PLURIPOTENT STEM CELL CULTURE ON MICRO-CARRIERS

FIELD OF THE INVENTION

The present invention claims priority to application serial number 61/116,447, filed November 20, 2008.

FIELD OF THE INVENTION

The present invention is directed to methods for the growth, expansion and differentiation of pluripotent stem cells on micro-carriers.

BACKGROUND

Pluripotent stem cells, such as, for example, embryonic stem cells have the ability to differentiate into all adult cell types. As such, embryonic stem cells may be a source of replacement cells and tissue for organs that have been damaged as a result of disease, infection, or congenital abnormalities. The potential for embryonic stem cells to be employed as a replacement cell source is hampered by the difficulty of propagating the cells *in vitro* while maintaining their pluripotency.

Current methods of culturing undifferentiated embryonic stem cells require complex culture conditions, such as, for example, culturing the embryonic stem cells in the presence of a feeder cell layer. Alternatively, media obtained by exposure to feeder cell cultures may be used to culture embryonic stem cells. Culture systems that employ these methods often use cells obtained from a different species than that of the stem cells being cultivated (xenogeneic cells). Additionally, these culture systems may be supplemented with animal serum.

Embryonic stem cells provide a potential resource for research and drug screening. At present, large-scale culturing of human embryonic stem cell lines is problematic and provides substantial challenges. Current *in vitro* methods to propagate pluripotent stem cells are carried out in tissue flasks on planar surfaces pre-coated with extracellular matrix (ECM) proteins or feeder cells. Planar cultures also require frequent subculturing

because their limited surface area cannot support long-term growth of pluripotent stem cells. Micro-carrier-based methods of pluripotent stem cell culture may provide a solution. Micro-carriers have a high surface-area-to-volume ratio and, therefore, eliminate the surface area restriction of growing pluripotent stem cells on planar surfaces.

For example, Fok *et al* disclose stirred-suspension culture systems for the propagation of undifferentiated ESC - micro-carrier and aggregate cultures (Stem Cells 2005;23:1333–1342.)

In another example, Abranches *et al* disclose the testing of Cytodex 3® (GE Healthcare Life Sciences, NJ), a microporous micro-carrier made up of a dextran matrix with a collagen layer at the surface for its ability to support the expansion of the mouse S25 ES cell line in spinner flasks (Biotechnol. Bioeng. 96 (2007), pp. 1211–1221.)

In another example, US20070264713 disclose a process for cultivating undifferentiated stem cells in suspension and in particular to a method for cultivating stem cells on micro-carriers in vessels.

In another example, WO2006137787 disclose a screening tool is used which comprises particulate matter or micro-carriers, such as beads, attached to a solid support, such as a micro titer plate, for the cultivation of cells on said micro-carriers.

In another example, WO2008004990 disclose a method of promoting the attachment, survival and/or proliferation of a stem cell in culture, the method comprising culturing a stem cell on a positively-charged support surface.

In another example, WO2007012144 disclose a bioreactor, comprising: a support surface; and a synthetic attachment polypeptide bound to the support surface wherein the synthetic attachment polypeptide is characterized by a high binding affinity for an embryonic stem cell or a multipotent cell.

SUMMARY

The present invention provides methods for the growth, expansion and differentiation of pluripotent stem cells on micro-carriers.

In one embodiment, the present invention provides a method for the propagation of pluripotent stem cells, comprising the steps of:

a. Attaching a population of pluripotent stem cells to a first volume of micro-carriers,

b. Culturing the pluripotent stem cells on the first volume of micro-carriers,

c. Removing the pluripotent stem cells from the first volume of micro-carriers, and

d. Attaching the population of pluripotent stem cells to a second volume of micro-carriers.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Rho-kinase inhibitor promotes attachment and growth of human embryonic stem cells to micro-carriers. Images of H9 cells grown in static culture for 2 days on HILLEX®II micro-carriers (Solohill, MI). The cells were cultured in mouse embryonic fibroblast conditioned medium (MEF-CM) with or without 10μM Rho Kinase inhibitor, Y27632 ((Sigma-Aldrich, MO) A and B, respectively).

Figure 2: H9 cells grown on micro-carriers. H9 cells were allowed to attach to various micro-carriers and placed on a rocking platform at 37°C. Plastic micro-carriers, ProNectinF micro-carriers, HILLEX®II micro-carriers (Solohill, MI), and Plastic Plus micro-carriers, were used (A, B, C, D respectively). Growth after 3 days showed cells on HILLEX®II (Solohill, MI) with best cell attachment to the micro-carriers. Arrows identify cells forming aggregates without attachment to the micro-carriers.

Figure 3: H9 cell proliferation on micro-carriers. H9 cells were attached to HILLEX®II micro-carriers, ProNectinF micro-carriers, Plastic Plus micro-carriers, and Plastic micro-carriers (Solohill, MI) and placed in a 6 well dish on a rocking platform at 37°C in the presence of 10μM Y27632 (Sigma-Aldrich, MO) and MEF-CM. The initial cell seeding density is the value at day 0. Day 3 and day 5 cell numbers are shown.

Figure 4: H1 cell images after attachment to micro-carriers. Images of cells at days 3, 5 and 7 are shown attached to ProNectinF micro-carriers, Plastic Plus micro-carriers, and Plastic micro-carriers. The cells were grown in MEF-CM with 10μM Y27632 (Sigma-Aldrich, MO) in a 12 well dish on a rocking platform at 37°C. Cells formed aggregates independent of binding to Plastic Plus and Plastic micro-carriers (arrows in G, H).

Figure 5: H1 cell images after attachment to micro-carriers. Images of cells at days 3, 5 and 7 are shown attached to Cytodex 1® micro-carriers, Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) and HILLEX®II micro-carriers (Solohill, MI). The cells were grown in MEF-CM with 10μM Y27632 (Sigma-Aldrich, MO) in a 12 well dish on a rocking platform at 37°C.

Figure 6: H1 cell proliferation on micro-carriers. H1 cells were allowed to attach to HILLEX®II micro-carriers (Solohill, MI), Cytodex 1® micro-carriers (GE Healthcare Life Sciences, NJ), Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ), ProNectinF micro-carriers (Solohill, MI), Plastic Plus micro-carriers (Solohill, MI), and Plastic micro-carriers (Solohill, MI) and placed in a 12 well dish on a rocking platform at 37°C in the presence of 10μM Y27632 (Sigma-Aldrich, MO) and MEF-CM. The initial cell seeding density is the value at day 0. Day 3, 5, and 7 cell numbers are shown. The initial seeding density was 13,333 cells/cm^2, as indicated by the line.

Figure 7: H9 cell proliferation on micro-carriers in various concentrations of Rho kinase inhibitors. Cells were grown in a 12 well plate on a rocking platform and counted at day 4 and 7 to determine attachment and proliferation rate. A. H9 cells were grown in MEF-CM with 1, 2.5, 5, or 10μM Y27632 (Sigma-Aldrich, MO). B. H9 cells were grown in MEF-CM with 0.5, 1, 2.5, or 5μM Glycyl-H 1152 dihydrochloride (Tocris, MO).

Figure 8: H1 cells were grown in decreasing concentrations of Rho kinase inhibitors. H1p38 cells were grown in the presence of Y27632 (Sigma-Aldrich, MO) or Glycyl-H 1152 dihydrochloride (Tocris, MO) for two days at decreasing concentrations (10 μM /5 μM, 2.5 μM /0.5 μM or 1.0 μM /0.5 μM) or at 0.25 μM Glycyl-H 1152 dihydrochloride (Tocris, MO) continuously. Cells were allowed to attach to HILLEX®II (Solohill, MI),

Cytodex 1®, or Cytodex 3® ((GE Healthcare Life Sciences, NJ) A, B, C, respectively). Cells were counted at 3, 5 and 7 days post seeding.

Figure 9: Determination of cell attachment to micro-carriers at different seeding densities in spinner flasks. H1 cells were seeded onto Cytodex 3® (GE Healthcare Life Sciences, NJ) micro-carriers at the densities listed on the left; Low (0.4 x 10^4 cells/cm^2), Mid (1.2 x 10^4 cells/cm^2) or High (3 x 10^4 cells/cm^2). At 3, 5 and 7 days the cells were imaged and the percentage of micro-carriers with cells attached was determined (embedded in image).

Figure 10: Cell growth on micro-carriers in spinner flasks is affected by the initial seeding densities. H1 cells were seeded onto Cytodex 3® (GE Healthcare Life Sciences, NJ) micro-carriers at the densities listed on the left; Low (0.4 x 10^4 cells/cm^2), Mid (1.2 x 10^4 cells/cm^2) or High (3 x 10^4 cells/cm^2). At 3, 5 and 7 days the cells were dissociated from the micro-carriers and counted.

Figure 11: Determination of cell growth rate on micro-carriers at different seeding densities in spinner flasks. H1 cells were seeded onto Cytodex 3® (GE Healthcare Life Sciences, NJ) micro-carriers at different densities (day 0); Low (0.4 x 10^4 cells/cm^2), Mid (1.2 x 10^4 cells/cm^2) or High (3 x 10^4 cells/cm^2). At 3, 5 and 7 days the cells were dissociated from the micro-carriers and counted. The fold increase in cell number is shown versus initial seeding density.

Figure 12: H1 cells grown on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) were imaged after 7 days in culture. The cells received MEF-CM without Rho kinase inhibitor from day3 onward. The cells remained attached to the micro-carriers.

Figure 13: H9 cells growth and dissociation of H9 cells on HILLEX®II micro-carriers (Solohill, MI). A, B 10x and 20x images of H9 cells grown for 6 days on HILLEX®II micro-carriers (Solohill, MI). C, 20 x image of cells dissociated from HILLEX®II micro-carriers (Solohill, MI) for 10 minutes with 0.05% Trypsin/EDTA. D, 20x image of cells dissociated from HILLEX®II micro-carriers (Solohill, MI) for 10 minutes with TrypLE™ Express.

Figure 14: Dissociation of H9 cells from micro-carriers. H9 cells grown on HILLEX®II (Solohill, MI) on a rocking platform, were dissociated with TrypLE™ Express or 0.05% Trypsin/EDTA. The number of cells and their viability is shown, A and B respectively.

Figure 15: Dissociation of H1 cells from micro-carriers. H1 cells grown on Cytodex 3® (GE Healthcare Life Sciences, NJ) in a spinner flask were dissociated with TrypLE™ Express (Invitrogen, CA), Accutase™ or Collagenase (10mg/ml). The number of cells and their viability is shown, A and B respectively.

Figure 16: H9 cells grown on HILLEX®II (Solohill, MI) micro-carriers do not transfer between micro-carriers.

Figure 17: H9 at passage 43 were grown for 5 passages on HILLEX®II (Solohill, MI) micro-carriers in a spinner flask. Cells were counted every 2 to 3 days and passaged when cells reached 1-2 x 10^5 cells/cm^2.

Figure 18: H9 cells at passage 43 were grown for 5 passages on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) in a spinner flask. Cells were counted every 2 to 3 days and passaged when cells reached 1-2 x 10^5 cells/cm^2.

Figure 19: Fluorescent-activated cell sorting (FACS) shows pluripotency of H9 cells grown in spinner flasks. A, The majority of H9 p43 cells grown on HILLEX®II (Solohill, MI) micro-carriers express of pluripotency proteins. Passage1 and 3 cells were not evaluated for TRA-1-81. B, The majority of H9 p43 cells grown on Cytodex 3® (GE Healthcare Life Sciences, NJ) micro-carriers express of pluripotency proteins. Passage1 cells were not evaluated for TRA-1-81.

Figure 20: H1 p49 cells were grown for 5 passages on Cytodex 1® micro-carriers (GE Healthcare Life Sciences, NJ) in a spinner flask. Cells were counted every 2 to 3 days and passaged when cells reached 4-8 x 10^4 cells/cm^2.

Figure 21: H1 cells at passage 49 were grown for 5 passages on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) in a spinner flask. Cells were counted every 2 to 3 days and passaged when cells reached 1-2 x 10^5 cells/cm^2.

Figure 22: Fluorescence activated cell sorting (FACS) shows pluripotency of H1 cells grown in spinner flasks.

Figure 23: Population doublings of H1 and H9 cells on micro-carriers. Population doubling times were calculated from day 3 to the day of passaging (day 5,6 or 7).

Figure 24: H9 cells cultured on micro-carriers in defined media. The cells were cultured on HILLEX®II (HII, (Solohill, MI)) or Cytodex 3® (C3, (GE Healthcare Life Sciences, NJ)). Cells were cultured on micro-carriers in one of the following media; mTESR (StemCell Technologies, Vancouver, Canada), StemPro or MEF-CM. 10 μM Y27632 (Y, (Sigma-Aldrich, MO)) or 2.5 μM Glycyl-H 1152 dihydrochloride (H, (Tocris, MO)) was added to the media. Growth rate at 3, 5 and 7 days post seeding was determined.

Figure 25: H1 cells at passage 38 were cultured on micro-carriers in defined media. The cells were cultured on HILLEX®II (HII, (Solohill, MI)) or Cytodex 3® (C3, (GE Healthcare Life Sciences, NJ)) micro-carriers. Cells were cultured on micro-carriers in one of the following medias; mTESR (StemCell Technologies, Vancouver, Canada), StemPro and MEF-CM. 10 μM Y27632 (Y, (Sigma-Aldrich, MO)) or 2.5 μM Glycyl-H 1152 dihydrochloride (H, (Tocris, MO)) was added to the media. Growth rate at 3, 5 and 7 days post seeding was determined.

Figure 26: H1 cells at passage 50 were cultured on HILLEX®II (Solohill, MI)) micro-carriers with defined medium in a spinner flask. A, Images of H1 p50 cells grown in MEF-CM after 3, 7, or 9 days in a spinner flask. B, Images of H1 p50 cells grown in mTESR (StemCell Technologies, Vancouver, Canada) after 3, 7, or 9 days. Arrows identify cell clusters not attached to the micro-carriers.

Figure 27: Differentiation of human embryonic stem cells passaged five times in spinner flasks. A, H9 cells at passage 43 were passaged five times on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ). B, H1 cells at passage 49 were passaged five times on Cytodex 1® micro-carriers (GE Healthcare Life Sciences, NJ). Both cell types were released from the micro-carriers and seeded onto MATRIGEL (BD Biosciences, CA) coated plates. At 80-90% confluency the cells were exposed to a protocol that is capable

of differentiating embryonic stem cells to definitive endoderm. The cells were then analyzed by FACS for the percentage of cells expressing CXCR4, a definitive endoderm marker. The percent of CXCR4 positive cells is in the upper right corner of the plot.

Figure 28: Differentiation of H1 cells on micro-carriers to definitive endoderm. Here FACS plots display the percentage of cells expressing the definitive endoderm marker CXCR4. Percent positive is in the upper right corner. Cells were all expanded on micro-carriers in spinner flasks prior to treatment. A, H1cells at passage 40 were grown on Cytodex 1® micro-carriers (GE Healthcare Life Sciences, NJ) for 6 days after passage 5 prior to differentiation. B, H1cells at passage 40 were grown on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) for 8 days after passage1 prior to differentiation. C, H1 cells at passage 50 were grown on HILLEX®II micro-carriers (Solohill, MI) for 6 days after passage 1 prior to differentiation.

Figure 29: Differentiation of H1 cells on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) to definitive endoderm. A, H1cells at passage 40 were grown on micro-carriers for eight days. B, H1 cells at passage 40 were grown on micro-carriers for 11 days. Both cell population were then differentiated to definitive endoderm on a rocking platform at 37°C. Here FACS plots display the percentage of cells expressing the definitive endoderm marker CXCR4. Percent positive is in the upper right corner.

Figure 30: Differentiation of cells of the human embryonic stem cell line H1, cultured on micro-carriers to definitive endoderm. FACS results for the percent positive CXCR4 cells are shown on the Y-axis. H1 cells were grown on HILLEX®II, Cytodex 1® or Cytodex 3® micro-carriers prior to and during differentiation.

Figure 31: Differentiation of cells of the human embryonic stem cell line H1 cultured on micro-carriers to pancreatic endoderm cells. CT values are shown on the Y-axis for pancreatic endodermal markers, Ngn3, Nkx6.1 and Pdx1. H1 cells were differentiated on HILLEX®II (HII), Cytodex 1®(C1) or Cytodex 3®(C3) micro-carriers in either DMEM-High Glucose (HG) or DMEM-F12 (F12) media. The differentiation protocol lasted 13 days.

Figure 32: Differentiation of cells of the human embryonic stem cell line H1 cultured on micro-carriers to hormone producing pancreatic cells. Percent positive cells were determined by FACS shown on the Y-axis for pancreatic hormone cell markers, Synaptophysin, Glucagon and Insulin. H1 cells were seeded at two different concentrations $10x10^5$ (10) or $20x10^5$ (20) onto Cytodex 3®(C-3) micro-carriers. The cells were differentiated in DMEM-High Glucose (HG) during days four to nine and further differentiated in either HG or DMEM-F12 (F12) media from days 10 through 24.

Figure 33: Differentiation of H1 cells on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) to endocrine cells. H1 cells were differentiated to pancreatic endocrine cells through pancreatic endoderm (Day 14), pancreatic endocrine cells (Day 21) to insulin-expressing cells (Day28). Gene expression levels of Pdx1, Glucagon, and Insulin were measured (A,B,C respectively). H1 cells grown and differentiated on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) (C3) were compared to those grown and differentiated on MATRIGEL (BD Biosciences, CA) coated 6 well dishes (planar). The gene expression values for cells grown on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) was performed in triplicate.

Figure 34: H9 cells were differentiated on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) to definitive endoderm (DE). FACS plots of CXCR4 expression. Percent of definitive endoderm marker CXCR4 positive cells is stated in upper right corner. A, H9 cells at passage 39 were grown on a MATRIGEL (BD Biosciences, CA) coated 6 well dishes and differentiated to DE. B, C Duplicate samples of H9 cells on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) from spinners were placed in a 12 well dish and incubated on a rocking platform.

Figure 35: Differentiation of H9 cells on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) to insulin-expressing cells. H9 cells were differentiated to pancreatic endocrine cells through pancreatic endoderm (Day 14), Endocrine cells (Day 22) to Insulin-expressing cells (Day29). Gene expression level of Pdx1, Glucagon, and Insulin was measured (A, B, C respectively). H9 cells grown and differentiated on Cytodex 3®

micro-carriers (GE Healthcare Life Sciences, NJ) (C3) were compared to those grown and differentiated on MATRIGEL (BD Biosciences, CA) coated 6 well dishes (planar).

Figure 36: Maintenance of pluripotency in human embryonic stem cells cultured for 5 passages on Cytodex 3® micro-carriers, then transferred and cultured on the planar substrates indicated and cultured in the presence of a Rho kinase inhibitor. Panel A depicts the expression of the pluripotency markers CD9, SSEA3, SSEA4, Tra-160, and Tra-181 as detected by flow cytometry. Panel B depicts the expression of the pluripotency markers Nanog, Pou5F1, SOX2, and ZFP42 and markers of differentiation: FOXA2, FOXD3, GATA2, GATA4, and Brachyury as detected by real-time PCR.

Figure 37: Formation of definitive endoderm by human embryonic stem cells cultured for 5 passages on Cytodex 3® micro-carriers, then transferred and cultured on the planar substrates indicated and cultured in the presence of a Rho kinase inhibitor. Panel A depicts the expression of CXCR4 as detected by flow cytometry. Panel B depicts the expression of the markers indicated as detected by real-time PCR.

Figure 38: Formation of definitive endoderm by human embryonic stem cells cultured for 5 passages on Cytodex 3® micro-carriers, then transferred and cultured on a PRIMARIA™ planar substrate. Expression of the genes indicated was determined by flow cytometry.

Figure 39: Human embryonic stem cells cultured on planar substrates maintain pluripotency. mRNA samples from TrypLE ™, Accutase™, or Collagenase passaged H1 human ES cells were collected and assayed for mRNA pluripotency gene expression. Cells were grown for either one passage for 4 days in culture on MATRIGEL in MEF conditioned media (A) or one passage on Primaria™ in MEF conditioned media supplemented with Rock Inhibitor (B), or two passages on Primaria™ in MEF conditioned media supplemented with Rock Inhibitor (C).

Figure 40: H1 human embryonic stem cells grown for greater than 7 passages on PRIMARIA (greater than p45) passaged with Accutase™ or TrypLE™ at 1:4, 1:8, or 1:16 split ratios on PRIMARIA in the presence of Rho Kinase inhibitor Glycyl-H 1152

dihydrochloride were tested for pluripotency (A), and the ability to differentiate to Definitive Endoderm (B). The control is H1p48 human embryonic stem cells grown on 1:30 MATRIGEL passaged with collagenase. 10mA= passaged with 10 minute exposure to Accutase™. 10mT= passaged with 10 minute exposure to TrypLE™.1:4, 1:8, or 1:16 indicate the passage ratio. P(X) indicate passage number since moving from MEF feeders to Primaria™ plastic.

Figure 41: H1 human embryonic stem cells grown for greater than 7 passages on PRIMARIA (greater than p45) passaged with Accutase™ or TrypLE™ at 1:4 ratio on PRIMARIA in the presence of Rho Kinase inhibitor Glycyl-H 1152 dihydrochloride were tested for mRNA expression of pluripotency and differentiation markers. The control is the starting population of cells at passage 37. 10min Accutase™= passaged with 10 minute exposure to Accutase™. P(X) indicate passage number since moving from MEF feeders to PRIMARIA™ plastic.

Figure 42: H1 human embryonic stem cells grown for greater than 7 passages on PRIMARIA™ (greater than p45) passaged with Accutase™ or TrypLE™ at 1:8 ratio on PRIMARIA in the presence of Rho Kinase inhibitor Glycyl-H 1152 dihydrochloride were tested for mRNA expression of pluripotency and differentiation markers. The control is the starting population of cells at passage 37. 10min Accutase™ passaged with 10 minute exposure to Accutase™. P(X) indicate passage number since moving from MEF feeders to PRIMARIA™ plastic.

Figure 43: H1 human embryonic stem cells grown for greater than 7 passages on PRIMARIA™ (greater than p45) passaged with Accutase™ or TrypLE™ at 1:16 ratio on PRIMARIA in the presence of Rho Kinase inhibitor Glycyl-H 1152 dihydrochloride were tested for mRNA expression of pluripotency and differentiation markers. The control is the starting population of cells at passage 37. 10min Accutase™= passaged with 10 minute exposure to Accutase™. P(X) indicate passage number since moving from MEF feeders to PRIMARIA™ plastic.

Figure 44: Images of H1 cells grown on Primaria™ planar substrates (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ) then transferred to micro-carriers 3 days after

seeding. A-C H1 cells were seeded onto Cytodex 3® (GE Healthcare Life Sciences, NJ) micro-carriers. D-F Cells were seeded onto HILLEX®II micro-carriers (Solohill, MI). A,D H1 cells were passaged on Primaria™ planar substrate (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ) plates with 10 minutes of TrypLE™ Express (Invitrogen, CA) treatment prior to transferring onto micro-carriers. A,E H1 cells were passaged on Primaria™ planar substrates (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ) plates with 10 minutes of Accutase™ treatment prior to transferring onto micro-carriers. C,F H1 cells at passage 46 were passaged on MATIRGEL (BD Biosciences, CA) coated plates with collagenase (1mg/ml) prior to transferring onto micro-carriers.

Figure 45: Pluripotentency of H1 cells grown on Primaria™ planar substrates (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ) then transferred to Cytodex 3® (GE Healthcare Life Sciences, NJ) and HILLEX®II micro-carriers. FACS analysis shows expression of pluripotent cell-surface proteins. Cells were treated with Accutase™ or TrypLE™ Express (Invitrogen, CA) for 3 to 10 minutes during passaging on Primaria™ (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ).

Figure 46: Differentiation of H1 cells propagated on Primaria™ (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ) then transferred to Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ). FACS analysis of cell surface expression of CXCR4, definitive endoderm marker. Cells were treated with Accutase™ or TrypLE™ Express (Invitrogen, CA) for 3 to 10 minutes during passaging on Primaria™ (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ).

Figure 47: FACS analysis of human embryonic stem cells cultured on planar substrates consisting of mixed cellulose esters prior to culture on micro-carriers.

Figure 48: FACS analysis of the expression of markers characteristic of the definitive endoderm lineage from human embryonic stem cells cultured on planar substrates consisting of mixed cellulose esters prior to culture and differentiation on micro-carriers.

DETAILED DESCRIPTION

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections that describe or illustrate certain features, embodiments or applications of the present invention.

Definitions

Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny cells, including self-renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate *in vitro* into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts.

Stem cells are classified by their developmental potential as: (1) totipotent, meaning able to give rise to all embryonic and extraembryonic cell types; (2) pluripotent, meaning able to give rise to all embryonic cell types; (3) multipotent, meaning able to give rise to a subset of cell lineages, but all within a particular tissue, organ, or physiological system (for example, hematopoietic stem cells (HSC) can produce progeny that include HSC (self- renewal), blood cell restricted oligopotent progenitors and all cell types and elements (e.g., platelets) that are normal components of the blood); (4) oligopotent, meaning able to give rise to a more restricted subset of cell lineages than multipotent stem cells; and (5) unipotent, meaning able to give rise to a single cell lineage (e.g. , spermatogenic stem cells).

Differentiation is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, for example, a nerve cell or a muscle cell. A differentiated or differentiation-induced cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term “committed”, when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less

differentiated cell type. De-differentiation refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, i.e., which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest.

Various terms are used to describe cells in culture. "Maintenance" refers generally to cells placed in a growth medium under conditions that facilitate cell growth and/or division that may or may not result in a larger population of the cells. "Passaging" refers to the process of removing the cells from one culture vessel and placing them in a second culture vessel under conditions that facilitate cell growth and/or division.

A specific population of cells, or a cell line, is sometimes referred to or characterized by the number of times it has been passaged. For example, a cultured cell population that has been passaged ten times may be referred to as a P10 culture. The primary culture, i.e., the first culture following the isolation of cells from tissue, is designated P0. Following the first subculture, the cells are described as a secondary culture (P1 or passage 1). After the second subculture, the cells become a tertiary culture (P2 or passage 2), and so on. It will be understood by those of skill in the art that there may be many population doublings during the period of passaging; therefore the number of population doublings of a culture is greater than the passage number. The expansion of cells (i.e., the number of population doublings) during the period between passaging depends on many factors, including but not limited to the seeding density, substrate, medium, growth conditions, and time between passaging.

"β-cell lineage" refer to cells with positive gene expression for the transcription factor PDX-1 and at least one of the following transcription factors: NGN-3, Nkx2.2, Nkx6.1, NeuroD, Isl-1, HNF-3 beta, MAFA, Pax4, or Pax6. Cells expressing markers characteristic of the β cell lineage include β cells.

"Cells expressing markers characteristic of the definitive endoderm lineage" as used herein refer to cells expressing at least one of the following markers: SOX-17, GATA-4, HNF-3 beta, GSC, Cer1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA-6, CXCR4, C-Kit, CD99, or OTX2. Cells expressing markers characteristic of the definitive endoderm lineage include primitive streak precursor cells, primitive streak cells, mesendoderm cells and definitive endoderm cells.

"Cells expressing markers characteristic of the pancreatic endoderm lineage" as used herein refer to cells expressing at least one of the following markers: PDX-1, HNF-1beta, PTF-1 alpha, HNF-6, or HB9. Cells expressing markers characteristic of the pancreatic endoderm lineage include pancreatic endoderm cells.

"Cells expressing markers characteristic of the pancreatic endocrine lineage" as used herein refer to cells expressing at least one of the following markers: NGN-3, NeuroD, Islet-1, PDX-1, NKX6.1, Pax-4, Ngn-3, or PTF-1 alpha. Cells expressing markers characteristic of the pancreatic endocrine lineage include pancreatic endocrine cells, pancreatic hormone expressing cells, and pancreatic hormone secreting cells, and cells of the β-cell lineage.

"Definitive endoderm" as used herein refers to cells which bear the characteristics of cells arising from the epiblast during gastrulation and which form the gastrointestinal tract and its derivatives. Definitive endoderm cells express the following markers: CXCR4, HNF-3 beta, GATA-4, SOX-17, Cerberus, OTX2, goosecoid, c-Kit, CD99, and Mixl1.

"Extraembryonic endoderm" as used herein refers to a population of cells expressing at least one of the following markers: SOX-7, AFP, or SPARC.

"Markers" as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells

of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

"Mesendoderm cell" as used herein refers to a cell expressing at least one of the following markers: CD48, eomesodermin (EOMES), SOX-17, DKK4, HNF-3 beta, GSC, FGF17, or GATA-6.

"Pancreatic endocrine cell" or "pancreatic hormone expressing cell" as used herein refers to a cell capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.

"Pancreatic hormone secreting cell" as used herein refers to a cell capable of secreting at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.

"Pre-primitive streak cell" as used herein refers to a cell expressing at least one of the following markers: Nodal, or FGF8.

"Primitive streak cell" as used herein refers to a cell expressing at least one of the following markers: Brachyury, Mix-like homeobox protein, or FGF4.

Micro-Carriers

"Micro-carriers" refers to particles, beads, or pellets useful for attachment and growth of anchorage dependent cells in culture. The micro-carriers have the following properties: (a) They are small enough to allow them to be used in suspension cultures (with a stirring rate that does not cause significant shear damage to the micro-carriers or the cells); (b) They are solid, or have a solid core with a porous coating on the surface; and (c) Their surfaces (exterior and interior surface in case of porous carriers) may be positively or negatively charged. In one aspect, the micro-carriers have an overall particle diameter between about 150 and 350 μm, and have a positive charge density of between about 0.8 and 2.0 meq/g. Useful micro-carriers include, without limitation, Cytodex 1®, Cytodex 2®, or Cytodex 3® (GE Healthcare Life Sciences, NJ).

In another aspect, the micro-carrier is a solid carrier. Solid carriers are particularly suitable for adhesion cells, e.g., anchorage-dependent cells. The carrier particle can also be a porous micro-carrier.

"Porous micro-carriers" refers to particles useful for attachment and growth of anchorage-dependent cells in culture. The porous micro-carriers have the following properties: (a) they are small enough to allow them to be used in suspension cultures (with a stirring rate that does not cause significant shear damage to the micro-carriers or the cells); (b) they have pores and interior spaces of sufficient size to allow cells to migrate into the interior spaces of the particle and (c) their surfaces (exterior and interior) may be positively or negatively charged. In one series of embodiments, the carriers (a) have an overall particle diameter between about 150 and 350 μm; (b) have pores having an average pore opening diameter of between about 15 and about 40 μm; and (c) have a positive charge density of between about 0.8 and 2.0 meq/g. In some embodiments, the positive charge is provided by DEAE (N,N,-diethylaminoethyl) groups. Useful porous micro-carriers include, without limitation, Cytopore 1® and Cytopore 2® (GE Healthcare Life Sciences, Piscataway N.J.). Micro-carriers may be any shape, but are typically roughly spherical in shape, and can be either macro- or micro-porous, or solid.

Both porous and solid types of micro-particulate carriers are commercially available from suppliers. Examples of commercially available micro-carriers include Cytodex 1® and Cytodex 3® (GE Healthcare Life Sciences, NJ), which are both dextran-based micro-carriers from GE Healthcare Life Sciences. Porous micro-carriers on the market include Cytoline as well as Cytopore products also from GE Healthcare Life Sciences. Biosilon (NUNC) and Cultispher (Percell Biolytica) are also commercially available. In a further aspect, the micro-carriers can be comprised of, or coated with polycarbonate or mixed cellulose esters.

Micro-carriers suitable for use in the present invention can be comprised of natural or synthetically-derived materials. Examples include collagen-based micro-carriers, dextran-based micro-carriers, or cellulose-based micro-carriers, as well as glass, ceramics, polymers, or metals. The micro-carrier can be protein-free or protein-coated,

for example, with collagen. In a further aspect the micro-carrier can be comprised of, or coated with, compounds that enhance binding of the cell to the micro-carrier and enhance release of the cell from the micro-carrier including, but not limited to, poly(monostearoylglyceride co-succinic acid), poly-D,L-lactide-co-glycolide, sodium hyaluronate, collagen, fibronectin, laminin, elastin, lysine, n-isopropyl acrylamide, vitronectin.

Micro-Carriers for Cell Culture

Micro-carrier culture is a technique, which makes possible the practical high yield culture of anchorage-dependent, cells, for example, human embryonic stem cells. Micro-carriers have been specifically developed for the culture of cells, such as human embryonic stem cells, in culture volumes ranging from a few milliliters to greater than one thousand liters. The micro-carrier is biologically inert and provides a strong but non-rigid substrate for stirred micro-carrier cultures. The micro-carriers may be transparent, allowing microscopic examination of the attached cells. Cytodex 3® (GE Healthcare Life Sciences, NJ) consists of a thin layer of denatured collagen chemically coupled to a matrix of crosslinked dextran. The denatured collagen layer on Cytodex 3® (GE Healthcare Life Sciences, NJ) is susceptible to digestion by a variety of proteases, including trypsin and collagenase, and provides the ability to remove cells from the micro-carriers while maintaining maximum cell viability, function, and integrity.

Protein free micro-carriers can be used to culture human embryonic stem cells. For example, micro-carriers for use in manufacturing and laboratory or research use sold under the tradename HILLEX® (SoloHill Engineering, Inc., MI.) are modified polystyrene beads with cationic trimethyl ammonium attached to the surface to provide a positively charged surface to the micro-carrier. The bead diameter ranges from about 90 to about 200 microns in diameter.

Micro-carrier-based methods of cell culture provided many advantages including ease of downstream processing in many applications. Micro-carriers are typically roughly spherical in shape, and can be either porous or solid. The use of micro-carriers for cell attachment facilitates the use of stirred tank and related reactors for growth of anchorage-

dependent cells. The cells attach to the readily suspended micro-carriers. The requirement for suspendability limits the physical parameters of the micro-carriers. Thus, micro-carriers commonly have a mean diameter in the range of 50-2000 microns. In some applications solid-type micro-carriers range from about 100 to about 250 microns whereas porous-type micro-carriers range from about 250 to about 2500 microns. These size ranges allow for selection of micro-carriers, which are large enough to accommodate many anchorage-dependent cells, while small enough to form suspensions with properties suitable for use in stirred reactors.

Among the factors considered in using micro carriers and the like are: attachment efficiency, immunogenicity, biocompatibility, ability to biodegrade, time to reach confluence, the growth parameters of attached cells including maximum attainable density per unit surface area, detachment techniques where required, and the efficiency of the detachment, scalability of the culture conditions as well as homogeneity of the culture under scaled-up conditions, the ability to successfully scale-up detachment procedures, and whether the micro-carriers will be used for implantation. These considerations can be influenced by the surface properties of the micro-carrier, as well as by the porosity, diameter, density, and handling properties of the micro-carrier.

For example, the density of the micro-carriers is a consideration. Excessive density may cause the micro-carriers to settle out of the suspension, or tend to remain completely towards the bottom of the culture vessel, and thus may result in poor bulk mixing of the cells, culture medium and gaseous phases in the reactor. On the other hand, a density that is too low may result in excessive floating of the micro-carrier. A density of 1.02 to 1.15 g/cm^3 is typical of many micro-carriers.

The small diameter of micro-carriers and the volume of particles that can be added to a reactor allows the micro-carriers to contribute substantial surface area in vast excess to that found in roller bottles or other methods of growing anchorage-dependent cells, e.g. on plates. Porous micro-carriers provide even greater surface area per unit volume or weight. These porous micro-carriers possess large cavities that are available for the growth of anchorage-dependent cells. These cavities increase the surface area greatly,

and may protect cells from detrimental mechanical effects, such as shear stress, for example from mixing or from gas sparging.

The micro-carrier surface may be textured to enhance cell attachment and proliferation. The micro-carrier surface texture be achieved by techniques including, but not limited to, molding, casting, leeching and etching. The resolution of the features of the textured surface may be on the nanoscale. The textured surface may be used to induce a specific cell alignment on the micro-carrier surface. The surface of the pores within the porous micro-carriers may also be textured to enhance cell attachment and proliferation. Pore surface texture be achieved by techniques such as but not limited to molding, casting, leeching and etching.

The micro-carrier surface may be plasma-coated to impart a specific charge to micro-carrier surfaces. These charges may enhance cell attachment and proliferation.

In other embodiments, the micro-carriers are composed of, or coated with, thermoresponsive polymers such as poly-N-isopropylacrylamide, or have electromechanical properties.

Both porous and solid types of microparticulate carriers are commercially available from suppliers. Examples of commercially available solid micro-carriers include Cytodex 1® and Cytodex 3® (GE Healthcare Life Sciences, NJ), which are both dextran-based micro-carriers from GE Healthcare Life Sciences. Porous micro-carriers on the market include Cytoline as well as Cytopore products also from GE Healthcare Life Sciences. Biosilon (NUNC) and Cultispher (Percell Biolytica) are also commercially available.

The micro-carriers may also contain a bioactive agent. The micro-carrier may also contain a bioactive agent that may regulate the growth or function of cells or the tissue milieu these factors may include but are not limited to fibroblast growth factors, erythropoietin, vascular endothelial cell growth factors, platelet derived growth factors, bone morphogenic proteins, transforming growth factors, tumor necrosis factors, epidermal growth factors, insulin-like growth factors. Complete factors, mimetics or active fragments thereof may be used.

The micro-carriers may be inoculated with a second cell type and co-cultured with the pluripotent stem cells. In one embodiment the two (or more) cell types may be adherent to an individual micro-carrier in equal or un-equal proportions. The two or more cell types can be inoculated onto the micro-carrier at the same time point or they may be inoculated at different times. The micro-carriers can be treated in such a manner to preferentially adhere specific cell types onto specific regions of the micro-carrier. In a further embodiment, the micro-carrier with adherent single or multiple cell types can be co-cultured in a culture vessel with a second cell type cultured in suspension.

Second cell types may include, for example, epithelial cells (e.g., cells of oral mucosa, gastrointestinal tract, nasal epithelium, respiratory tract epithelium, vaginal epithelium, corneal epithelium), bone marrow cells, adipocytes, stem cells, keratinocytes, melanocytes, dermal fibroblasts, keratinocytes, vascular endothelial cells (e.g., aortic endothelial cells, coronary artery endothelial cells, pulmonary artery endothelial cells, iliac artery endothelial cells, microvascular endothelial cells, umbilical artery endothelial cells, umbilical vein endothelial cells, and endothelial progenitors (e.g., CD34+, CD34+/CD117+ cells)), myoblasts, myocytes, hepatocytes, smooth muscle cells, striated muscle cells, stromal cells, and other soft tissue cells or progenitor cells, chondrocytes, osteoblasts, islet cells, nerve cells including but not limited to neurons, astrocytes, Schwann cells, enteric glial cells, oligodendrocytes.

Pluripotent Stem Cells

Characterization of Pluripotent Stem Cells

Pluripotent stem cells may express one or more of the stage-specific embryonic antigens (SSEA) 3 and 4, and markers detectable using antibodies designated Tra-1-60 and Tra-1-81 (Thomson *et al.*, Science 282:1145, 1998). Differentiation of pluripotent stem cells in vitro results in the loss of SSEA-4, Tra- 1-60, and Tra-1-81 expression (if present) and increased expression of SSEA-1. Undifferentiated pluripotent stem cells typically have alkaline phosphatase activity, which can be detected by fixing the cells with 4% paraformaldehyde and then developing with Vector Red as a substrate, as described by

the manufacturer (Vector Laboratories, Burlingame Calif.) Undifferentiated pluripotent stem cells also typically express OCT4 and TERT, as detected by RT-PCR.

Another desirable phenotype of propagated pluripotent stem cells is a potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Pluripotency of stem cells can be confirmed, for example, by injecting cells into severe combined immunodeficient (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining them histologically for evidence of cell types from the three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers.

Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published karyotypes of the corresponding primate species. It is desirable to obtain cells that have a "normal karyotype," which means that the cells are euploid, wherein all human chromosomes are present and not noticeably altered.

Sources of Pluripotent Stem Cells

The types of pluripotent stem cells that may be used include established lines of pluripotent cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10-12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, GA). Also suitable are pluripotent stem cells derived from non-pluripotent cells, such as, for example, an adult somatic cells.

Attaching Pluripotent Stem Cells to the Micro-Carriers Suitable for Use in the Present Invention

Pluripotent stem cells may be cultured on a planar substrate by any method in the art, prior to attaching to micro-carriers. For example, pluripotent stem cells may be cultured on planar substrates, treated with an extracellular matrix protein (e.g. MATRIGEL). Alternatively, pluripotent stem cells may be cultured on planar substrates seeded with a feeder cell layer.

In one embodiment, the pluripotent stem cells are embryonic stem cells. In an alternate embodiment, the embryonic stem cells are human.

In one aspect of the present invention, pluripotent stem cells are released from a planar substrate by treating the pluripotent stem cells with a protease that will release the cells from the planar substrate. The protease may be, for example, collagenase, TrypLE™ Express , Accutase™, trypsin, and the like.

In one embodiment, the pluripotent stem cells are released from the micro-carrier substrate by treating the cells with Accutase™ for about five to about ten minutes.

In one embodiment, the pluripotent stem cells are released from the micro-carrier substrate by treating the cells with 0.05% trypsin/EDTA for about ten to about twenty minutes.

In one embodiment, the pluripotent stem cells are released from the micro-carrier substrate by treating the cells with TrypLE™ Express for about five to about twenty minutes.

In one embodiment, the pluripotent stem cells are released from the micro-carrier substrate by treating the cells with 10 mg/ml Collagenase for about five to about ten minutes.

The released pluripotent cells are added to medium containing micro-carriers at a specific density. In one embodiment, the pluripotent stem cells were seeded at about 4,000 to about 30,000 cells per cm^2 of micro-carriers.

The released pluripotent cells are added to medium containing micro-carriers. In one embodiment, the attachment of the pluripotent stem cells is enhanced by treating the pluripotent stem cells with a Rho kinase inhibitor. The Rho kinase inhibitor may be Y27632 (Sigma-Aldrich, MO). Alternatively, the Rho kinase inhibitor is Glycyl-H 1152 dihydrochloride.

In one embodiment, the pluripotent stem cells are treated with Y27632 at a concentration from about 1 μM to about 10 μM. In one embodiment, the pluripotent stem cells are treated with Y27632 at a concentration of about 10 μM.

In one embodiment, the pluripotent stem cells are treated with Glycyl-H 1152 dihydrochloride at a concentration from about 0.25 μM to about 5 μM. In one embodiment, the pluripotent stem cells are treated with Glycyl-H 1152 dihydrochloride at a concentration of about 2.5 μM.

The medium containing the micro-carriers may be agitated. Agitation as used in the present invention may be the movement of the culture medium. Such agitation may be achieved manually, or, alternatively, by use of apparatus, such as, for example, a rocking platform, a spinner flask, and the like. In one embodiment, the medium containing the micro-carriers is agitated by the use of manual movement. The dish containing the micro-carriers and cells is moved back and forth for less than 30 seconds.

The medium containing the micro-carriers may be agitated. In one embodiment, the medium containing the micro-carriers is agitated by the use of a spinner flask. The spinner flask (Corning, Lowell, MA) is placed on a stir plate at 30-70 RPM depending on bead type.

In an alternate embodiment, the medium containing the micro-carriers is agitated by the use of a rocking platform (Vari-mix, Barnstead, Dubuque, Iowa). The rocking platform speed is about one rotation in 2 seconds.

Differentiating Pluripotent Stem Cells on Micro-Carriers

In one embodiment, the pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage on micro-carriers. Alternatively, the pluripotent stem cells may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage on micro-carriers. Alternatively, the pluripotent stem cells may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage on micro-carriers.

In an alternate embodiment, the pluripotent stem cells may be propagated on micro-carriers, then differentiated into cells expressing markers characteristic of the definitive endoderm lineage on planar surfaces. Alternatively, the pluripotent stem cells may be propagated on micro-carriers, then differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage on planar surfaces. Alternatively, the pluripotent stem cells may be propagated on micro-carriers, then differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage on planar surfaces.

Pluripotent stem cells treated in accordance with the methods of the present invention may be differentiated into a variety of other cell types by any suitable method in the art. For example, pluripotent stem cells treated in accordance with the methods of the present invention may be differentiated into neural cells, cardiac cells, hepatocytes, and the like.

For example, pluripotent stem cells treated in accordance with the methods of the present invention may be differentiated into neural progenitors and cardiomyocytes according to the methods disclosed in WO2007030870.

In another example, pluripotent stem cells treated in accordance with the methods of the present invention may be differentiated into hepatocytes according to the methods disclosed in US patent 6,458,589.

Formation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

Pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by any method in the art.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 23, 1534 - 1541 (2005).

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in Shinozaki *et al*, Development 131, 1651 - 1662 (2004).

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in McLean *et al*, Stem Cells 25, 29 - 38 (2007).

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 24, 1392 - 1401 (2006).

Markers characteristic of the definitive endoderm lineage are selected from the group consisting of SOX17, GATA4, HNF-3 beta, GSC, CER1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4, CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, CXCR4, C-Kit, CD99, and OTX2. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the definitive endoderm lineage. In one aspect of the present invention, a cell expressing markers characteristic of the definitive endoderm lineage is a primitive streak precursor cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a mesendoderm cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a definitive endoderm cell.

In another example, pluripotent stem cells treated according to the methods of the present invention may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A in the absence of serum, then culturing the cells with activin A and serum, and then culturing the cells with activin A and serum of a different concentration. An example of this method is disclosed in Nature Biotechnology 23, 1534 - 1541 (2005).

In another example, pluripotent stem cells treated according to the methods of the present invention may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A in the absence of serum, then culturing the cells with activin A with serum of another concentration. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2005.

In another example, pluripotent stem cells treated according to the methods of the present invention may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A and a Wnt ligand in the absence of serum, then removing the Wnt ligand and culturing the cells with activin A with serum. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).

In another example, pluripotent stem cells treated according to the methods of the present invention may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

In another example, pluripotent stem cells treated according to the methods of the present invention may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

Formation of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage

Pluripotent stem cells may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage by any method in the art.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 24, 1392 - 1401 (2006).

For example, cells expressing markers characteristic of the definitive endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with a fibroblast growth factor and the hedgehog signaling pathway inhibitor KAAD-cyclopamine, then removing the medium containing the fibroblast growth factor and KAAD-cyclopamine and subsequently culturing the cells in medium containing retinoic acid, a fibroblast growth factor and KAAD-cyclopamine. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).

For example, cells expressing markers characteristic of the definitive endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with retinoic acid one fibroblast growth factor for a period of time, according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

For example, cells expressing markers characteristic of the definitive endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with retinoic acid (Sigma-Aldrich, MO) and exendin 4, then removing the medium containing DAPT (Sigma-Aldrich, MO) and exendin 4 and subsequently culturing the cells in medium containing exendin 1, IGF-1 and HGF. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).

For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing exendin 4, then removing the medium containing exendin 4 and subsequently

culturing the cells in medium containing exendin 1, IGF-1 and HGF. An example of this method is disclosed in D' Amour *et a*l, Nature Biotechnology, 2006.

For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing DAPT (Sigma-Aldrich, MO) and exendin 4. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing exendin 4. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into

cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

Markers characteristic of the pancreatic endocrine lineage are selected from the group consisting of NGN3, NEUROD, ISL1, PDX1, NKX6.1, PAX4, NGN3, and PTF-1 alpha. In one embodiment, a pancreatic endocrine cell is capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endocrine lineage. In one aspect of the present invention, a cell expressing markers characteristic of the pancreatic endocrine lineage is a pancreatic endocrine cell. The pancreatic endocrine cell may be a pancreatic hormone-expressing cell. Alternatively, the pancreatic endocrine cell may be a pancreatic hormone-secreting cell.

In one aspect of the present invention, the pancreatic endocrine cell is a cell expressing markers characteristic of the β cell lineage. A cell expressing markers characteristic of the β cell lineage expresses PDX1 and at least one of the following transcription factors: NGN3, NKX2.2, NKX6.1, NEUROD, ISL1, HNF-3 beta, MAFA, PAX4, or PAX6. In one aspect of the present invention, a cell expressing markers characteristic of the β cell lineage is a β cell.

The present invention is further illustrated, but not limited by, the following examples.

EXAMPLES

Example 1: Attachment and Proliferation of Human Embryonic Stem Cells on Micro-Carriers

To determine if human embryonic stem cells can attach and proliferate on micro-carriers, H9 cells passage 52 were released from MATRIGEL™ (BD Biosciences, CA) coated plates with TrypLE™ Express. They were then incubated with micro-carriers and MEF-CM. Suspensions of ProNectinF (PN), Plastic (P), PlasticPlus (PP), HILLEX®II (H), collagen (Col) and FACT III (SoloHill, MI) micro-carriers were prepared according to manufacturer's instructions. After 2 days at 37°C, **Table 1** describes the attachment and growth of the H9 cells on the micro-carriers based on daily images. Few cells attached and/or proliferated on most micro-carriers tested. H9 cells did attach and proliferate on HILLEX®II micro-carriers (Solohill, MI) but images showed fewer cell-bead aggregates after 2 days in static culture (**Figure 1B**).

To improve the attachment and proliferation of human embryonic stem cells on micro-carriers, a small molecule inhibitor of Rho-associated coiled coil forming protein serine/threonine kinase, Rho kinase inhibitor was added to the media. Specifically, Y27632, Y, (Sigma-Aldrich, MO) was used. MEF-CM plus 10 μM Y27632 (Sigma-Aldrich, MO) was changed daily. In the presence of 10 μM Y27632 (Sigma-Aldrich, MO) the H9 cells attached and formed aggregates with all micro-carriers tested (**Table 2**). By analysis of images, human embryonic stem cells grown on HILLEX®II micro-carriers (Solohill, MI) appeared to attach and proliferate better than human embryonic stem cells on other micro-carriers tested. Additionally, H9 cells attached better to HILLEX®II (Solohill, MI) in the presence of the Rho kinase inhibitor (**Figure 1A** compared to **1B**).

Expansion of human embryonic stem cells for a cell therapy application is necessary to meet product demand. Currently the best techniques for expansion include spinner flasks

and bioreactors. Both of these techniques require physical movement of the micro-carriers in suspension. To determine the effect of motion on the growth of the human embryonic stem cells on micro-carriers, 6 or 12 well dishes were placed on a rocking platform in a 37°C incubator. After growth for 3 days, the cell aggregates began to release from some of the micro-carriers. **Figure 2 A, B, D** illustrates that the cell aggregates disassociated from the Plastic Plus, Plastic, or Pronectin micro-carriers. In contrast, the cells remained attached to the HILLEX® II micro-carriers (Solohill, MI) and proliferated **Figure 2C**. **Example 4** describes the dissociation method used prior to cell counting in a Guava PCA-96 with Viacount Flex (Guava Technologies, Hayward, CA). Measuring the growth rate of the cells on micro-carriers reveals a dip in cell number at day 3 compared to the starting number at seeding. This is likely due to poor initial attachment of the cells to the micro-carriers followed by an expansion afterwards until the experiment was terminated at day 5. H9 cells on HILLEX®II micro-carriers (Solohill, MI) have the highest proliferation rate compared to the other bead types, likely due to better attachment of the cells to the HILLEX® II micro-carriers (Solohill, MI) (**Figures 2, 3**). This demonstrates that the HILLEX® II micro-carriers (Solohill, MI) can support growth of H9 cells in suspension. This was further validated after repeat passaging, see **Example 5**.

The H1 human embryonic cell line was also tested for growth on micro-carriers for large-scale expansion. Because a Rho kinase inhibitor, Y27632 (Sigma-Aldrich, MO), was necessary for attachment of the H9 cell line, it was also assumed to be necessary for H1 cells. Cytodex 1®, Cytodex 3® (GE Healthcare Life Sciences, NJ), HILLEX®II, Plastic, ProNectinF, Plastic Plus micro-carriers (SoloHill Ann Arbor, MI) were prepared according to the manufacturer's instructions. The H1 human embryonic stem cells at passage 47 were seeded at about 13,333 cells/cm^2 of micro-carriers in MEF-CM plus 10μM Y27632 (Sigma-Aldrich, MO). Cells and micro-carriers were placed in a 12 well non-tissue culture treated dish at 15cm^2 per 12 well on a rocking platform at 37°C to allow movement of the micro-carriers and medium. After 3, 5 and 7 days, one well was imaged, harvested, and counted. The ability of cells to attach depended on the bead type. Similar results were observed with the H1 line as with the H9 line. Specifically, cells

seeded onto Plastic, Plastic Plus or ProNectinF micro-carriers did not attach and/or proliferate well (**Figure 4**). Cells seeded onto HILLEX®II (Solohill, MI), Cytodex 1®, or Cytodex 3® (GE Healthcare Life Sciences, NJ) micro-carriers attached and proliferated well (**Figure 5**). Cells were detached according to **Example 4** and counted for yield. Cells grown on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) exhibited the highest cell number after 7 days in culture (**Figure 6**).

Example 2: Optimal Concentrations of Y27632 and Other Rho Kinase Inhibitors for Cell Attachment and Growth

To determine the concentration of Rho kinase inhibitor that best supports attachment and growth of the human embryonic stem cells on micro-carriers, the following experiments were conducted.

A starting aliquot of 13,333 cells/cm^2 H9 cells at passage 44 was seeded onto 15cm^2 of micro-carriers in a single well of a 12 well non-tissue culture treated plate. The cells were placed at 37°C for at least 60 minutes before placing them onto a rocking platform at 37°C. Prior to adding the cells, HILLEX®II (Solohill, MI) and Cytodex 3® (GE Healthcare Life Sciences, NJ) micro-carriers were prepared as directed by the manufacturer. Cells were grown in MEF-CM plus a range of Rho kinase inhibitor concentrations, Y27632 at 10, 5, 2.5 or 1 μM, or (S)-(+)-4-Glycyl-2-methyl-1-[(4-methyl-5-isoquinolinyl) sulfonyl]-hexahydro-1H-1,4-diazepine dihydrochloride (Glycyl-H 1152 dihydrochloride (H), Tocris, MO) at 5, 2.5, 1 or 0.5 μM). The medium was changed daily and one well of cells was counted at 4 and 7 days after seeding for yield and viability (**Figure 7, A and B**). Overall, 10 or 5 μM Y27632 (Sigma-Aldrich, MO) showed the best cell proliferation (day 7) while 2.5 and 1.0 μM appeared to have the best attachment (day 4). Concentrations of 1 and 0.5 μM Glycyl-H 1152 dihydrochloride (Tocris, MO) showed the best cell proliferation (day 7) while 5 μM appeared to have the best attachment (day 4).

Next a dose titration of the Rho kinase inhibitor was attempted since it has been characterized as a promoting apoptosis. H1 cells at passage 48 were dissociated from

MATRIGEL™ (BD Biosciences, CA) coated plates with TrypLE™ Express. The cells were then seeded onto 15cm^2 of micro-carriers into a single well of a 12 well non-tissue culture treated plate. HILLEX®II (Solohill, MI), Cytodex 1®, or Cytodex 3® (GE Healthcare Life Sciences, NJ) micro-carriers were tested with decreasing amounts of Rho kinase inhibitor: 10 μM Y27632 (Sigma-Aldrich, MO) was used on day one followed by 0.5 μM on day two (Y10/5 μM); 2.5 μM Glycyl-H 1152 dihydrochloride (Tocris, MO) was used on day one followed by 0.5 μM on day two (H2.5/0.5 μM); 1 μM Glycyl-H 1152 dihydrochloride (Tocris, MO) was used on day one followed by 0.5 μM Glycyl-H 1152 dihydrochloride (Tocris, MO) on day two (H1/0.5 μM); or continuous addition of 0.25 μM Glycyl-H 1152 dihydrochloride (Tocris, MO) was applied daily in MEF-CM (H0.25 μM). H1 cells and micro-carriers were agitated every 45 minutes for 3 hours at 37°C before being placed on a rocking platform at 37°C. Cells were counted after 3, 5 and 7 days on the rocking platform at 37°C (**Figure 8**). Overall the best concentration of Glycyl-H 1152 dihydrochloride (Tocris, MO) was 1-2.5 μM on day one and 0.5 μM on day 2 followed by withdrawal of the compound. The cells exhibited similar growth rates at these concentrations of Glycyl-H 1152 dihydrochloride (Tocris, MO) compared to 10 μM Y27632 (Sigma-Aldrich, MO) for all micro-carriers tested. Maintaining the cells in 0.25 μM Glycyl-H 1152 dihydrochloride (Tocris, MO) resulted in poor cell yield. Using a minimal amount of Rho-kinase inhibitor also helps reduce costs for the process and maybe beneficial to cell proliferation. These data also show that human embryonic stem cells did not require Rho kinase inhibitor in order to remain attached to micro-carriers and to proliferate.

Example 3: Effect of Cell Density on Attachment and Growth on Micro-Carriers

Improving the seeding density is a method to reduce the total number of cells needed. To determine the proper seeding density, the number of micro-carriers per 4x objective field was counted. H1 cells were seeded at 0.4x10^4 cells/cm^2 (low), 1.2 x10^4 cells/cm^2 (mid), or 3 x10^4 cells/cm^2 (high) densities into a 10 cm plate with Cytodex 3 micro-carriers (GE Healthcare Life Sciences, NJ) in MEF-CM plus 10μM Y27632 (Sigma-Aldrich, MO). The plate was then agitated every 45 minutes for 6 hours at 37°C. The cells and micro-

carriers were transferred to a spinner flask (described in **Example 5**) at 37°C at 30 rpm in 50ml MEF-CM plus 10 μM Y27632 (Sigma-Aldrich, MO). After 24 hours 25ml of MEF-CM with 5 μM Y27632 (Sigma-Aldrich, MO) was added. After 24 hours the speed of rotation was increased to 40 rpm. On day 3 and 5 of culture, 50ml of 75ml was removed and replaced with MEF-CM. Images were taken of an aliquot from the spinner flasks at 6 hours, 3 days, 5 days and 7days. The percentage of micro-carriers with cells attached is stated in the lower right corner in **Figure 9** images. At 3 days post seeding, the number of micro-carriers coated with cells corresponds to the original seeding density but at days 5 and 7 the number of micro-carriers coated with cells did not increase for the lower density seeded cells. This suggests that 0.4 $x10^4$ cells/cm^2 is not a sufficient number of cells to allow incorporation of micro-carriers into the aggregates. At 3 $x10^4$cells/cm^2 the number of micro-carriers with cells attached is similar to 1.2 $x10^4$ cells/cm^2 seeded at days 5 and 7 (**Figure 9).** When looking at the cell number, it is clear that more cells are attached to micro-carriers from high density cell seeding (**Figure 10**). Analysis of the fold change compared to the starting seeding cell number reveals a higher number of cells attached at 3 and 5 days in the high density seeded cultures (**Figure 11**). By day 7, the control and high density seeded cultures have similar fold change in cell number from their starting seeding density. From these data, we conclude that 1.2 $x10^4$ cells/ cm^2 is the minimum cell number for efficient attachment and growth of H1 cells on micro-carriers. Moving to higher seeding densities may aid in decreasing the number of days required for cell expansion.

Example 4: Dissociation of Cells from Micro-Carriers

In order to determine growth rates it was necessary to dissociate the cells from the micro-carriers. Removal of the Rho kinase inhibitor Y27632 (Sigma-Aldrich, MO) did not cause the H1 cells to dissociate from the micro-carriers (**Example 2**, **Figure 12**). H9 cells on HILLEX®II micro-carriers (Solohill, MI) were imaged at 10x and 20x magnification before dissociation of the cells from the micro-carriers (**Figure 13 A, B** respectively). Enzymatic treatment of the H9 cells on HILLEX®II micro-carriers (Solohill, MI) allowed for detachment of viable cells (**Figure 13 C, D and 14)**. The H9 cells were grown for 6 days in a 6 well dish with HILLEX®II micro-carriers (Solohill,

MI) on a rocking platform at 37°C. The cells attached to micro-carriers were placed in a 15ml conical tube and the medium was aspirated after allowing the micro-carriers to settle. The settled micro-carriers were washed three times with 4 ml PBS (without magnesium and calcium ions) allowing the micro-carriers to settle by gravity sedimentation. The PBS was aspirated and 1ml of PBS was added. The micro-carriers with cells were transferred into a single well of a 12 well non-tissue culture treated plate. The plate was allowed to rest at an angle to allow the micro-carriers to settle. The PBS was aspirated and 1 ml TrypLE™ Express (Invitrogen, CA) or 0.05% Trypsin/EDTA was added to the well. The plate was placed at 37°C on the rocking platform for 10 or 20 minutes. The plate was removed and 3ml DMEM/F12 or MEF-CM was added to the well. The medium was vigorously pipetted, releasing the cells (**Figure 13 C, D**). Observation of the micro-carriers under a microscope determined detachment of the cells from the micro-carriers. The cells were then centrifuged at 200 x g for 5 minutes. The medium was aspirated and the pellet was resuspended in 1ml DMEM/F12 or MEF-CM medium. The cells were then counted on a Guava PCA-96 (Guava Technologies, Hayward, CA) with Viacount dye. Specifically, a 200 µl volume of cells in appropriate dilution of medium, was incubated with 2 µl of Viacount for 10 minutes. The viability and cell number were determined (**Figure 14**). Both TrypLE™ Express and Trypsin/EDTA dissociated the cells effectively from micro-carriers.

Since TrypLE™ Express released the cells from the micro-carriers and is available as a GMP product, it was tested against other possible dissociation agents, specifically Collagenase and Accutase™ (Sigma-Aldrich, MO). H1 p48 cells were grown in a spinner flask (**Example 5**) for 10 days. The micro-carriers were then collected and transferred to a 50ml conical tube. The cells were washed in PBS as above and transferred to a 12 well plate. PBS was aspirated and 1ml of TrypLE™ Express, Accutase™ or Collagenase (10mg/ml) was added to the well and placed on a rocking platform at 37°C for 5 or 10 minutes. The cells/micro-carriers were vigorously resuspended in DMEM/F12, and then the dissociated cells and micro-carriers were passed through a 40µm cell strainer over a 50ml conical tube. The well was washed with an additional 2 ml medium, also added to the strainer before centrifuging at 200 x g for 5

minutes. The cells were then resuspended in 1ml DMEM/F12 and diluted for cell counting, as above. Cell viability was similar with all tested enzymes. Accutase™ and TrypLE™ Express released similar cell numbers over 5 and 10 minute incubations (**Figure 15**). This illustrates the suitability of Accutase™ and TrypLE™ Express as cell dissociation regents for human embryonic stem cells on micro-carriers.

Example 5: Propagation of Undifferentiated Pluripotent Stem Cells on Micro-Carriers

In order to expand cells on micro-carriers, cells must be able to detach or be enzymatically dissociated from the micro-carriers and reattach to new micro-carriers. Typical methods of cell propagation on micro-carriers rely on the property of cells to detach and reattach. The following experiment showed that this was not a characteristic of human embryonic stem cells. Specifically, H9 p43 cells were seeded onto HILLEX®II micro-carriers (Solohill, MI) and incubated in a 125ml spinner flask (see below). Phenol red present in the medium and was taken up by the HILLEX®II micro-carriers (Solohill, MI). After 8 days of growth, a 10ml aliquot of the cells on micro-carriers was placed in a new spinner flask containing phenol red-free MEF-CM, 440mg of HILLEX®II micro-carriers, and 5μM Y27632 (Sigma-Aldrich, MO). After 5 days incubation at 37°C with 30rpm rotation, the micro-carriers were removed and images were acquired (**Figure 16**). The dark micro-carriers shown are the micro-carriers covered with H9 cells grown in medium containing phenol red. The light micro-carriers are the newly added micro-carriers. It was expected that the H9 cells would detach and reattach to new micro-carriers, however, instead the cells formed aggregates with the new micro-carriers. No light micro-carriers had cells attached that are not also in aggregates with the dark micro-carriers, suggesting that the cells were not able to detach and reattach to micro-carriers. In order to propagate cells grown on micro-carriers, the cells must be enzymatically dissociated from the micro-carriers (see **Example 4**).

Since it is now established how human embryonic stem cells can be propagated on micro-carriers it needs to be determined how human embryonic stem cells propagate in larger scale spinner flasks. Spinner flasks allow the expansion of cells in high-density systems.

This is space conserving and is considered the first step to expanding cells in bioreactors. To test the ability of human embryonic stem cells to proliferate in spinner flasks, H9 passage 43 cells were seeded into 125ml spinner flasks. Cells were initially attached to the micro-carriers in a 10cm plate before transferring to the spinner flask. Specifically, H9 cells were released from the two six-well dishes by a five minute incubation with TrypLE™ Express at 37°C. Prior to passaging with TrypLE™ Express the cells had been passaged with Collagenase (1mg/ml) and seeded onto 1:30 Growth Factor Reduced MATRIGEL™ (BD Biosciences, CA) coated plates. The cells were resuspended in DMEM/F12 and counted on a Guava instrument with Viacount. After centrifugation, $3x10^6$ cells were seeded into a 10cm plate containing MEF-CM plus 10 μM Y27632 (Sigma-Aldrich, MO) and 250 cm^2 of HILLEX® II micro-carriers (Solohill, MI), prepared according to the manufacturer's instructions. The dish was placed at 37°C and gently rotated and agitated once every 45 minutes for 4.5 hours. Then the cells, micro-carriers and medium were transferred to a 125ml spinner flask. The spinner flask was then filled to 50ml with MEF-CM plus 10 μM Y27632 (Sigma-Aldrich, MO) and placed on a stir plate at 37°C at 40rpm. The following day the medium was changed and filled to 75ml with MEF-CM plus 5μM Y27632 (Sigma-Aldrich, MO). The rate of stirring was increased to 70rpm. Medium was changed every other day without addition of Y27632 compound (Sigma-Aldrich, MO). The cells were passaged according to the methods disclosed in **Example 4,** and $3x10^6$ cells were reseeded onto 250cm^2 of new micro-carriers. The cultures were passaged when they reached a confluency of $1\text{-}2x10^5$ cells/cm^2. This was conducted for 5 passages (**Figure 17**). At each passage pluripotent marker expression was evaluated showing 80-95% of cells expressed the pluripotency markers CD9, SSEA4, SSEA3, TRA-1-60 and TRA-1-81 (**Figure 19A**). A similar experiment was conducted with H9 p43 cells on Cytodex 3® micro-carriers ((GE Healthcare Life Sciences, NJ), **Figure 18, 19B**). Overall, the cells proliferated well on both HILLEX®II (Solohill, MI) and Cytodex 3® (GE Healthcare Life Sciences, NJ) micro-carriers and remained pluripotent. Karyotypic analysis was conducted after 5 passages in spinner flasks and showed an abnormal trisomy in chromosome 12 in 1.5% of the cells. Since these cells were nearing passage 50 at the conclusion of the experiment,

it may be a common occurrence to observe such abnormalities. Beginning with a lower cell passage number may allow this premise to be tested.

Similar experiments were conducted with the H1 line at p48 and p49. All parameters remained the same except the rotation speed and seeding density. The rotation speed for the spinner flask was 30 rpm over-night on day 1 and increased to 40 rpm for all additional days. The seeding density for Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) was about 11,000 cells/cm^2 while the seeding density for Cytodex 1® micro-carriers (GE Healthcare Life Sciences, NJ) was about 7,000 cells/ cm^2. The cell number seeded was held constant at $3x10^6$ cells per spinner flask. The weight of micro-carriers was held constant at 100mg for Cytodex 1® and Cytodex 3®. One advantage of Cytodex 1® and Cytodex 3® over HILLEX®II micro-carriers is their larger surface area. **Figures 20** and **21** show the expansion of H1 cells on Cytodex 1® and Cytodex 3® respectively. The cells remained pluripotent over the five passages (**Figure 22**). Karyotype analysis of H1 cells on Cytodex 3® micro-carriers revealed duplication of the Y chromosome in 10% of the cells tested. These H1 cells were passaged onto micro-carriers at p48 and were analyzed 5 passages later. H1p55 cells grown on MATRIGEL™ (BD Biosciences, CA) on a planar surface had a normal karyotype. Analysis of the doubling rates for these cells between day 3 and the day of passaging (day 5, 6 or7) showed no overall change in doubling times (**Figure 23**). H1 cells grown on Cytodex 1® micro-carriers and H9 grown on HILLEX®II micro-carriers (Solohill, MI) showed the most consistent doubling times (**Table 3**).

Example 6: Proliferation of Human Embryonic Stem Cells on Micro-Carriers in Defined Medium

To manufacture a therapeutic product, it is desirable to remove any animal components from the human embryonic stem cell culture medium. Currently human embryonic stem cells are maintained on MATRIGEL™ (BD Biosciences, CA) in medium conditioned using mouse embryonic fibroblasts (MEF-CM). Both MATRIGEL™ (BD Biosciences, CA) and MEF-CM are derived from mouse cells. Additionally, MEF-CM is an expensive and time-consuming medium to generate. To determine if human embryonic

stem cells can be sustained on micro-carriers with defined medium, H9 cells were seeded onto Cytodex 3® (GE Healthcare Life Sciences, NJ) and HILLEX®II (Solohill, MI) micro-carriers in the presence of Rho kinase inhibitors, 10μM Y27632 (Sigma-Aldrich, MO) or 2.5 μM Glycyl-H 1152 dihydrochloride (Tocris, MO) in Stem Pro (Invitrogen, CA), mTESR (StemCell Technologies, Vancouver, Canada) or MEF-CM. The cells were placed in a 12 well dish on a rocking platform at 37°C. The cells were counted at days 3, 5 and 7. H9 p39 cells grown in MEF-CM on both bead types showed typical expansion characteristics (**Figure 24**). Similar cells grown in mTESR (StemCell Technologies, Vancouver, Canada) proliferated well on Cytodex 3® micro-carriers in the presence of 10 μM Y27632 (Sigma-Aldrich, MO) but exhibited a slow growth rate on HILLEX®II micro-carriers. Cells of the human embryonic stem cell line H9 at passage 64 (H9p64) cells that had been acclimated to StemPro medium for over 20 passages proliferated well on both HILLEX®II and Cytodex 3® in the presence of 10 μM Y27632 (Sigma-Aldrich, MO). Surprisingly, these cells did not proliferate well in the presence of 2.5 μM Glycyl-H 1152 dihydrochloride (Tocris, MO) on Cytodex 3® micro-carriers. Therefore the micro-carrier type, Rho kinase inhibitor, and medium all play a role in determining the ability of human embryonic stem cells to proliferate.

H1 human embryonic stem cells at passage 38 were seeded onto either Cytodex 3® or HILLEX®II micro-carriers in the presence of Rho kinase inhibitors, 10μM Y27632 (Sigma-Aldrich, MO) or 2.5μM Glycyl-H 1152 dihydrochloride in mTESR (StemCell Technologies, Vancouver, Canada) or MEF-CM in a 12 well dish. The cells were placed on a rocking platform at 37°C. The cells were counted at days 3, 5 and 7. Cells grown in MEF-CM on both micro-carrier types showed typical expansion characteristics in the presence of Y27632 (Sigma-Aldrich, MO) but exhibited poor growth with Glycyl-H 1152 dihydrochloride (Tocris, MO) on Cytodex 3® (**Figure 25**). mTESR medium (StemCell Technologies, Vancouver, Canada) allowed the H1 cells to proliferate on HILLEX®II micro-carriers in the presence of both Rho kinase inhibitors but exhibited low growth rate on Cytodex 3® micro-carriers.

Given that H1 p50 cells proliferated well in mTESR (StemCell Technologies, Vancouver, Canada) on HILLEX®II micro-carriers, $3x10^6$ cells were seeded onto 250 cm^2 HILLEX®II micro-carriers. Cells were incubated at 37°C in a 10 cm^2 dish for 5 hours with agitation by hand every 45 minutes. mTESR (StemCell Technologies, Vancouver, Canada) plus 10μM Y27632 (Sigma-Aldrich, MO) was changed every other day. This was conducted in parallel with cells grown in MEF-CM (**Example 5**). Unlike cells grown in MEF-CM, the cells grown in mTESR medium (StemCell Technologies, Vancouver, Canada) began to detach from the HILLEX®II micro-carriers after 7 days (**Figure 26A vs. 26B**). This indicates that additional supplements needed to be added to mTESR (StemCell Technologies, Vancouver, Canada) in order for the human embryonic stem cells to remain attached and proliferate on HILLEX®II micro-carriers (Solohill, MI).

Example 7: Differentiation of Human Embryonic Stem Cells on Micro-Carriers

Since the human embryonic stem cells can be expanded on micro-carriers, the differentiation potential of these cells must be determined. Cells of the human embryonic stem cell line H9 at passage 43 were passaged five times on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ). At passage 5, the cells were grown for 6 days on the micro-carriers before being dissociated from the micro-carriers with TrypLE™ Express (see **Example 4**). The cells were then plated on 1:30 MATRIGEL™: DMEM/ F12 coated plates. After the cells became 80 to 90% confluent on the plates they were exposed to differentiating agents. Differentiation of the human embryonic stem cells to definitive endoderm was conducted by treating the cells for 2 days with 2% Albumin Bovine Fraction V Fatty Acid Free (FAF BSA, MP Biomedicals, Ohio) in RPMI plus 100ng/ml Activin A (PeproTech, NJ), 20ng/ml Wnt3a (R&D Biosciences, MN) and 8ng/ml bFGF (PeproTech, NJ). The cells were treated for an additional 2 days in 2% FAF BSA in RPMI plus 100ng/ml Activin A (PeproTech, NJ) and 8ng/ml bFGF (PeproTech, NJ). Medium was changed daily. FACS analysis conducted for the definitive endoderm cell surface marker CXCR4, showed that 87% of the cells expressed the protein (**Figure 27A**). A similar experiment was conducted with cells of the human embryonic stem cell line H1 at passage 49 grown on Cytodex 1® micro-carriers (GE

Healthcare Life Sciences, NJ) for 5 passages, revealing that 91% of the cells differentiated on the micro-carriers expressed CXCR4 (**Figure 27B**). This demonstrates that the cells grown on micro-carriers are capable of differentiating into definitive endoderm, the first step to becoming insulin-producing cells.

Three types of micro-carriers, Cytodex 1®, Cytodex 3® (GE Healthcare Life Sciences, NJ) and HILLEX®II (Solohill, MI), allow attachment and growth of H1 cells. Differentiation of H1 cells on these three micro-carriers was conducted. The cells were grown on these micro-carriers in spinner flasks (**Example 5***)* for various passage numbers (1 to 5). Six to eight days after the last passage aliquots of the micro-carriers plus cells in suspension were transferred to 6 or 12 well plates. A total of $15cm^2$ of micro-carriers plus cells per 12 well plate well or $30cm^2$ micro-carriers plus cells per 6 well plate was transferred. Differentiation medium was then added to the plate wells and the plate was placed on a rocking platform at 37°C. Differentiation of the human embryonic stem cells to definitive endoderm was conducted by treating the cells for 2 days with 2% Albumin Bovine Fraction V Fatty Acid Free (MP Biomedicals, Ohio) in RPMI plus 100ng/ml Activin A (PeproTech, NJ), 20ng/ml Wnt3a (R&D Biosciences, MN) and 8ng/ml bFGF (PeproTech, NJ). The cells were treated for an additional 2 days in 2% FAF BSA in RPMI plus 100ng/ml Activin A (PeproTech, NJ) and 8ng/ml bFGF (PeproTech, NJ). Medium was changed daily. FACS analysis was conducted for the definitive endoderm cell surface marker CXCR4 (**Figure 28**). Cells grown on Cytodex 1®, and Cytodex 3® micro-carriers supported differentiation to definitive endoderm (87% and 92% respectively while HILLEX®II micro-carriers did not support differentiation as to the same extent as the other micro-carriers tested in this experiment (42%).

To determine if the cell density affects differentiation of the cells on micro-carriers, cells of the human embryonic stem cell line H1 at passage 40 were grown on Cytodex 3® micro-carriers in a spinner flask for either 8 days or 11 days. Then the equivalent of about 15 cm^2 of micro-carriers plus cells was placed in a 6 well dish and placed on a rocking platform. The cells were then incubated in definitive endoderm differentiating medium as above. After 4 days the cells were analyzed by FACS for CXCR4 expression. 87% of the cells grown for 6 days in spinner flask expressed CXCR4 while 56% of cells

grown for 11 days in the spinner flask expressed CXCR4 (**Figure 29**). This demonstrates that the number of days that the cells are in culture is important prior to differentiation, specifically, if the cell density is too high it may not allow the cells to efficiently differentiate.

To determine if human embryonic stem cells could be differentiated into pancreatic endoderm cells on all three micro-carrier types determined sufficient for attachment and growth, cells of the human embryonic stem cell line H1 at passage 41 (H1p41) were seeded on to Cytodex 1®, Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) and HILLEX®II micro-carriers (Solohill, MI) (see **Example 1**). Micro-carriers were prepared according to the manufactures instructions. $30cm^2$ of micro-carriers were transferred to low attachment 6 well plates. The H1 cells were dissociated from two $10cm^2$ plates with TrypLE™ Express according to manufacturer's instructions. Cell were seeded at $5x10^5$ cells per well. Attachment of the cells to the beads was carried out according to the methods described in **Example 3**. Briefly, the cells and micro-carriers were incubated in MEF conditioned media with 10μM Y27632 at 37°C for four hours with brief agitation each hour. The cells on HILLEX®II and Cytodex 1® micro-carriers were placed on a rocking platform. The cells on Cytodex 3® micro-carriers were allowed to sit undisturbed overnight. The media was changed daily and no longer included Y27632.

Due to poor attachment in this experiment, the majority of cells in the Cytodex 1® plate were no longer attached to the micro-carriers. However, longer attachment time and/or slower rocking speed may improve the cell attachment. After 7 days, the cells were differentiated to definitive endoderm with 2% fatty acid free (FAF) BSA (Proliant, IA) in RPMI and the following growth factors: bFGF (8ng/ml, (PeproTech, NJ)), Activin A (100ng/ml, (PeproTech, NJ)), Wnt3a (20ng/ml, (R&D Biosciences, MN)). For the second through fourth day of differentiation, the cells were treated with the same media lacking Wnt3a. FACS analysis of duplicate samples after 4 days revealed CXCR4 levels of 77-83% positive cells. Definitive endoderm expression was equivalent between cells grown on the different micro-carriers. See **Figure 30**.

The cells were then differentiated further for 2 days with FGF7 (50ng/ml, (R&D Systems, MN)), KAAD-Cyclopamine (0.25μM, (Calbiochem, NJ)) in DMEM/F12 or DMEM-HG plus 2% FAF BSA (Proliant, IA). This was followed by four days of treatment with Noggin (100ng/ml, (R&D Biosciences, MN)), FGF7 (50ng/ml, (R&D Systems, MN)), Retinoic Acid (2 μM, (Sigma-Aldrich, MO)), and KAAD-Cyclopamine (0.25 μM, (Calbiochem, NJ)) in DMEM/F12 or DMEM-HG with 1% B-27 supplement (Invitrogen, CA). The cells were then differentiated for three days with Noggin (100ng/ml, (R&D Biosciences, MN)), DAPT (1 μM, (Sigma-Aldrich, MO)), Alk5 inhibitor II (1 μM, (Axxora, CA)) in DMEM/F12 or DMEM-HG with 1% B-27 supplement (day 13, pancreatic endoderm, (Invitrogen, CA)). **Figure 31** shows the expression level by Q-PCR for the pancreatic specific genes, NKX6.1, PDX1 and NGN3. CT values clearly show that cells differentiated on HILLEX®II micro-carriers do not differentiate efficiently to express the necessary beta cell precursor cell markers. Although the cells differentiated efficiently to definitive endoderm on all three micro-carrier types further differentiation to pancreatic progenitors is not efficient on HILLEX®II micro-carriers.

To determine if the human embryonic stem cells could be further differentiated into insulin producing cells, H1 p45 cells were grown on Cytodex 3® micro-carriers (GE Healthcare Life Sciences, NJ) and differentiated similar to above. Briefly, H1 cells were dissociated from 10cm^2 plates with TrypLE™ Express according to manufacturer's instructions. Cells were seeded at 1 or 2x10^6 cells per 6 well plate well. Attachment of the cells to the beads is described in **Example 3**. Briefly, the cells and micro-carriers were incubated in MEF conditioned media with 10μM Y27632 at 37°C for four hours with brief agitation each hour. The cells were then allowed to incubate overnight undisturbed. On day 2 the media was replaced with MEF conditioned media plus 5uM Y27632 and the plates were placed on a rocking platform. The media was changed each subsequent day without Y27632. On day five, the media was replaced with definitive endoderm differentiation media, 2% fatty acid free (FAF) BSA (Proliant, IA) in RPMI with the following growth factors: : bFGF (8ng/ml, (PeproTech, NJ)), Activin A (100ng/ml, (PeproTech, NJ)), Wnt3a (20ng/ml, (R&D Biosciences, MN)). For the

second and third day of differentiation, the cells were treated with the same media lacking Wnt3a. FACS analysis of duplicate samples after 3 days revealed CXCR4 levels of 97-98% positive cells. The cells were then differentiated further for 2 days with FGF7 (50ng/ml, (R&D Systems, MN)), KAAD-Cyclopamine (0.25μM, (Calbiochem, NJ)) in DMEM-High Glucose (HG) plus 2% FAF BSA (Proliant, IA). This was followed by four days of treatment with Noggin (100ng/ml, (R&D Biosciences, MN)), FGF7 (50ng/ml, (R&D Systems, MN)), Retinoic Acid (2 μM, (Sigma-Aldrich, MO)), and KAAD-Cyclopamine (0.25 μM, (Calbiochem, NJ)) in DMEM-HG with 1% B-27 supplement (Invitrogen, CA). The cells were then differentiated for three days with Noggin (100ng/ml, (R&D Biosciences, MN)), DAPT (1 μM, (Sigma-Aldrich, MO)), Alk5 inhibitor II (1 μM, (Axxora, CA)) in DMEM-HG or DMEM-F12 with 1% B-27 supplement (Invitrogen, CA). This was followed by differentiation in DMEM-HG or DMEM-F12 with Alk5 inhibitor II (1 μM, (Axxora, CA)) for seven days. Final differentiation was for five days in DMEM-HG or DMEM-F12 respectively. This is a total of 24 days of differentiation leading to expression of pancreatic endocrine hormones. **Figure 32** shows the FACS analysis results of the cells at this end point. Cells with the highest seeding density and differentiated in DMEM-HG from days 6 through 24 had the highest levels of insulin expression (**Figure 32**).

Alternatively, to determine if the human embryonic stem cells could be differentiated into insulin producing cells, H1 p44 cells were grown in a spinner flask for 7 days on Cytodex 3® micro-carriers (see **Example 5**). The cells plus micro-carriers were transferred to a 12 well plate at 15cm^2/well and placed on a rocking platform at 37°C. The cells were differentiated to definitive endoderm as above but with DMEM/F12 instead of RMPI. FACS analysis after 4 days revealed CXCR4 levels of 75 to 77% positive cells in a triplicate analysis. The cells were then differentiated further with 3 days of treatment with FGF7 (50ng/ml, (R&D Systems, MN)), KAAD-Cyclopamine (0.25μM, (Calbiochem, NJ)) in DMEM/F12 plus 2% Albumin Bovine Fraction V Fatty Acid Free. This was followed by four days of treatment with Noggin (100ng/ml, (R&D Biosciences, MN)), FGF7 (50ng/ml, (R&D Systems, MN)), Retinoic Acid (2 μM, (Sigma-Aldrich, MO)), and KAAD-Cyclopamine (0.25 μM, (Calbiochem, NJ)) in DMEM/F12 with 1%

B-27 supplement (Invitrogen, CA). The cells were then differentiated for three days with Noggin (100ng/ml, (R&D Biosciences, MN)), Netrin4 (100ng/ml, (R&D Biosciences, MN)), DAPT (1 μM, (Sigma-Aldrich, MO)), Alk5 inhibitor II (1 μM, (Axxora, CA)) in DMEM/F12 with 1% B-27 supplement (day 15, pancreatic endoderm, (Invitrogen, CA)). This was followed by six days of treatment with Alk5 inhibitor II (1 μM, (Axxora, CA)) in DMEM/F12 with 1% B-27 supplement (day 21, pancreatic endocrine cells, (Invitrogen, CA)). The final treatment for seven days was DMEM/F12 with 1% B-27 supplement (day 28, insulin-expressing cells, (Invitrogen, CA)). **Figure 33** shows the expression level by Q-PCR for the pancreatic specific genes, insulin, Pdx1 and glucagon. The data on micro-carriers is compared to previous data of H1 p42 cells differentiated on a MATRIGEL™ (BD Biosciences, CA) coated planar surface. The expression level of these pancreas specific genes is similar or better for micro-carrier differentiated cells compared to cells differentiated on planar surfaces.

Similar experiments were conducted with H9p38 cells passaged onto Cytodex 3® micro-carriers and expanded in a spinner flask. An aliquot of 15cm^2 of micro-carriers plus cells was placed in a 12 well plate and placed on a rocking platform with differentiation medium. This was compared to cells plated on a 6 well plate coated with MATRIGEL™ (BD Biosciences, CA). Differentiation of the cells to definitive endoderm in RPMI and supplements was achieved, with an average of 83% of the cells expressing CXCR4 (samples in duplicate) compared to 72% of cells expressing CXCR4 on a planar substrate (**Figure 34**). Further differentiation to pancreatic endoderm (day 15), pancreatic-endocrine cells (day 22) and insulin-expressing cells (day29) showed similar expression levels of insulin and glucagon between cells grown on micro-carriers to those grown on a planar substrate (**Figure 35**). The medium components were identical to those listed for the above H1 differentiation experiment with one additional day in the endocrine cell differentiating components. At the insulin-expressing stage, cells showed a surprising decrease in insulin expression compared to day 22. Since the decrease was noted in both micro-carrier and planar samples, it is likely not due to the attachment substrate. This shows that H9 cells can also be successfully differentiated to at least pancreatic endocrine cells on micro-carriers.

Overall, two different human embryonic stem cell lines, H1 and H9, can be differentiated to pancreatic endocrine cells on Cytodex 3® micro-carriers, illustrating the potential to expand and differentiate these cells in a large-scale culture system (**Figures 17, 21, 33, and 35**). Human embryonic stem cells were able to attach and proliferate to at least three micro-carrier bead types and the cells could be differentiated to at least definitive endoderm (**Figures 28**). These results illustrate a method by which human embryonic stem cells can be expanded and differentiated for therapeutic uses.

Example 8: Human embryonic stem cells passaged as single cells in a 3D micro-carrier based culture can be transferred to culture on an ECM free surface while maintaining pluripotency

H1 human embryonic stem cells were cultured on micro-carriers according to the methods described in **Example 5**. Cells were removed from micro-carriers and plated to Nunc4, Nunc13, CELLBIND™, or PRIMARIA™ tissue culture polystyrene (TCPS) planar surfaces with MEFCM16 supplemented with 3μM Glycyl-H 1152 dihydrochloride. The cells were seeded at a density of 100,000 cells/cm^2 in six well plates and then cultured for one additional passage on the respective surface. Cells were then either lifted with TrypLE and tested by flow cytometry for pluripotency markers, or lysed in the well with RLT for mRNA purification and qRT-PCR, or differentiated to definitive endoderm. Differentiation was induced by treating the cells with RPMI media supplemented with 2% BSA, 100ng/ml Activin A, 20ng/ml Wnt3a, 8ng/ml bFGF, and 3μM Glycyl-H 1152 dihydrochloride for 24 hours. Media was then changed to RPMI media supplemented with 2% BSA, 100ng/ml Activin A, 8ng/ml bFGF, and 3μM Glycyl-H 1152 dihydrochloride for an additional 48 hours with daily media change.

As measured by pluripotency markers, using either flow cytometry or qRT-PCR, cells cultured on micro-carriers and transferred to culture on Nunc4, Nunc13, CellBIND, or Primaria tissue culture polystyrene (TCPS) planar surfaces maintained pluripotency after two passages on the respective planar surface (**Figure 36**). Furthermore, the cells maintained the capacity to differentiate to a definitive endoderm fate as measured by either flow cytometry or qRT-PCR (**Figure 37**). Similar results were also obtained in

side-by-side tests of H1 and H9 human embryonic stem cells passaged on Cytodex 3® micro-carriers and differentiated to definitive endoderm (**Figure 38**).

These results indicate that human embryonic stem cells can be passaged on micro-carriers and then subsequently cultured on another surface while maintaining pluripotency. The cells may also be transferred to another surface and efficiently induced to differentiate.

Example 9: Human embryonic stem cells can be transferred directly from a cluster/colony style culture on mitotically inactivated fibroblast feeders to culture as single cells on ECM free surfaces for at least 10 passages without loss of pluripotency and without manual removal of fibroblast feeders.

Human embryonic stem cell lines are currently derived using a method that promotes a colony outgrowth of a single cell or a cluster of a few cells from a blastocyst. This colony outgrowth is then serially passaged and propagated until enough cluster/colonies of cells are available that they constitute a cell line. Once a cell line has been derived, in order to maintain the pluripotent and karyotypically stable characteristics of human embryonic stem cells, the current standard in the art for high quality, reproducible culture of human embryonic stem cells is to maintain the clusters/colonies of human embryonic stem cells on a feeder layer of mitotically inactive fibroblasts and to pass the cells using manual disruption or gentle enzymatic bulk passage with collagenase or neutral protease or a blend thereof. These passage methods maintain human embryonic stem cells clusters and promote colony style growth of human embryonic stem cell. After a stable human embryonic stem cell line is established the cells can be transitioned to an extracellular matrix (ECM) substrate such as MATRIGEL™. However, whether the cells are grown on fibroblast feeders or on an ECM substrate, the recommended passage method for human embryonic stem cells specifically instructs technicians not to fully dissociate human embryonic stem colonies.

The current best practice for large scale culture of mammalian cells is to use a 3-dimensional culture vessel that tightly maintains homeostatic, uniform conditions and can incorporate micro-carriers for support of adhesion dependent cells. However, the current standard methods used for human embryonic stem cell culture- growth on fibroblast

feeders or an ECM substrate and cluster/colony style culture pose a technical hurdle to successfully growing and maintaining a pluripotent human embryonic stem cell culture on micro-carriers, since these methods are not easily transferable to large scale culture on micro-carriers. In order to effectively grow human embryonic stem cells on micro-carriers the human embryonic stem cell culture must be able to be passaged as single cells, and not as colonies or clusters, as is currently the standard in the art. Furthermore, the human embryonic stem cells should be able to grow without a layer of feeder cells or ECM substrate.

We describe below a method which addresses these technical hurdles. We demonstrate how to convert human embryonic stem cell cultures from clusters/colonies on a mitotically inactive fibroblast feeder layer directly to a single cell culture system that does not require an underlying fibroblast feeder layer or a surface coated with MATRIGEL or other an extracellular matrix substrate. This method utilizes bulk passage of human embryonic stem without any manual removal of fibroblast feeder cells or selection of pluripotent cells from the total cell population to convert the culture directly from colony style, fibroblast feeder based culture to feeder free/matrix free culture on PRIMARIA in the presence of the Rho Kinase (ROCK) inhibitor, Glycyl-H 1152 dihydrochloride. This method can be completed in a sealed vessel to adhere to regulatory requirements and produces a highly homogeneous human embryonic stem culture that retains pluripotency and the ability to differentiate to definitive endoderm, and does not contain a fibroblast cell population.

Method: Cells were routinely passaged by aspirating media, washing with PBS, and then treating the cells with a dissociation enzyme (collagenase, Accutase™,or TrypLE). Collagenase was used at 1mg/ml concentration; Accutase™ or TrypLE were used at 1X stock concentration. All enzymes were used after reaching room temperature. A solution of 2% BSA in DMEM/F12 was added to each well and cells were uniformly suspended in the solution after treating the cells with enzyme. Cells were then centrifuged for 5 minutes at 200g, the cell pellet and additional 2% BSA in DMEM/F12 solution was added to resuspend cells and the cell suspension was distributed to three 50ml sterile conical tubes and centrifuged for 5min at 200g.

Using a sequential method, we removed the fibroblast feeders by high density passaging the cluster/colony style human embryonic stem cells to a Primaria surface by treating the MEF based culture with either Accutase™, TrypLE™, or collagenase. At the first passage, cells were plated to T-25 flasks coated with a 1:30 dilution of MATRIGEL™ in mouse embryonic fibroblast (MEF) conditioned media (CM)or the cells were plated to T-25 PRIMARIA™ culture flasks in MEF-CM plus 3uM Glycyl-H 1152 dihydrochloride. All cells were plated at a split ratio of 1 to 3.5 and cells were exposed to enzyme for 10 minutes. Cell number for cells lifted with TrypLE™ or Accutase™ was determined by counting trypan blue stained cells with a hemocytometer. After plating the cells the media was changed daily, and cells plated in MEF-CM+ 3μM Glycyl-H 1152 dihydrochloride were fed daily with MEF-CM + 1μM Glycyl-H 1152 dihydrochloride and samples were assayed for expression of mRNA markers of pluripotency and differentiation. hESCs passaged twice as single cells under Matrix free conditions maintained gene expression of pluripotency genes and inhibited expression of differentiation genes (**Figure 39**).

2nd passage: Cells were passaged at a ratio of 1 to 4 using a 10 minutes exposure to TrypLE™ or Accutase™. We also introduced a shorter enzyme exposure time that was determined empirically by treating the cells and monitoring for detachment. We observed that 3 minute exposure to TrypLE™ and 5 minute exposure to Accutase™ was sufficient to lift the cells. After treating the cells with enzyme, the cells were passaged as described above and aliquots of cell mRNA were taken for qRT-PCR at the time of passaging.

3rd passage: Upon reaching confluence cells were washed with PBS, disrupted with enzyme for 3 or 10 minutes (TrypLE™) or 5 or 10 minutes (Accutase™), suspended in 2%BSA in DMEM/F12 and centrifuged, washed again with 2%BSA in DMEM/F12, centrifuged, and then resuspended and plated in their respective media. At this passage cells were plated at 1:4 ratio and also at 2 additional split ratios- 1:8 and 1:16. Aliquots of cell mRNA were taken for qRT-PCR at each passage.

4 passages+: The conditions adopted for time of exposure to enzyme and passage ratio at passages 2 and 3 were maintained from passage 4 onward. Each time the culture grew to confluence cells were washed with PBS, disrupted with enzyme for the specified time, suspended in 2%BSA in DMEM/F12 and centrifuged, washed again with 2% BSA in DMEM/F12, centrifuged, and then resuspended in their respective media at the specified plating ratio. The media for cells plated to PRIMARIA was supplemented with 3μM Glycyl-H 1152 dihydrochloride at the time of plating. After plating, media was changed daily and cells plated in MEF-CM + 3μM Glycyl-H 1152 dihydrochloride were fed daily with MEF-CM + 1μ Glycyl-H 1152 dihydrochloride. Aliquots of cell mRNA were taken for qRT-PCR at the time of passaging.

At the completion of greater than 8 passages, cells were assayed for pluripotency by flow cytometry for pluripotency surface markers (**Figure 40**) and by qRT-PCR for pluripotency and differentiation markers (**Figures 41, 42, and 43**). Cells were also differentiated to definitive endoderm by treating the cells with RPMI media supplemented with 2% BSA, 100ng/ml Activin A, 20ng/ml Wnt3a, 8ng/ml bFGF, and 3uM Glycyl-H 1152 dihydrochloride for 24 hours. Media was then changed to RPMI media supplemented with 2% BSA, 100ng/ml Activin A, 8ng/ml bFGF, and 3μM Glycyl-H 1152 dihydrochloride for an additional 48 hours with daily media change. Samples differentiated to definitive endoderm were then tested for the presence of the definitive endoderm marker CXCR4 by flow cytometry (**Figure 40**).

These results indicate that bulk passage from colony style, fibroblast feeder based culture to feeder free/matrix free culture on PRIMARIA in the presence of the Rho Kinase (ROCK) inhibitor, Glycyl-H 1152 dihydrochloride results in a highly homogeneous human embryonic stem cell culture that retains pluripotency and the ability to differentiate to definitive endoderm, and does not contain a fibroblast cell population.

Example 10: Human Embryonic Stem Cells Transferred from Tissue Culture Plastic to Micro-Carriers

H1 cells were cultured on PRIMARIA™ (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ) tissue culture plates (method in **Example 9**) and released by treatment with

TrypLE™ Express for 3-5 minutes and seeded into 6 well non-tissue culture treated plates with Cytodex 3® (GE Healthcare Life Sciences, NJ) or HILLEX®II (Solohill, MI) micro-carriers in MEF-CM plus 10μM Y27632 (Sigma-Aldrich, MO). As a control, H1p46 cells grown on MATRIGEL (BD Biosciences, CA) coated plates and passaged with Collagenase (1mg/ml) were released and seeded onto micro-carriers in a similar manner. The plates were incubated at 37°C for 5 hours, agitating by hand every 45 minutes. The plates were then placed on a rocking platform at 37°C. Medium was changed every day with MEF-CM plus 10μM Y27632 (Sigma-Aldrich, MO). Images show good attachment of cells to the micro-carriers at 3 days (**Figure 44**). After 7 days the cells were released (described in **Example 4** *infra*) and analyzed by FACS for the pluripotency markers CD9, SSEA-4, SSEA-3, TRA-1-60, TRA-1-81 (**Figure 45**). The majority of pluipotency markers were expressed on 90-100% of the cells. There are no clear differences between cells passaged with Accutase™ (Millipore, MA) and TrypLE™ Express (Invitrogen, CA) nor between growth on with Cytodex 3® (GE Healthcare Life Sciences, NJ) and HILLEX®II (Solohill, MI) micro-carriers. Overall, the cells remained pluripotent when transferred from PRIMARIA™ (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ) cell culture plastic onto micro-carriers.

Next these H1 cells on the micro-carriers were differentiated to definitive endoderm. The method is described in **Example 7**. After 4 days of differentiation, the H1 cells were released from the micro-carriers and underwent FACS analysis showing greater than 82% of the cells expressing CXCR4. See **Figure 46**. The cells were efficiently differentiated into definitive endoderm regardless of the micro-carrier type or passaging enzyme on PRIMARIA™ (cat. no. 353846, Becton Dickinson, Franklin Lakes, NJ). This proves the flexibility of the expansion system and allows for cells to be grown without matrix on plastic and micro-carriers.

Example 11: Human Embryonic Stem Cells Transferred from Planar Substrates Consisting of Mixed Cellulose Esters to Micro-Carriers

H1 cells were cultured on planar substrates consisting of mixed cellulose esters for 12 passages, according to the methods disclosed U.S. Patent Application No. 61/116,452. The cells were released from the planar substrate by treatment with TrypLE™ Express

for 3-5 minutes and seeded into 6 well non-tissue culture treated plates with CYTODEX 3® (GE Healthcare Life Sciences, NJ) or HILLEX®II (Solohill, MI) micro-carriers in MEF-CM plus 10mM Y27632 (Sigma-Aldrich, MO). As a control, H1p44 cells grown on MATRIGEL™ coated plates (BD Biosciences, CA), passaged with Collagenase (1mg/ml) were released and seeded onto micro-carriers in a similar manner. The plates were incubated at 37°C for 5 hours, agitating by hand every 45 minutes. The plates were then placed on a rocking platform at 37°C. Media was changed daily. After 7 days the cells were released (described in **Example 4**) and analyzed by FACS for the pluripotency markers CD9, SSEA-4, SSEA-3, TRA-1-60, TRA-1-81 (**Figure 47**). The majority of pluripotency markers were expressed on greater than 90% of the cells. There were no clear differences between cells grown on CYTODEX 3® and HILLEX®II micro-carriers. H1p44 control cells were not tested for pluripotency after growth on HILLEX®II micro-carriers, since pluripotency had been confirmed by other experiments (see **Example 5**). Overall, the cells maintained pluripotency when transferred from planar substrates consisting of mixed cellulose esters onto micro-carriers.

Next, H1 cells on the micro-carriers were differentiated to definitive endoderm, according to the methods described in **Example 7**. After 4 days of differentiation, the H1 cells were released from the micro-carriers and underwent FACS analysis showing greater than 65% of the cells expressing CXCR4 (**Figure 48**). The cells were efficiently differentiated into definitive endoderm regardless of the micro-carrier type. There appeared to be a lower number of cells differentiating into definitive endoderm on the HILLEX®II (Solohill, MI) micro-carriers. The ability of the cells to differentiate proves the flexibility of the expansion system. Additionally cells can be grown and differentiated directly on membranes and micro-carriers eliminating any need for an animal component matrix.

Table 1: Attachment of H9 cells to micro-carrier beads in MEF-CM in static cultures.

Bead	company	surface coating	attachment 0-5*
ProNectin F	SoloHill™ -polystyrene	Recombinant fibronectin	0
Plastic	SoloHill™ -polystyrene	none	0
Plastic Plus	SoloHill™ -polystyrene	Cationic	0
HillexII	SoloHill™ -polystyrene	Cationic trimethyl ammonium	2
Collagen	SoloHill™ -polystyrene	Porcine collagen	0
FACTIII	SoloHill™ -polystyrene	Cationic porcine collagen	0
Glass	SoloHill™ -polystyrene	High silica glass	0
Cytodex 1	GE -dextran		0
Cytodex 3	GE -dextran	denatured collagen	0
	* 5 is most efficient cell attachment		

Table 2: Attachment of H1 and H9 cells to micro-carrier beads in MEF-CM with 10μM Rho kinase inhibitor, Y27632.

Bead	company	surface coating	attachment 0-5*
ProNectin F	SoloHill™ -polystyrene	Recombinant fibronectin	1
Plastic	SoloHill™ -polystyrene	none	1
Plastic Plus	SoloHill™ -polystyrene	Cationic	1
HillexII	SoloHill™ -polystyrene	Cationic trimethyl ammonium	4
Collagen	SoloHill™ -polystyrene	Porcine collagen	1
FACTIII	SoloHill™ -polystyrene	Cationic porcine collagen	1
Glass	SoloHill™ -polystyrene	High silica glass	1
Cytodex 1	GE -dextran		4
Cytodex 3	GE -dextran	denatured collagen	4
	* 5 is most efficient cell attachment		

Table 3: The population doublings for H1 and H9 cells grown 5 passages on Cytodex 1®, Cytodex 3®, or HILLEX®II.

Cell line-micro-carrier	Population doubling	Standard Deviation
H9-HII	27hrs	4.1
H9-C3	32.4hrs	12.8
H1-C1	20.3hrs	3.7
H1-C3	25hrs	12.8

Publications cited throughout this document are hereby incorporated by reference in their entirety. Although the various aspects of the invention have been illustrated above by reference to examples and preferred embodiments, it will be appreciated that the scope of the invention is defined not by the foregoing description but by the following claims properly construed under principles of patent law.

What is claimed is:

1. A method for the propagation of pluripotent stem cells, comprising the steps of:

    a. Attaching a population of pluripotent stem cells to a first volume of micro-carriers,

    b. Culturing the pluripotent stem cells on the first volume of micro-carriers,

    c. Removing the pluripotent stem cells from the first volume of micro-carriers, and

    d. Attaching the population of pluripotent stem cells to a second volume of micro-carriers.

2. The method of claim 1, wherein the steps of culturing, removing and attaching the pluripotent stem cells on micro-carriers are is repeated using subsequent volumes of micro-carriers.

3. The method of claim 1, wherein the first volume of micro-carriers is selected from the group consisting of dextran micro-carriers and polystyrene micro-carriers.

4. The method of claim 1, wherein the second volume of micro-carriers is selected from the group consisting of dextran micro-carriers and polystyrene micro-carriers.

5. The method of claim 1, wherein the pluripotent stem cells are attached to the first volume of micro-carriers in medium containing a Rho kinase inhibitor.

6. The method of claim 1, wherein the pluripotent stem cells are attached to the second volume of micro-carriers in medium containing a Rho kinase inhibitor.

7. The method of claim 1, wherein the pluripotent stem cells are removed from the first volume of micro-carriers by enzymatic treatment.

8. The method of claim 1, wherein the pluripotent stem cells are removed from the second volume of micro-carriers by enzymatic treatment.

9. The method of claim 1, wherein the first volume of micro-carriers is removed from the pluripotent stem cells prior to attaching the cells to the second volume of micro-carriers.

10. A method to differentiate pluripotent stem cells to insulin-expressing cells on micro-carriers comprising the steps of:

    a. Attaching the pluripotent stem cells to a volume of micro-carriers,

    b. Differentiating the pluripotent stem cells to cells expressing markers characteristic of the definitive endoderm lineage,

    c. Differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage,

    d. Differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing markers characteristic of the pancreatic endocrine lineage, and

    e. Differentiating the cells expressing markers characteristic of the pancreatic endocrine lineage into insulin-expressing cells.