METHODS AND COMPOSITIONS FOR CELL ATTACHMENT AND CULTIVATION ON PLANAR SUBSTRATES

The present invention claims priority to application serial number 61/116,452, 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 planar substrates lacking an adlayer and a feeder cell layer.

BACKGROUND

Cultivation of mammalian cells is one of many processes in the life and health sciences. Vessels for mammalian cell culture and analysis involving anchorage-dependent cells are often made of glass or a polymer, such as, for example, polystyrene, that frequently requires additional surface treatment to allow the cells to attach to the surface of the vessel. Such treatments may include applying an adlayer on the surface, for example, by adsorption, grafting or plasma polymerization techniques. Alternatively, the surface treatment may be via chemical modification of the vessel surface itself, which can be achieved by, for example, atmospheric corona, radio frequency vacuum plasma, DC glow discharge, and microwave plasma treatments.

Current methods of culturing pluripotent stem cells, in particular, embryonic stem (ES) cells require complex culture conditions, such as, for example, culturing the embryonic stem cells on a solid substrate surface with a feeder cell layer, or on a solid substrate surface with an adlayer of extracellular matrix protein. Culture systems that employ these methods often use feeder cells or extracellular matrix proteins obtained from a different species than that of the stem cells being cultivated (xenogeneic material). Media obtained by exposure to feeder cells, that is, media conditioned by cells other than undifferentiated ES cells, may be used to culture the ES cells, and media may be supplemented with animal serum.

For example, Reubinoff *et al.* (Nature Biotechnol. 18:399-404, 2000) and Thompson *et al.* (Science 282:1145-1147, 1998) disclose the culture of ES cell lines from human blastocysts using a mouse embryonic fibroblast feeder cell layer.

In another example, Xu *et al.* (Nature Biotechnology 19: 971-974, 2001) discloses the use of MATRIGEL® and laminin for treating solid substrate surfaces before feeder-cell free cultivation of human ES cells without differentiation. In another example, Vallier *et al.* (J. Cell Sci. 118:4495-4509, 2005) discloses the use of fetal bovine serum for treating solid substrate surfaces before feeder-cell free cultivation of human ES cells without differentiation.

In another example, WO2005014799 discloses conditioned medium for the maintenance, proliferation and differentiation of mammalian cells. WO2005014799 state: "The culture medium produced in accordance with the present invention is conditioned by the cell secretion activity of murine cells, in particular, those differentiated and immortalized transgenic hepatocytes, named MMH (Met Murine Hepatocyte)."

In another example, Wanatabe *et al.* (Nature Biotechnol. 35:681-686, 2007) state "a ROCK inhibitor permits survival of dissociated human embryonic stem cells", and demonstrate reduced dissociation-induced apoptosis, increases cloning efficiency (from approximately 1% to approximately 27%) and facilitation of subcloning after gene transfer, using mouse embryonic fibroblasts as feeder cells, collagen and MATRIGEL® as extracellular matrix protein, and Y-27632 or Fasudil for inhibition of ROCK. Furthermore, dissociated human ES cells treated with Y-27632 were protected from apoptosis in serum-free suspension culture.

In another example, Peerani *et al.* (EMBO Journal 26:4744-4755, 2007) state "Complexity in the spatial organization of human embryonic stem cell (hESC) cultures creates heterogeneous microenvironments (niches) that influence hESC fate. This study demonstrates that the rate and trajectory of hESC differentiation can be controlled by engineering hESC niche properties. Niche size and composition regulate the balance between differentiation-inducing and –inhibiting factors. Mechanistically, a niche size-dependent spatial gradient of Smad1 signaling is generated as a result of antagonistic interactions between hESCs and hESC-derived

extra-embryonic endoderm (ExE). These interactions are mediated by the localized secretion of bone morphogenetic protein-2 (BMP2) by ExE and its antagonist, growth differentiation factor-3 (GDF3) by hESCs. Micropatterning of hESCs treated with small interfering (si) RNA against GDF3, BMP2 and Smad1, as well treatments with a Rho-associated kinase (ROCK) inhibitor demonstrate that independent control of Smad1 activation can rescue the colony size-dependent differentiation of hESCs. Our results illustrate, for the first time, a role for Smad1 in the integration of spatial information and in the niche-size dependent control of hESC self-renewal and differentiation."

In another example, Koyanagi, M *et al* (J Neurosci Res. 2008 Feb 1; 86(2): 270-80) state "Rho-GTPase has been implicated in the apoptosis of many cell types, including neurons, but the mechanism by which it acts is not fully understood. Here, we investigate the roles of Rho and ROCK in apoptosis during transplantation of embryonic stem cell-derived neural precursor cells. We find that dissociation of neural precursors activates Rho and induces apoptosis. Treatment with the Rho inhibitor C3 exoenzyme and/or the ROCK inhibitor Y-27632 decreases the amount of dissociation-induced apoptosis (anoikis) by 20-30%. Membrane blebbing, which is an early morphological sign of apoptosis; cleavage of caspase-3; and release of cytochrome c from the mitochondria are also reduced by ROCK inhibition. These results suggest that dissociation of neural precursor cells elicits an intrinsic pathway of cell death that is at least partially mediated through the Rho/ROCK pathway. Moreover, in an animal transplantation model, inhibition of Rho and/or ROCK suppresses acute apoptosis of grafted cells. After transplantation, tumor necrosis factor-alpha and pro-nerve growth factor are strongly expressed around the graft. ROCK inhibition also suppresses apoptosis enhanced by these inflammatory cytokines. Taken together, these results indicate that inhibition of Rho/ROCK signaling may improve survival of grafted cells in cell replacement therapy."

The use of xenogeneic material may be unsuitable for certain applications utilizing pluripotent stem cells. Alternative materials may be used. For example, Stojkovic *et al.* (Stem Cells 23:895-902, 2005) discloses the use of human serum for treating solid substrate surfaces before feeder-cell free cultivation of human ES cells without differentiation.

An alternative culture system employs serum-free medium supplemented with growth factors capable of promoting the proliferation of embryonic stem cells.

For example, Cheon *et al.* (BioReprod DOI:10.1095/biolreprod.105.046870; 19 Oct 2005) disclose a feeder-cell free, serum-free culture system in which ES cells are maintained in unconditioned serum replacement medium supplemented with different growth factors capable of triggering ES cell self-renewal.

In another example, Levenstein *et al.* (Stem Cells 24:568-574, 2006) disclose methods for the long-term culture of human ES cells in the absence of fibroblasts or conditioned medium, using media supplemented with basic fibroblast growth factor (FGF).

In another example, US20050148070 discloses a method of culturing human ES cells in defined media without serum and without fibroblast feeder cells, the method comprising: culturing the stem cells in a culture medium containing albumin, amino acids, vitamins, minerals, at least one transferrin or transferrin substitute, at least one insulin or insulin substitute, the culture medium essentially free of mammalian fetal serum and containing at least about 100 ng/ml of a FGF capable of activating a FGF signaling receptor, wherein the growth factor is supplied from a source other than just a fibroblast feeder layer, the medium supported the proliferation of stem cells in an undifferentiated state without feeder cells or conditioned medium.

In another example, US20050233446 discloses a defined media useful in culturing stem cells, including undifferentiated primate primordial stem cells. In solution, the media is substantially isotonic as compared to the stem cells being cultured. In a given culture, the particular medium comprises a base medium and an amount of each of basic FGF, insulin, and ascorbic acid necessary to support substantially undifferentiated growth of the primordial stem cells.

In another example, US6800480 states: "In one embodiment, a cell culture medium for growing primate-derived primordial stem cells in a substantially undifferentiated state is provided which includes a low osmotic pressure, low endotoxin basic medium that is effective to support the growth of primate-derived primordial stem cells. The basic medium is combined with a nutrient serum effective to support the growth of primate-derived primordial stem cells and a substrate selected from the group

consisting of feeder cells and an extracellular matrix component derived from feeder cells. The medium further includes nonessential amino acids, an anti-oxidant, and a first growth factor selected from the group consisting of nucleosides and a pyruvate salt."

In another example, US20050244962 states: "In one aspect the invention provides a method of culturing primate embryonic stem cells. One cultures the stem cells in a culture essentially free of mammalian fetal serum (preferably also essentially free of any animal serum) and in the presence of fibroblast growth factor that is supplied from a source other than just a fibroblast feeder layer. In a preferred form, the fibroblast feeder layer, previously required to sustain a stem cell culture, is rendered unnecessary by the addition of sufficient fibroblast growth factor."

In another example, WO2005065354 discloses a defined, isotonic culture medium that is essentially feeder-free and serum-free, comprising: a. a basal medium; b. an amount of basic fibroblast growth factor sufficient to support growth of substantially undifferentiated mammalian stem cells; c. an amount of insulin sufficient to support growth of substantially undifferentiated mammalian stem cells; and d. an amount of ascorbic acid sufficient to support growth of substantially undifferentiated mammalian stem cells.

In another example, WO2005086845 discloses a method for maintenance of an undifferentiated stem cell, said method comprising exposing a stem cell to a member of the transforming growth factor-beta (TGFβ) family of proteins, a member of the fibroblast growth factor (FGF) family of proteins, or nicotinamide (NIC) in an amount sufficient to maintain the cell in an undifferentiated state for a sufficient amount of time to achieve a desired result.

Pluripotent stem cells provide a potential resource for research and drug screening. At present, large-scale culturing of human ES cell lines is problematic and provides substantial challenges. A possible solution to these challenges is to passage and culture the human ES cells as single cells. Single cells are more amenable to standard tissue culture techniques, such as, for example, counting, transfection, and the like.

For example, Nicolas *et al.* provide a method for producing and expanding human ES cell lines from single cells that have been isolated by fluorescence-activated cell

sorting following genetic modification by lentivirus vectors (Stem Cells Dev. 16:109-118, 2007).

In another example, US patent application US2005158852 discloses a method "for improving growth and survival of single human embryonic stem cells. The method includes the step of obtaining a single undifferentiated hES cell; mixing the single undifferentiated cell with an extracellular matrix to encompass the cell; and inoculating the mixture onto feeder cells with a nutrient medium in a growth environment".

In another example, Sidhu *et al.* (Stem Cells Dev. 15:61-69, 2006) describe the first report of three human ES cell clones, hES 3.1, 3.2 and 3.3, derived from the parent line hES3 by sorting of single-cell preparations by flow cytometry.

However, passage and culture of human ES cells as single cells leads to genetic abnormalities and the loss of pluripotency. Culture conditions are important in the maintenance of pluripotency and genetic stability. Generally, passage of human ES cell lines is conducted manually or with enzymatic agents such as collagenase, liberase or dispase.

For example, Draper *et al.* note the presence of "karyotypic changes involving the gain of chromosome 17q in three independent human embryonic stem cell lines on five independent occasions." (Nature Biotechnol. 22:53-54, 2004).

In another example, Buzzard *et al.* state, "we have only ever detected one karyotype change event…the culture methods used may have had some bearing on our results, given that our methods are distinctly different from those used by most other groups. Typically we passage human ES cells after 7 days by first dissecting the colony with the edge of a broken pipette…No enzymatic or chemical methods of cell dissociation are incorporated into this method. We speculate that this may explain the relative cytogenetic resilience of hES (human ES) cells in our hands." (Nature Biotechnol. 22:381-382, 2004).

In another example, Mitalipova *et al.* state: "bulk passage methods... can perpetuate aneuploid cell populations after extended passage in culture, but may be used for shorter periods (up to at least 15 passages) without compromising the karyotypes…it

may be possible to maintain a normal karyotype in hES cells under long-term manual propagation conditions followed by limited bulk passaging in experiments requiring greater quantities of hES cells than manual passage methods, alone, can provide". (Nature Biotechnol. 23:19-20, 2005).

In another example, Heng *et al.* state "the results demonstrated that the second protocol (trypsinization with gentle pipetting) is much less detrimental to cellular viability than is the first protocol (collagenase treatment with scratching). This in turn translated to higher freeze-thaw survival rates." (Biotechnology and Applied Biochemistry 47:33-37, 2007).

In another example, Hasegawa *et al.* state, "we have established hESC sublines tolerant of complete dissociation. These cells exhibit high replating efficiency and also high cloning efficiency and they maintain their ability to differentiate into the three germ layers." (Stem Cells 24:2649-2660, 2006).

In another example, US Patent application 61/030,544 provides methods and compositions for cell attachment to, cultivation on and detachment from a solid substrate surface containing from at least about 0.9% nitrogen to about at least 11% nitrogen and from at least about 12% oxygen to at least about 30% oxygen, and lacking an adlayer and feeder cells. In one embodiment of the present invention, the cells are treated with a compound capable of inhibiting Rho kinase activity.

There is a significant need for methods and compositions for the culture of cells, including pluripotent stem cells in the absence of feeder cells and an adlayer, while maintaining the pluripotency of the cells. The present invention provides methods for the growth, expansion and differentiation of pluripotent stem cells on planar substrates lacking an adlayer and a feeder cell layer, wherein the cells do not require treatment with a compound capable of inhibiting Rho kinase activity in order to bind to the planar substrate.

SUMMARY

In one embodiment, the present invention provides methods for the attachment, cultivation and differentiation of pluripotent stem cells to a planar substrate containing up to about 12% N, from at least about 12% O to at least about 55% O, a

contact angle from about 18 degrees to about 32 degrees, and lacking an adlayer and a feeder cell layer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of the Rho kinase inhibitor H-1152 on the attachment of the human embryonic stem cell line H1 to planar substrates. Panel a): depicts cell attachment on mixed cellulose ester membranes (membrane No. 2 in Table 1). Panel b): depicts cell attachment on nylon membranes (membrane No. 4 in Table 1). Panel c): depicts cell attachment on cellulose acetate membranes (membrane No. 5 in Table 1). Panel d): depicts cell attachment on polycarbonate membranes (membrane No. 7 in Table 1). Panel e): depicts cell attachment on polyethylene terephthalate membranes (membrane No. 12 in Table 1).

Figure 2: shows the effect of the Rho kinase inhibitor Y-26732 on the attachment of the human embryonic stem cell line H9 to mixed cellulose ester membrane (membrane No. 1 in Table 1). Panel a): depicts cell attachment in a control well. Panel b): depicts cell attachment for cells treated with 10μM Y-26732. Panel c): depicts cell attachment for cells treated with 20μM Y-26732.

Figure 3: shows the proliferation curves of the human embryonic stem cell line H1 on MATRIGEL® coated surface (solid line) and on mixed cellulose ester membranes (membrane No. 1 in Table 1) (dashed line).

Figure 4: shows the G-banded chromosomes from representative cells of the human embryonic stem cells of the line H1. Panel a): depicts the chromosomes from a cell cultured on MATRIGEL® coated surface for 10 passages. Panel b): depicts the chromosomes from a cell cultured on mixed cellulose ester membranes (membrane No.1 in Table 1) for 10 passages.

Figure 5: shows the effect of the Rho kinase inhibitor Y26732 on cells of the human embryonic stem cell line H9 on the attachment to polycarbonate membranes (membrane No. 7 in Table 1). Panel a): depicts cell attachment in a control well. Panel b): depicts cell attachment following treatment with 10μM Y-26732. Panel c): depicts cell attachment following treatment with 20μM Y-26732.

Figure 6 shows the effect of the Rho kinase inhibitor H-1152 on the attachment of cells of the human embryonic stem cell line H1 to polycarbonate membranes (membrane No. 7 in Table 1). Panel a): depicts cell attachment in a control well. Panel b): depicts cell attachment when 0.03μM of H-1152 was added to the medium. Panel c): depicts cell attachment when 0.1μM of H-1152 was added to the medium. Panel d): depicts cell attachment when 0.3μM of H-1152 was added to the medium. Panel e): depicts cell attachment when 1μM of H-1152 was added to the medium. Panel f): depicts cell attachment when 3μM of H-1152 were added to the medium.

Figure 7 shows the detachment of cells of the human embryonic stem cell line H1 from polycarbonate membranes (membrane No. 9 in Table 1) following the removal of Rho kinase inhibitor H-1152 from the cell culture medium. Panel a): depict the attachment of cells when 3μM of H-1152 were maintained in the culture medium. Panel b): depict detachment of cells when H-1152 was removed from the culture medium.

Figure 8 shows the effect of membrane pore size and Rho kinase inhibitor treatment on the attachment of the human embryonic stem cell line H1 to the planar substrates comprising the following: polycarbonate membrane No. 10 in Table 1 in panel a and c; and polycarbonate membrane No. 11 in Table 1 in panel b and d). Panels a and b): depicts the attachment of cells when 3μM of H-1152 were maintained in the culture medium. Panels c and d): depicts the detachment of cells when H-1152 was removed from the culture medium.

Figure 9 shows the maintenance of the expression of markers associated with pluripotency in cells of the human embryonic stem cell line H1 cultured on polycarbonate membranes (membrane No. 8 in Table 1) for three passages. Expression of the genes indicated in the figure was determined by real-time PCR. The solid bars represent data obtained from the undifferentiated human embryonic stem cell line H1. Hashed bars represent data obtained from the cells cultured on polycarbonate membranes.

Figure 10 shows the ability of cells of the human embryonic stem cell line H1 to form embryoid bodies following culture for 12 passages on polycarbonate membranes

(membrane No. 8 in Table 1). The figure shows representative data from a single experiment.

Figure 11 shows scanning electron micrographs of the planar substrates of the present invention.

Figure 12 shows scanning electron micrographs of the ULTRAWEB™ planar substrate.

Figure 13 shows the effect of the defined medium mTESR™ on the binding of cells of the human embryonic stem cell line H1 to various planar substrates.

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

"Adlayer" as used herein refers to a layer that is formed on a surface of a solid substrate, by attaching molecules to the surface by either covalent (also known as grafting) or non-covalent (also known as adsorption) bonds. Molecules used in making an adlayer can, for example, be proteinaceous molecules, which may include, for example, extracellular matrix proteins, amino acids and the like, and non-biological molecules, such as, for example, polyethyleneimine.

"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: NGN3, NKX2.2, NKX6.1, NEUROD, ISL1, 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, refers to cells expressing at least one of the following markers: SOX17, GATA4, HNF3 beta, GSC, CER1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, 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, refers to cells expressing at least one of the following markers: PDX1, HNF1 beta, PTF1 alpha, HNF6, NKX6.1, or HB9. Cells expressing markers characteristic of the pancreatic endoderm lineage include pancreatic endoderm cells, primitive gut tube cells, and posterior foregut cells.

"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: HNF3 beta, GATA4, SOX17, Cerberus, OTX2, goosecoid, C-Kit, CD99, and MIXL1.

"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.

"Extraembryonic endoderm" as used herein refers to a population of cells expressing at least one of the following markers: SOX7, AFP, or SPARC.

"Extracellular matrix proteins" refers to proteinaceous molecules normally found between cells in the body or in the placenta. Extracellular matrix proteins can be derived from tissue, body fluids, such as, for example, blood, or media conditioned by non-recombinant cells or recombinant cells or bacteria.

"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, HNF3 beta, GSC, FGF17, or GATA6.

"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.

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.

“Surface” as used herein refers to the outermost layer of molecules of a solid substrate vessel or matrix intended for use in cell culture or analysis. The elemental composition, the roughness, and the wettability of the surface can be analyzed by X-Ray Photoelectron Spectroscopy (XPS), Atomic Force Microscopy (AFM), and contact angle measurement, respectively.

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.

Planar Substrates of the Present Invention

Planar substrates suitable for use in the present invention may be comprised of any material that is capable of providing a support onto which pluripotent cells may attach. For example, the planar substrate may be comprised of polycarbonate. Alternatively, the planar substrate may be comprised of polyethylene terephthalate (PETE). Alternatively, the planar substrate may be comprised of nylon. Alternatively, the planar substrate may be comprised of cellulose acetate. Alternatively, the planar substrate may be comprised of a mixed cellulose ester. Examples of planar substrates suitable for use in the present invention may be found in Table 1.

In one embodiment, the present invention provides methods for the attachment, cultivation and differentiation of pluripotent stem cells to a planar substrate containing up to about 12% N, from at least about 12% O to at least about 55% O, a contact angle from about 18 degrees to about 32 degrees, and lacking an adlayer and a feeder cell layer. The planar substrate containing from at least about 8% N to at least about 12% N, and from at least about 12% O to at least about 55% O may be a rough fibrous surface, or, alternatively, a smooth surface.

In one embodiment, the present invention provides a method to attach pluripotent stem cells to a planar substrate containing up to about 12% N, from at least about 12% O to at least about 55% O, a contact angle from about 18 degrees to about 32 degrees, and lacking an adlayer and a feeder cell layer, comprising the steps of:

a. Obtaining a suspension of pluripotent stem cells, and

b. Adding the cell suspension to the planar substrate and allowing the cells to attach.

In one embodiment, the pluripotent stem cells are maintained in culture after the cells attach to the surface. In one embodiment, the pluripotent stem cells are differentiated on the planar substrate after the cells attach to the surface.

In one embodiment, the attachment of pluripotent stem cells to a planar substrate containing up to about 12% N, from at least about 12% O to at least about 55% O, a contact angle from about 18 degrees to about 32 degrees, and lacking an adlayer and a feeder cell layer is enhanced by treating the cells with a capable of inhibiting Rho kinase activity. The compound capable of inhibiting Rho kinase activity may be removed from the cells after they have attached.

The compound capable of inhibiting Rho kinase activity is selected from the group consisting of: Y-27632, Fasudil, H-1152 and Hydroxyfasudil.

In one embodiment, the compound capable of inhibiting Rho kinase activity may be used at a concentration from about 0.1μM to about 100μM. In one embodiment, the at least one compound capable of inhibiting Rho kinase activity is used at a concentration of about 10μM.

Characterization of the Planar Substrates of the Present Invention

In one embodiment, the elemental composition of the surface of the planar substrates of the present invention may be analysed by X-Ray Photoelectron Spectroscopy (XPS). XPS, also known as Electron Spectroscopy for Chemical Analysis (ESCA), is used as a method to determine what elements or atoms are present in the surface of a solid substrate (all elements in concentrations greater than 0.1 atomic percent can be detected, except hydrogen and helium), and to determine the bonding environment of such elements or atoms.

In one embodiment, the roughness of the surface of the planar substrates of the present invention may be analyzed by Atomic Force Microscopy (AFM). Surface atoms or molecules with a lateral resolution down to 1 Å and a vertical resolution down to 0.1 Å can be imaged by AFM.

In one embodiment, the wettability of the surface of the planar substrates of the present invention may be analyzed by measuring the contact angle. For example,

contact angle measurement by the static sessile drop method provides information on the interaction between the surface of a solid substrate and a liquid. The contact angle describes the shape of a liquid drop resting on the surface of the solid substrate, and is the angle of contact of the liquid on the surface of the solid substrate, measured within the liquid at the contact line where liquid, solid, and gas meet. A surface with a water contact angle larger than 90° is termed hydrophobic, and a surface with water contact angle less than 90° is termed hydrophilic. On extremely hydrophilic surfaces, that is, surfaces that have a high affinity for water, a water droplet will completely spread (an effective contact angle of 0°).

In one embodiment, the negative charge density of the surface of the planar substrates of the present invention may be analyzed by measuring the reactivity of the surface with crystal violet. Crystal violet carries a positive charge, which enables it to bind to negatively charged molecules and parts of molecules, for example, negatively charged functional groups present on a polymer surface. A surface with a high crystal violet reactivity has a higher density of negative charges than a surface with a low crystal violet reactivity, given that the surfaces have the same roughness and thus area.

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 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 OCT-4 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 to 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 cell.

In one embodiment, human embryonic stem cells are prepared as described by Thomson *et al.* (U.S. Pat. No. 5,843,780; Science 282:1145, 1998; Curr. Top. Dev. Biol. 38:133 ff., 1998; Proc. Natl. Acad. Sci. U.S.A. 92:7844, 1995).

Culture of Pluripotent Stem Cells

In one embodiment, pluripotent stem cells are cultured on a layer of feeder cells or extracellular matrix protein that support the pluripotent stem cells in various ways, prior to culturing according to the methods of the present invention. For example, pluripotent stem cells are cultured on a feeder cell layer that supports proliferation of pluripotent stem cells without undergoing substantial differentiation. The growth of pluripotent stem cells on a feeder cell layer without differentiation is supported using (i) Obtaining a culture vessel containing a feeder cell layer; and (ii) a medium conditioned by culturing previously with another cell type, or a non-conditioned medium, for example, free of serum or even chemically defined.

In another example, pluripotent stem cells are cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of pluripotent stem cells without undergoing substantial differentiation. The growth of pluripotent stem cells in feeder-cell free culture without differentiation is supported using (i) an adlayer on a solid substrate surface with one or more extracellular matrix proteins; and (ii) a medium conditioned by culturing previously with another cell type, or a non-conditioned medium, for example, free of serum or even chemically defined.

In an alternate embodiment, pluripotent stem cells are cultured on a planar surface comprising a mixed cellulose ester in a medium conditioned by culturing previously with another cell type, or a non-conditioned medium, for example, free of serum or even chemically defined.

Culture medium: An example of cell culture medium suitable for use in the present invention may be found in US20020072117. Another example of cell culture medium suitable for use in the present invention may be found in US6642048. Another example of cell culture medium suitable for use in the present invention may be found in WO2005014799. Another example of cell culture medium suitable for use in the present invention may be found in Xu *et al* (Stem Cells 22: 972-980, 2004). Another example of cell culture medium suitable for use in the present invention may be found in US20070010011. Another example of cell culture medium suitable for use in the present invention may be found in Cheon *et al.* (BioReprod DOI:10.1095/biolreprod.105.046870; 19 Oct 2005). Another example of cell culture medium suitable for use in the present invention may be found in Levenstein *et al.* (Stem Cells 24: 568-574, 2006). Another example of cell culture medium suitable for

use in the present invention may be found in US20050148070. Another example of cell culture medium suitable for use in the present invention may be found in US20050233446. Another example of cell culture medium suitable for use in the present invention may be found in US6800480. Another example of cell culture medium suitable for use in the present invention may be found in US20050244962. Another example of cell culture medium suitable for use in the present invention may be found in WO2005065354. Another example of cell culture medium suitable for use in the present invention may be found in WO2005086845.

Suitable culture media may also be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco # 11965-092; Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco # 10829-018; Ham's F12/50% DMEM basal medium; 200 mM L-glutamine, Gibco # 15039-027; non-essential amino acid solution, Gibco 11140-050; β-mercaptoethanol, Sigma # M7522; human recombinant basic fibroblast growth factor (bFGF), Gibco # 13256-029.

Differentiation of Pluripotent Stem Cells

In one embodiment of the present invention, pluripotent stem cells are propagated in culture, while maintaining their pluripotency. Changes in pluripotency of the cells with time can be determined by detecting changes in the levels of expression of markers associated with pluripotency. Alternatively, changes in pluripotency can be monitored by detecting changes in the levels of expression of markers associated with differentiation or markers associated with another cell type.

In an alternate embodiment, pluripotent stem cells are propagated in culture and then treated in a manner that promotes their differentiation into another cell type. The other cell type may be a cell expressing markers characteristic of the definitive endoderm lineage. Alternatively, the cell type may be a cell expressing markers characteristic of the pancreatic endoderm lineage. Alternatively, the cell type may be a cell expressing markers characteristic of the pancreatic endocrine lineage. Alternatively, the cell type may be a cell expressing markers characteristic of the β-cell lineage.

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.

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 Biotechnol. 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 Biotechnol. 24:1392-1401, 2006.

Markers characteristic of the definitive endoderm lineage are selected from the group consisting of SOX17, GATA4, HNF3 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.

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 Biotechnol. 24:1392-1401, 2006.

Markers characteristic of the pancreatic endoderm lineage are selected from the group consisting of PDX1, HNF1 beta, PTF1 alpha, HNF6, HB9 and PROX1. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endoderm lineage. In one aspect of the present invention, a cell expressing markers characteristic of the pancreatic endoderm lineage is a pancreatic endoderm cell.

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

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

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by the methods disclosed in D'Amour *et a*l., Nature Biotechnol. 24: 1392-1401, 2006.

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, HNF3 beta, MAFA, PAX4, and 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 of Human Embryonic Stem Cells to the Planar Substrates of the Present Invention.

The Rho kinase inhibitor Y26732 has been shown to enhance the attachment of human embryonic stem cells on surface modified plates (see U.S. Patent Application No. 61/030,544). The purpose of the studies of the present invention was to determine the ability of human embryonic stem cells to attach to other planar surfaces. The planar surfaces tested in the present invention are shown in Table 1.

Prior to testing, cells of the human embryonic stem cell line H1 cells were expanded on tissue culture plates coated with a 1:30 dilution of growth factor-reduced MATRIGEL®. Cells were seeded onto 100 mm culture dishes in 10 ml MEF conditioned media supplemented with 20 ng/ml bFGF (MEF-CM/bFGF). The cells were cultured at 37°C in a humidified with a 5% CO_2 atmosphere. The media was changed everyday with fresh MEF-CM/bFGF. Once the cells reached approximately 80% confluence, the cells were passaged by treatment with 1mg/ml LIBERASE for 5 minutes at 37°C. The digestion was stopped by removing enzyme from the dish and rinsing the cells with MEF-CM/bFGF. The cells were collected by manual scraping in 10 ml MEF-CM/bFGF and transferred to a 50-ml conical tube. The cells were centrifuged at 200x g (1000rpm) on a tabletop centrifuge to form a pellet. After the

supernatant was removed, the cells were re-suspended in 40 ml MEF-CM/bFGF and evenly distributed in four 100 mm culture dished coated with a 1:30 dilution of growth factor-reduced MATRIGEL®.

Cells of the human embryonic stem cell line H1 were seeded onto the various planar substrates set forth in Table 1, at a density of 100,000 cells/ cm^2. The planar substrates lacked an adlayer and a fibroblast feeder cell layer. The cells were cultured in MEF-CM/bFGF as described above. The effect of the Rho kinase inhibitor H-1152 on the attachment of the cells to the planar substrates was determined. 3μM H-1152 was added to the medium used to seed the cells. Cells were allowed to attach for 24 hrs. After this time, the cells were fixed with 4% paraformaldehyde for 5 minutes at room temperature. The cells were then stained with 1% hematoxylin, and the number of cells was determined via light microscopy. Wells containing vehicle were included as a control.

The cells of the human embryonic stem cell line H1 attached to the following membranes in a Rho kinase inhibitor independent manner: mixed cellulose ester membrane (membrane No. 2, Figure 1, panel a); nylon membrane (membrane No. 4, Figure 1, panel b), and cellulose acetate membrane (membrane No. 5, Figure 1, panel c). The attachment of cells to these membranes was enhanced by addition of 3μM H-1152 (See Figure 1, panels a-c).

Cells of the human embryonic stem cell line H1 required the presence of 3μM H-1152 to attach to the following planar substrates: Polycarbonate membrane (membrane No. 7, Figure 1, panel d) and Polyethylene terephthalate membrane (membrane No. 12, Figure 1, panel e). Removal of H-1152 from the culture medium led to detachment of H1 cells from both types of membranes. No attachment was observed to these membranes in the absence of H-1152.

Example 2: The Effect of Rho Kinase Treatment on the Attachment of Human Embryonic Stem Cells to Planar Substrates Comprising Mixed Cellulose Esters (Membrane No. 1).

Cells of the human embryonic stem cell line H9 were cultured on MATRIGEL® coated dishes prior to experimental manipulation. Cells were seeded a mixed

cellulose ester membrane (membrane No. 1) at a density of 150,000 cells/ cm^2 in MEF conditioned medium. The planar substrate lacked an adlayer and a fibroblast feeder cell layer. The effect of the Rho Kinase inhibitor treatment on the attachment to the planar substrate was examined. Cells were treated with 0, 10, or 20μM Y26732. After 24 hours, the cells were fixed with 4% paraformaldehyde, rinsed with PBS, air dried, stained with crystal violet dye. The number of cells was determined via light microscopy. Wells containing vehicle were included as a control.

Cells were observed to attach to the planar substrate in the absence of Y26732 (Figure 2, panel a). Addition of Y26732 increased the attachment of cells to the planar substrate, at 10 and 20μM (Figure 2, panels b and c). Removal of Y26732 from the culture medium for 24 hr did not result in the detachment of the cells from the planar substrate.

Example 3: The Effect of Culture on Planar Substrate Membrane No. 1 on the Proliferation Rate of Human Embryonic Stem Cells.

The proliferation rate of cells of the human embryonic stem cell line cultured on MATRIGEL® coated dished and cells cultured on Membrane No. 1 was compared. Cells were seeded at equal densities on both substrates. Cells were released from the substrates by TrypLE treatment to create a single cell suspension to determine cell number. Samples of cells were taken at the times indicated in Figure 3. Cells were observed to proliferate at comparable rates. The doubling time is about 1.151 day and 1.138 day on MATRIGEL® and on Membrane No. 1, respectively.

Example 4: Human Embryonic Stem Cells Maintain their Pluripotency for Three Passages on Planar Substrates Comprising Mixed Cellulose Esters (Membrane No. 1).

Cells of the human embryonic stem cell line H1 were seeded on a planar substrate comprising mixed cellulose ester membranes (membrane No. 1) at a density of 75,000 cells/cm^2 in MEF-CM containing 20ng/ml bFGF. The cells were cultured for 5 or 6 days before passaging to reach approximately 75 to 90% confluency according to the methods described above. The culture medium was changed everyday. After culturing for 3 passages, the cells were collected and the expression of markers associated with pluripotency was determined by flow cytometry. As shown in Table

2, over 95% of cells maintained expression of cell surface markers associated with pluripotency, including Tra1-60, Tra1-81, SSEA-3, and SSEA-4, indicating the cells were still pluripotent.

Example 5: Human Embryonic Stem Cells Maintain a Stable Karyotype for Ten Passages on Planar Substrates Comprising Mixed Cellulose Esters (Membrane No. 1).

Cells of the human embryonic stem cell line H1 were cultured either on MATRIGEL® coated culture plates or on mixed cellulose esters membrane for 10 passages. The cells were cultured according to the methods described above. The karyotype was determined by cytogenetic analysis by analyzing twenty G-banded metaphase cells. As shown in Figure 4, the G-banded chromosomes of a representative cell cultured on MATRIGEL® coated culture plates (Figure 4, panel a) and those of another cell cultured on mixed cellulose membrane (Figure 4, panel b) demonstrate a normal male karyotype.

The karyotype was also determined by examining two hundred interphase nuclei by fluorescence in situ hybridization (FISH) using a chromosome 12p probe and a 17q probe to identify very small populations of cells with changes in chromosome 12 and 17 copy number that can not be detected by routine cytogenetics. In cells cultured on MATRIGEL® and on mixed cellulose esters membranes, no abnormal cells with trisomy 12 and/or 17 were detected.

Example 6: Human Embryonic Stem Cells Are Able To Differentiate To Insulin-Producing Cells on Planar Substrates Comprising Mixed Cellulose Esters (Membrane No. 1).

Cells of the human embryonic stem cell line H1 were seeded on a planar substrate comprising mixed cellulose esters (Membrane No. 1) at a density of 150,000 cells/ cm^2 in MEF conditioned medium containing 20ng/ml bFGF. The cells were differentiated to insulin-producing cells by treating the cells according to the differentiation protocol outlined in Table 3. The cells were cultured in MEF conditioned medium containing 20ng/ml bFGF for 3 to 4 days to reach approximately 75 to 90% confluency. The cells were treated in DMEM-F12 medium containing 2% Fatty-Acid Free Bovine Serum Albumin (FAF-BSA), 100 ng/ml activin A, and 20

ng/ml Wnt3A for two days, followed by treatment with DMEM-F12 medium, 2% Fatty-Acid Free Bovine Serum Albumin (FAF-BSA), and 100 ng/ml activin A for another two days. Next, the cells were treated in DMEM-F12 medium containing 2% BSA, 20 ng/ml FGF7, and 250 nM Cyclopamine-KAAD for three days, followed the treatment in DMEM-F12 medium containing 1% B27 supplement, 20 ng/ml FGF7, 250 nM Cyclopamine-KAAD, 2 μM retinoic acid (RA), and 100 ng/ml Noggin for 4 days. The cells were treated in DMEM-F12 medium containing 1% B27 supplement, 1 μM ALK5 inhibitor 2 (Axxora Cat. No: ALX-270-445-M001), 100 ng/ml Noggin, 100 ng/ml Netrin-4, 50 ng/ml Exendin-4, and 1 μM DAPT for 3 days. The cells were cultured in DMEM-F12 medium, 1% B27 supplement, and 1 μM ALK5 inhibitor 2 for 7 days and in DMEM-F12 medium containing 1% B27 supplement for another 7 days.

At the end of the differentiation protocol, RNA samples were collected to determine the expression of markers characteristic of the pancreatic endocrine lineage. A CT number for insulin of about 17 was observed. The corresponding CT value for GAPDH was about 19; these data suggests that the cells expressed high levels of insulin following treatment.

Example 7: Human Embryonic Stem Cells Attach to Planar Substrates Comprising Polycarbonate Membranes in a Rho Kinase Inhibitor Dependent Manner.

Cells of the human embryonic stem cell line H9 were seeded onto a planar substrate comprising polycarbonate (Membrane No. 7) at a density of 150,000 cells/cm^2 in MEF conditioned medium. The effect of Rho kinase inhibitor treatment on attachment was examined: The Rho Kinase inhibitor Y26732 was added to the culture medium at concentration of 0, 10, or 20μM. After 24 hours, the cells on the membrane were fixed with 4% paraformaldehyde at room temperature, rinsed with PBS, air dried, stained with crystal violet dye. The number of cells was determined via light microscopy. Wells containing vehicle were included as a control.

The cells do not attach to the membrane in control dishes (Figure 5, panel a). Addition of Y26732 resulted in the attachment of the cells on the membranes (Figure 5, panels b and c).

In a separate experiment, the effect of the Rho kinase inhibitor H-1152 on the attachment of cells of the human embryonic stem cell line H1 to Membrane No. 7 was determined. The cells were seeded on a planar substrate comprising polycarbonate membranes (membrane No. 7) at a density of 150,000 cells/cm^2 in MEF-CM containing 20ng/ml bFGF. The Rho Kinase inhibitor H-1152 was added to the culture medium at concentration of 0, 0.03, 0.1, 0.3, 1, and 3μM. After 24 hours, the cells on the membrane were fixed with 4% paraformaldehyde, rinsed with PBS, air dried, stained with crystal violet dye. The number of cells was determined via light microscopy. Wells containing vehicle were included as a control.

The cells do not attach to the membrane in the control dish (Figure 6, panel a) and in the dishes with 0.03 or 0.1μM of H-1152 (Figure 6, panels b and c). However, attachment was observed in cultures treated with 0.3, 1, and 3μM of H-1152 (Figure 6, panels d-f).

Example 8: Removal of the Rho Kinase Inhibitor from the Culture Medium Results in the Detachment of Human Embryonic Stem Cells from Planar Substrates Comprising Polycarbonate Membranes.

Cells of the human embryonic stem cell line H1 were seeded on to a planar substrate comprising polycarbonate (Membrane No. 9) at a density of 100,000 cells/ cm^2, in MEF conditioned medium containing 20ng/ml bFGF and 3μM of the Rho kinase inhibitor H-1152. The cells were cultured for 24 hr. After this time, the culture medium was replaced with MEF conditioned medium containing 20ng/ml bFGF, lacking H-1152. After 24 hours, the cells on the membrane were fixed with 4% paraformaldehyde, rinsed with PBS, air dried, stained with crystal violet dye. The number of cells was determined via light microscopy. Wells containing H-1152 were included as a control. Removal of H-1152 from the culture medium resulted in the detachment of cells from the planar substrate (Figure 7).

Example 9: Porosity of the Planar Substrate Affects the Attachment of Human Embryonic Stem Cells.

Cells of the human embryonic stem cell line H1 at passage 42 were seeded onto the following planar substrates: Membrane No. 10 (pore size 0.4 μm); and Membrane

No. 11 (pore size 3 μm). Cells were seeded at a density of 100,000 cells/cm^2, in MEF conditioned medium containing 20ng/ml bFGF. The effect of Rho kinase inhibition on the attachment of the cells to the planar substrates was also examined. The cell culture medium was supplemented with .3μM H-1152. After 24 hours, the culture medium was replaced with MEF conditioned medium containing 20ng/ml bFGF, lacking H-1152. After another 24 hours culture, the cells on the membrane were fixed with 4% paraformaldehyde, rinsed with PBS, air dried, stained with crystal violet dye. Wells containing 1μM H-1152 were included as a control. The number of cells was determined via light microscopy. Wells containing vehicle were included as a control.

A greater number of cells attached to Membrane No. 10 (Figure 8, panel a) than Membrane No. 11 (Figure 8, panel b). Presence of 1μM H-1152 in the culture medium is required to maintain the attachment of H1 cells on the membranes (Figure 8, panel a and b). Removal of H-1152 from the culture medium resulted in detachment of cells from Membrane No. 10 and Membrane No. 11 (Figure 8, panel c and d).

Example 10: Human Embryonic Stem Cells Maintain their Pluripotency After Multiple Passages on Planar Substrates Comprising Polycarbonate Membranes.

Cells of the human embryonic stem cell line H1 were seeded on to a planar substrate comprising polycarbonate membrane (Membrane No. 8). Cells were cultured in MEF conditioned medium containing 20ng/ml bFGF, supplemented with 3μM H-1152. The cell culture medium was changed daily. Cells were passaged by the removal of H-1152 from the medium, and the cells were removed from the planar substrate by gentle swirling. The cells were cultured for 3 passages and collected for flow cytometry and quantitative RT-PCR analysis. As shown in Table 4, over 95% of the cells expressed cell surface markers associated with pluripotency, including Tra1-60, Tra1-81, SSEA-3, and SSEA-4, as determined by flow cytometry. Figure 9 shows the results of quantitative RT-PCR, indicating multiple genes expressed in the H1 cultured on polycarbonate membranes for 3 passages are at comparable levels as in undifferentiated H1 cells.

In a separate study, cells of the human embryonic stem cell line H1 were seeded on to a planar substrate comprising polycarbonate membrane (Membrane No. 8). Cells were cultured in MEF conditioned medium containing 20ng/ml bFGF, supplemented with 1μM H-1152. The cell culture medium was changed daily. Cells were passaged by the removal of H-1152 from the medium, and the cells were removed from the planar substrate by gentle swirling. The cells were cultured for 9 passages and collected for flow cytometry. As shown in Table 5, over 95% of the cells express cell surface markers associated with pluripotency, including Tra1-60, Tra1-81, SSEA-3, and SSEA-4.

An alternative method to assess pluripotency is via the ability of the cells to form embryoid bodies. Cells of the human embryonic stem cell line H1 were seeded on to planar substrates comprising polycarbonate membranes (Membrane No. 8). The cells were cultured in MEF conditioned medium containing 20ng/ml bFGF, supplemented with 3μM H-1152. The cell culture medium was changed daily. Cells were passaged by the removal of H-1152 from the medium, and the cells were removed from the planar substrate by gentle swirling. The cells were cultured for 12 passages.

Embryoid body formation was achieved by the following protocol. The H1 cells were collected and cultured in DMEM/F12 medium supplemented with 20% fetal bovine serum in Ultra Low Cluster Plate (Corning Cat. No: 3471). The cells were fed every other day by changing 50% of the medium. Embryoid bodies were formed after 14 days (Figure 10).

Example 11: Human Embryonic Stem Cells are Capable of Forming Definitive Endoderm after Cultured on Planar Substrates Comprising Polycarbonate Membranes.

Cells of the human embryonic stem cell line H1 were seeded on to a planar substrate comprising polycarbonate (Membrane No. 8). The cells were initially cultured in MEF conditioned medium containing 20ng/ml bFGF, supplemented with 3μM H-1152. The cells were then cultured in MEF conditioned medium containing 20ng/ml bFGF, supplemented with 1μM H-1152 for 10 passages prior to experimental manipulation.

The cells were then seeded onto 100 mm tissue culture plates, coated with a 1:30 dilution of MATRIGEL®. The cells were cultured in MEF conditioned medium containing 20ng/ml bFGF for 3 days. Next, the cells were treated in DMEM/F12, supplemented with 2% fatty acid free Bovine Serum Albumin, 100ng/ml activin A, and 20ng/ml Wnt3a for two days and then treated with DMEM/F12, supplemented with 2% fatty acid free Bovine Serum Albumin, and 100ng/ml activin A for another two days. After this time, the cells were released by TRYPLE treatment to form a single cell suspension and the expression of markers characteristic of the definitive endoderm lineage was determined by flow cytometry.

Over 90% of the cells are CD99 and CXCR4 (CD184) double positive and 12% of the cells are CD9 positive CXCR4 negative, as shown in Table 6. These data suggest that the cells retain the capacity to differentiate into definitive endoderm.

Example 12: Physical Properties of the Planar Substrates of the Present Invention.

The surface chemistry was determined on the planar substrates of the present invention. Tables 7-10 depict the X-ray Photoelectron spectroscopy (XPS) analysis and contact angle. For XPS, an analysis depth of approximately 50-100 Å was used. Typically, 95% of the signal originates from within this depth.

Membranes 1-3 contained similar concentrations of oxygen, carbon (mainly as C-O, and C-(C,H), probably O-C-O), and nitrogen (as NO_3, NO_2, and possibly C-N, and R_4-N^+). Membrane 3 also contained trace concentrations of Na^+ and SO_x and a higher concentration of C-(C,H). Membrane 4 contained C-(C,H), C-(O,N), and (O,N)-C=O and possibly a trace of sodium. Membrane 5 contained mainly C-O and also C-(C,H) and O-C-O and/or O-C=O. Trace concentrations of Na^+ and SO_x were also detected. Membranes 6-11 contained C-(C,H), C-O, O-C=O, C-N, CO3, p-p*, and trace concentrations of R_4-N^+, SO_x, and either Na^+ or Cr^{3+}. The surface of membrane 6 may also contain a trace concentration of chlorine. Trace concentrations of chromium were detected only on membranes 10 and 11, while Na^+ was detected on membranes 6 – 9. The surface of membrane 12 contained C-(C,H), C-O, O-C=O, and pi-pi* consistent with PET. Trace concentrations of nitrogen and sodium were also detected.

Figure 11 shows the scanning electron micrographs of the planar substrates of the present invention. Two types of morphologies were observed. One type was characterized by an open network of fibers. The second type was characterized by a smooth sheet with circular holes dispersed across the surface.

Table 10 shows the contact angle measurements from the surfaces of the present invention. Surfaces 1 through 5 had contact angle measurements from about 18° to about 32°. Pluripotent stem cells did not require the presence of an inhibitor of Rho kinase activity in order to attach to surfaces 1-5.

Surfaces 6 through 12 had contact angle measurements greater than 32°. Pluripotent stem cells required the presence of an inhibitor of Rho kinase activity in order to attach to these surfaces.

Example 13: Attachment of Pluripotent Stem Cells to a Planar Substrate Consisting of a Polyamine.

The planar substrate consisting of polyamine was manufactured according to the methods disclosed in US6743273, and Schindler M *et al*, Biomaterials 26(28): 5624-5631; 2005. The planar substrate is available commercially, sold under the trademark ULTRAWEB™. ULTRAWEB™ synthetic surfaces are composed of randomly orientated electrospun polyamide nanofibers with an average fiber diameter of 280nm. The fiber size distribution is between 200 and 400nm. The first ULTRAWEB™ surface tested had a slightly hydrophilic surface (catalogue #3870XX1) while the second surface, surface (catalogue #3871XX1) was slightly hydrophilic and was coated with a polyamine material which providedthe nanofibers with a free amine groups for a net positive charge. Both surfaces are highly effective at protein absorption through hydrophobic interactions. 5 micron resolution and 10,000X magnification scanning electron micrographs are shown in Figure 12. However, cells of the human embryonic stem cell line H1 were unable to attach to either of the UTRAWEB™ surfaces tested.

Example 14: The Effect of the use of Defined Medium on the Attachment of Pluripotent Stem Cells to the Planar Substrates of the Present Invention.

Cells of the human embryonic stem cell line H1 were seeded onto the following planar substrates: Membrane 1 (mixed cellulose ester), Membrane 4 (nylon), Membrane 5 (cellulose acetate) and nitrocellulose. Cells were seeded at a 1:3 dilution in the defined medium mTESR™ and cultured for 24 hours. Parallel cultures un MEF-conditioned medium were included as controls. Culture of the cells in mTESR™ did not affect the ability of the cells to attach to the planar surfaces. Cells were able to attach to membranes 1, 4, and 5, and nitrocellulose. The membrane that showed the greatest binding of cells using mTESR™ was Membrane 4, followed by Membrane 5, which was equal to nitrocellulose, followed by Membrane 1.

Table 1: Characterization of membranes suitable for use in the present invention.

Membrane	Vendor	Catalog Number	Chemical composition	Hydrophobicity	Porosity	Surface	BSA binding capacity	Attachment of hES cells
1	Millipore	PIHA03050	Mixed cellulose esters	Hydrophilic	0.45 micrometer	Rough, fibrous	Greater than 160 micrograms per centimeter square	Rho kinase independent
2	Pall	66276	Mixed cellulose esters	Hydrophilic	0.8 micrometer	Rough, fibrous		Rho kinase independent
3	Sterlitech	MCE4525100	Mixed cellulose esters	Hydrophilic	0.45 micrometer	Rough, fibrous	Approximately 160 micrograms per centimeter square	Rho kinase independent
4	Sterlitech	NY4525100	Nylon	Hydrophilic	0.45 micrometer	Rough, fibrous	Greater than 120 micrograms per centimeter square	Rho kinase independent
5	Sterlitech	CA0225100	Cellulose Acetate	Hydrophilic	0.2 micrometer	Rough, fibrous	3.8 micrograms per centimeter square	Rho kinase independent
6	Sterlitech	PCT0425100	Polycarbonate	Hydrophilic	0.4 micrometer	Smooth, thin, glass-like	Less than 5 micrograms per centimeter square	Rho kinase dependent
7	Millipore	PIHP03050	Polycarbonate	Hydrophilic	0.4 micrometer	Smooth, thin, glass-like	Less than 5 micrograms per centimeter square	Rho kinase dependent
8	Millipore	ATTP04700	Polycarbonate	Hydrophilic	0.8 micrometer	Smooth, thin, glass-like	Less than 5 micrograms per centimeter square	Rho kinase dependent
9	Corning	3420	Polycarbonate	Hydrophilic	3 micrometer	Smooth, thin, glass-like	Less than 5 micrograms per centimeter square	Rho kinase dependent
10	Nunc	137060	Polycarbonate	Hydrophilic	0.4 micrometer	Smooth, thin, glass-like	Less than 5 micrograms per centimeter square	Rho kinase dependent

11	Nunc	137435	Polycarbonate	Hydrophilic	3 micrometer	Smooth, thin, glass-like	Less than 5 micrograms per centimeter square	Rho kinase dependent
12	Millipore	PISP30R48	Polyethylene terephthalate	Hydrophilic	3 micrometer	Smooth, thin, glass-like	Less than 5 micrograms per centimeter square	Rho kinase dependent

Table 2: Expression of cell surface markers associated with pluripotency on human embryonic stem cell line H1 after propagated on mixed cellulose esters membranes for 3 passages, as determined by flow cytometry.

Surface markers	Percentage of the positive cells
Tra1-60	98.4%
Tra1-81	98.8%
SSEA-3	97.5%
SSEA-4	98.1%

Table 3: Protocol to treat human embryonic stem cells to induce differentiation to insulin-producing cells.

Time	Treatment
2 days	DMEM-F12 medium 2% Fatty-Acid Free Bovine Serum Albumin (FAF-BSA) 100 nanogram per milliliter ActivinA 20 nanogram per milliliter Wnt3A
2 days	DMEM-F12 medium 2% Fatty-Acid Free Bovine Serum Albumin (FAF-BSA) 100 nanogram per milliliter ActivinA
3 days	DMEM-F12 medium 2% BSA 20 nanogram per milliliter FGF7 250 nanomole Cyclopamine-KAAD
4 days	DMEM-F12 medium 1% B27 supplement 20 nanogram per milliliter FGF7 250 nanomole Cyclopamine-KAAD 2 micromole Retinal Acid (RA) 100 nanogram per milliliter Noggin
3 days	DMEM-F12 medium 1% B27 supplement 1 micromole ALK5 inhibitor 2 100 nanogram per milliliter Noggin 100 nanogram per milliliter Netrin-4 50 nanogram per milliliter Exendin-4 1micromole DAPT
7 days	DMEM-F12 medium 1% B27 supplement 1 micromole ALK5 inhibitor 2
7 days	DMEM-F12 medium 1% B27 supplement

Table 4: Expression of cell surface markers associated with pluripotency on human embryonic stem cell line H1 after cultured on polycarbonate membranes for 3 passages, as determined by flow cytometry.

Surface markers	Percentage of the positive cells
Tra1-60	97.0%
Tra1-81	96.0%
SSEA-3	97.6%
SSEA-4	97.2%

Table 5: Expression of cell surface markers associated with pluripotency on human embryonic stem cell line H1 after propagated on polycarbonate membranes for 9 passages, as determined by flow cytometry.

Surface markers	Percentage of the positive cells
Tra1-60	96.8%
Tra1-81	96.9%
SSEA-3	95.0%
SSEA-4	99.7%

Table 6: Expression of cell surface markers associated with definitive endoderm on human embryonic stem cell line H1. The cells were propagated on polycarbonate membranes for 10 passages and treated for definitive endoderm differentiation.

	CD9 negative cells	CD9 positive cells	CD99 negative cells	CD99 positive cells
CXCR4 positive cells	69.4%	7.6%	0.9%	76.8%
CXCR4 negative cells	12.0%	11.0%	5.1%	17.1%

Table 7: Surface Chemistry (atomic concentration in %).

Sample	C	N	O	Na	S	Cr
1	39.3	8.8	51.9	-	-	-
2	38.2	9.6	52.1	-	-	-
3	41.3	8.5	49.1	0.7	0.5	-
4	75.9	11.9	12.2	?	-	-
5	59.5	-	40.3	0.2	0.1	-
6*	82.0	3.5	14.2	0.2	0.1	-
7	79.1	0.8	19.6	0.3	0.2	-
8	82.2	3.1	14.3	0.2	0.2	-
9	77.2	1.1	21.5	0.1	0.1	-
10	76.2	2.9	20.0	-	0.6	0.3
11	80.4	0.6	18.5	-	0.2	0.3
12	70.2	0.2	29.5	$0.1^{?}$	-	-

Table 8: Concentrations (in %) of Carbon Functional Groups

	C-(C,H)		C-(O,N)		O-C-O / O-C=O		CO_3		pi-pi*	
Sample	**B.E.**	**Atom%**	**B.E.**	**Atom%**	**B.E.**	**Atom%**	**B.E.**	**Atom%**	**B.E.**	**Atom%**
1	284.8	2.3	287.0	29.7	288.4	7.3	-	-	-	-
2	284.8	1.7	287.1	30.0	288.5	6.5	-	-	-	-
3	284.8	7.6	287.0	27.1	288.4	6.6	-	-	-	-
4	284.8	51.8	285.9	12.9	287.7	11.2	-	-	-	-
5	284.8	14.9	286.3	28.8	288.5	15.8	-	-	-	-
6	284.8	55.5	286.1	18.2	287.6	2.8	290.9	4.2	292.4	1.3
7	284.8	56.5	286.4	13.6	288.3	1.4	290.4	2.8	291.3	4.7
8	284.8	55.8	286.1	17.4	287.5	2.8	290.8	4.1	292.1	2.1
9	284.8	54.1	286.4	13.0	288.5	2.1	290.4	3.1	291.2	4.9
10	284.8	52.5	286.3	13.6	288.6	3.4	290.6	4.2	291.9	2.6
11	284.8	60.8	286.4	11.7	288.6	$0.2^{?}$	290.7	4.8	292.0	2.8
12	284.8	39.2	286.4	15.5	288.8	13.2	290.5	$0.5^{?}$	291.6	1.9

Table 9: Concentrations (in %) of Nitrogen Functional Groups

	C-N		R_4-N^+		NO_2		NO_3	
Sample	**B.E.**	**Atom%**	**B.E.**	**Atom%**	**B.E.**	**Atom%**	**B.E.**	**Atom%**
1	400.0	0.1[?]	401.6	0.1[?]	404.5	0.5	407.5	8.1
2	-	-	-	-	404.6	0.4	407.5	9.2
3	400.0	0.1[?]	401.7	0.1[?]	404.4	0.4	407.4	7.8
4	400.0	11.9	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	400.0	3.5	-	-	-	-	-	-
7	400.0	0.4	401.7	0.3	406.1	0.1[?]	-	-
8	400.0	2.9	401.8	0.1[?]	-	-	408.3	0.1[?]
9	400.0	0.6	401.7	0.4	405.8	0.1[?]	-	-
10	400.0	2.1	401.5	0.8	-	-	-	-
11	400.0	0.2[?]	401.8	0.4	-	-	-	-
12	400.0	0.1[?]	401.7	0.1[?]	-	-	-	-

Table 10: Contact angle measurements of the plates of the present invention

	Contact Angle Reading Water (°)				Contact Angle Reading (°)				
Sample Number	1	2	3	Ave	1	2	3	Ave	Comment
1	110.9	-	-	110.9	18.32	-	-	18.32	
2	55.4	59.96	60.44	58.6	28.13	-	-	28.13	
3	25.54	19.79	-	22.67	29.76	25.53	-	27.65	
4	29.36	31.24	32.89	31.16	31.09	25.88	-	28.49	
5	-	-	-	-	-	-	-	-	Unable to measure
6	90.42	-	-	-	32.07	-	-	32.07	
7	63.59	-	-	-	49.49	-	-	49.49	
8 gloss	58.8	-	-	58.8	41.29	-	-	41.29	Sample had glossy appearance
8 matt	72.69	-	-	72.69	43.3	-	-	43.3	Sample had matt appearance
9	71.89	-	-	71.89	33.82	-	-	33.82	
10	-	-	-	-	-	-	-	-	Unable to measure
11	-	-	-	-	-	-	-	-	Unable to measure
12	76.96	65.187	-	71.07	46.31	-	-	46.31	-

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 to attach pluripotent stem cells to a planar substrate containing up to about 12% N, from at least about 12% O to at least about 55% O, a contact angle from about 18 degrees to about 32 degrees, and lacking an adlayer and a feeder cell layer, comprising the steps of:

    a. Obtaining a suspension of pluripotent stem cells, and

    b. Adding the cell suspension to the planar substrate and allowing the cells to attach.

2. The method of claim 1, wherein the cells are maintained in culture after they have attached to the substrate.

3. The method of claim 1, wherein the cells are differentiated after they have attached to the substrate.

4. The method of claim 1, wherein the attachment of the pluripotent stem cells to the substrate is enhanced by treating the suspension of cells with a compound capable of inhibiting Rho kinase activity.

5. The method of claim 4, wherein the compound capable of inhibiting Rho kinase activity is removed after the pluripotent stem cells attach to the substrate.

6. The method of claim 4, wherein the compound capable of inhibiting Rho kinase activity is selected from the group consisting of: Y-27632, Fasudil, H-1152 and Hydroxyfasudil.