for Immunofluorescence: Oct-4A at 1:800 dilution (Cell Signaling, #2890); Sox17 antibody at 1:50 dilution (R&D Systems, Cat# BAF1924). Donkey anti-Rabbit IgG (H+L) Alexa Fluor® 568 conjugate at 1:500 dilution (Thermo Fisher Scientific, Cat #A10042); Donkey anti-Mouse IgG Alexa Fluor® 488 conjugate at 1:500 dilution (Thermo Fisher Scientific, Cat #A21202); and 4',6-Diamidino-2-phenylindole (DAPI), dilactate stain at 1:500 dilution (Sigma, Cat #D9564).

His-tagged CER1 (a kind gift from Shoen Kume, School of Life Science and Technology, Tokyo Institute of Technology) was cloned into the pCOLD vector at the 5' Nde1 site and the 3' Xho1 site and expressed in BL21/DE3 (pLysS). The protein was lysed in buffer containing 1% NP-40, centrifuged to remove debris, and purified following the standard batch purification protocol under native conditions (Qiagen QIAexpressionist). After elution in imidazole containing buffer, the protein was dialyzed twice against 2 liters of buffer (50 mM Hepes/NaOH, pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 5% glycerol) and stored frozen at -70 °C.

The following was used for MAPK inhibition: CyT49 cells were treated with 10 $\mu$ M U0126 (Calbiochem # CAS 109511-58-2).

## **Methods:**

### **Cell Culture**

CyT49, a human embryonic stem cell line (NIH registry 0041), were cultured in DMEM/F12 supplemented with 20% knockout serum replacement, glutamax, nonessential amino acids,  $\beta$ -mercaptoethanol, penicillin/streptomycin (all from Life Technologies), 4 ng/mL basic fibroblast growth factor (FGF; Peprotech, Rocky Hill, NJ, USA) and 10 ng/mL Activin A (R&D Systems, Minneapolis, MN) on BD Matrigel and maintained at 37°C in a humidified (5% CO<sub>2</sub>) incubator.

#### *Conditioned Media:*

For media exchange experiments, two separate sets of CyT49 cells were used: sample A and sample B. Sample A was plated at twice the density of sample B. Stem cells normally double in 24 hours, making sample A twice as dense as sample B, 24 hours later. Media from A was removed, centrifuged, filtered through a 0.2 $\mu$  filter and supplemented with Activin A (100 ng/ml; R&D Systems, Minneapolis, MN), correct amount of Fetal Bovine Serum, and Wnt3A (25 ng/ml, R&D Systems) when appropriate. This conditioned media was then added to sample B 24 hours later, depending on the media exchange experiment.

#### *Cell Density Experiments:*

CyT49 cells were plated at 1 $\times 10^6$ , 0.5 $\times 10^6$ , 0.25 $\times 10^6$ , 0.125 $\times 10^6$ , or 0.625 $\times 10^5$  and differentiated to day 3 and day 7 according to the King Lab protocol, discussed below.

## **Differentiation Protocol**

CyT49 cells and H1 cells were differentiated according to the King Lab protocol, as described previously<sup>17</sup>. Media was added to differentiating CyT49 stem cells at 24 hour periods as follows: Day 0: RPMI-1640 media (Invitrogen) supplemented with Activin A (100 ng/ml; R&D Systems), Wnt3A (25 ng/ml, R&D Systems), and 0.2% Fetal Bovine Serum; Days 1-2: RPMI-1640 media (Invitrogen) supplemented with Activin A (100 ng/ml) and 0.5% Fetal Bovine Serum; Day 3: RPMI-1640 media (Invitrogen) supplemented with Activin A (100 ng/ml) and 2% Fetal Bovine Serum; Days 4-6: DMEM/F12 media (Invitrogen) supplemented with KGF (50ng/ml) and 2% Fetal Bovine Serum; Days 7-10: DMEM media (Invitrogen) supplemented with 1% B-27 (Invitrogen, Cat# 17504-044), Noggin (100ng/ml, R&D Systems 1967-ng), Retinoic acid (2umol, Sigma R2625), SANT-1 (0.25umol, Millipore 559303); Days 11-14: DMEM media (Invitrogen) supplemented with 1% B-27, Noggin (100ng/ml), Alk5 inhibitor II (1umol, ENZO ALX-270-445), TPB (50nmol, Millipore 565740).

## **RNA Quantification**

### *qRT-PCR*

mRNA was extracted from CyT49 cells using the RNeasy Mini kit (Qiagen). 1 µg of the total mRNA was used for quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. cDNA was used for quantitative PCR (qPCR) using the iQ5 real-time PCR detection system with IQ SYBR Green (Bio-Rad).

Oligonucleotides used are shown below in **Table 1**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalization control.

**Table 1:** Oligonucleotide Sequences used for qPCR RNA quantification

Gene	Abbreviation	Sequence
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH F	TCG ACA GTC AGC CGC ATC TTC TTT
	GAPDH R	ACC AAA TCC GTT GAC TCC GAC CTT
POU domain, class 5, transcription factor 1	OCT4 F	TGG GCT CGA GAA GGA TGT G
	OCT4 R	GCA TAG TCG CTG CTT GAT GC
Transcription factor SOX-2	SOX2 F	GCC GAG TGG AAA CTT TTG TCG
	SOX2 R	GGC AGC GTG TAC TTA TCC TTC T
Krueppel-like factor 4	KLF4 F	CCT GGC GAG TCT GAC ATG G
	KLF4 R	CGT GGA GAA AGA TGG GAG CA
Cerberus	CER1 F	CAT CCA GGG ACT CAG ATA GTG
	CER1 R	GCA GGT CTC CCA ATG TAC TTC
Hepatocyte nuclear factor 3-beta	FOXA2 F	GGG AGC GGT GAA GAT GGA
	FOXA 2 R	TCA TGT TGC TCA CGG AGG AGT
Homeobox protein goosecoid-2	GSC F	AAC GCG GAG AAG TGG AAC AAG
	GSC R	CTG TCC GAG TCC AAA TCG C
Transcription factor SOX-17	SOX17 F	TCC ACG TAG GGC CTC TTC TG
	SOX17 R	TGG CGC AGC AGA ATC CAG A
Brachyury	Brach F	TGC TTC CCT GAG ACC CAG TT
	Brach R	GAT CAC TTC TTT CCT TTG CAT CAA G

### *RT-PCR Array*

mRNA was extracted from CyT49 cells using the RNeasy Mini kit (Qiagen). 1 µg of the total mRNA was used for quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. RT-PCR array assays were performed using the Human Stem Cell RT<sup>2</sup> Profiler<sup>TM</sup> PCR Array (PAHS-405ZA-2; 96 genes total) according to the manufacturer's protocol.

### **Quantitative Western Blots**

Samples were solubilized in Lysis Buffer (150mM NaCl, 50mM Tris-HCl, 1% NP-40, 1% sodium deoxycholate, 10 µg/mL phenylmethanesulfonyl fluoride (PMSF), 10 µg/mL leupeptin, 1 µg/mL microcystin) and diluted in Laemmli sample buffer. Equal amounts of lysates were loaded onto 10% SDS-PAGE gels and run at 150 Volts for approximately 1 hour in Running Buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3). Protein was transferred to nitrocellulose at 300mA in Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membrane was incubated for 30 minutes in blocking buffer (10mM Hepes pH 7.4, 0.5M NaCl, 3% Bovine Serum Albumin, 10% Fetal Bovine Serum). Primary antibodies were diluted in buffer containing 10mM Hepes pH 7.4, 0.5M NaCl, 0.2% Tween-20, 1% Bovine Serum Albumin, 3% Fetal Bovine Serum at 4°C overnight. The membrane was washed three times in PBS-T and incubated with Li-COR secondary antibodies, either IRDye 800CW or IRDye 680LT (Li-COR,

Lincoln, NE, USA) diluted 1:10,000 in antibody diluent. Protein expression was imaged using the Li-COR Odyssey Infrared CLx Imaging System and analyzed by ImageJ.

### **Cell Imaging**

Cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 minutes, washed twice with PBS, followed by permeabilization with PBS containing 0.2% Triton for 5 minutes. Cells were incubated in blocking buffer for 1 hour followed by overnight incubation with primary antibody. The next day, cells were washed 3X with PBS, followed by incubation for 1 hour in the appropriate secondary antibody. After an additional three washes with PBS, the samples were mounted and images were acquired on a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Inc., Pleasanton, CA, USA) using a MicroMax digital camera (Roper-Princeton Instruments, Trenton, NJ, USA) controlled by MetaFluor software (Universal Imaging, Corp., Bedford Hills, New York, USA).

## RESULTS

### 3.1 MAPK inhibition creates a model system to block DE formation

To study the effects of secreted proteins on DE formation, an effective method to block DE formation was required. Inhibitors of multiple signaling pathways were incubated individually with CyT49 cells during DE formation and expression of the pluripotency transcription factor, *OCT4*, was measured by Western blot (Data not shown). Increased expression of OCT4 was observed in day 4 differentiated cells incubated with the MAPK inhibitor, U0126 (**Figure 4A, B**). PCR array data also demonstrated that treatment of cells with U0126 prevented expression of DE specific genes (BMP2, Fzd, FoxA2) while maintaining expression of pluripotent genes (Sox2, SDF-1, FGF2, FGF4) (**Figure 4C**). Western blot experiments show that treatment of pluripotent hESCs with U0126 results in maintained expression of OCT4 and NANOG compared to pluripotent hESCs treated with DMSO alone (**Figure 5A; compare lanes 2 vs. 3**). Treatment of cells with U0126 maintained high expression of OCT4 and prevented expression of SOX17 compared to DMSO control (**Figure 5B,C**). Taken together, these results suggest that inhibition of MAPK prevents differentiation of hESCs and provides a model to test the role of CER1 and other secreted proteins in DE formation.

### 3.2 Cell Density influences hESC pluripotency

To better define the role of MAPK inhibition in maintenance of pluripotency, we next explored cell proliferation. The hypothesis driving this set of experiments was that MAPK activity is required for differentiation through the canonical proliferation

pathway. Cells were plated at decreasing number ( $1 \times 10^6$ ,  $0.5 \times 10^6$ ,  $0.25 \times 10^6$ ,  $0.125 \times 10^6$ , or  $0.625 \times 10^5$ ) and the ability to form DE was assessed by Western blot and Immunofluorescence. Cells plated at high density ( $1 \times 10^6$  or  $0.5 \times 10^6$ ) had lower OCT4 expression at Day 7 when compared to cells plated at low density ( $0.25 \times 10^6$ ; **Figure 6A**). The composite image shows a clear distinction between OCT4 positive and OCT4 negative cells. In addition, cells plated at high density had a higher expression of HNF4 $\alpha$  and FoxA2 (DE markers) at Day 7 of differentiation when compared to cells plated at a low density (Figure 6B, C). Therefore, cells plated at a higher density could efficiently exit pluripotency, form DE, and express markers of pancreatic endocrine precursors, while cells plated at lower densities ( $0.25 \times 10^6$ ,  $0.125 \times 10^6$ , or  $0.625 \times 10^5$ ) maintained high levels of OCT4, expressed diminished levels of DE markers (HNF4 $\alpha$ , FOXA2). In addition, conditioned media from cells differentiated at a high density ( $1 \times 10^6$ ) was added to cells plated at a low density ( $0.125 \times 10^6$ ; **Figure 7**). There was an increased expression of *OCT4* in the low density cells treated with conditioned media from high density cells in comparison to the low density controls (**Figure 7; lane 1 vs. lane 4**).

### 3.3 CER1 expression increases as hESCs differentiate into DE

Next, we wanted to determine the expression profile of CER1 in differentiating cells. CyT49 cells were incubated with either DMSO, 1 $\mu$ M U0126, or no Wnt3a, and CER1 expression was measured by western blot. CER1 expression was low in pluripotent hESCs, and increased 4- fold upon DE formation in the presence of DMSO (**Figure 8**). Prevention of DE formation by the addition of U0126 or omission of Wnt3a resulted in a decrease in CER1, with expression levels comparable with day 0 control cells.

### 3.4 Secreted factors influence differentiation of hECS into DE

To explore the possible effects of secreted factors on stem cell differentiation, conditioned media experiments were conducted. CyT49 cells were treated with either normal media (phi) or conditioned media (Day4 + Cond. Media). By day 4 of differentiation, the cells were lysed and gene expression levels were measured with RT-PCR. The relative amounts of gene expression were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Treatment with conditioned media resulted in increased expression of pluripotency genes (*OCT4*, *SOX2*, *KLF4*) and decreased expression of DE genes (*CER1*, *FOXA2*, *GSC*) when compared to the day 4 controls (**Figure 9**). Together, these media exchange experiments suggest that a secreted factor is influencing hESC differentiation into DE.

### 3.5 CER1 expression regulates DE formation

Pluripotent hESCs express high levels of *OCT4* and *SOX2*, and while DE cells highly express *FOXA2* and *SOX17*. CyT49 cells treated with conditioned media maintained high expression of *OCT4* and *SOX2* while maintaining low expression of *FOXA2* and *SOX17* (**Figure 10**). Depletion of CER1 from the conditioned media by the addition of a CER1 antibody resulted in increased expression of *FOXA2* and *SOX17* while *OCT4* and *SOX2* levels decreased. Treatment with the CER1 antibody resulted in expression of the respective genes at levels comparable to day 4 DE cells. Addition of recombinant CER1 to the media resulted in higher expression of genes associated with pluripotency (*OCT4* and *SOX2*) and decreased expression of genes associated with DE

(*FOXA2* and *SOX17*). Cells incubated with recombinant His-CER1 increased expression of OCT4 positive cells compared to CyT49 cells treated with DMSO (**Figure 11**). These results support the hypothesis that CER1 plays a regulatory role on DE formation.

## DISCUSSION

In this study, we identified the MAPK inhibitor U0126 as a chemical modulator of DE formation and used this model system to explore how proteins transcribed and secreted by hESCs regulate DE formation. Cells treated with U0126 failed to generate DE and expressed high levels of pluripotency markers (OCT4, SOX2, and NANOG), indicating a critical role of MAPK signaling during the exit from pluripotency. To explore the role of MAPK in hESC differentiation, we measured the ability of cells to exit pluripotency when plated at decreasing densities. Cells plated at high density ( $1 \times 10^6$  or  $0.5 \times 10^6$ ) had were able to efficiently exit pluripotency, form DE, and express markers of pancreatic endocrine cells, while cells plated at lower densities ( $0.125 \times 10^6$ , or  $0.625 \times 10^5$ ) maintained high levels of OCT4, SOX2, NANOG, expressed diminished levels of DE markers (SOX17, FOXA2), and had no expression of pancreatic endoderm markers. Together, these results indicate that MAPK activity not only drives cell proliferation, but also regulates hESC exit from pluripotency. Microarray data from CyT49 and H1 cells at DE (Day 4 of differentiation) show dramatically increased expression of transmembrane receptors and secreted proteins. One secreted protein whose expression is decreased upon treatment of cells with U0126 is Cerberus1 (CER1) a member of the DAN family of BMP antagonists that is highly expressed at DE<sup>30,32,36</sup>. Depletion of CER1 from the media of hESCs enhanced DE formation, while addition of recombinant CER1 to the media of differentiating cells prevented DE formation. Together, the data suggests that MAPK signaling regulates expression and/or secretion of CER1 that is a driver of DE formation.

hESC pluripotency is defined by unlimited self-renewal and the ability to differentiate into either of the 3 primary germ layers. For these cells to undergo differentiation, coordinated down-regulation of pluripotency markers, including OCT4, NANOG, and SOX2, and up-regulation of lineage specific genes must occur<sup>11,12</sup>. Even though all hESCs are pluripotent, each cell line displays an intrinsic differentiation bias – the potential to selectively generate cells from one of the three germ layers over the other<sup>37</sup>. As a result, specific protocols have been developed allowing hESCs to be pushed toward specific cell fates *in vitro*<sup>28</sup>. However, regardless of the protocol, different hESC cell lines have intrinsically different capacities to form DE. These variances in differentiation between cell lines suggest that factors secreted into the media are helping drive hESC cell fate independently of growth factors or proteins supplemented in the media. Microarray data from two different hESC lines show dramatic upregulation in expression of receptors and secreted proteins during DE, indicating that cells respond to changes in their environment by upregulating their own receptors and secreted proteins. In this study, we have explored this possible mechanism in hopes of further improving the use of hESCs for cell replacement therapy.

To explore the regulation of hESC cell fate, we created a new model system that allows us to modulate DE formation. Previous studies in our laboratory have shown that efficient DE formation requires down regulation of signaling through PI3- Kinase and signaling input from Activin A and Wnt3a, but much less has been documented on the role of Mitogen Activated Protein Kinase/Extracellular Regulated Kinase (MAPK/ERK) signaling pathway on DE formation<sup>16</sup>. While this pathway is known to regulate cell adhesion, proliferation, migration, and survival, there is controversy as to whether

MAPK signaling enhances differentiation or promotes pluripotency<sup>38</sup>. Armstrong found that inhibition of MAPK/ERK, resulted in apoptosis and differentiation<sup>39</sup> and Niwa found that inhibition of MAPK signaling resulted in increased expression of Nanog, a marker of pluripotency<sup>40</sup>. Here, we showed that inhibition of MAPK with U0126 resulted in maintenance of pluripotency markers (*OCT4*, *SOX2*, *NANOG*) and a loss of differentiation markers (*SOX17*) (**Figure 4A, 5A, B**). We see that *OCT4* levels are maintained but without the degradation of *ERK* (**Figure 4B**). Erk levels are maintained, but not phosphorylated (data not shown), no DE markers are upregulated, and by day 15, no hormone positive cells are formed. Together, these results further suggest that inhibition of MAPK with U0126 is an effective system to modulate DE formation. Furthermore, upregulation of receptors and secreted proteins seen in the PCR array analysis suggests that the cells may be using both to modulate cell fate in response to added growth factors and serum (**Figure 4A**). All in all, inhibition of MAPK has created a system where the cells are locked into pluripotency, allowing us to explore factors that influence cell fate.

Working under the hypothesis that there was a link between MAPK activity and expression of DE markers, we wanted to explore the possible effects of various cell densities on regulation of cell fate. Cells plated and differentiated in a low density had elevated expression of pluripotency markers (*OCT4*; **Figure 6A**) and a decreased expression of DE specific markers (*HNF4 $\alpha$* ) than those plated at a higher density suggesting that MAPK activity is essential for DE formation (**Figure 6B**). Conditioned media, media containing the secreted proteins from hESCs already differentiated to DE supplemented with standard, established serum and growth factors, experiments further

highlighted this possibility. Traditionally, experiments with conditioned media are used to understand how different hESCs may influence themselves, or other cell lines. For example, Yang et al., have shown that conditioned media can be used for maintenance of hESC pluripotency<sup>41</sup>, and Hannoun et al., have shown that conditioned media from human adipose tissue derived mesenchymal stem cells induces apoptosis and differentiation in human glioma cell lines<sup>42</sup>. In this study, media from cells differentiated at a high density was added to cells plated at a low density. There was an increased expression of *OCT4* in the low density cells treated with conditioned media from high density cells in comparison to the low density controls, suggesting that the cells themselves are secreting proteins, growth factors, or hormones into the media that may be influencing their cell fate (**Figure 7**).

To further understand the role hESCs may be having on cell fate decisions, we explored the secreted protein CER1, a member of the Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) superfamily of secreted ligands that regulate cell proliferation and differentiation<sup>30</sup>. CER1 is currently known as an important proponent for head induction and head formation in embryogenesis<sup>24,35,43</sup>. While secreted CER1 has been shown to bind BMP2, BMP4, and Activin A in *Xenopus* and mouse model systems<sup>24,32,35</sup>, Aykul and Martinez have shown that in humans, CER1 binds Nodal, Activin B, BMP-6, and BMP-7 with high affinity and effectively inhibit them from signaling<sup>31</sup>. For efficient differentiation of hESCs to occur, TGFB/Activin/Nodal must be activated and BMP signaling must be repressed. Binding of Activin/Nodal to receptors results in autophosphorylation, recruitment and phosphorylation of transcription factor proteins Smad 1/5/8. Upon phosphorylation, Smads form heterodimers and translocate to the

nucleus where they bind co-activators and repressors that regulate expression of genes that control proliferation, development, and maintenance of pluripotency<sup>30,31,34</sup>.

CER1 is upregulated as hESCs transition from pluripotency to DE (**Figure 8, lanes 1 and 2**) and because of this, the primary understood role of CER1 in hESC biology is as a marker of DE formation<sup>15,34,36</sup>. CER1 expression not only increases over time, but it has a 5.6x higher expression than *GSC* and 128x higher expression than *FOXA2* (**Figure 8**). In addition, silver stains of purified CER1 with column chromatography show large, visible bands and preliminary microarray data of two different hESC lines show significant increase of CER1 expression with DE formation (data not shown). This exceedingly high expression suggests that CER1 might be one of the secreted proteins that the hESCs are secreting in response to the changing stem cell environment.

To further explore the role of CER1 in DE formation, we conducted media exchange experiments to see if this secreted protein could influence cell fate decisions and found that cells treated with conditioned media remained pluripotent while removal of CER1 from the media with antibodies resulted in DE formation (**Figure 10, lanes 2 and 3; lanes 2 and 4**). Furthermore, cells incubated in the presence of recombinant, exogenous CER1 expressed higher levels of pluripotency markers (OCT4 and SOX2) (**Figure 10, lanes 2 and 5; Figure 8**). These results further confirm that CER1 is one of the secreted factors that is influencing hESC fate.

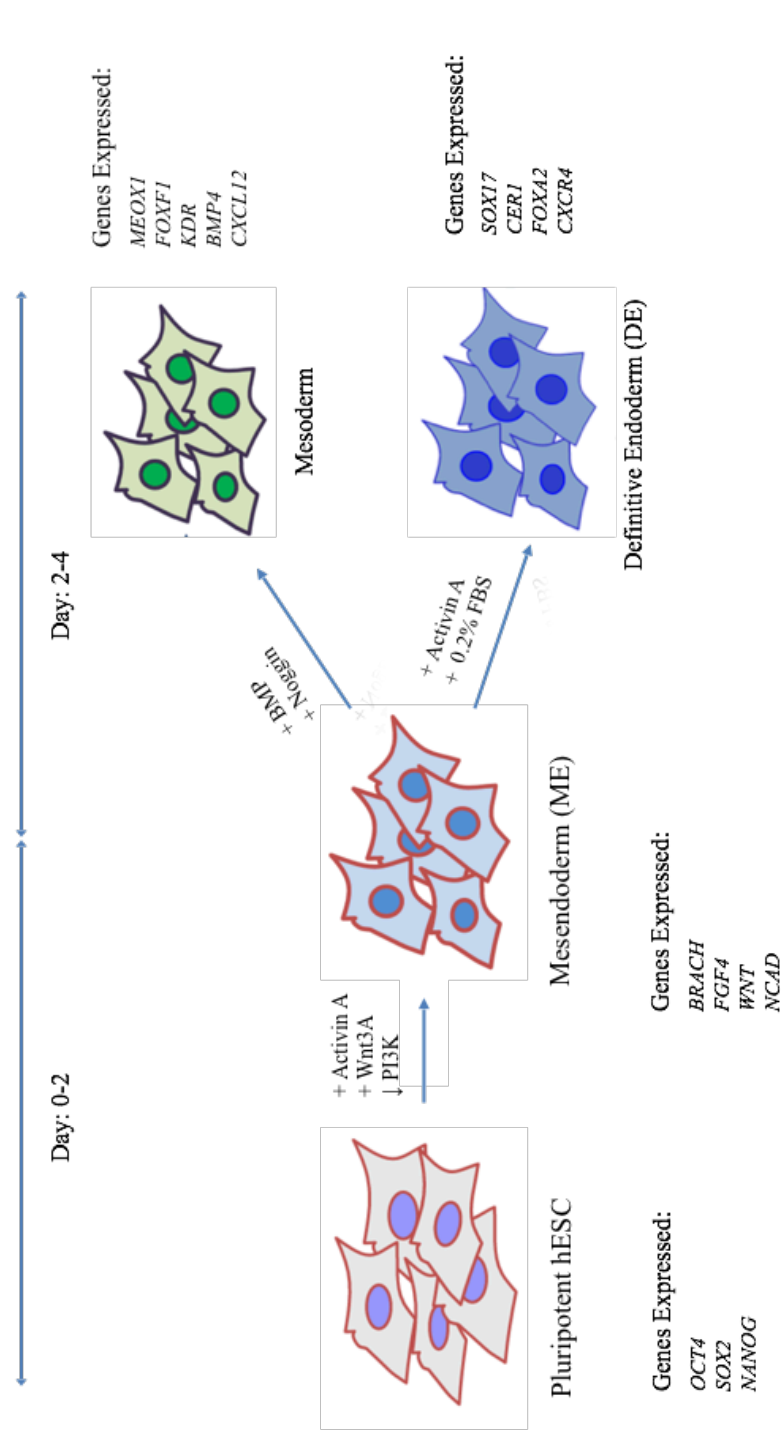
Looking at the structure of CER1, we can try to understand how this secreted protein may be modulating differentiation. This secreted cysteine knot protein has two active forms: the full-length form (which contains the N-terminal region) and the short-length form<sup>31,34</sup>. These results suggest that the N-terminal region on the long-form of

CER1 effectively binds and inhibits Nodal from signaling. Nodal signaling, in addition to BMP repression, must occur for efficient differentiation of hESCs to occur. The high levels of CER1 by day 4 of differentiation signal to the cell by binding and inhibiting Nodal, signaling to the cell that DE formation is complete.

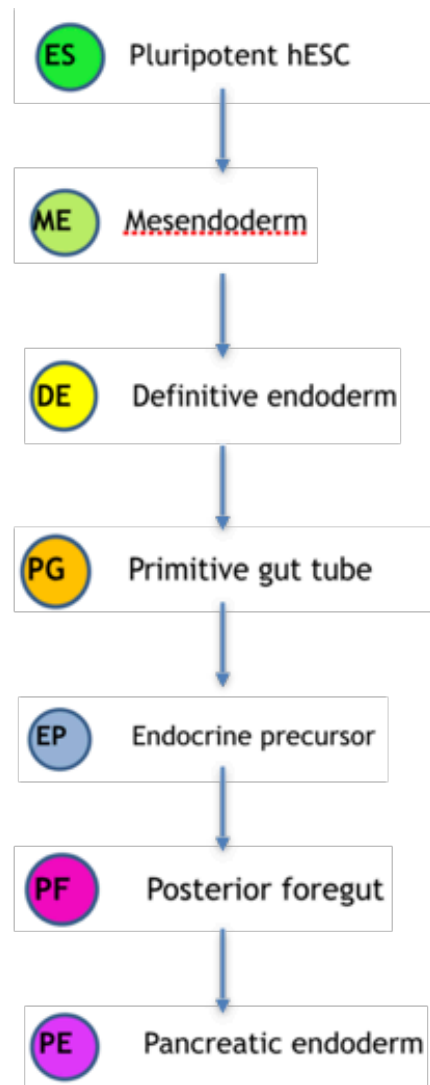
Future experiments will explore the possible role of the other DAND Family members in hESC fate. Although all DAN family members antagonize TGF $\beta$ /Wnt signaling, each acts and is expressed differentially during development<sup>30</sup>. For example, GREM1 has been shown to interact with VEGFR, playing a role in tumorigenesis and angiogenesis<sup>33</sup>. In this study, we see an increase in GREM1 with maintenance of pluripotency, suggesting that this protein and the other DAND family members might be antagonists of one another by competing for other secreted proteins (**Figure 8A**). We will also create knockout cell lines using CRISPR/CAS9 to further explore the role these proteins have on formation of DE and hESC fate.

Through chemical manipulation of DE formation by MAPK inhibition, we show that stem cell differentiation is driven by the cell's responses to its environment. The changes in the media from directed differentiation protocols notify the cells and the cells respond by upregulating and downregulating receptors and secreted proteins to drive its own fate. We show that hESCs secrete CER1 in response to the changes in their environment to modulate their fate. The other DAN family members may also be involved in coordinated regulatory events and will further be explored. These findings have helped us gain a better understanding of differentiation bias and will aid in enhancement of the production of insulin-secreting, glucose responsive cells across different hESC lines.

FIGURES

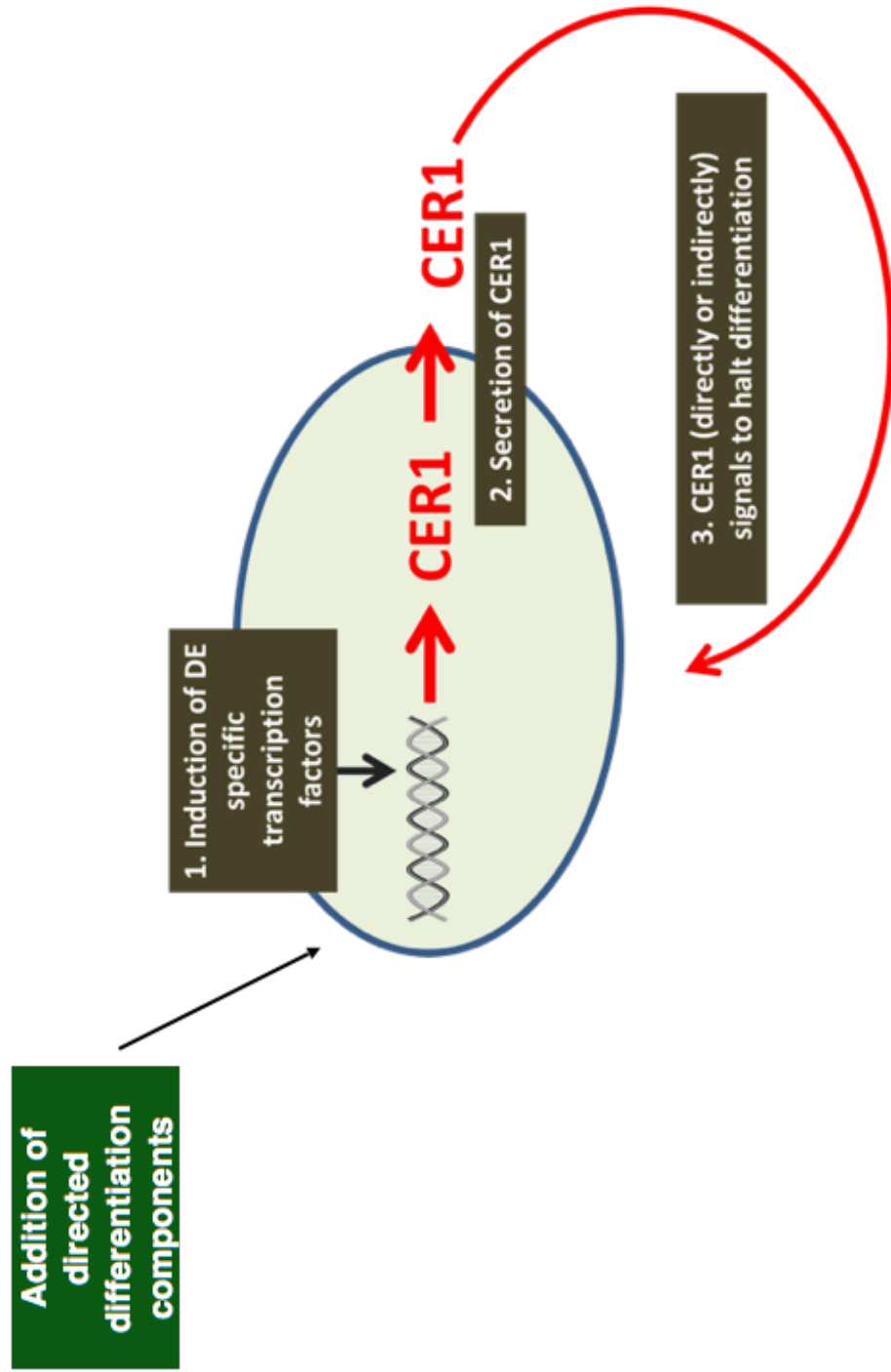


**Figure 1. Overview of early stem cell differentiation *in vivo*.** Schematic of differentiation protocol and expression of transcription factors that define pluripotent hESCs, Mesendoderm to transition to ME cells require removal of serum to block PI3K and addition of Wnt3a and high levels of Activin A. To transition from ME to DE requires maintained high levels of Activin A and increasing amounts of knockout replacement serum (KRS). For cells to become Mesoderm, BMP and Noggin are required.

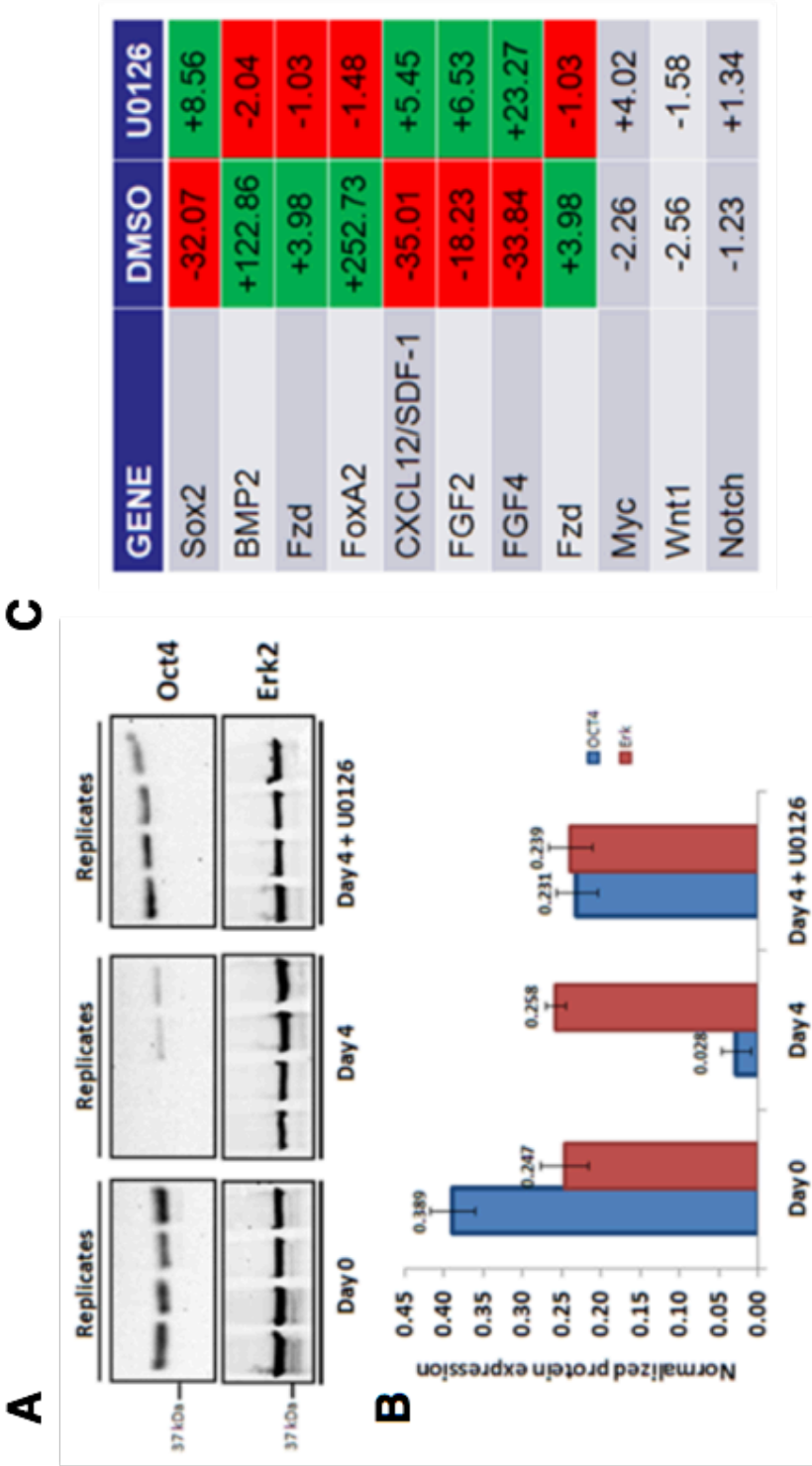


**Figure 2. Schematic of pluripotent human embryonic stem cell differentiation into endocrine precursor cells.**

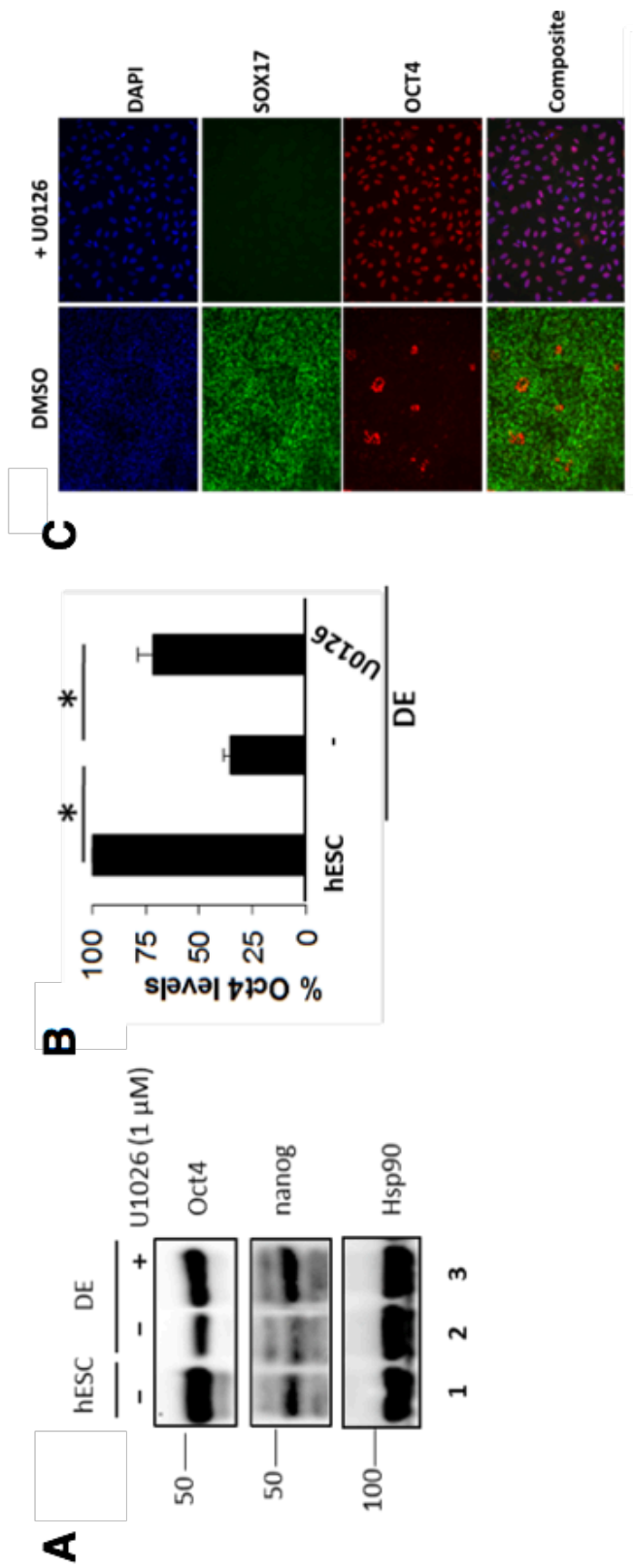
Once pluripotent hESCs become definitive endoderm, they progress through the other stages of differentiation until they become endocrine precursor cells and express the insulin hormone.



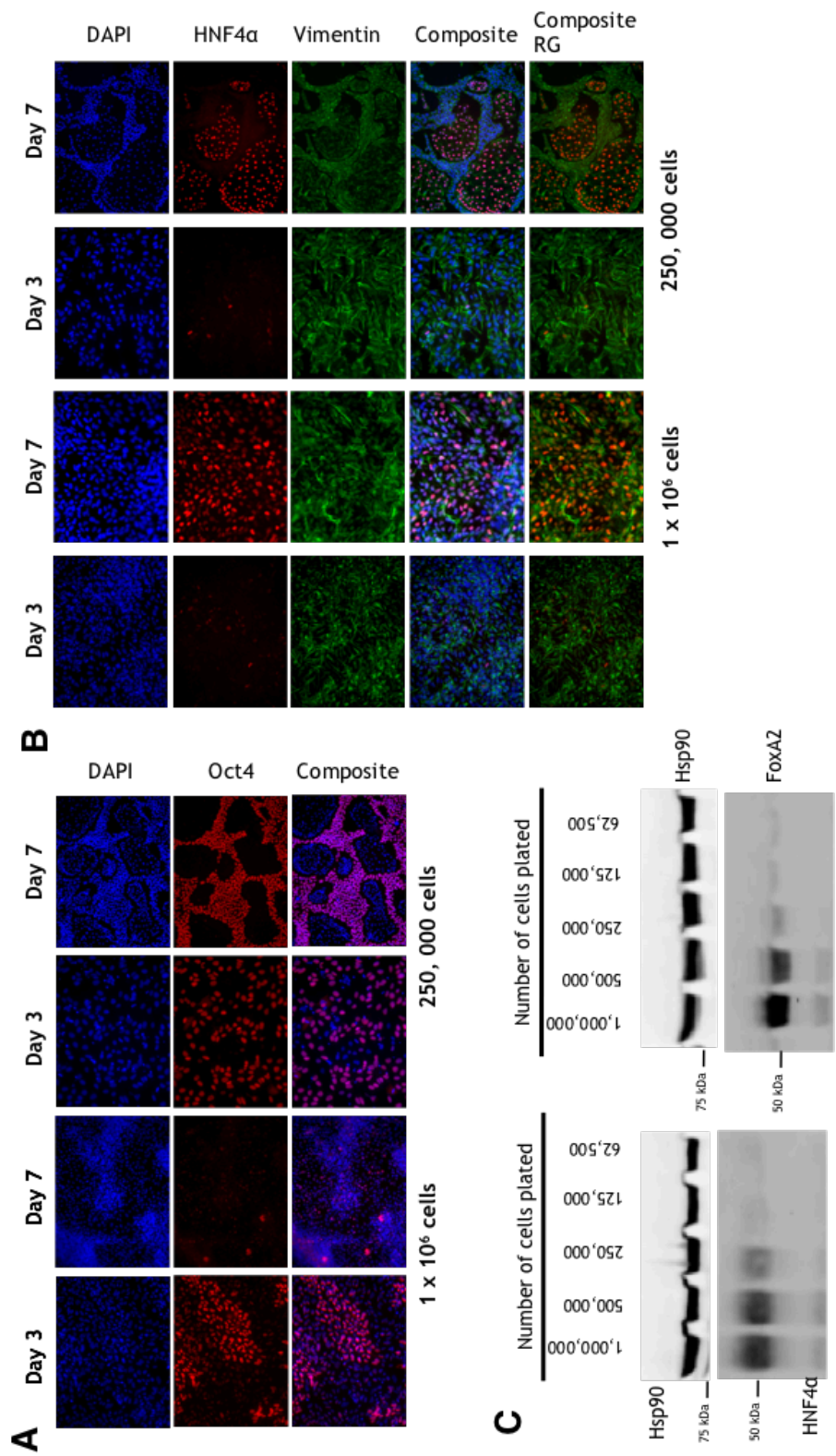
**Figure 3. Hypothesis: hESCs respond to changes in their environment by up regulating receptors and secreted proteins, like Cerberus1. Cerberus1 is then secreted, and either directly or indirectly modulates differentiation.**



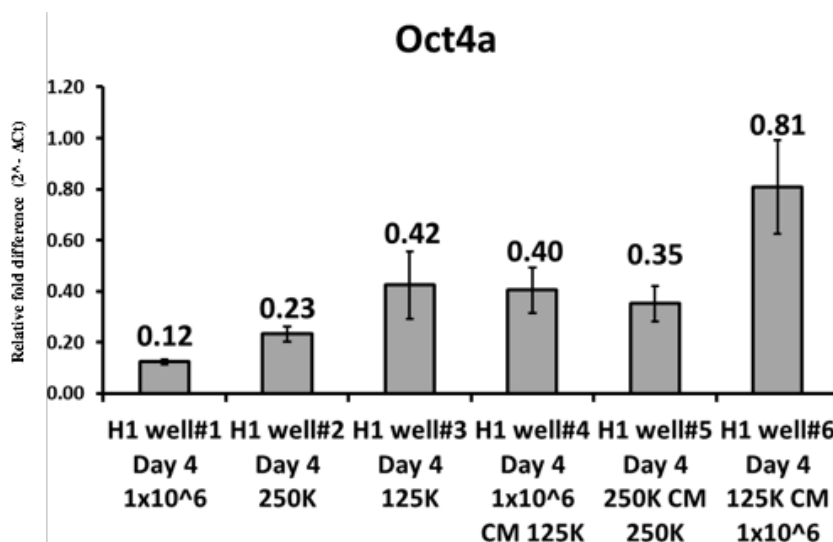
**Figure 4. Development of a model system to block DE formation**  
Cyt49 cells were incubated with DMSO or 1 $\mu$ M U0126 for four days to generate DE. (A) OCT4 protein was measured by western blot (n=4) and (B) quantified and normalized to Erk2. (C) PCR array highlights the effect of U0126 on expression of pluripotency genes (*SOX2*, *SDF1*, *FGF2*, *FGF4*) and DE genes (*BMP2*, *FZD*, *FOXA2*).



**Figure 5. MAPK inhibition prevents hESC exit from pluripotency**  
Cyt49 cells were incubated with DMSO or 1μM U0126 for four days to generate DE. (A) OCT4 and NANOG protein levels were measured with western blot (n=6). (B) OCT4 levels were quantified and normalized to HSP90. (C) Immunofluorescence of Cyt49 cells differentiated to DE in the presence of DMSO (left panel) or with U0126 (right panel). DAPI (blue), nuclear staining; SOX17 (green), DE marker; OCT4 (red), pluripotency marker. \*P<0.005.

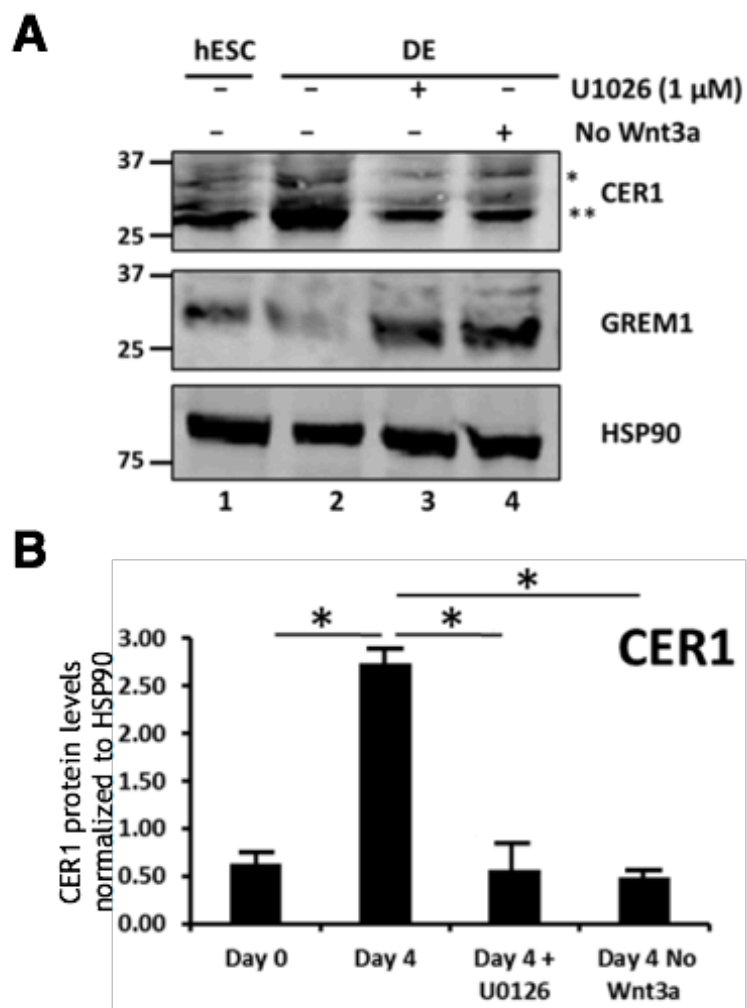


**Figure 6. Cell Density Influences hESC Pluripotency**  
Cyt49 cells plated at high density (1x10<sup>6</sup>; left two panels) and low density (250,000; right two panels) and differentiated to Day 3 and Day 7. (A) Immunofluorescence with DAPI (blue, top row), nuclear staining; HNF4α (red; top row), pluripotency marker. (B) Immunofluorescence with DAPI (blue, top row), nuclear staining; HNF4α (red; second row) DE marker, Vimentin (green; third row), intermediate filament marker. (C) Western Blot of Day 7 differentiated cells plated at 1x10<sup>6</sup>, 500k, 250k, 125k, and 62.5k. HNF4α and FoxA2 (DE markers) and HSP90 (control) protein levels were measured (n=4).



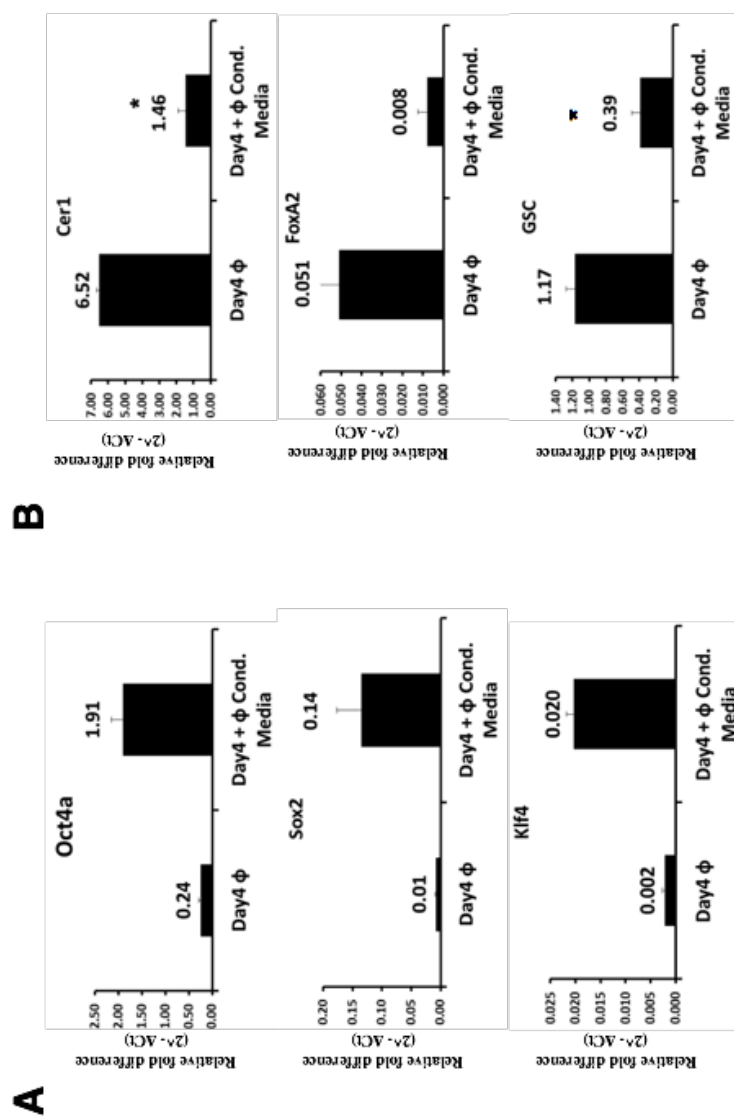
**Figure 7. Increased *OCT4* expression when cells plated at a low density cells were treated with conditioned media from cells plated at a high density.**

H1 cells were plated at different densities:  $1 \times 10^6$ ,  $0.25 \times 10^6$ , and  $0.125 \times 10^6$  and the following media exchange experiment was conducted: conditioned media from  $1 \times 10^6$  (lane 1) was added to cells plated at a low density ( $0.125 \times 10^6$ ; lane 4), conditioned media from  $0.25 \times 10^6$  (lane 2) was added to  $0.25 \times 10^6$  plated cells (lane 5), and conditioned media from  $0.125 \times 10^6$  was added to  $1 \times 10^6$ . *OCT4* expression was measured by RT-PCR and normalized to *GAPDH*.

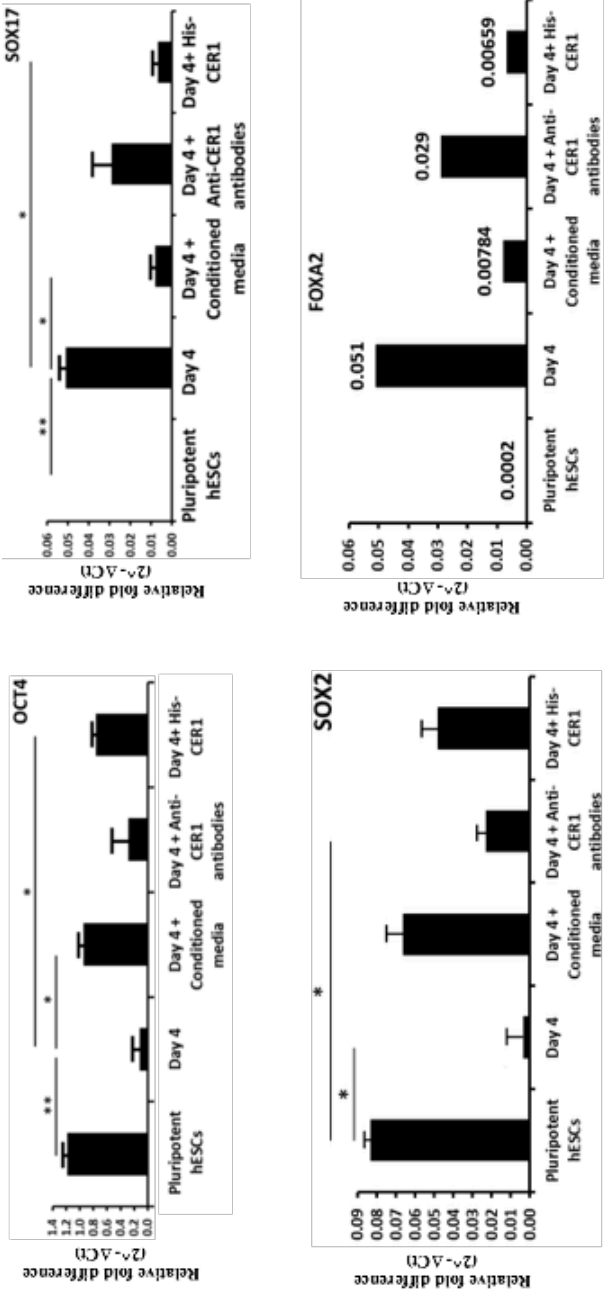


**Figure 8. CER1 expression increases as hESCs differentiate into DE**

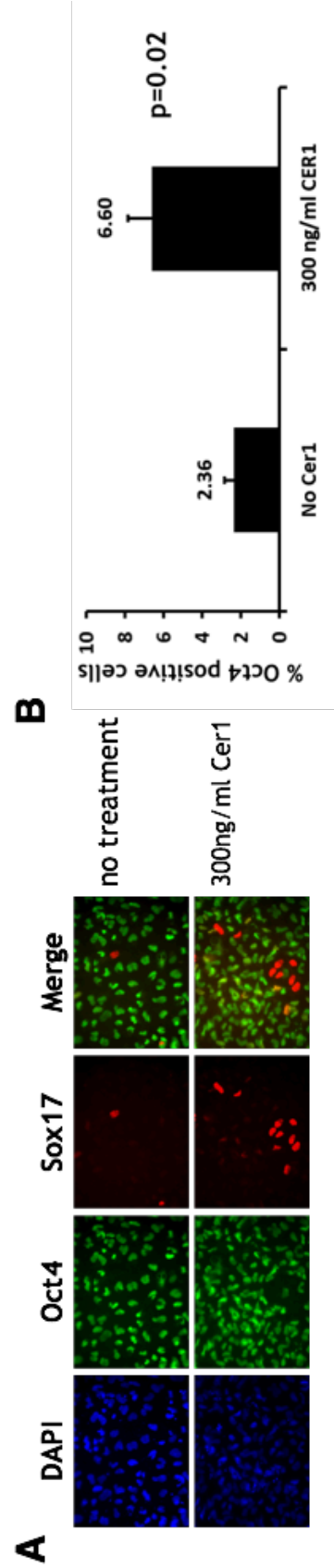
Cyt49 cells were incubated with DMSO, 1 $\mu$ M U0126, or in media without Wnt3a (lane 4) for four days to generate DE. (A) CER1 levels were measured with western blot and (B) quantified and normalized to HSP90. \*P<0.01



**Figure 9. Gene expression of Cyt49 cells after media exchange experiment**  
 Cyt49 cells were treated with:  $\phi$  (normal ES media, supplemented with FBS and Activin) or  $\phi$  + Conditioned Media (Cond. media). Relative gene expression values were analyzed using *GAPDH* as a normalization control. Reverse transcription polymerase chain reaction (RT-PCR) analysis of (A) Pluripotency genes (B) Definitive Endoderm Genes by day 4 of differentiation in Cyt49 hESCs. T-test ( $n=3$ ) was used to determine the significance \* $p<0.005$  \*\* $p<0.05$ ;



**Figure 10. CER1 expression regulates DE formation.** Cy49 cells were differentiated to DE with normal differentiation media, conditioned media, conditioned media and CER1 antibodies, or conditioned media and His-CER1 (recombinant CER1). Pluripotency markers, *OCT4* and *SOX2*, and DE markers, *SOX17* and *FOXA2*, were analyzed by RT-PCR.  $P < 0.05$



**Figure 11. Addition of recombinant CER1 influences hESC pluripotency**

CyT49 cells were differentiated to DE with normal differentiation media or with normal differentiation media with 300ng/ml His-CER1 (recombinant CER1) addition. (A) Immunofluorescence of CyT49 cells differentiated to DE without His-CER1 (top row) and CyT49 cells differentiated to DE with His-CER1 (bottom row). DAPI (blue), nuclear staining; OCT4 (green) pluripotency marker; Sox17 (red), DE marker. (B) Quantification of the percentage of OCT4 positive cells with and without addition of 300ng/ml of His-CER1. Values normalized to DAPI. p=0.02.

**Table 1:** Oligonucleotide Sequences used for qPCR RNA quantification

Gene	Abbreviation	Sequence
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH F	TCG ACA GTC AGC CGC ATC TTC TTT
	GAPDH R	ACC AAA TCC GTT GAC TCC GAC CTT
POU domain, class 5, transcription factor 1	OCT4 F	TGG GCT CGA GAA GGA TGT G
	OCT4 R	GCA TAG TCG CTG CTT GAT GC
Transcription factor SOX-2	SOX2 F	GCC GAG TGG AAA CTT TTG TCG
	SOX2 R	GGC AGC GTG TAC TTA TCC TTC T
Krueppel-like factor 4	KLF4 F	CCT GGC GAG TCT GAC ATG G
	KLF4 R	CGT GGA GAA AGA TGG GAG CA
Cerberus	CER1 F	CAT CCA GGG ACT CAG ATA GTG
	CER1 R	GCA GGT CTC CCA ATG TAC TTC
Hepatocyte nuclear factor 3-beta	FOXA2 F	GGG AGC GGT GAA GAT GGA
	FOXA 2 R	TCA TGT TGC TCA CGG AGG AGT
Homeobox protein goosecoid-2	GSC F	AAC GCG GAG AAG TGG AAC AAG
	GSC R	CTG TCC GAG TCC AAA TCG C
Transcription factor SOX-17	SOX17 F	TCC ACG TAG GGC CTC TTC TG
	SOX17 R	TGG CGC AGC AGA ATC CAG A
Brachyury	Brach F	TGC TTC CCT GAG ACC CAG TT
	Brach R	GAT CAC TTC TTT CCT TTG CAT CAA G

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