Laminin/Entactin Complex: A Feeder-Free Surface for Culture of Human Embryonic Stem Cells

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Application Note

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Introduction

Embryonic stem (ES) cells are pluripotent with immense proliferation potential and unique self-renewal capacity[1]. These cells have the ability to differentiate into all three germ layers—ectoderm, endoderm, and mesoderm—and many terminally differentiated cell types[2]. A great deal of hope is associated with the potential application of human ES (hES) cells in functional genomics, cell therapy, and regenerative medicine. Their possible clinical application has the potential to revolutionize the practice of medicine by providing cures for diseases such as diabetes, Alzheimer's, and heart disease.

Isolation of hES cells was first described by Thomson and colleagues in 1998[1], when immunosurgically-isolated inner cell masses of human blastocysts were cultured on mitotically inactivated murine embryonic fibroblast (MEF) feeder cells in medium containing 20% fetal bovine serum. While MEF feeder layers have proved to be a robust surface for long-term culture of hES cells, there are undefined components secreted in the media causing batch-to-batch variability in feeder layers. There are also concerns about xenogeneic contamination of hES cells grown in this system. The compact hES cell colonies formed on feeder layers are difficult to transfect and genetically manipulate, and some quenching also occurs due to the feeder layer. Clinical applications using these cells require development of feeder-independent, defined culture conditions for hES cell propagation as well as standardized methods to promote differentiation into desired cell types. This, in turn, necessitates a thorough understanding of the environmental cues that determine hES cell fate choices (self-renewal, differentiation, survival or apoptosis).

In the last few years, several studies have demonstrated feeder-free culture of hES cell lines on extracellular matrix (ECM) substrates, particularly Corning® Matrigel® matrix, in MEF-conditioned media (MEF-CM) as well as in defined media.[3, 4, 5] Most recently mTeSR®1, a defined media developed at WiCell™ Research Institute, has been successfully used to culture WiCell cell

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lines H1, H7, H9, and H14 on Corning® Matrigel® matrix[6]. Corning Matrigel matrix is a reconstituted basement membrane isolated from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, predominantly composed of laminin, collagen IV, entactin, and heparin sulfate proteoglycan. Several groups have compared the growth of hES cells on Corning Matrigel matrix and the ECM proteins laminin, fibronectin, and collagen IV. A combination of ECM proteins is required for hES cells to perform comparably to hES cultures on Corning Matrigel matrix.[3, 7, 8] Moreover, purified proteins are generally more expensive and not well established for long-term performance as compared to Corning Matrigel matrix.

Corning Laminin/Entactin Complex High Concentration is also prepared from EHS mouse sarcoma. However, this material is a further purified formulation than Corning Matrigel matrix. In this study, we demonstrate the maintenance of undifferentiated hES cells on Corning Laminin/Entactin Complex High Concentration. The H9 hES cell line grown on this surface in mTeSR™1 media showed undifferentiated colony morphology, expressed markers of undifferentiated hES cells, and maintained pluripotency. Cells were able to differentiate in vitro into the cells of all three-germ layers and maintained normal karyotype after multiple passages.

Materials and Methods

Cell Culture

H9 hES cells (WiCell™ Research Institute) were maintained on Corning Matrigel hESC-qualified matrix-coated Falcon® Multiwell plates in mTeSR®1 media (STEMCELL Technologies, Canada). These cells were cultured on Corning Laminin/Entactin Complex High Concentration either in mTeSR1 media or MEF-CM using DMEM/F12 containing 20% KnockOut™ Serum Replacement, 10 mM MEM non-essential amino acids, 1 mM L-Glutamine, 10 ng/mL basic-fibroblast growth factor (bFGF), and 0.1 mM b-mercaptoethanol supplemented with 8 ng/mL bFGF. Cells growing in MEF-CM were passaged using collagenase IV, and those growing in mTeSR1 media were passaged using dispase. Cells were passaged using a 1:4-1:6 split ratio. Media was changed every day during one passage cycle, except once per week when a double volume of media was added to skip changing media on one day. Differentiation media contained 20% fetal calf serum instead of KnockOut Serum Replacement and did not contain bFGF.

Reagents

Corning Matrigel hESC-qualified matrix (Cat. No. 354277), Corning Laminin/Entactin Complex High Concentration (Cat. No. 354259), Falcon 6-well Multiwell plate (Cat. No. 353046) and bFGF (Cat. No. 354060) were obtained from Corning Incorporated. DMEM/F12, KnockOut serum replacement, and MEM non-essential amino acids were purchased from Life Technologies. L-Glutamine was purchased from Sigma-Aldrich.

For immunohistochemistry, SSEA-1 (Cat. No. MAB2155), SSEA-4 (Cat. No. MAB1435) and Oct-3/4 (Cat. No. AF1759) antibodies were purchased from R&D Systems. Alexa Fluor® 488-conjugated anti-mouse IgM (Cat. No. A21042), donkey anti-mouse IgG (Cat. No. A21202), and Alexa Fluor 594 rabbit anti-goat IgG (Cat. No. A11080) secondary antibodies were purchased from Invitrogen. For flow cytometry, SSEA-4 (Cat. No. SC-21704), Oct-3/4 (Cat. No. SC5279), and appropriate isotype controls were purchased from Santa Cruz Biotechnology. Alexa Fluor 488 rabbit anti-mouse IgG secondary antibody (Cat. No. A11059) was from Invitrogen. GATA-4 (560327) and Nestin (Cat. No. 611658) antibodies were obtained were obtained from BD Biosciences.

Coating Procedure

Corning® Laminin/Entactin Complex High Concentration was thawed on ice, aliquots were prepared and stored at -70°C. Repeated freeze thawis not recommended. For coating, Corning Laminin/Entactin Complex High Concentration was diluted to a final concentration of 50 µg/mL using ice cold DPBS (coating solution). The coating solution was kept on ice at all times. One mL of the coating solution was added to each well of a Falcon® 6-well Multiwell plate. Plates were incubated at room temperature for 1-2 hours. The coating solution was aspirated from the plate, and 2 mL media was added to each well. Plates were incubated in a 37°C, 5% CO_2 incubator for 30 minutes prior to the addition of the cells. Coated plates with coating solution can be stored at 4°C and should be used within one week of coating. Coating solution should be aspirated just before using the plates.

Immunohistochemical (IHC) analysis

Cells were fixed in 4% paraformaldehyde (prepared fresh in PBS) for 20 minutes at room temperature, and washed three times with PBS for 5 minutes each. For SSEA-4 staining, cells were blocked with 1% BSA and 10% normal donkey serum in PBS (blocking buffer) at room temperature for 45 minutes. For SSEA-1, donkey serum was substituted with normal goat serum. For Oct-3/4, cells were permeabilized and blocked with 0.1% Triton X-100, 1% BSA, 10% normal rabbit serum in PBS at room temperature for 45 minutes. After blocking, cells were probed with anti-SSEA-1, anti-SSEA-4, or Oct-3/4 primary antibody at a final concentration of 2.5 µg/mL, in blocking buffer. Cells were washed three times with 2 mL of 1%BSA, PBS for 5 minutes each. After washing, cells were incubated with secondary antibody, diluted 1:1000 in 1%BSA, PBS, for 60 minutes, at room temperature in the dark. Cells were then washed 3 times with PBS for 5 minutes each and plates were stored wrapped in aluminum foil at 4°C in 2 mL PBS until visualized under a fluorescence microscope.

Flow cytometry analysis

Cells were grown for at least 5 passages before FACS analysis. Cells were dissociated using 1 mL of 0.25% Trypsin/EDTA for 5 minutes at 37°C, and collected in a tube containing 1 mL 2.5% fetal bovine serum (FBS) in PBS.

For Oct-3/4 staining, cells were fixed with 1% paraformaldehyde for 10 minutes at 37°C, washed with BD perm/wash buffer (BD Cat. No. 554723), and permeabilized in BD perm/wash buffer for 15 minutes on ice. At the end of the permeabilization step, cells were collected by centrifugation and resuspended in BD perm/wash buffer. For detection of surface marker SSEA-4, live cells were resuspended in 2.5% FBS in PBS. For staining, 1-5x10^5 fixed (Oct-3/4) or live (SSEA-4) cells were probed for 1 hour at 4°C with the specific primary or an appropriate isotype control antibody at a concentration of 1 µg/10^6 cells. Cells were washed and probed with a 1:500 dilution of an Alexa Fluor® 488 labeled secondary antibody for 30 minutes in the dark at 4°C. Cell were then washed, resuspended in 0.2 mL 2.5%FBS in PBS and analyzed using a BD FACSCalibur™ flow cytometer. Acquisition was set for 20,000 events per sample. Data were analyzed with BD CellQuest™ 3.0 software.

Embryoid body (EB) formation and in vitro differentiation into 3 germ layers

Expression of 3-germ layer differentiation markers was analyzed by quantitative RT-PCR. Cells were grown on Corning® Laminin/Entactin Complex High Concentration for at least 5 passages, treated with dispase, washed 4 times with DMEM/F12 and collected in differentiation media by gently scrapping with a 5 mL pipette (no trituration). Cell clumps were transferred to Corning 6-well Low Attachment Culture Dishes, and EB formation was monitored under the microscope. For quantitative gene expression analysis, EBs were collected at day 12, RNA was isolated using the RNeasy® kit (Qiagen), reverse transcription and real time PCR was performed using TaqMan® One-Step RT-PCR Master Mix Reagents Kit (ABI Cat. No. 4309169). Primers for real time PCR were also purchased from ABI; b-actin was used as a normalization control. Expression of TUBB3 (ectoderm), HAND1 (mesoderm), and, FOXA2 (endoderm) markers were quantified against undifferentiated control hES cells cultured on Corning Laminin/Entactin Complex High Concentration. For differentiation into cardiomyocytes and neurons, 5 day old EBs were transferred to 0.1% gelatin coated dishes, cultured further in differentiation media and monitored regularly under the microscope. Cells were fixed after 15 days and stained with GATA-4 and nestin antibodies.

Karyotype analysis

Cells were grown on Corning Laminin/Entactin Complex High Concentration in mTeSR1 media for 26 passages, and treated with colcemid. Cells were collected by 0.05% trypsin treatment and processed according to the recommendations of the Dana Farber/ Harvard Cancer Center Core Laboratory. Karyotype analysis was performed at the cytogenetics core of the Dana Farber Cancer Center, Boston, MA.

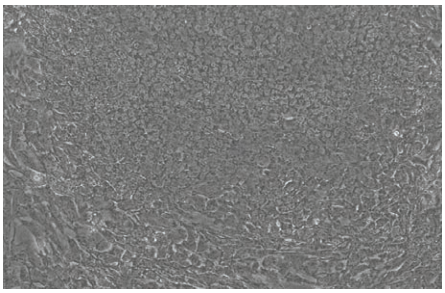

Figure 1a.

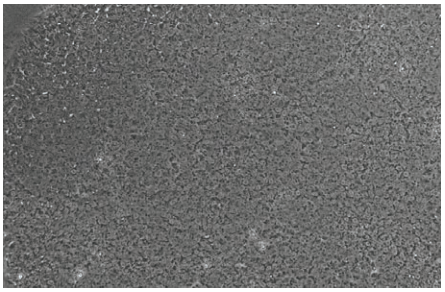

Figure 1b.

Phase contrast images of H9 cells grown on Corning Laminin/Entactin Complex High Concentration coated plates in a) MEF-CM, b) mTeSR1 maintenance media. Images were captured at 4X magnification.

Results

Optimal concentration of Corning® Laminin/Entactin Complex High Concentration for attachment and growth of undifferentiated hES cell colonies

Falcon® 6-well Multiwell Plate were coated with 5, 10, 15, 25, and 50 µg/mL Corning Laminin/Entactin Complex High Concentration. H9 cells were seeded onto these coated dishes in mTeSR®1 media and colonies were monitored under the microscope every day. H9 cells attached on 25 µg/mL Corning Laminin/Entactin Complex High Concentration coated plates; however attachment was significantly better at 50 µg/mL. A concentration below 25 µg/mL showed poor attachment and differentiation (data not shown). For subsequent experiments, plates were coated with 50 µg/mL Laminin/Entactin complex, which supported undifferentiated expansion of hES cells in mTeSR1 as well as in MEF-CM (Figures 1a and b). In contrast to colonies in TeSR™1 media that exhibited well defined borders, cells in MEF-CM exhibited colonies with undefined borders and more isolated cells at the periphery (Figures 1a and b).

Immunohistochemical analysis of undifferentiated hES cell markers

Cells were grown for 5 passages on Corning Laminin/Entactin Complex High Concentration, fixed and stained with Oct-3/4, SSEA-4 and SSEA-1 antibodies and visualized under the fluorescent microscope. As shown in Figures 2a and c, cells stained positive for Oct-3/4 when grown for multiple passages on this surface either in mTeSR1 or MEF-CM. Similarly, SSEA-4, a marker of undifferentiated hES cells, was also expressed abundantly under these culture conditions (Figures 2b and d). Expression of SSEA-1 was not detectable (data not shown), further demonstrating undifferentiated hES cells.

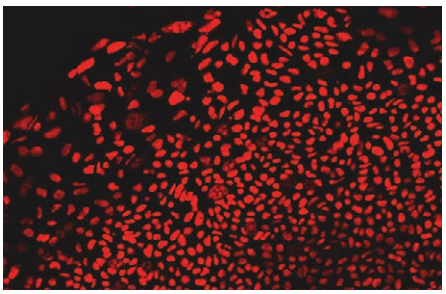

Figure 2a.

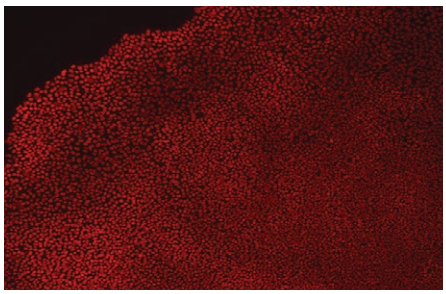

Figure 2c.

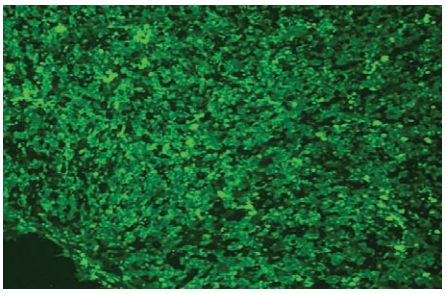

Figure 2b.

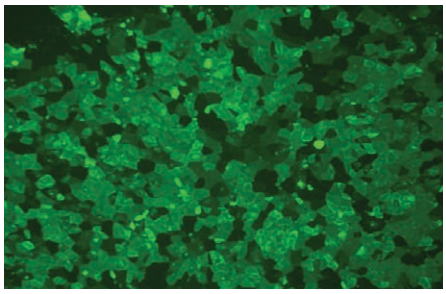

Figure 2d.

Expression of undifferentiated hES cell markers in H9 cells cultured on Corning Laminin/Entactin Complex High Concentration. Cells stained positive for undifferentiated markers Oct-3/4 (red), and SSEA-4 (green) in MEF-CM (a and b), and mTeSR1 maintenance media (c and d). Images were captured at 4X (b, c) and 10X (a, d) magnification.

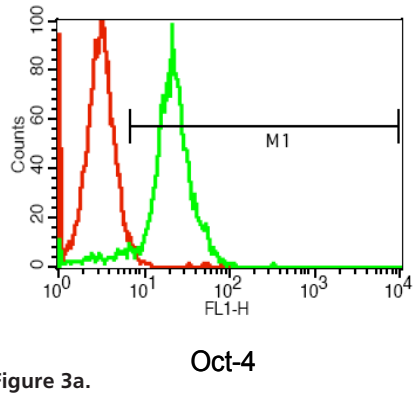

Figure 3a.

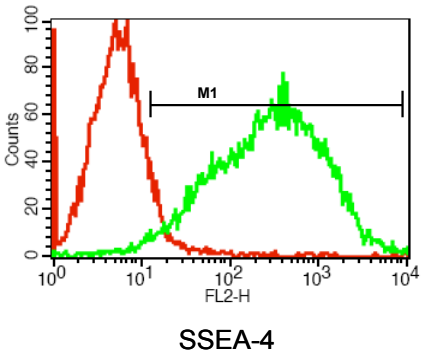

Figure 3b.

Flow cytometry analysis of H9 cells cultured on Corning Laminin/Entactin Complex High Concentration coated surface in mTeSR1 maintenance media; a) 95% cells stained positive for Oct-3/4 (green), and b) 91% cells stained positive for SSEA-4 (green). Isotype control is shown in red.

Flow cytometry analysis revealed expression of undifferentiated hES cell markers

Cells were grown for 5 passages on Corning® Laminin/Entactin Complex High Concentration in mTeSR®1 media, stained for undifferentiated hES cell markers and analyzed by flow cytometry. FACS analysis revealed ~95% Oct-3/4 positive, and ~91% SSEA-4 positive cells, indicating undifferentiated hES cells (Figures 3a and b). The levels of undifferentiated hES cells were comparable to those grown on Corning Matrigel® hESC-qualified matrix (data not shown). Thus, Corning Laminin/Entactin Complex High Concentration, a more defined ECM complex is equivalent to Corning Matrigel matrix for hES cell culture.

Embryoid body formation and in vitro differentiation potential

To determine pluripotency, hES cells grown on Corning Laminin/Entactin Complex High Concentration for multiple passages were allowed to differentiate in suspension culture. Embryoid bodies were formed under these culture conditions (Figure 4). Quantitative RT-PCR analysis performed on 9 day old EBs confirmed expression of three germ layer markers in embryoid bodies. Cells grown for 32 passages on Corning Laminin/Entactin Complex High Concentration formed EBs with expression of HAND1 (heart and neural crest derivatives expressed 1 -mesoderm), TUBB3 (tubulin beta 3 -ectoderm), and FOX-A2 (forkhead box A2 -endoderm) as shown in Figure 5.

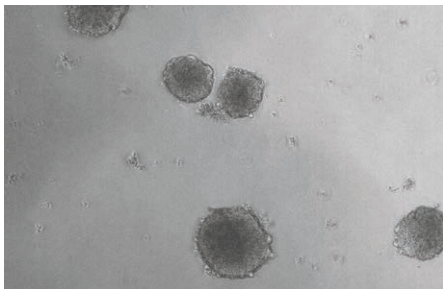

Figure 4.

H9 cells cultured on Corning Laminin/Entactin Complex High Concentration in the presence of mTeSR1 maintenance media formed embryoid bodies on a low-attachment surface in differentiation media. Images were captured at 4X magnification.

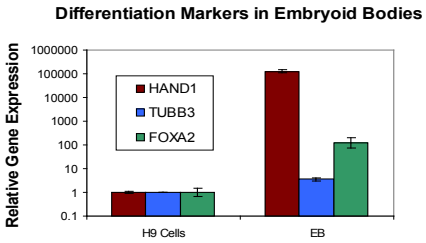

Figure 5.

Quantitative RT-PCR analysis of 3-germ layer markers in embryoid bodies derived from H9 cells cultured on Corning Laminin/Entactin Complex High Concentration for 32 passages in mTeSR1 media.

After 5 days of suspension culture, EBs were transferred to gelatin coated plates. EBs attached rapidly to the gelatin coated surface and cultures continued to multiply and differentiate. Cells were allowed to differentiate further for another 15 days. Beating cardiomyocytes could be visualized under the microscope after 10 days (still image Figure 6a), and neurons also became clearly visible (Figure 6b). Differentiation into beating cardiomyocytes and neurons was successfully demonstrated at passage 15 and passage 32. Cardiomyocytes stained positive for GATA-4 (Figure 6c), and differentiated neurons were identified with nestin staining (Figure 6d).

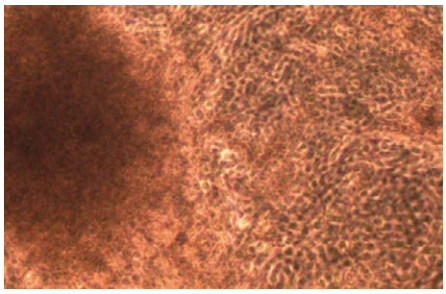

Figure 6a.

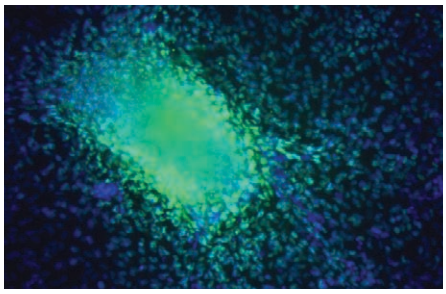

Figure 6c.

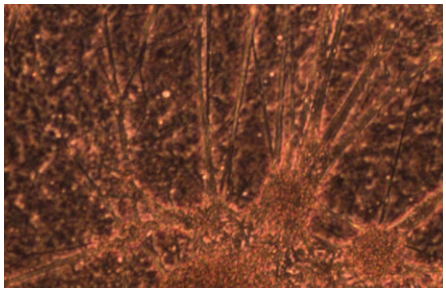

Figure 6b.

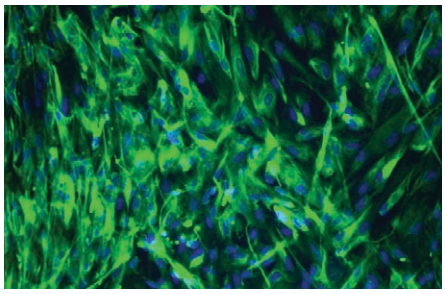

Figure 6d.

Differentiation of H9 cell line. a) Still image of H9–derived beating cardiomyocytes, b) H9-derived neurons, c) GATA-4 expression in cardiomyocytes and, d) Nestin expressing neuronal cells. Images a, c, and d were captured at 10X magnification; nerons (b) were imaged at 4X magnification.

Cells maintained stable karyotype after culture on Corning® Laminin/ Entactin Complex High Concentration

Cells were grown on Corning Laminin/Entactin Complex High Concentration in mTeSR®1 media for 26 passages and karyotype analysis was performed. These cells had a normal karyotype. G-banding chromosome analysis is represented in Figure 7. Therefore, Laminin/Entactin support undifferentiated expansion of hES cells, and also maintains the differentiation potential and karyotype stability of these cells.

Figure 7.

G banding chromosome analysis. Karyotype analysis of H9 cells grown on Corning Laminin/ Entactin Complex High Concentration in mTeSR1 media for 26 passages.

Conclusions

Corning® Laminin/Entactin Complex High Concentration represents an alternative surface that supports the feeder-free expansion of undifferentiated hES cells. Cultures remained undifferentiated throughout the testing period of more than 6 months, maintaining normal morphology and stable karyotype. Cells expressed markers of undifferentiated hES cells and differentiated into all three germ layers. While clinical application requires an animal-free culture environment, many basic research questions can be answered with this feeder-free culture environment.

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