The Generation of Definitive Endoderm from Human Embryonic Stem Cells on 3D Biodegradable Poly(lactic-*co*-glycolic Acid) Scaffolds and its Comparison to those Generated on 2D Monolayer Cultures

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Abstract: The generation of insulin producing cells from human embryonic stem cells (hESCs) has shown great promise as a cellular replacement therapy for the treatment of Type 1 Diabetes. Mature functional β-cell surrogates however, have yet to be successfully generated *in vivo*. One approach to potentially improve current differentiation protocols is the use of 3 dimensional (3D) scaffolds, which has been shown to enhance cellular function and differentiation potential. The present study aimed to explore the feasibility of using single cell preparations of pluripotent hESCs seeded onto laminin or Matrigel coated 3D poly(lactic-*co*-glycolic) acid (PLGA) scaffolds to derive definitive endoderm, the first vital stage of endoderm tissue differentiation. Our results demonstrated that hESCs which were induced to differentiate on laminin or Matrigel coated 3D scaffolds can be successfully coaxed to differentiate into definitive endoderm. The cells that were cultured on laminin or Matrigel coated 3D scaffolds expressed significantly higher levels of the key endoderm transcription factors *SOX17 and FOXA2* in comparison to those differentiated on 2D monolayers. On Matrigel coated 3D scaffolds, the differentiated cells expressed lower levels of the endoderm surface marker *CXCR4* and anterior endoderm marker *CER* in comparison to its monolayer counterpart. Together, the results of this study demonstrated the positive effect of 3D cultures on endoderm commitment from hESCs over traditional monolayer cultures. Furthermore, the definitive endoderm produced on Matrigel coated scaffolds may have a more posterior phenotype in comparison to those derived from monolayers. This may have an effect on later stages of pancreatic differentiation and warrants further detailed investigations.

Keywords: Embryonic stem cells, differentiation, poly(lactic-*co*-glycolic acid), scaffold, endoderm, pancreas.

INTRODUCTION

Insulin producing β-cell replacement therapies in the form of whole pancreas and human islet transplantation may allow people with Type 1 Diabetes to cease the administration of insulin [1,2]. A lack of suitable donor organs however, prevents the majority of patients from receiving such treatments. A number of alternative sources of β-cells have been investigated, which include the ontogeny based differentiation of human embryonic stem cells (hESCs) into insulin producing cells. Considerable advances have been made in this field, however mature functional β-cells have yet to be convincingly generated *in vitro*. This may be due to the inability of existing protocols to reproduce the appropriate *in vivo* environment in an *in vitro* setting. One aspect that remains elusive is the *in vitro* replication of the 3 dimensional (3D) environment of the developing human body, where a myriad of cell types interact and influence each other via physical and chemical cues to drive cellular differentiation [3].

A 3D niche can be mimicked *in vitro* through the use of 3D scaffolds. hESCs that were cultured on 3D biodegradable scaffolds have been found to display enhanced cellular function and differentiation potential in comparison to those grown on conventional 2D monolayer cultures [4,5]. In addition, hESCs that were grown in a 3D environment showed tendencies to form 3D structures that are similar to those found *in vivo* [5,6]. Despite these advantages however, all pancreatic differentiation protocols published to date have utilized conventional 2D monolayer cultures and/or the formation of embryoid bodies. The effects of the directed

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differentiation of pluripotent hESCs towards the pancreatic lineage on 3D scaffolds has yet to be examined.

Previously, we investigated the adhesion of hESCs on 3D scaffolds made from poly(lactic-*co*-glycolic acid) (PLGA), an FDA approved polymer, and their interactions with surrounding matrices during differentiation towards the endoderm lineage [7, 9]. The present study aimed to explore the feasibility of using single cell preparations of hESCs seeded onto laminin or Matrigel coated 3D PLGA scaffolds to derive definitive endoderm, the first vital stage of endoderm tissue differentiation. Our results demonstrated that hESCs which were cultured on laminin or Matrigel coated 3D scaffolds can be successfully coaxed to differentiate into definitive endoderm following the protocol described by Kroon *et al.* (2008) [8]. 3D culturing of hESCs however, had a positive effect on endoderm commitment in comparison to conventional monolayer cultures. Some differences in gene expression profile were also observed between the cells cultured in 2D and 3D environments, which underlined the impacts of 3D cultures on the differentiation potential of hESCs.

MATERIALS AND METHODS

Cell Culture

Pluripotent hESCs from the cell line HES3 and its green fluorescence protein (GFP) expressing clone Envy (both were generous gifts from Embryonic Stem Cell International via Prof. Ed Stanley of Monash Immunology and Stem Cell Laboratories) were maintained on gamma irradiated human fetal fibroblast feeder layers and cultured as previously described [9].

Differentiation of hESCs to Definitive Endoderm

Single cell preparations of hESCs [7] were seeded onto 25μg/mL mouse laminin (Invitrogen, CA) or Growth Factor Reduced Matrigel (1:200 dilution) (BD Biosciences, Bedford, MA) coated tissue culture dishes or 3D PLGA scaffolds at a density of 7.5 x 10^4cells/cm^2. The cells were cultured in human fetal fibroblast conditioned media (HFF-CM) supplemented with 10ng/mL Fgf2 (Invitrogen) for 48 hours at 37ºC with the media changed daily. Following a brief wash with phosphate buffered saline (PBS, containing Ca^{2+} and Mg^{2+}) (Invitrogen), differentiation to definitive endoderm was induced following stage 1 of the protocol described by Kroon *et al.* [8]. Briefly, the cells were cultured in RPMI media (Invitrogen) supplemented with 100ng/mL activin A (R&D Systems, Minneapolis, MN) and 25ng/mL Wnt3a (R&D Systems) for 24 hours. The media was then changed to RPMI supplemented with 0.2% v/v fetal bovine serum (Invitrogen) and 100ng/mL activin A and the cells were cultured in this media for a further 48 hours. Samples were collected at various time points during the 72 hour differentiation period for analysis. For each of the 2D and 3D comparison experiments, hESCs were harvested from the same flask and treated equally in order to reduce variance.

RNA Extraction and Real-Time Quantitative RT-PCR

Illustra RNAspin Mini RNA Isolation Kits (GE Healthcare, Little Chalfont, UK) were used to extract total RNA from samples. Total RNA was reverse transcribed to cDNA using the Superscript III First Strand Synthesis Supermix (Invitrogen). Oligo(dT) primers were used, and the reactions were carried out in a MyoCycler® thermal cycler (Bio-Rad, Hercules, CA). Real-time quantitative RT-PCR (qRT-PCR) was carried out using the ABsolute^{TM} Blue QPCR SYBR Green Master Mix (Thermo Scientific, Waltham, MA) and was performed on a Mx3000P® (Stratagene, Cedar Creek, TX). Relative gene expression was calculated by evaluating the efficiency of individual primers and normalising the quantified values against the input determined by two housekeeping genes, *β-actin* and *HSP90*. Sequences for *BRACHYURY, N-Cadherin, E-Cadherin, SOX17*, *FOXA2*, *CER, CXCR4*, *MEOX1, SOX7, SOX1* and *ZIC1* were obtained from D'Amour *et al.* [10]. Sequences for *β-actin* and *HSP90* were selected from primer bank [11] as follows: *β-actin* for, GGACCTGACTGACTACCTC and rev, GCCA TCTCTTGCTCGAAG; and *HPSP90* for, GACTTGTGTCT TCACCTTGCT and rev, GGTGGAGTTGTCCCGAAGTG.

Western Blot Analysis

Cells cultured on 3D PLGA scaffolds and 2D monolayers were lysed and protein concentrations were quantified using Bradford assay [12]. An equal amount of protein from each sample was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked using 5% bovine serum albumin in TBST (Sigma Aldrich, St. Louis, MO) overnight at 4ºC. Membranes were then incubated with primary antibodies SOX17 (1:1000) (R&D Systems) or ACTIN (1:5000) (Sigma Aldrich) for 2 hours at room temperature and subsequently incubated with appropriate secondary antibodies (Dako, Carpinteria, CA) for 1 hour. All membranes were visualised by using ECL and developed onto ECL hyperfilm.

Statistics

Statistical analysis of qRT-PCR data was performed using Student's t-test with the GraphPad Prism software (GraphPad Prism version 4.00 for Windows, GraphPad Softward, San Diego, CA). The difference was considered to be significant if the p-value was ≤ 0.05.

RESULTS AND DISCUSSION

This study has successfully derived definitive endoderm from single cell preparations of pluripotent hESCs cultured on laminin or Matrigel coated 3D PLGA scaffolds. Differences in the endoderm gene expression profile between cells differentiated in 2D or 3D cultures were observed, notably the elevated expression of *SOX17* and *FOXA2* on both matrices and reduced expression of *CER* and *CXCR4* on Matrigel in a 3D environment. This illustrated the impact of 3D PLGA scaffolds on early hESC differentiation towards the pancreatic lineage.

Differentiation of hESCs to Definitive Endoderm can be Efficiently and Effectively Generated on Laminin or Matrigel Coated 3D PLGA Scaffolds

We have previously shown that definitive endoderm can be successfully derived from single cell preparations of

hESCs that were induced to differentiate on laminin or Matrigel coated 2D monolayer cultures [9]. To determine whether definitive endoderm can also be efficiently generated on laminin or Matrigel coated 3D PLGA scaffolds, single cell preparations of hESCs were induced to differentiate in 2D or 3D cultures following a well established differentiation protocol. The use of single cells allows the proper infiltration of cells into the pores of the scaffolds, and to provide relatively uninhibited access of media and growth factors to the cells. The single defined extracellular matrix (ECM) of laminin was used alongside the commonly chosen undefined ECM of Matrigel, because laminin is preferential to other ECM proteins for the adhesion and maintenance of pluripotent hESCs [7,13].

The results of the qRT-PCR analysis of relevant gene expression by hESCs that were differentiated on Matrigel coated 3D PLGA scaffolds are shown (Fig. **1**). At 36 hours of differentiation, the cells had proceeded through the mesendoderm to endoderm transition as indicated by the transient expression of the mesendoderm marker *BRACHYURY* [10]. This was accompanied by a decrease in expression of the pluripotency related *E-Cadherin*, and an increase in expression of markers characteristic of definitive endoderm including *N-Cadherin*, *SOX17* and *FOXA2* by the end of 72 hours of differentiation (Fig. **1A**). A similar trend in results was also displayed by hESCs differentiated on laminin coated 3D PLGA scaffolds (data not shown), and those differentiated on laminin or Matrigel coated 2D monolayers [9]. These results concur with the expected gene expression patterns during the formation of definitive endoderm [10], indicating that the hESCs which were differentiated in 3D cultures followed a normal developmental trend and responded to growth factors as rapidly as those cultured on monolayers.

The 3D samples at 72 hours of differentiation expressed high levels of the endoderm genes *CER* and *CXCR4* with low levels of expression of the extraembryonic endoderm marker *SOX7* and the ectoderm and mesoderm lineage

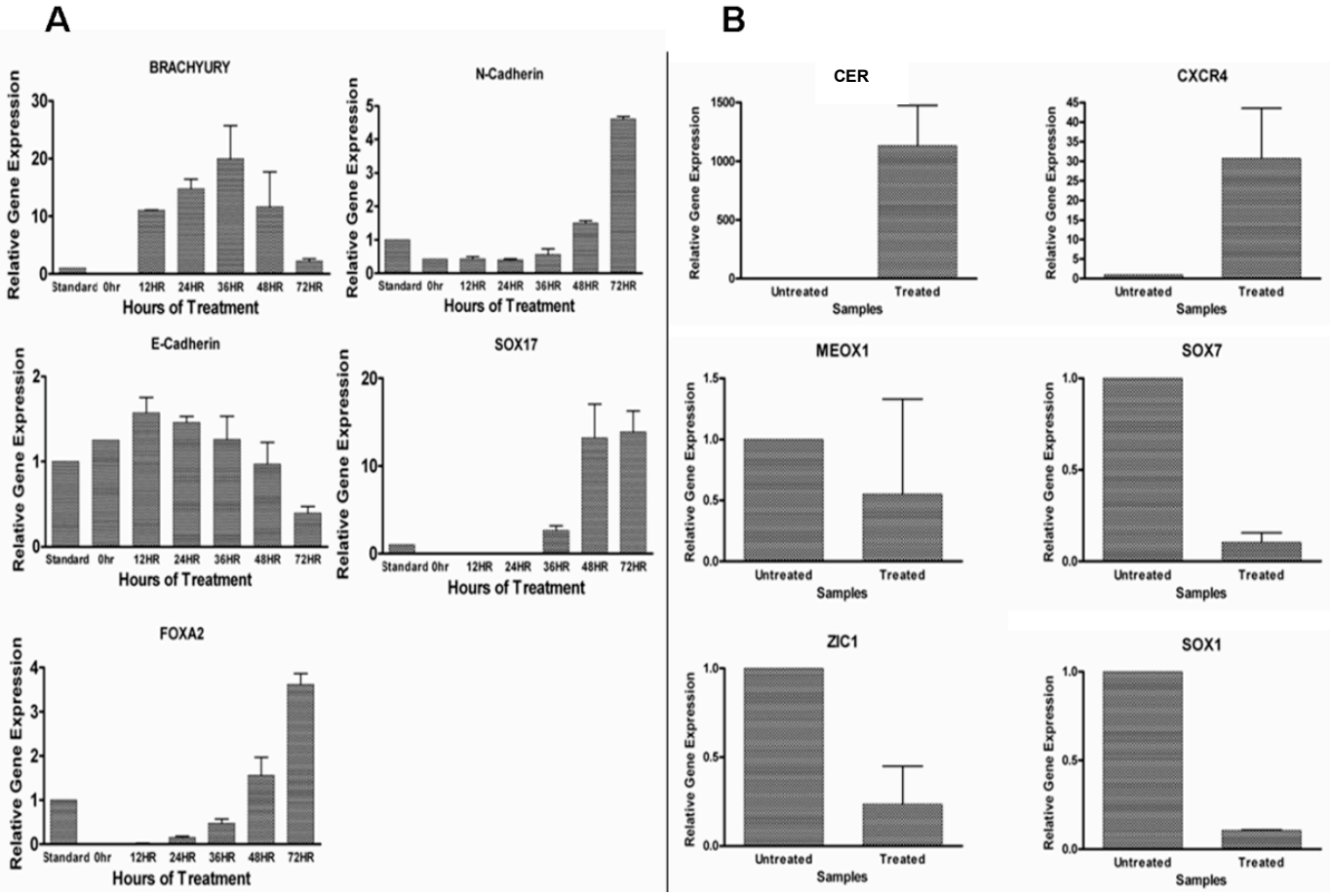

Fig. (1). Gene expression profile of definitive endoderm derived from hESCs seeded onto Matrigel coated 3D PLGA scaffolds. (**A**) The temporal expression of key developmental genes during hESC differentiation to definitive endoderm on Matrigel coated 3D PLGA scaffolds showed a decrease in expression of pluripotency related genes, and an increase in expression of markers characteristic of definitive endoderm. Data presented as the average relative gene expression ± standard deviation in comparison to a standard, which consisted of a mix of human fetal tissue cDNA that expressed low levels of all the genes of interest. (**B**) The gene expression status of additional endoderm markers (*CER* and *CXCR4*) and markers of other lineages (*MEOX1, SOX7, SOX1, ZIC1*) at the end of the 72 hour definitive endoderm differentiation period from cells cultured on Matrigel coated 3D PLGA scaffolds. Data presented as the average relative gene expression ± standard deviation relative to the untreated control, which consisted of hESCs cells cultured on Matrigel coated 3D PLGA scaffolds in human fetal fibroblast conditioned media without the supplementation of growth factors. All results were collected from 3 independent experiments with 2 internal replicates per experiment.

markers *SOX1, MEOX1* and *ZIC1* (Fig. (**1B**), data shown here are from 3D Matrigel cultures). Expression of the extraembryonic marker *AFP* was not detected in either 2D or 3D samples (data not shown). These were in comparison to control cells cultured in HFF-CM without the supplementation of growth factors. The results confirmed that a majority of the cells on 3D scaffolds and 2D monolayers were of the definitive endoderm lineage, and displayed a similar gene expression profile to definitive endoderm derived previously on 2D monolayers [10,14].

Comparison of Definitive Endoderm Genes between Cells Differentiated in 3D and 2D Cultures

To investigate the impact of 3D culturing on hESC differentiation to definitive endoderm, we compared the expression levels of the four endoderm genes *SOX17, FOXA2, CXCR4* and *CER* between cells cultured on either laminin or Matrigel coated 3D PLGA scaffolds to their monolayer counterparts. After 72 hours of differentiation, the expression levels of the key endoderm transcription factors *SOX17* and *FOXA2* were significantly higher ($p<0.05$) in the laminin or Matrigel coated 3D samples in comparison to monolayer cultures (Fig. **2**). No statistically significant differences were found in the expression levels of the surface marker *CXCR4* [15] and the anterior endoderm marker *CER* [16,17] between the laminin coated 3D and 2D samples. Interestingly however, expression of these two genes were significantly lower ($p<0.05$) in Matrigel coated 3D samples in comparison to their corresponding 2D samples. This suggested that the impact of 3D culturing can be affected by the ECM on which the cells are cultured. Although the major component of Matrigel is laminin, it is an undefined ECM that also contains collagen IV and other basement membrane factors. Whether certain factors within Matrigel can affect the phenotype of the definitive endoderm cells that were formed on Matrigel coated 3D PLGA scaffolds is unconfirmed. Nevertheless, these results highlighted an impact of ECM and 3D culturing on ESC differentiation and tissue engineering applications.

Western blot analysis of SOX17 protein was conducted to verify whether the difference in gene expression was translated to its protein expression. Higher levels of SOX17 protein expression were detected in cells differentiated on both laminin and Matrigel coated 3D scaffolds in comparison to 2D monolayers (Fig. **2**). This provided further evidence that although the definitive endoderm cells produced on both 3D and 2D cultures followed a similar trend during their development, 3D cultures had a notable

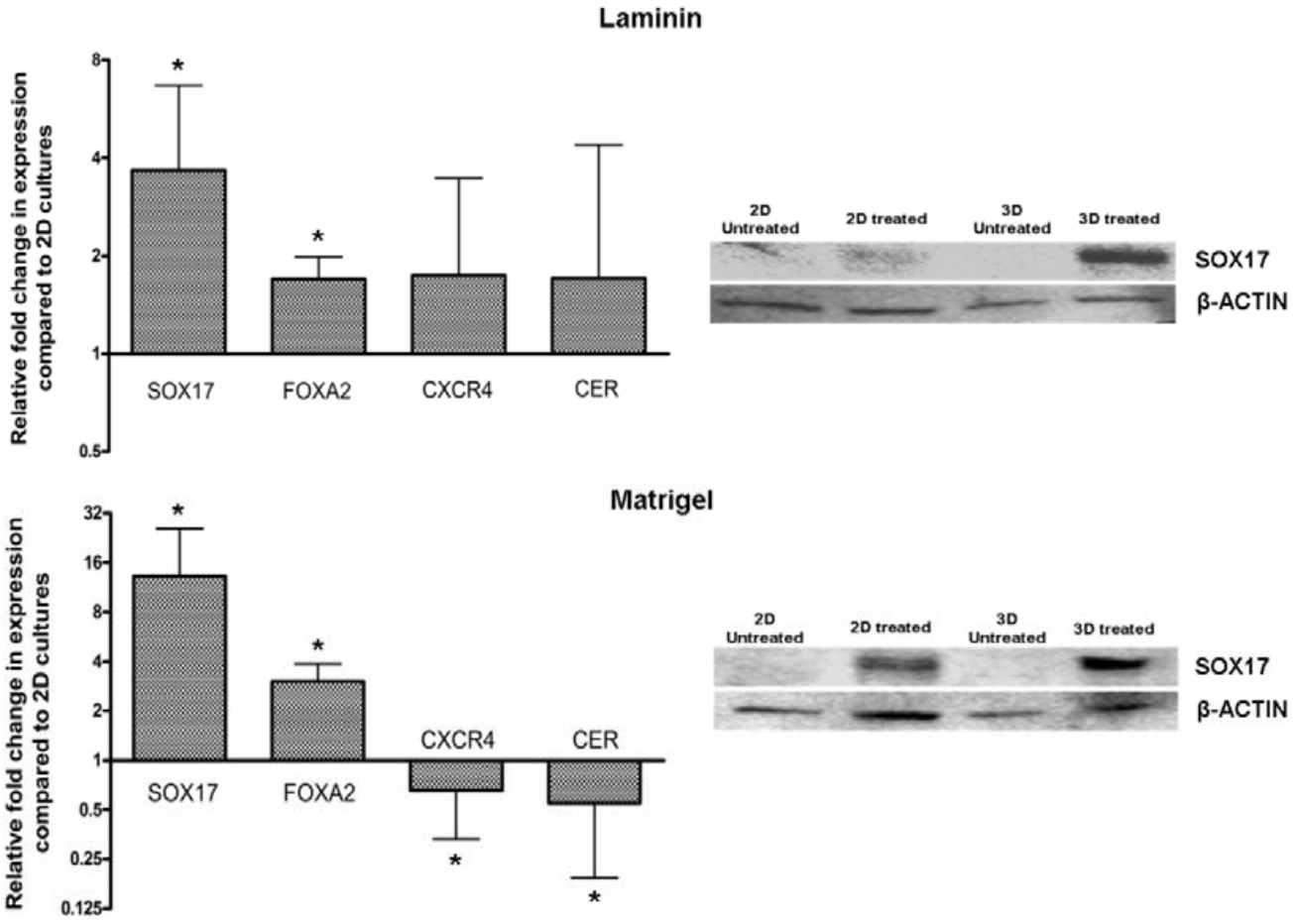

Fig. (2). A comparison of the gene and protein expression levels of key endodermal markers after 72 hours of induced hESC differentiation to definitive endoderm in 2D and 3D environments. (Left) Gene expression analysis of relative fold changes of key endoderm markers by cells cultured on 3D scaffolds in comparison to those cultured on 2D monolayers. Data presented as average fold change ± standard deviation. Results were collected from 3 independent experiments with 2 internal replicates per experiment. Differences were statistically significant if $p \leq 0.05$. (Right) Western blot analysis of SOX17 protein expression from cells differentiated in 2D and 3D environments.

positive impact on the differentiating hESCs. This was reflected in differences in the levels of gene and protein expression of the key endoderm transcription factors between the cells cultured in the two systems.

We hypothesised that the differences in the endoderm gene expression profile by the hESCs that were cultured on Matrigel coated 3D scaffolds may be because the cells had differentiated faster and progressed into the posterior foregut stage of development. However, there were no differences in gene expression of the gut markers *HNF1β* and *HNF4α* [18] between cells that were differentiated in 2D and 3D environments (data not shown). It is possible that the definitive endoderm derived on Matrigel coated 3D scaffolds adapted a more posterior phenotype under the combined influence of the ECM and the 3D structure. If true, this may have an impact on later stages of pancreatic differentiation and as such, warrants further detailed investigations.

In summary, this study has shown that hESCs can be successfully differentiated into definitive endoderm on laminin or Matrigel coated 3D PLGA scaffolds using a currently well established protocol. The cells cultured on 3D scaffolds exhibited elevated *SOX17* and *FOXA2* levels, and in the case of cells cultured on Matrigel, reduced *CER* and *CXCR4* levels in comparison to those cultured on 2D monolayers. The results showed that 3D cultures can make a positive impact on endoderm differentiation of hESCs. This highlights the underrated potential of 3D culturing on hESC differentiation in general, and more detailed investigations should be made in the future to determine the effect that 3D cultures have on the further pancreatic differentiation of hESCs.

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