CELL CULTURE MEDIUM

BACKGROUND

[0001] This application claims priority from U.S. Provisional Patent Application Serial No. 61/424,273, filed December 17, 2010; U.S. Provisional Patent Application Serial No. 61/488,353, filed May 20, 2011; U.S. Provisional Patent Application Serial No. 61/533,063, filed September 9, 2011; U.S. Provisional Patent Application Serial No. 61/537,940, filed September 22, 2011; U.S. Provisional Patent Application Serial No. 61/565,380, filed November 30, 2011; and U.S. Provisional Patent Application Serial No. 61/565,849, filed December 1, 2011. The entireties of those provisional applications are fully incorporated by reference herein.

[0002] This application relates to cell biology, cell differentiation, cell therapy, molecular biology, proteins, recombinant human proteins, nucleic acids, and laminins.

[0003] Basal laminae (basement membranes) are sheet-like, cell-associated extracellular matrices that play a central role in cell growth, cellular differentiation, cell phenotype maintenance, tissue development, and tissue maintenance. They are present in virtually all tissues, and appear in the earliest stages of embryonic development.

[0004] Basal laminae are central to a variety of architectural and cell-interactive functions. For example:

[0005] 1. They serve as architectural supports for tissues, providing adhesive substrata for cells.

[0006] 2. They create perm-selective barriers between tissue compartments that impede the migration of cells and passively regulate the exchange of macromolecules. These properties are illustrated by the kidney glomerular basement membrane, which functions as an important filtration structure, creating an effective blood-tissue barrier that is not permeable to most proteins and cells.

[0007] 3. Basal laminae create highly interactive surfaces that can promote cell migration and cell elongation during embryogenesis and wound repair. Following an injury, they provide a surface upon which cells regenerate to restore normal tissue function.

[0008] 4. Basal laminae present information encoded in their structure to contacting cells that is important for cellular differentiation, prevention of apoptosis,

and tissue maintenance. This information is communicated to the cells through various receptors that include the integrins, dystroglycan, and cell surface proteoglycans. Signaling is dependent not only on the presence of matrix ligands and corresponding receptors that interact with sufficient affinities, but also on such topographical factors as ligand density in a three-dimensional matrix "landscape", and on the ability of basal lamina components to cluster receptors. Because these matrix proteins can be long-lived, basal laminae create a "surface memory" in the basal lamina for resident and transient cells.

[0009] The basal lamina is largely composed of laminin and type IV collagen heterotrimers that in turn become organized into complex polymeric structures. Additional components include proteoglycans such as agrin and perlecan and nidogens (entactins). To date, six type IV collagen polypeptide chains and at least twelve laminin subunit chains have been identified. These chains possess shared and unique functions and are expressed with specific temporal (developmental) and spatial (tissue-site specific) patterns.

[0010] Laminins are a family of heterotrimeric glycoproteins that reside primarily in the basal lamina. They function via binding interactions with neighboring cell receptors on the one side, and by binding to other laminin molecules or other matrix proteins such as collagens, nidogens or proteoglycans. The laminin molecules are also important signaling molecules that can strongly influence cellular behavior and function. Laminins are important in both maintaining cell/tissue phenotype, as well as in promoting cell growth and differentiation in tissue repair and development.

[0011] Laminins are large, multi-domain proteins, with a common structural organization. The laminin molecule integrates various matrix and cell interactive functions into one molecule.

[0012] A laminin protein molecule comprises one α-chain subunit, one β-chain subunit, and one γ-chain subunit, all joined together in a trimer through a coiled-coil domain. **FIG. 1** depicts the resulting structure of the laminin molecule. The twelve known laminin subunit chains can form at least 15 trimeric laminin types in native tissues. Within the trimeric laminin structures are identifiable domains that possess binding activity towards other laminin and basal lamina molecules, and membrane-bound receptors. **FIG. 2** shows the three laminin chain subunits separately. For example, domains VI, IVb, and IVa form globular structures, and domains V, IIIb, and IIIa (which contain cysteine-rich EGF-like elements) form rod-like structures.

Domains I and II of the three chains participate in the formation of a triple-stranded coiled-coil structure (the long arm).

[0013] There exist five different alpha chains, three beta chains and three gamma chains that in human tissues have been found in at least fifteen different combinations. These molecules are termed laminin-1 to laminin-15 based on their historical discovery, but an alternative nomenclature describes the isoforms based on their chain composition, e.g. laminin-111 (laminin-1) that contains alpha-1, beta-1 and gamma-1 chains. Four structurally defined family groups of laminins have been identified. The first group of five identified laminin molecules all share the β1 and γ1 chains, and vary by their α-chain composition (α1 to α5 chain). The second group of five identified laminin molecules, including laminin-521, all share the β2 and γ1 chain, and again vary by their α-chain composition. The third group of identified laminin molecules has one identified member, laminin-332, with a chain composition of α3β3γ2. The fourth group of identified laminin molecules has one identified member, laminin-213, with the newly identified γ3 chain (α2β1γ3).

[0014] There have been no reports of isolated laminin-521 that is free of other laminin chains. Thus far, there are no studies on the function of laminin-521. Attempts to purify laminin-521 from cell sources by affinity chromatography using laminin chain antibodies have been unsuccessful in eliminating, for example, laminin β1 chain, which is a component of laminin-411 and laminin-511. It would be desirable to provide compositions that contain laminin-521 (aka LN-521) and methods for making laminin-521.

[0100] Human embryonic stem (hES) cells hold promise for the development of regenerative medicine for a variety of diseases, such as spinal cord and cardiac injuries, type I diabetes and neurodegenerative disorders like Parkinson's disease. A stem cell is an undifferentiated cell from which specialized cells are subsequently derived. Embryonic stem cells possess extensive self-renewal capacity and pluripotency with the potential to differentiate into cells of all three germ layers. They are useful for therapeutic purposes and may provide unlimited sources of cells for tissue replacement therapies, drug screening, functional genomics and proteomics.

[0015] A prerequisite for the development of stem cell derived cells for regenerative medicine are methods that allow long-term cultures of pluripotent stem cells, chemically defined and repeatable differentiation protocols, as well as xeno-free cell culture systems. However, culturing of pluripotent human embryonic stem

cells (hES cells) and induced pluripotent stem cells (hiPS cells) has encountered a number of problems. One major problem has been that hES cells grow slowly in clusters that need to be manually split for cell propagation. Dissociation of the cells usually leads to extensive cell death, the cloning efficiency of hES cells after complete dissociation being ≤1 %.

[0016] Maintenance of pluripotent hES cells has required complex culture substrata, such as extracellular matrix protein mixtures like the mouse tumor derived Matrigel or fibroblast feeder cell layers, that may be immunogenic and toxic and that generate extensive batch to batch variability reducing reliability of the experiments. Thus far, the most successful feeder cell free substrate used for hES cell cultures is Matrigel, a complex tumor and BM-like extract obtained from murine Engelbreth-Holm-Swarm (EHS) sarcoma tumor tissues. Matrigel mainly contains murine LN-111, type IV collagen, perlecan and nidogen, but also varying amounts of other materials, including growth factors and cellular proteins and, therefore, its composition is undefined and varies from batch-to-batch. This variability can cause irreproducibility of scientific results, and due to the animal origin of the substratum makes Matrigel unacceptable for the expansion and maintenance of hES cells for human cell therapy.

[0017] However, successful development of more or less defined coating materials that support self-renewal of hES and hiPS cells has recently been reported. It has been reported that recombinant vitronectin supports adhesion and self-renewal of hES cells. An acrylate coating containing a variety of peptides from various ECM proteins has also been previously developed, and it has been shown that a synthetic methacrylate-based polymer also facilitated adhesion and self-renewal of hES cells.

[0018] One of the main problems with large-scale propagation of hES cells is that they poorly survive replating after dissociation into single cell suspension. This, in turn, makes passaging tedious and large-scale automated expansions impossible. However, hES cells released into single cell suspension using trypsin treatment in the presence of a rho-kinase (ROCK) inhibitor[10] or blebbistatin[11] can be plated and expanded from single clones, but the molecules are not components of the natural stem cell niche, and they affect the actin cytoskeleton and thus can cause cell damage. Therefore, the use of the ROCK inhibitor may not be a preferred solution for long-term expansion of hES cells aimed for cell therapy purposes.

[0019] For the purposes of regenerative medicine, there is a desire to develop methods that allow derivation and long-term cultures of pluripotent stem cells under chemically defined, xeno-free, pathogen-free, and stable batch-to-batch conditions. Moreover, such methods should allow fast and economically efficient scale-up to acquire large quantities of pluripotent hES/hiPS cells in a short period of time. Preferably, the methods should also allow clonal survival of human ES cells in media containing no synthetic inhibitor of apoptosis, that could facilitate scientific and clinical applications involving cell sorting or gene knock-out in the cells.

BRIEF DESCRIPTION

[0020] The present disclosure provides isolated laminin-521 (also known as LN-521 or laminin-11) and methods for producing isolated laminin-521. In further aspects, the present disclosure provides recombinant host cells that express laminin-521 chains and secrete recombinant laminin-521.

[0021] In other aspects, the present disclosure provides GMP quality laminin-521 for culturing cells for differentiation and maintenance for the purpose of developing cells for human cell therapy. The present disclosure also provides pharmaceutical compositions, comprising isolated laminin-521 together with a pharmaceutically acceptable carrier. Such pharmaceutical compositions can optionally be provided with other extracellular matrix components.

[0022] The present disclosure also provides methods to effectively generate amounts of isolated laminin-521 for various uses. In preferred embodiments of those uses, recombinant laminin-521 is used. Kits comprising an amount of isolated laminin-521, or pharmaceutical compositions thereof, effective for the desired effect, and instructions for the use thereof, are also disclosed.

[0023] The present disclosure also provides methods for culturing stem cells in monolayer cultures which facilitates cellular homogeneity, removal of the stem cells from a cell culture plate or other cellular support in single cell suspension, and replating stem cells in single cell suspension for passaging and expansion in significant dilutions that enable expansion of stem cell cultures and large scale production of such cells.

[0024] In further aspects, the present disclosure describes culturing pluripotent stem cells on laminin-521 in monolayer culture, removing stem cells from the cell culture plates along with other cell supporting materials in single cell suspension,

and replating the stem cells from single cell suspension as single cells on a matrix containing laminin-521 such that this process can be performed in automated robotic systems with high efficiency.

[0025] In further aspects, the present disclosure provides improved medical devices and grafts, wherein the improvement comprises providing medical devices and grafts with an effective amount of isolated laminin-521.

[0026] In further aspects, the disclosure provides improved cell culture devices, and methods for preparing improved cell culture devices, for the growth and maintenance of phenotypes of cells in culture, by providing an effective amount of isolated laminin-521 to a cell culture device for cell attachment, and subsequent cell stasis, proliferation, differentiation, and/or migration.

[0027] In other aspect, the disclosure provides compositions, such as human recombinant laminin-521, and methods for culturing and rapid expanding of human embryonic stem cells in vitro in undifferentiated state. The methods comprise single cell dissociation of human embryonic stem cells and plating them on laminin-521 coated cell culture dishes in medium without any Rho-associated kinase (ROCK) inhibitor (e.g. without Y-27632) (Watanabe et al., *Nature Biotechnology* **25**, 681 - 686 (2007)). The improvement provides a possibility to expand human embryonic stem cells in pluripotent state faster than in conventional human embryonic stem cell cultures.

[0028] In further aspects, the disclosure provides new materials, such as human recombinant laminin-521, which permit human embryonic cell survival after dissociation into single cell suspension. The improvement provides better cloning survival of human embryonic stem cells, which may be advantageous for isolating clones (e.g. after genetic manipulations) or obtaining homogeneous and uniformal human embryonic stem cell populations.

[0029] Disclosed in some embodiments is isolated recombinant laminin-521, comprising: an alpha chain comprising a polypeptide with at least 80% identity to a polypeptide sequence of SEQ ID NO: 1; a beta chain comprising a polypeptide with at least 70% identity to a polypeptide sequence of SEQ ID NO: 2; and a gamma chain comprising a polypeptide with at least 70% identity to a polypeptide sequence of SEQ ID NO: 3; wherein the alpha, beta, and gamma chains are assembled into recombinant laminin-521.

[0030] In further embodiments, the alpha chain polypeptide has at least 90% identity to the polypeptide sequence of SEQ ID NO: 1. The beta chain polypeptide may also have at least 90% identity to the polypeptide sequence of SEQ ID NO: 2. The gamma chain polypeptide may also have at least 90% identity to the polypeptide sequence of SEQ ID NO: 3.

[0031] In other embodiments, the alpha chain has the polypeptide sequence of SEQ ID NO: 1. Furthermore, the beta chain may have the polypeptide sequence of SEQ ID NO: 2. Even more specifically, the gamma chain may have the polypeptide sequence of SEQ ID NO: 3.

[0032] The beta chain polypeptide may have at least 80% identity to the polypeptide sequence of SEQ ID NO: 2. The gamma chain polypeptide may have at least 80% identity to the polypeptide sequence of SEQ ID NO: 3.

[0033] Also disclosed in embodiments is a pharmaceutical composition, comprising: a) the isolated recombinant laminin-521 of claim 1; and b) a pharmaceutically acceptable carrier.

[0034] Also disclosed in embodiments is a composition that enables self-renewal of pluripotent stem cells grown *in vitro*, comprising a growth medium and a coating thereover, the coating comprising recombinant laminin 521 (laminin-11).

[0035] The composition may further comprise a growth factor.

[0036] The composition may be devoid of any differentiation inhibitors. The composition may also be devoid of any feeder cells. The composition may also be devoid of any differentiation inductors. In some embodiments, the composition is devoid of any differentiation inhibitors, feeder cells, or differentiation inductors.

[0037] Also disclosed in embodiments is a method for maintaining the pluripotency of pluripotent stem cells *in vitro*, comprising: providing a substrate comprising a growth medium and a coating thereover, the coating comprising recombinant laminin 521 (laminin-11); dissociating pluripotent stem cells into a single cell suspension; and placing the pluripotent stem cells in the single cell suspension on the coating.

[0038] The growth medium may comprise a growth factor or growth factors. Exemplary growth factors include basic fibroblast growth factor and insulin growth factor.

[0039] Sometimes, the growth medium and the coating are devoid of any differentiation inhibitors. For example, leukemia inhibitor factor is not present.

[0040] The composition may be devoid of any feeder cells, such as mouse fibroblasts or human foreskin fibroblasts. The composition may be devoid of any differentiation inductors, such as Noggin or keratinocyte growth factor. In some embodiments, the composition is devoid of any differentiation inhibitors, feeder cells, or differentiation inductors.

[0041] The pluripotent stem cells may be placed on the laminin-521 coating as a monolayer.

[0042] The pluripotent stem cells may be placed on the coating at a density of 200 cells / mm^2 or less. Alternatively, the pluripotent stem cells may be placed on the coating at a density of 200 cells / mm^2 or more. The pluripotent stem cells can alternatively be placed on the coating such that no two stem cells contact each other.

[0043] Also disclosed in embodiments is isolated recombinant laminin-521 produced by a method comprising: providing host cells that express recombinant laminin-521, wherein the recombinant laminin-521 comprises: a first chain comprising a polypeptide with at least 80% identity to a polypeptide sequence of SEQ ID NO: 1, a second chain comprising a polypeptide with at least 70% identity to a polypeptide sequence of SEQ ID NO: 2, and a third chain comprising a polypeptide with at least 70% identity to a polypeptide sequence of SEQ ID NO: 3, wherein the first, second, and third chains are assembled into recombinant laminin-521; growing the host cells in a cell culture medium under conditions to stimulate expression of the recombinant laminin-521 chains; passing the host cell culture medium through a column, wherein the column contains a compound that binds to the recombinant laminin-521; washing the column to remove unbound materials; and eluting the bound recombinant laminin-521 from the column.

[0044] Described in other embodiments are methods of maintaining the pluripotency of pluripotent stem cells in vitro, comprising: receiving a substrate having a coating thereon, the coating containing an intact laminin; placing pluripotent stem cells and a cell culture medium on the substrate; and activating the PI3-kinase/Akt pathway.

[0045] The intact laminin may be laminin-521 or laminin-511. In some embodiments, the cell culture medium does not contain any growth factors, such as beta fibroblast growth factor (bFGF). The coating may also contain a cadherin. The pluripotent stem cells may be placed on the coating at a density of 200 cells / mm2 or less.

[0046] The present disclosure also relates to cell culture media that can be used to maintain stem cells in a pluripotent state. Described in various embodiments is a cell culture medium that provides nutrition to pluripotent stem cells, comprising from greater than zero to 3.9 ng/mL of basic fibroblast growth factor (bFGF).

[0047] The cell culture medium may comprise 3.5 ng/mL or less of bFGF, including from 0.5 to 3.5 ng/mL of bFGF.

[0048] The cell culture medium may further comprise at least one inorganic salt, at least one trace mineral, at least one energy substrate, at least one lipid, at least one amino acid, at least one vitamin, or at least one additional growth factor. In particular embodiments, the cell culture medium further comprises at least one inorganic salt, at least one trace mineral, at least one energy substrate, at least one lipid, at least one amino acid, and at least one vitamin.

[0049] The cell culture medium may not contain any one of (1) albumin, (2) insulin or an insulin substitute, or (3) transferrin or a transferrin substitute.

[0050] The cell culture medium may further comprise albumin, insulin, lithium chloride, GABA, TGF beta 1, pipecolic acid, L-glutamine, MEM non-essential amino acid solution, and DMEM/F12 solution.

[0051] The cell culture medium may further comprise at least one additional growth factor, at least one trace mineral, and at least one lipid.

[0052] Also disclosed is a system for maintaining pluripotent stem cells, comprising: a cell culture medium comprising from greater than zero to 3.9 ng/mL of basic fibroblast growth factor (bFGF); and a substrate for providing support to the stem cells.

[0053] The substrate may contain laminin-521 or laminin-511. The substrate may also contain a cadherin.

[0054] These and other non-limiting characteristics of the disclosure are more particularly disclosed below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0055] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0056] The following is a brief description of the drawings, which are presented for the purposes of illustrating the exemplary embodiments disclosed herein and not for the purposes of limiting the same.

[0057] **FIG. 1** is a rotary shadowing electron microscopy picture of a recombinant laminin molecule.

[0058] **FIG. 2** shows the structural motifs of laminin α, β, and γ chains. The N-terminal, internal, and C-terminal globular domains are depicted as white ovals. The coiled-coil forming domains (I and II) are shown as white rectangles. The rod-like structures (domains V, IIIb, and IIIa) are depicted as grey rectangles.

[0059] **FIG. 3** shows the results of characterization of human recombinant laminin-521 using SDS-PAGE. An immunoblot of recombinant laminin-521 was made under non-reducing (labeled as ox) and reducing (labeled as red) conditions. Proteins on 3-8% gels were transferred onto PVDF membranes followed by staining with antibodies to laminin α5 (2F7), β2 (MAB2066), β1 (MAB1921) and γ1 (H-19).

[0060] **FIGS. 4** and **5** show the Crystal Violet staining of human ES cells adherent to dishes coated in Matrigel (Mg), mouse laminin-111 (mLN111), human recombinant-laminin-511 (r-laminin-511 or r-LN-511), human recombinant-laminin-521 (r-laminin-521 or r-LN-521), or a mixture of r-laminin-511 and r-laminin-521 (mix) after one day in culture. Both Figures are magnified 5x. In **FIG. 4**, the cells were cultured without ROCK inhibitor. In **FIG. 5**, the cells were cultured with ROCK inhibitor Y-27632.

[0061] **FIG. 6** is a graph showing the adhesion of human ES cells to human r-laminin-521 (labeled as LN521), human r-laminin-511 (LN511), and Matrigel (Mg) coated dishes after one hour in culture without ROCK inhibitor. Error bars show the standard error of measurement. (n=3).

[0062] **FIG. 7** is a graph showing the adhesion of human ES cells to dishes coated in either Matrigel (Mg), mouse laminin-111 (mLN111), human r-laminin-511 (LN511), human r-laminin-521 (LN521), or a mixture of r-laminin-511 and r-laminin-521 (mix), after one day in culture and without ROCK inhibitor. Also included are results for dishes with ROCK inhibitor and either Matrigel (In&Mg), mouse laminin-111 (In&mLN111), human r-laminin-511 (In&LN511), human r-laminin-521 (In&LN521), or a mixture of r-laminin-511 and r-laminin-521 (In&mix), also after one

day in culture. The cells for both experiments were plated at the same density on the same cell culture dish. Error bars show standard error of measurement (n=3).

[0063] **FIG. 8** shows the growth curves for human ES cells cultured on r-laminin-521 (LN-521_1 and LN-521_2) using single cell dissociation passaging and for human ES cells cultured on Matrigel (Mg_1 and Mg_2) using passaging in small clumps. The latter cells were passaged as described in Rodin et al., *Nature Biotechnol.*, vol. 28, pp. 611-615 (2010).

[0064] **FIG. 9** is a FACS analysis of human ES cells after several single cell dissociation passages on r-laminin-521 for OCT4, a marker of pluripotency. The percentage of positive cells is listed in parentheses.

[0065] **FIG. 10** is a FACS analysis of human ES cells after several passages on Matrigel using splitting in small clumps for OCT4. The percentage of positive cells is listed in parentheses.

[0066] **FIG. 11** shows the results of a real-time quantitative RT-PCR analysis which was used to compare numbers of mRNA transcripts of the pluripotency markers Oct4 and Nanog in human ES cells cultured on human r-laminin-521 (LN521) after several single cell dissociation passaging and in the cells cultured on Matrigel (Mg) after several passaging of the cells in clumps. Error bars show 95% confidence intervals.

[0067] **FIG. 12** is a Western blot comparing the levels of phosphorylated myosin light chain (P-MLC) in stem cells grown on Matrigel (Mg), LN-111, LN-511, and LN-521 one hour after plating.

[0068] **FIG. 13** is a Western blot comparing the levels of phosphorylated myosin light chain (P-MLC) in stem cells grown on Matrigel (Mg), LN-111, LN-511, and LN-521 six hours after plating.

[0069] **FIG. 14** is a graph comparing the relative migration of human embryonic stem cells grown on Matrigel (Mg), LN-111, LN-511, and LN-521 between five and seven hours after plating.

[0070] **FIG. 15** is a graph comparing the relative migration of human embryonic stem cells grown on LN-521 with IgG and IgM blocking antibodies applied (IgGM) and with a function blocking antibody to integrin β1 (b1) applied. This shows that blocking the integrin significantly reduced motility.

[0071] **FIG. 16** is a graph comparing the relative survival of hES cells on LN-521 in the presence of water, DMSO, LY294002 (Akt inhibitor), wortmannin (PI3K/Akt inhibitor), and PD 98059 (MEK1 inhibitor). This showed that activation of PI3K/Akt was necessary for hES cell survival on LN-521. The medium contained bFGF.

[0072] **FIG. 17** is a graph comparing the relative survival of hES cells on LN-521 in the presence of DMSO, LY294002 (Akt inhibitor), and PD 98059 (MEK1 inhibitor). No exogenous bFGF was present.

[0073] **FIG. 18** is a Western blot comparing cells treated with LY 294002 against control cells (DMSO) and collected one hour after plating on LN-521.

[0074] **FIG. 19** is a Western blot comparing cells treated with PD98059 against control cells (DMSO) and collected one hour after plating on LN-521.

[0075] **FIG. 20** is a graph showing the relative levels of Akt2 phosphorylation on cell lysates collected one hour after plating on Matrigel (Mg), LN-111, LN-511, or LN-521, obtained using ELISA.

[0076] **FIG. 21** is a graph showing the relative levels of Akt1 phosphorylation on cell lysates collected one hour after plating on Matrigel (Mg), LN-111, LN-511, or LN-521, obtained using ELISA.

[0077] **FIG. 22** is a graph showing the growth curve of HS181 hES cells cultured in a low bFGF medium (O3_3.9) compared to a higher bFGF medium (O3_100).

[0078] **FIG. 23** is a graph showing the relative mRNA expression level for two pluripotency markers (Oct4 and Nanog) in HS181 hES cells cultured in a low bFGF medium (O3_3.9) compared to a higher bFGF medium (O3_100).

[0079] **FIG. 24** is a picture showing an early stage derivation of a new human embryonic stem HS841 cell line on laminin-521/E-Cadherin matrix.

DETAILED DESCRIPTION

[0080] A more complete understanding of the compositions and methods disclosed herein can be obtained by reference to the accompanying drawings. These figures are merely schematic representations based on convenience and the ease of demonstrating the present disclosure, and are, therefore, not intended to define or limit the scope of the exemplary embodiments.

[0081] Although specific terms are used in the following description for the sake of clarity, these terms are intended to refer only to the particular structure of the embodiments selected for illustration in the drawings, and are not intended to define

or limit the scope of the disclosure. In the drawings and the following description below, it is to be understood that like numeric designations refer to components of like function.

[0082] All publications, patents, and patent applications discussed herein are hereby incorporated by reference in their entirety.

[0083] Unless otherwise stated, the techniques utilized in this application may be found in any of several well-known references such as: Molecular Cloning: A Laboratory Manual (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), Gene Expression Technology (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, Calif.), "Guide to Protein Purification" in Methods in Enzymology (M. P. Deutshcer, ed., (1990) Academic Press, Inc.); PCR Protocols: A Guide to Methods and Applications (Innis, et al. 1990. Academic Press, San Diego, Calif.), Culture of Animal Cells: A Manual of Basic Technique, Second Ed. (R. I. Freshney. 1987. Liss, Inc. New York, N.Y.), Gene Transfer and Expression Protocols, pp. 109-128, ed. E. J. Murray, The Humana Press Inc., Clifton, N.J.), or the Ambion 1998 Catalog (Ambion, Austin, Tex.).

[0084] As used herein, the term "isolated nucleic acid sequence" refers to a nucleic acid sequence that is free of gene sequences which naturally flank the nucleic acid in the genomic DNA of the organism from which the nucleic acid is derived (i.e., genetic sequences that are located adjacent to the gene for the isolated nucleic molecule in the genomic DNA of the organism from which the nucleic acid is derived). The "isolated" sequence may, however, be linked to other nucleotide sequences that do not naturally flank the recited sequence, such as a heterologous promoter sequence, or other vector sequences. It is not necessary for the isolated nucleic acid sequence to be free of other cellular material to be considered "isolated", as a nucleic acid sequence according to the disclosure may be part of an expression vector that is used to transfect host cells (see below).

[0085] The present disclosure provides recombinant expression vectors comprising a full length laminin β2 chain nucleic acid sequence (SEQ ID NO: 4) of the human laminin β2 chain. In some embodiments, the expression vectors comprise a nucleic acid encoded by SEQ ID NO: 4, operatively linked to a heterologous promoter (i.e. is not the naturally occurring promoter for the given β2 laminin chain). A promoter and a laminin β2 chain nucleic acid sequence are "operatively linked"

when the promoter is capable of driving expression of the laminin β2 chain DNA into RNA.

[0086] As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA into which additional DNA segments may be cloned. Another type of vector is a viral vector, wherein additional DNA segments may be cloned into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors), are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In the present disclosure, the expression of the laminin polypeptide sequence is directed by the promoter sequences of the disclosure, by operatively linking the promoter sequences of the disclosure to the gene to be expressed. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably, as the plasmid is the most commonly used form of vector. However, the disclosure is intended to include other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

[0087] The vector may also contain additional sequences, such as a polylinker for subcloning of additional nucleic acid sequences, or a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the methods of the disclosure, and any such sequence may be employed, including but not limited to the SV40 and bovine growth hormone poly-A sites. Also contemplated as an element of the vector is a termination sequence, which can serve to enhance message levels and to minimize readthrough from the construct into other sequences. Additionally, expression vectors typically have selectable markers, often in the form of antibiotic resistance genes, that permit selection of cells that carry these vectors.

[0088] In further embodiments, the present disclosure provides host cells transfected with the laminin β2 chain-expressing recombinant expression vectors disclosed herein. As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid of the present disclosure, such as a recombinant expression vector, has been introduced. Such cells may be prokaryotic, which can be used, for example, to rapidly produce a large amount of the expression vectors of the disclosure, or may be eukaryotic, for functional studies.

[0089] The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0090] The host cells can be transiently or stably transfected with one or more of the expression vectors of the disclosure. Such transfection of expression vectors into prokaryotic and eukaryotic cells can be accomplished via any technique known in the art, including but not limited to standard bacterial transformations, calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. (See, for example, Molecular Cloning: A Laboratory Manual (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press; Culture of Animal Cells: A Manual of Basic Technique, 2.sup.nd Ed. (R. I. Freshney. 1987. Liss, Inc. New York, N.Y.).

[0091] In another aspect, the present disclosure provides an isolated full length human laminin β2 chain polypeptide consisting of the amino acid sequence of SEQ ID NO: 2.

[0092] As used herein, an "isolated polypeptide" refers to a polypeptide that is substantially free of other proteins, including other laminin chains, and gel agents, such as polyacrylamide and agarose. In preferred embodiments, the isolated laminin polypeptide is free of detectable contaminating laminin chains. Thus, the protein can either be isolated from natural sources, or recombinant protein can be isolated from the transfected host cells disclosed above.

[0093] In another aspect, the present disclosure provides isolated laminin-521. As used herein, the term "laminin-521" refers to the protein formed by joining α5, β2 and γ1 chains together. The term should be construed as encompassing both

recombinant laminin-521 and heterotrimeric laminin-521 from naturally occurring sources. In preferred embodiments, the laminin-521 comprises recombinant laminin-521 (or "r-laminin-521").

[0094] As used herein, the term "r-laminin-521" refers to recombinant heterotrimeric laminin-521, expressed by a host cell that has been transfected with one or more expression vectors comprising at least one nucleic acid sequence encoding a laminin-521 chain selected from the α5, β2 and γ1 chains, or processed or secreted forms thereof. Such r-laminin-521 can thus comprise α5, β2, and γ1 sequences from a single organism, or from different organisms. Various laminin-521 chain DNA sequences are known in the art, and the use of any such sequence to prepare the r-laminin-521 of the disclosure is contemplated. (See, for example, Pouliot, N. et al., Experimental Cell Research 261(2):360-71, (2000); Kikkawa, Y. et al., Journal of Cell Science 113 (Pt 5):869-76, (2000); Church, H J. et al., Biochemical Journal 332 (Pt 2):491-8, (1998); Sorokin, L M. et al., Developmental Biology 189(2):285-300, (1997); Miner, J H. et al., Journal of Biological Chemistry 270(48):28523-6, (1995); Sorokin, L. et al., European Journal of Biochemistry 223(2):603-10, (1994)). In preferred embodiments, the r-laminin-521 is formed from recombinant human α5, β2, and γ1 polypeptide chains.

[0095] The disclosure encompasses those laminin molecules wherein only one or two chains that make up the recombinant heterotrimeric laminin-521 are encoded by endogenous laminin-521 chains. In preferred embodiments, each of the α5, β2, and γ1 polypeptide chains are expressed recombinantly.

[0096] The laminin-521 is an intact protein. The term "intact" refers to the protein being composed of all of the domains of the α-chain, β-chain, and γ-chain, with the three chains being joined together to form the heterotrimeric structure. The protein is not broken down into separate chains, fragments, or functional domains. The term "chain" refers to the entirety of the alpha, beta, or gamma chain of the laminin protein. The term "fragment" refers to any protein fragment which contains one, two, or three functional domains that possesses binding activity to another molecule or receptor. However, a chain should not be considered a fragment because each chain possesses more than three such domains. Similarly, an intact laminin protein should not be considered a fragment. Examples of functional domains include Domains I, II, III, IV, V, VI, and the G domain.

[0097] Laminin-521 is a secreted protein, which is capable of being directed to the endoplasmic reticulum (ER), secretory vesicles, and the extracellular space as a result of a signal sequence. If the secreted protein is released into the extracellular space, the secreted protein can undergo extracellular processing to produce a "mature" protein. Such processing events can be variable, and thus may yield different versions of the final "mature protein". For example, the lengths of the α5, β2, and γ1 chains may vary between proteins. However, the final mature protein still has the same functionality, even though the chain lengths vary. The isolated laminin-521 of the present disclosure includes heterotrimers comprising both the full length polypeptide chains and any such naturally processed laminin-521 polypeptide chains.

[0098] As used herein, a laminin-521 polypeptide chain refers to a polypeptide chain according to one or more of the following:

[0099] (a) a polypeptide chain that comprises a polypeptide structure selected from the group consisting of: R1-R2-R3, R1-R2-R4, R3, R4, R1-R3, R1-R4, R2-R3, and R2-R4, wherein R1 is an amino terminal methionine; R2 is a signal sequence that is capable of directing secretion of the polypeptide, wherein the signal sequence may be the natural signal sequence for the particular laminin chain, that of another secreted protein, or an artificial sequence; R3 is a secreted laminin chain selected from the group consisting of a α5 chain, a β2 chain, and a γ1 chain; and R4 is a secreted α5, β2, or γ1 laminin chain that further comprises an epitope tag (such as those described below), which can be placed at any position within the laminin chain amino acid sequence; or

[0100] (b) a polypeptide chain that is encoded by a polynucleotide that hybridizes under high or low stringency conditions to the coding regions, or portions thereof, of one or more of the recombinant laminin-521 chain DNA sequences disclosed herein (SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6), or complementary sequences thereof; or

[0101] (c) a polypeptide chain that has at least 70% identity to one or more of the disclosed laminin-521 polypeptide chain amino acid sequences (SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3), preferably at least 80% identity, and most preferably at least about 90% identity.

[0102] "Stringency of hybridization" is used herein to refer to washing conditions under which nucleic acid hybrids are stable. The disclosure also includes nucleic

acids that hybridize under high stringency conditions (as defined herein) to all or a portion of the coding sequences of the laminin chain polynucleotides disclosed herein, or their complements. The hybridizing portion of the hybridizing nucleic acids is typically at least 50 nucleotides in length. As known to those of ordinary skill in the art, the stability of hybrids is reflected in the melting temperature (T_M) of the hybrids. T_M decreases approximately 1-1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. As used herein, high stringency refers to an overnight incubation at 42°C in a solution comprising 50% formamide, 5× SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5× Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1× SSC at about 65°C

[0103] Also contemplated are laminin-521-encoding nucleic acid sequences that hybridize to the polynucleotides of the present disclosure at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6× SSPE (20× SSPE=3M NaCl; 0.2M NaH_2PO_4 ; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA; followed by washes at 50°C with 1× SSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5× SSC).

[0104] Note that variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

[0105] As used herein, "percent identity" of two amino acids or of two nucleic acids is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci.

USA 87:2264.2268, 1990), modified as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403-410, 1990). BLAST nucleotide searches are performed with the NBLAST program, score 100, wordlength=12, to determine nucleotide sequences identity to the nucleic acid molecules of the disclosure. BLAST protein searches are performed with the XBLAST program, score=50, wordlength=3, to determine an amino acid sequence identity to a polypeptide of the disclosure. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Altschul et al. (Nucleic Acids. Res. 25:3389-3402, 1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used.

[0106] Further embodiments of the present disclosure include polynucleotides encoding laminin-521 chain polypeptides having at least 70% identity, preferably at least 80% identity, and most preferably at least 90% identity to one or more of the polypeptide sequences contained in SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

[0107] As used herein, "α5 polynucleotide" refers to polynucleotides encoding a laminin α5 chain. Such polynucleotides can be characterized by one or more of the following: (a) polynucleotides that encode polypeptides which share at least 70% identity, preferably 80% identity, and most preferably at least 90% identity with a sequence selected of SEQ ID NO: 5; (b) polynucleotides that hybridize under low or high stringency conditions to the coding sequence of SEQ ID NO: 5 or complementary sequences thereof; or (c) polynucleotides encoding a laminin α5 chain polypeptide with a general structure selected from the group consisting of R1-R2-R3, R1-R2-R4, R3, R4, R1-R3, R1-R4, R2-R3, and R2-R4, wherein R1 and R2 are as described above, R3 is a secreted α5 chain, and R4 is a secreted α5 chain that comprises an epitope tag.

[0108] As used herein, "β2 polynucleotides" refers to polynucleotides encoding a β2 laminin chain of the same name. Such polynucleotides can be characterized by one or more of the following: (a) polynucleotides that encode polypeptides which share at least 70% identity, preferably at least 80%, and most preferably at least 90% identity with the sequence of SEQ ID NO: 4; (b) polynucleotides that hybridize under low or high stringency conditions to the coding sequences of SEQ ID NO: 4, or

complementary sequences thereof; or (c) polynucleotides encoding a polypeptide with a general structure selected from R1-R2-R3, R1-R2-R4, R3, R4, R1-R3, R1-R4, R2-R3, and R2-R4, wherein R1 and R2 are as described above, R3 is a secreted β2 chain, and R4 is a secreted β2 chain that comprises an epitope tag.

[0109] As used herein, "γ1 polynucleotides" refers to polynucleotides encoding a γ1 laminin chain of the same name. Such polynucleotides can be characterized by one or more of the following: (a) polynucleotides that encode polypeptides which share at least 70% identity, preferably at least 80%, and most preferably at least 90% identity with the sequence of SEQ ID NO: 6; (b) polynucleotides that hybridize under low or high stringency conditions to the coding sequence of SEQ ID NO: 6 or complementary sequences thereof; or (c) polynucleotides that encode a polypeptide with a general structure selected from R1-R2-R3, R1-R2-R4, R3, R4, R1-R3, R1-R4, R2-R3, and R2-R4, wherein R1 and R2 are as described above, R3 is a secreted γ1 chain, and R4 is a secreted γ1 chain that comprises an epitope tag.

[0110] As used herein, the term "epitope tag" refers to a polypeptide sequence that is expressed as part of a chimeric protein, where the epitope tag serves as a recognition site for binding of antibodies generated against the epitope tag, or for binding of other molecules that can be used for affinity purification of sequences containing the tag.

[0111] In preferred embodiments, cDNAs encoding the laminin α5, β2 and γ1 chains, or fragments thereof, are subcloned into an expression vector. Alternatively, laminin α5, β2 and/or γ1 gene sequences, including one or more introns, can be used for sub-cloning into an expression vector.

[0112] In other aspects, the present disclosure provides laminin-521 expressing-cells that have been transfected with an expression vector containing promoter sequences that are operatively linked to nucleic acid sequences encoding at least one polypeptide sequence comprising a sequence selected from the group consisting of the α5, β2 and γ1 chains of laminin-521, wherein the transfected cells secrete heterotrimeric laminin-521 containing the recombinant laminin chain. In preferred embodiments, the cells are systematically transfected with recombinant expression vectors containing promoter sequences that are operatively linked to nucleic acid sequences encoding polypeptide sequences comprising the α5, β2 and γ1 chains of laminin-521, which are even more preferably all human chains. After the

multiple transfections, the cells express recombinant laminin-521 chains, which form the heterotrimeric r-laminin-521.

[0113] Transfection of the expression vectors into eukaryotic cells can be accomplished via any technique known in the art, including but not limited to calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. Transfection of bacterial cells can be done by standard methods.

[0114] In preferred embodiments, the cells are stably transfected. Methods for stable transfection and selection of appropriate transfected cells are known in the art. In other preferred embodiments, a CMV promoter driven expression vector is used in a human kidney embryonic 293 cell line.

[0115] Any cell capable of expressing and secreting the r-laminin-521 can be used. Preferably, eukaryotic cells are used, and most preferably mammalian cells are used, including but not limited to kidney and epithelial cell lines. The promoter sequence used to drive expression of the individual chains or r-laminin-521 may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). Carbohydrate and disulfide post-translational modifications are believed to be required for laminin-521 protein folding and function. This makes the use of eukaryotic cells preferable for producing functional r-laminin-521, although other systems are useful for obtaining, for example, antigens for antibody production. In most preferred embodiments, the mammalian cells do not express the laminin β2 chain endogenously. In other preferred embodiments, the cells do not express all of the laminin-521 chains endogenously.

[0116] The protein may comprise additional sequences useful for promoting purification of the protein, such as epitope tags and transport signals. Examples of such epitope tags include, but are not limited to FLAG (Sigma Chemical, St. Louis, Mo.), myc (9E10) (Invitrogen, Carlsbad, Calif.), 6-His (Invitrogen; Novagen, Madison, Wis.), and HA (Boehringer Manheim Biochemicals). Examples of such transport signals include, but are not limited to, export signals, secretory signals, nuclear localization signals, and plasma membrane localization signals.

[0117] In some embodiments, at least one of the laminin chain polypeptide sequences, or fragments thereof, is operatively linked to a nucleic acid sequence

encoding an "epitope tag", so that at least one of the chains is expressed as a fusion protein with an expressed epitope tag. The epitope tag may be expressed as the amino terminus, the carboxy terminus, or internal to any of the polypeptide chains comprising r-laminin-521, so long as the resulting r-laminin-521 remains functional.

[0118] In other embodiments, one of the r-laminin-521 chains is expressed as a fusion protein with a first epitope tag, and at least one other r-laminin chain is expressed as a fusion protein with a second different epitope tag. This permits multiple rounds of purification to be carried out. Alternatively, the same epitope tag can be used to create fusion proteins with more than one of the r-laminin chains.

[0119] In further embodiments, the epitope tag can be engineered to be cleavable from the r-laminin-521 chain(s). Alternatively, no epitope tag is fused to any of the r-laminin-521 chains, and the r-laminin-521 is isolated by standard techniques, including but not limited to affinity chromatography using laminin-521 specific antibodies or other laminin-521 binding molecules.

[0120] Media from cells transfected with a single laminin chain are initially analyzed on Western blots using laminin chain-specific antibodies. The expression of single laminin chains following transfection is generally intracellular. Clones showing reactivity against individual transfected chain(s) are verified by any appropriate method, such as PCR, reverse transcription-PCR, or nucleic acid hybridization, to confirm incorporation of the transfected gene. Preferably, analysis of genomic DNA preparations from such clones is done by PCR using laminin chain-specific primer pairs. Media from transfected clones producing all three chains are further analyzed for r-laminin-521 secretion and/or activity, by any appropriate method, including Western blot analysis and cell binding assays.

[0121] In preferred embodiments, purification of r-laminin-521 is accomplished by passing media from the transfected cells through an antibody affinity column. In some embodiments, antibodies against a peptide epitope expressed on at least one of the recombinant chains are attached to an affinity column, and bind the r-laminin-521 that has been secreted into the media. The r-laminin-521 is removed from the column by passing excess peptide over the column. Eluted fractions are analyzed by any appropriate method, including gel electrophoresis and Western blot analysis. In further embodiments, the peptide epitope can be cleaved after purification. In other embodiments, two or three separate r-laminin chains are expressed as fusion proteins, each with a different epitope tag, permitting two or three rounds of

purification and a doubly or triply isolated r-laminin-521. The epitope tag can be engineered so as to be cleavable from the r-laminin-521 chain(s) after purification. Alternatively, no epitope tag is fused to any of the r-laminin-521 chains, and the r-laminin-521 is isolated by standard techniques, including but not limited to affinity chromatography using laminin-521 specific antibodies or other laminin-521 binding molecules.

[0122] In other embodiments, purification of r-laminin-521 is accomplished by passing media from the transfected cells through a gel-filtration chromatography column. Eluted fractions are analyzed by any appropriate method, including gel electrophoresis and Western blot analysis. Fractions containing r-laminin-521 are collected and purity of the specimen is evaluated by any appropriate method, including gel electrophoresis and Western blot analysis. In some embodiments, the protein solution can be passed through a gel-filtration chromatography column again to gain higher purity of the protein. In some embodiments, to achieve higher purity of r-laminin-521 solution, the media or r-laminin-521 solution from the previous purification steps can be passed through an ion-exchange column. Eluted fractions are analyzed by any appropriate method, including gel electrophoresis and Western blot analysis. Fractions containing r-laminin-521 are collected and purity of the specimen is evaluated by any appropriate method, including mentioned above.

[0123] The laminin-521 polypeptide chains of the present disclosure also include (i) substitutions with one or more of the non-conserved amino acid residues, where the substituted amino acid residues may or may not be one encoded by the genetic code, or (ii) substitution with one or more amino acid residues having substituent groups, or (iii) fusion of the mature polypeptide with another compound, such as a compound to increase the stability and/or solubility of the polypeptide (for example, polyethylene glycol), or (iv) fusion of the polypeptide with additional amino acids, such as an IgG Fc fusion region peptide, or leader or secretory sequence, or a sequence facilitating purification. Such variant polypeptides are deemed to be within the scope of those skilled in the art from the teachings herein.

[0124] For example, polypeptide variants containing amino acid substitutions of charged amino acids with other charged or neutral amino acids may produce proteins with improved characteristics, such as less aggregation. Aggregation of pharmaceutical formulations both reduces activity and increases clearance due to the aggregate's immunogenic activity. (Pinckard et al., Clin. Exp. Immunol. 2:331-

340 (1967); Robbins et al., Diabetes 36: 838-845 (1987); Cleland et al., Crit. Rev. Therapeutic Drug Carrier Systems 10:307-377 (1993).)

[0125] In particular embodiments, the isolated laminin-521 comprises three chains. The first chain comprises a polypeptide with at least 80% identity to a polypeptide sequence of SEQ ID NO: 1 (i.e. the α5 laminin chain). The second chain comprises a polypeptide with at least 70% identity to a polypeptide sequence of SEQ ID NO: 2 (i.e. the β2 laminin chain). The third chain comprises a polypeptide with at least 70% identity to a polypeptide sequence of SEQ ID NO: 3 (i.e. the γ1 laminin chain). These first, second, and third chains are assembled into recombinant laminin-521.

[0126] In more specific embodiments, the polypeptide of the first chain has at least 80% identity to the polypeptide sequence of SEQ ID NO: 1, the polypeptide of the second chain has at least 80% identity to the polypeptide sequence of SEQ ID NO: 2, and the polypeptide of the third chain has at least 80% identity to the polypeptide sequence of SEQ ID NO: 3.

[0127] In more specific embodiments, the polypeptide of the first chain has at least 90% identity to the polypeptide sequence of SEQ ID NO: 1, the polypeptide of the second chain has at least 90% identity to the polypeptide sequence of SEQ ID NO: 2, and the polypeptide of the third chain has at least 90% identity to the polypeptide sequence of SEQ ID NO: 3.

[0128] In particular embodiments, the first chain comprises the polypeptide sequence of SEQ ID NO: 1, the second chain comprises the polypeptide sequence of SEQ ID NO: 2, and the third chain comprises the polypeptide sequence of SEQ ID NO: 3.

[0129] In particular embodiments, the first chain is the polypeptide sequence of SEQ ID NO: 1, the second chain is the polypeptide sequence of SEQ ID NO: 2, and the third chain is the polypeptide sequence of SEQ ID NO: 3.

[0130] The present disclosure further provides pharmaceutical compositions comprising isolated laminin-521 and a pharmaceutically acceptable carrier. In preferred embodiments, the pharmaceutical composition comprises isolated r-laminin-521. According to these aspects of the disclosure, other agents can be included in the pharmaceutical compositions, depending on the condition being treated. The pharmaceutical composition may further comprise one or more other compounds, including but not limited to any of the collagens, other laminin types,

fibronectin, vitronectin, cadherins, integrins, α-dystroglycan, entactin/nidogen, α-dystroglycan, glycoproteins, proteoglycans, heparan sulfate proteoglycan, glycosaminoglycans, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, or nerve growth factors, and peptide fragments thereof.

[0131] Pharmaceutical preparations comprising isolated laminin-521 can be prepared in any suitable form, and generally comprise the isolated laminin-521 in combination with a pharmaceutically acceptable carrier. The carriers can be injectable carriers, topical carriers, transdermal carriers, and the like. The preparation may advantageously be in a form for topical administration, such as an ointment, gel, cream, spray, dispersion, suspension or paste. The preparations may further advantageously include preservatives, antibacterials, antifingals, antioxidants, osmotic agents, and similar materials in composition and quantity as is conventional. Suitable solutions for use in accordance with the disclosure are sterile, are not harmful for the proposed application, and may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc. For assistance in formulating the compositions of the present disclosure, one may refer to Remington's Pharmaceutical Sciences, 15th Ed., Mack Publishing Co., Easton, Pa. (1975).

[0132] In further aspects, the present disclosure comprises medical devices with improved biocompatibility, wherein the exterior surfaces of the devices are coated with isolated laminin-521 or pharmaceutical compositions thereof, alone or in combination with other proteins or agents that serve to increase the biocompatibility of the device surface. The coated device stimulates cell attachment (such as endothelial cell attachment), and provides for diminished inflammation and/or infection at the site of entry of the device.

[0133] Such medical devices can be of any material used for implantation into the body, and preferably are made of or coated with a biocompatible metal, such as stainless steel or titanium. Alternatively, the device is made of or coated with a ceramic material or a polymer, such as polyester, polyglycolic acid, or a polygalactose-polyglycolic acid copolymer.

[0134] If the device is made of a natural or synthetic biodegradable material in the form of a mesh, sheet or fabric, isolated laminin-521 or pharmaceutical compositions thereof may be applied directly to the surface thereof. Appropriate cells may then be

cultured on the matrix to form transplantable or implantable devices, including dental abutment pieces, needles, metal pins or rods, indwelling catheters, colostomy tubes, surgical meshes and any other appliance for which coating with isolated laminin-521 is desirable. Alternatively, the devices may be implanted and cells may be permitted to attach in vivo.

[0135] Coupling of the isolated laminin-521 may be non-covalent (such as by adsorption), or by covalent means. The device may be immersed in, incubated in, or sprayed with the isolated laminin-521 or pharmaceutical compositions thereof.

[0136] The dosage regimen for various treatments using the isolated laminin-521 of the present disclosure is based on a variety of factors, including the type of injury or condition, the age, weight, sex, medical condition of the individual, the severity of the condition, and the route of administration. Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Laminins are extremely potent molecules, and one or a few molecules per cell could produce an effect. Thus, effective doses in the pico-gram per milliliter range are possible if the delivery is optimized.

[0137] In other aspects, the present disclosure provides a new material, isolated laminin-521, which permits human embryonic stem cell survival after dissociation into single cell suspension. The stem cells were dissociated into single cell suspension by Trypsin-EDTA treatment, pelleted by centrifugation, resuspended into O3 medium, filtered through a 40 μm sieve, and plated at a density of 30 Kcells/cm^2 on cell culture dishes precoated by either isolated laminin-521, laminin-511, or Matrigel. After one day in culture, the cells plated on Matrigel died, as is known in the art. The human embryonic stem cells plated on laminin-521 and in most cases on laminin-511 survived and started to proliferate, forming small colonies of pluripotent cells.

[0138] In further aspects, the present disclosure provides a method to expand human embryonic stem cells in pluripotent state. It has been shown that human embryonic stem cells plated in single cell suspension on laminin-521 survived and proliferated at a higher rate than that of classical methods known in the art. After 3 passages (1 month) the cells passaged in a single cell suspension underwent the same number of cell divisions as that of cell cultures passaged in pieces on Matrigel

after 20 passages (3 months). Therefore, the new method was advantageous in terms of time and labor, which may provide significant economical profits.

[0139] Laminin-521 is normally expressed and secreted by human pluripotent embryonic stem cells and can also be found in the kidneys, neuromuscular junctions, lungs, and placenta.

[0140] The availability of pure laminin-521 would enable studies of the effects of the protein on cellular differentiation and maintenance of cellular phenotypes. Thus, numerous research and therapeutic purposes including, but not limited to, treating injuries to tissues, promoting cell attachment, expansion and migration, ex vivo cell therapy, improving the biocompatibility of medical devices, and preparing improved cell culture devices and media, would be furthered if pure intact isolated laminin-521 were available. Also, the effects of pure laminin-521 on stem cells would be important to study as this protein is expressed in the early mammalian embryo.

[0141] Thus, there is a need in the art for isolated laminin-521 for research and therapeutic purposes, and methods for making isolated laminin-521. Laminin-521 can serve as a matrix for long-term self-renewal and fast multiplication of dissociated human ES and induced pluripotent stem (iPS) cells under completely chemically defined, feeder-free, and animal-protein-free (xeno-free) conditions. A LN-521 based system is easy to use and easy to automate, and allows fast scale-up of human ES/iPS cultures.

[0142] The present disclosure also relates to a cell culture medium that can be used to provide nutrition to cells, particularly stem cells. In this regard, stem cells typically require two things to be cultured: (1) a substrate or coating that provides a structural support for the stem cell; and (2) a cell culture medium to provide nutrition to the stem cell. The substrate or coating (1) is generally placed on, for example, a petri dish or some other container.

[0143] As used herein, the term "self-renewal" refers to the ability of the stem cell to go through numerous cycles of cell division and remain undifferentiated (i.e. pluripotent). Pluripotency itself refers to the ability of the stem cell to differentiate into any cell type. The term "proliferation" refers to the ability of the stem cell to divide. Survival refers to the ability of the stem cell to live, whether differentiated or undifferentiated, and does not require the stem cell to maintain its ability to divide or to differentiate.

[0144] The cell culture medium of the present disclosure is particularly suitable for being used with a substrate that contains laminin-521 and/or laminin-511. These laminins activate α6β1 integrins, which in turn leads to activation of the PI3K/Akt pathway. This increases the pluripotency, self-renewal, and/or proliferation of the stem cells. It is contemplated that the substrate may consist of laminin-521 or laminin-511, either intact, as separate chains, or as fragments thereof. Recombinant laminin-521 and recombinant laminin-511 are commercially available. Many different molecules can activate the PI3K/Akt pathway, though with different efficiencies. For example, TGF beta 1 and bFGF activate this pathway. The use of laminin-521 and/or laminin-511 allows the quantity of such molecules to be reduced in the cell culture medium. Laminin-521 conveys the highest dose of signal via α6β1 integrin, activating the PI3K/Akt pathway. The use of laminin-521 allows for single-cell suspension passaging without the addition of cell-detrimental rho-kinase (ROCK) inhibitor to increase cell survival after single-cell enzymatic dissociation. Previously, single-cell enzymatic passage of human ES cells without using artificial apoptosis inhibitors was impossible. The simplicity of the passaging procedure means the experimental variance is reduced and allows the process to be automated for high-throughput cell culture and results without the extensive training and costs of cell culture staff. In addition, human ES and iPS cells plated on laminin-521 or laminin-511 grow as a monolayer, which makes the culture homogeneous since cells are equally exposed to the matrix and the cell culture medium. Such human ES cell cultures, grown in a chemically defined, xeno-free environment on laminin-521, passaged as single cells in the absence of ROCK inhibitor expand continuously for months at an even better growth rate compared to cells grown on Matrigel passaged as clumps. These pluripotent long-term expanded cells homogeneously express Oct4 and remain karyotypically normal. Thus, one can obtain human ES and iPS cells with sustained survival and proliferation capacity.

[0145] The average contact area and spreading homogeneity is much larger for cells cultured on laminin-511 compared to other available substrata. Human ES cells grown on laminin-511 over 3 months maintain pluripotency and can generate teratomas after engraftment into SCID mice. Laminin-511 also supports the self-renewal of mouse ES cells for over 5 months without the presence of LIF or feeder cells, when other known matrices are unable to do so for longer than a couple of weeks.

[0146] The stem cells to be grown with this cell culture medium can be induced pluripotent stem cells, embryonic stem cells, adult stem cells, fetal stem cells, amniotic stem cells, and generally any pluripotent stem cell.

[0147] Typically, cell culture media include a large number and a large amount of various growth factors and cytokines to inhibit differentiation and improve proliferation. One advantage of the cell culture medium of the present disclosure is that it does not contain as many growth factors or cytokines, or such high amounts.

[0148] Most generally, the cell culture medium of the present disclosure requires lower amounts of basic fibroblast growth factor (bFGF) than typically used. It is contemplated that the cell culture medium may comprise from greater than zero to 3.9 nanograms per milliliter (ng/mL) of bFGF. The bFGF is human bFGF so that the cell culture medium is totally human and defined. In some more specific embodiments, the cell culture medium may comprise 3.5 or lower ng/mL of bFGF. In other embodiments, the cell culture medium may comprise from 0.5 to 3.5 ng/mL of bFGF. In some embodiments, the cell culture medium may have zero bFGF, i.e. no bFGF is present.

[0149] Generally, the cell culture medium includes a liquid phase in which at least one inorganic salt, at least one trace mineral, at least one energy substrate, at least one lipid, at least one amino acid, at least one vitamin, and at least one growth factor (besides bFGF) are dissolved. Table 1 below includes a list of various such ingredients which may be present in the cell culture medium of the present disclosure, and the minimum and maximum concentrations if the ingredient is present. The values are presented in scientific notation. For example, "4.1E-01" should be interpreted as 4.1×10^{-01}.

Table 1.

Ingredient	molar mass (g/mol)	Min. Conc. (mM)	Max. Conc. (mM)	Min. Conc. (ng/mL)	Max. Conc. (ng/mL)
INORGANIC SALTS					
Calcium chloride (Anhydrous)	110.98	4.1E-01	1.6E+00	4.6E+04	1.8E+05
HEPES	238.3	5.9E+00	1.8E+01	1.4E+06	4.2E+06
Lithium Chloride (LiCl)	42.39	4.9E-01	1.5E+00	2.1E+04	6.2E+04
Magnesium chloride (Anhydrous)	95.21	1.2E-01	3.6E-01	1.1E+04	3.4E+04

Magnesium Sulfate ($MgSO_4$)	120.37	1.6E-01	4.8E-01	1.9E+04	5.8E+04
Potassium chloride (KCl)	74.55	1.6E+00	4.9E+00	1.2E+05	3.6E+05
Sodium bicarbonate ($NaHCO_3$)	84.01	9.0E+00	4.4E+01	7.6E+05	3.7E+06
Sodium chloride (NaCl)	58.44	4.7E+01	1.4E+02	2.8E+06	8.3E+06
Sodium phosphate, dibasic (Anhydrous)	141.96	2.0E-01	5.9E-01	2.8E+04	8.3E+04
Sodium phosphate, monobasic monohydrate (NaH_2PO_4-H_2O)	137.99	1.8E-01	5.3E-01	2.4E+04	7.3E+04
TRACE MINERALS					
Ferric Nitrate ($Fe(NO_3)_3$-$9H_2O$)	404	4.9E-05	1.9E-04	2.0E+01	7.5E+01
Ferrous sulfate heptahydrate ($FeSO_4$-$7H_2O$)	278.01	5.9E-04	1.8E-03	1.6E+02	4.9E+02
Copper(II) sulfate pentahydrate ($CuSO_4$-$5H_2O$)	249.69	2.0E-06	8.0E-06	5.1E-01	2.0E+00
Zinc sulfate heptahydrate ($ZnSO_4$-$7H_2O$)	287.56	5.9E-04	1.8E-03	1.7E+02	5.1E+02
Ammonium Metavanadate NH_4VO_3	116.98	5.5E-06	1.6E-05	6.4E-01	1.9E+00
Manganese Sulfate monohydrate ($MnSO_4$-H_2O)	169.02	9.9E-07	3.0E-06	1.7E-01	5.0E-01
$NiSO_4$-$6H_2O$	262.85	4.9E-07	1.5E-06	1.3E-01	3.8E-01
Selenium	78.96	8.9E-05	2.7E-04	7.0E+00	2.1E+01
Sodium Meta Silicate Na_2SiO_3 -$9H_2O$	284.2	4.8E-04	1.4E-03	1.4E+02	4.1E+02
$SnCl_2$	189.62	6.2E-07	1.9E-06	1.2E-01	3.5E-01
Molybdic Acid, Ammonium salt	1235.86	9.9E-07	3.0E-06	1.2E+00	3.7E+00
$CdCl_2$	183.32	6.1E-06	1.8E-05	1.1E+00	3.4E+00
$CrCl_3$	158.36	9.9E-07	3.0E-06	1.6E-01	4.7E-01
$AgNO_3$	169.87	4.9E-07	1.5E-06	8.3E-02	2.5E-01
$AlCl_3$ -$6H_2O$	241.43	2.4E-06	7.3E-06	5.9E-01	1.8E+00
Barium Acetate ($Ba(C_2H_3O_2)_2$)	255.42	4.9E-06	1.5E-05	1.3E+00	3.8E+00
$CoCl_2$ -$6H_2O$	237.93	4.9E-06	1.5E-05	1.2E+00	3.5E+00
GeO_2	104.64	2.5E-06	7.5E-06	2.6E-01	7.8E-01
KBr	119	4.9E-07	1.5E-06	5.9E-02	1.8E-01
KI	166	5.0E-07	1.5E-06	8.3E-02	2.5E-01
NaF	41.99	4.9E-05	1.5E-04	2.1E+00	6.2E+00
RbCl	120.92	4.9E-06	1.5E-05	5.9E-01	1.8E+00

$ZrOCl_2$ -$8H_2O$	178.13	4.9E-06	1.5E-05	8.7E-01	2.6E+00
ENERGY SUBSTRATES					
D-Glucose	180.16	6.9E+00	2.1E+01	1.2E+06	3.7E+06
Sodium Pyruvate	110.04	2.0E-01	5.9E-01	2.2E+04	6.5E+04
LIPIDS					
Linoleic Acid	280.45	9.4E-05	2.8E-04	2.6E+01	7.9E+01
Lipoic Acid	206.33	2.0E-04	7.8E-04	4.1E+01	1.6E+02
Arachidonic Acid	304.47	6.5E-06	1.9E-05	2.0E+00	5.9E+00
Cholesterol	386.65	5.6E-04	1.7E-03	2.2E+02	6.5E+02
DL-alpha tocopherol-acetate	472.74	1.5E-04	4.4E-04	6.9E+01	2.1E+02
Linolenic Acid	278.43	3.5E-05	1.0E-04	9.7E+00	2.9E+01
Myristic Acid	228.37	4.3E-05	1.3E-04	9.8E+00	2.9E+01
Oleic Acid	282.46	3.5E-05	1.0E-04	9.8E+00	2.9E+01
Palmitic Acid	256.42	3.8E-05	1.1E-04	9.8E+00	2.9E+01
Palmitoleic acid	254.408	3.9E-05	1.2E-04	9.8E+00	2.9E+01
Stearic Acid	284.48	3.4E-05	1.0E-04	9.8E+00	2.9E+01
AMINO ACIDS					
L-Alanine	89.09	2.5E-02	2.1E-01	2.2E+03	1.8E+04
L-Arginine hydrochloride	147.2	2.7E-01	1.5E+00	4.0E+04	2.2E+05
L-Asparagine-H_2O	150.13	5.0E-02	2.1E-01	7.5E+03	3.1E+04
L-Aspartic acid	133.1	2.5E-02	2.1E-01	3.3E+03	2.7E+04
L-Cysteine-HCl-H_2O	175.63	3.9E-02	1.2E-01	6.9E+03	2.1E+04
L-Cystine dihydrochloride	313.22	3.9E-02	1.2E-01	1.2E+04	3.7E+04
L-Glutamic acid	147.13	2.5E-02	2.1E-01	3.7E+03	3.0E+04
L-Glutamine	146.15	1.5E+00	4.4E+00	2.1E+05	6.4E+05
Glycine	75.07	1.5E-01	4.4E-01	1.1E+04	3.3E+04
L-Histidine monohydrochloride monohydrate	209.63	5.9E-02	1.8E-01	1.2E+04	3.7E+04
L-Isoleucine	131.17	1.6E-01	4.9E-01	2.1E+04	6.4E+04
L-Leucine	131.17	1.8E-01	5.3E-01	2.3E+04	7.0E+04
L-Lysine hydrochloride	182.65	2.0E-01	5.9E-01	3.6E+04	1.1E+05
L-Methionine	149.21	4.5E-02	1.4E-01	6.8E+03	2.0E+04
L-Phenylalanine	165.19	8.5E-02	2.5E-01	1.4E+04	4.2E+04
L-Proline	115.13	1.1E-01	3.2E-01	1.2E+04	3.7E+04
L-Serine	105.09	1.5E-01	4.4E-01	1.5E+04	4.6E+04
L-Threonine	119.12	1.8E-01	5.3E-01	2.1E+04	6.3E+04
L-Tryptophan	204.23	1.7E-02	5.2E-02	3.5E+03	1.1E+04
L-Tyrosine disodium salt hydrate	225.15	8.4E-02	3.7E-01	1.9E+04	8.4E+04
L-Valine	117.15	1.8E-01	5.3E-01	2.1E+04	6.2E+04

VITAMINS					
Ascorbic acid	176.12	1.3E-01	3.8E-01	2.2E+04	6.7E+04
Biotin	244.31	5.6E-06	1.7E-05	1.4E+00	4.1E+00
B_{12}	1355.37	2.0E-04	5.9E-04	2.7E+02	8.0E+02
Choline chloride	139.62	2.5E-02	7.5E-02	3.5E+03	1.1E+04
D-Calcium pantothenate	238.27	1.8E-03	1.4E-02	4.4E+02	3.4E+03
Folic acid	441.4	2.4E-03	7.1E-03	1.0E+03	3.1E+03
i-Inositol	180.16	2.7E-02	1.1E-01	4.9E+03	1.9E+04
Niacinamide	122.12	6.5E-03	2.0E-02	7.9E+02	2.4E+03
Pyridoxine hydrochloride	205.64	3.8E-03	1.1E-02	7.8E+02	2.4E+03
Riboflavin	376.36	2.3E-04	6.8E-04	8.6E+01	2.6E+02
Thiamine hydrochloride	337.27	3.3E-03	3.6E-02	1.1E+03	1.2E+04
GROWTH FACTORS/PROTEINS					
GABA	103.12	0	1.5E+00	0	1.5E+05
Pipecolic Acid	129	0	1.5E-03	0	1.9E+02
bFGF	18000	0	2.17E-07	0	3.9E+00
TGF beta 1	25000	0	3.5E-08	0	8.8E-01
Human Insulin	5808	0	5.9E-03	0	3.4E+04
Human Holo-Transferrin	78500	0	2.1E-04	0	1.6E+04
Human Serum Albumin	67000	0	2.9E-01	0	2.0E+07
Glutathione (reduced)	307.32	0	9.6E-03	0	2.9E+03
OTHER COMPONENTS					
Hypoxanthine Na	136.11	5.9E-03	2.6E-02	8.0E+02	3.6E+03
Phenol red	354.38	8.5E-03	2.5E-02	3.0E+03	9.0E+03
Putrescine-2HCl	161.07	2.0E-04	5.9E-04	3.2E+01	9.5E+01
Thymidine	242.229	5.9E-04	1.8E-03	1.4E+02	4.3E+02
2-mercaptoethanol	78.13	4.9E-02	1.5E-01	3.8E+03	1.1E+04
Pluronic F-68	8400	1.2E-02	3.5E-02	9.8E+04	2.9E+05
Tween 80	1310	1.6E-04	4.9E-04	2.2E+02	6.5E+02

[0150] The liquid phase of the cell culture medium may be water, serum, or albumin.

[0151] Many of the ingredients or components listed above in Table 1 are not necessary, or can be used in lower concentrations.

[0152] It is contemplated that the cell culture medium may contain insulin or an insulin substitute. Similarly, the cell culture medium may contain transferrin or a transferrin substitute.

[0153] In more specific embodiments, it is contemplated that the cell culture medium may not (1) contain albumin, (2) insulin or insulin substitute, (3) transferrin or transferrin substitute, or any combination of these three components.

[0154] It should be noted that other cell culture mediums may contain growth factors such as interleukin-1 beta (IL-1β or catabolin), interleukin-6 (IL6), or pigment epithelium derived factor (PEDF). Such growth factors are not present in the cell culture medium of the present disclosure.

[0155] One specific formula for a cell culture medium is provided in Table 2:

Table 2.

Ingredient	Amount	Unit
bFGF	0.39	microgram (µg)
Albumin	1.34	milligram (mg)
Insulin	2	mg
Lithium Chloride	4.23	mg
GABA	0.01	mg
TGF beta 1	0.06	µg
Pipecolic acid	0.013	mg
L-glutamine	2.92	grams
MEM non-essential amino acid solution	1	mL
DMEM/F12	100	mL

[0156] In this regard, MEM non-essential amino acid solution is typically provided in a 100x concentrate. The MEM of Table 2 is used after dilution back to 1x, and contains the following amino acids in the following concentration listed in Table 3:

Table 3.

MEM Amino Acids	Concentration (ng/mL)
Glycine	7.50E+03
L-Alanine	8.90E+03
L-Asparagine	1.32E+04
L-Aspartic acid	1.33E+04
L-Proline	1.15E+04
L-Serine	1.05E+04

[0157] DMEM/F12 contains the following ingredients listed in Table 4:

Table 4.

DMEM/F12 Ingredients	Concentration (ng/mL)
Glycine	187.5
L-Alanine	44.5

DMEM/F12 Ingredients	Concentration (ng/mL)
L-Arginine hydrochloride	1475
L-Asparagine-H_2O	75
L-Aspartic acid	66.5
L-Cysteine hydrochloride-H_2O	175.6
L-Cystine 2HCl	312.9
L-Glutamic Acid	73.5
L-Glutamine	3650
L-Histidine hydrochloride-H_2O	314.8
L-Isoleucine	544.7
L-Leucine	590.5
L-Lysine hydrochloride	912.5
L-Methionine	172.4
L-Phenylalanine	354.8
L-Proline	172.5
L-Serine	262.5
L-Threonine	534.5
L-Tryptophan	90.2
L-Tyrosine disodium salt dihydrate	557.9
L-Valine	528.5
Biotin	0.035
Choline chloride	89.8
D-Calcium pantothenate	22.4
Folic Acid	26.5
Niacinamide	20.2
Pyridoxine hydrochloride	20
Riboflavin	2.19
Thiamine hydrochloride	21.7
Vitamin B_{12}	6.8
i-Inositol	126
Calcium Chloride ($CaCl_2$) (anhyd.)	1166
Cupric sulfate ($CuSO_4$-$5H_2O$)	0.013
Ferric Nitrate ($Fe(NO_3)_3$-$9H_2O$)	0.5
Ferric sulfate ($FeSO_4$-$7H_2O$)	4.17
Magnesium Chloride (anhydrous)	286.4
Magnesium Sulfate ($MgSO_4$) (anhyd.)	488.4
Potassium Chloride (KCl)	3118
Sodium Bicarbonate ($NaHCO_3$)	24380
Sodium Chloride (NaCl)	69955

DMEM/F12 Ingredients	Concentration (ng/mL)
Sodium Phosphate dibasic (Na_2HPO_4) anhydrous	710.2
Sodium Phosphate monobasic (NaH_2PO_4-H_2O)	625
Zinc sulfate ($ZnSO_4$-$7H_2O$)	4.32
D-Glucose (Dextrose)	31510
Hypoxanthine Na	23.9
Linoleic Acid	0.42
Lipoic Acid	1.05
Phenol Red	81
Putrescine 2HCl	0.81
Sodium Pyruvate	550
Thymidine	3.65

[0158] The combination of the laminin substrate with the cell culture medium of the present disclosure results in a cell culture system that can be cheaper, yet provides higher efficiency in maintaining pluripotent stem cells. In essence, all that is required is a laminin and a minimal amount of nutrition. It is particularly contemplated that the laminin used in combination with this cell culture medium is either LN-511 or LN-521.

[0159] The following examples are for purposes of further illustrating the present disclosure. The examples are merely illustrative and are not intended to limit devices made in accordance with the disclosure to the materials, conditions, or process parameters set forth therein.

EXAMPLES

EXAMPLE 1

[0160] Cloning of the Human Laminin β2 cDNA

[0161] The 5.6 kb fragment of human laminin β2 cDNA was PCR-amplified from human liver cDNA library (BD Biosciences) using primers 5'-GTGGTACCCACAGGCAGAGTTGAC-3' (SEQ ID NO: 7) and 5'-GCTCTAGAGCTCTTCAGTGCATAGGC-3' (SEQ ID NO: 8) thus introducing artificial XbaI and KpnI cutting sites on the ends of the fragment. To decrease the error rate during the PCR amplification, Phusion™ high-fidelity PCR Kit (Finnzymes) was used.

Subsequently, the fragment was digested with XbaI and KpnI and subcloned into pSK vector digested with the same restriction endonucleases (pSKHLAMB2 plasmid). To verify the integrity of the sequence, several clones of pSKHLAMB2 plasmid were sequenced. Sequencing was performed on an ABI PRISM™ 310 Genetic Analyzer (Perkin Elmer) using ABI PRISM® BigDye™ Terminator Cycle Sequencing kit (PE Applied Biosystems). Only complete matches with the NCBI database human laminin β2 sequence were selected for further cloning.

[0162] Expression Constructs

[0163] For expression of the human laminin β2 chain pSKHLAMB2 plasmid was digested with XbaI and KpnI and subcloned into XbaI-KpnI treated pcDNA 3.1(+) vector (Invitrogen).

[0164] The constructs used for expression of human laminin α5 (HLN5Full.pcDNA construct) and γ1 (HG1 construct) have been described previously (Doi, M. et al., J. Biol. Chem. 277(15), 12741-8 (2002)).

[0165] Antibodies

[0166] Anti-laminin β2 (MAB2066) monoclonal antibody (mAb) was purchased from R@D Systems. Anti-laminin α5 mAb (2F7) was purchased from Abnova. Anti-laminin β1 mAb (MAB1921) was purchased from Chemicon. Anti-laminin γ1 (H-190) rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc.

[0167] Production and Purification of Recombinant Laminin-521

[0168] r-laminin-521 was produced in human embryonic kidney cells (HEK293, ATCC CRL-1573) cultured in DMEM, 10% FCS in humidified 5% CO_2 atmosphere at 37°C. Wild-type cells were transfected using the standard calcium-phosphate method with the HG1 construct and stable colonies were selected using 100 mg/ml hygromycin (Cayla). All further cell culture and clonal expansion was carried out in continuous presence of relevant selection antibiotics. A highly expressing clone was then transfected with the human laminin β2 construct and stable clones were selected using 500 mg/ml G418 (Life Technologies). A clone highly expressing both laminin γ1 and laminin β2 was finally transfected with the HLN5Full.pcDNA construct and stable colonies were selected using 200 mg/ml zeocin (Cayla). The clones showing the highest secretion were expanded further.

[0169] For production of r-laminin-521, confluent cells were cultured in DMEM for up to five days. r-laminin-521 was affinity purified using anti-FLAG M2 matrix (Sigma). The collected medium was incubated in batch mode with the matrix overnight at 4°C with agitation. Bound r-laminin-521 was competitively eluted with 50 mg/ml FLAG peptide (Sigma) in TBS/E (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA) at room temperature. The elute was concentrated and the buffer was replaced by PBS using 30 kD cut-off ultrafiltration (Millipore). Finally the concentrated solution was passed through 0.2 mm filter to remove self-aggregated polymers.

[0170] *Characterization of Recombinant Laminin-521*

[0171] Secreted laminin in medium and after purification was characterized using 3-8% gradient SDS-PAGE. Proteins were visualized using Sypro staining (Bio-Rad) or transferred onto PVDF (**FIG. 3**). The membranes were probed with antibodies described above. After washing, the membranes were incubated with HRP-conjugated goat antibodies. The immunoreactivity was detected by a chemiluminescent kit (Life Science Products) according to the manufacturer's instructions.

Methods

[0172] Human ES cell cultures.

[0173] Human ES cells were cultured on r-laminin-521–coated laboratory dishes in chemically defined O3 medium (described in Rodin et al., *Nature Biotechnol.*, vol. 28, pp. 611-615 (2010)) at 37°C in 5% CO2. Cells were routinely passed once every 10–12 days by exposure to Trypsin-EDTA solution (GIBCO Invitrogen) for 5 minutes at 37°C. They were then gently pipetted to break into single-cell suspension and defined trypsin inhibitor (GIBCO Invitrogen) was added. The cell suspension was centrifuged at for 4 minutes, the supernatant discarded, the cell pellet resuspended in prewarmed O3 medium, and cells were then passed through a 40 μm sieve. After that cells were plated on new r-laminin-521–coated dishes at a concentration 30 Kcells/cm^2 (1:25-1:30 split ratio). Cells were fed once a day with fresh medium prewarmed in an incubator for 1 hour, except for the first day after a passage, when only a few drops of fresh medium were added. Control cells of the same line were

cultured on Matrigel (BD Biosciences) in O3 medium as described in Rodin et al., *Nature Biotechnol.*, vol. 28, pp. 611-615 (2010). Control cells were passaged in pieces.

[0174] Cell culture dish coating.

[0175] Ninety-six-well tissue cell culture plates were coated overnight at 4°C with sterile solutions of the ECM proteins mouse LN-111 (Invitrogen), human recombinant LN-511, and human recombinant LN-521, all at a concentration of 30 μg/ml (5 $\mu g/cm^2$). For control cells, BD Matrigel™ hESC qualified (BD Biosciences) was used according to the manufacturer's instructions.

[0176] Cell adhesion assay.

[0177] The assay was performed as described (Extracellular Matrix Protocols, 2000). *Briefly, 96-well plates were coated by extracellular matrix proteins as* described above and blocked by O3 medium containing bovine serum albumin. The ES cells were plated at a cell density of 600 cell / mm^2 upon extracellular matrix-coated plates and were left to adhere for either 1 hour or 1 day at the cell incubator. Non-adherent cells were washed away, and adherent cells were fixed for 20 min by 5% glutaraldehyde, stained by 0.1% Crystal Violet.

[0178] Real-time PCR quantification of mRNAs.

[0179] Total RNA was isolated and cDNA was synthesized as described in Rodin et al., *Nature Biotechnol.*, vol. 28, pp. 611-615 (2010). Real-time quantitative RT-PCR Taqman assays were performed using the Applied Biosystems 7300 Real-Time PCR System. All reactions were done in quadruplicate with predeveloped gene expression assay mix (Applied Biosystems) containing primers and a probe for the *mRNA of interest. Additional reactions for each experiment included predeveloped* gene expression assay mix for *GAPDH*, used to normalize the RNA input. All data were analyzed with 7300 System SDS Software version 1.4.

[0180] FACS analysis.

[0181] OCT4 expression was analyzed as described in Ludwig, T.E. *et al.* Derivation of human embryonic stem cells in defined conditions. Nat. Biotechnol. 24,

185–187 (2006). Cells were run on FACSCalibur Flow Cytometer (Becton Dickinson). Data were analyzed with CellQuest software (Becton Dickinson).

Results for Example 1

[0182] To find out how different cell culture coatings affect single cell survival of human ES cells in O3 medium without any additives, we completely dissociated HS181 cells and plated them on either Matrigel, mouse laminin-111, human r-laminin-511, human r-laminin-521, or a mixture of r-laminin-511 and r-laminin-521. The results are seen in **FIG. 4**. As expected, almost no cells remained attached to Matrigel or mouse laminin-111 by 24 hours after plating. In contrast, the cells on human r-laminin-521, the mixture, and at a less extent on human r-laminin-511 survived.

[0183] The experiment was repeated on human ES cells in O3 medium treated with ROCK inhibitor Y-27632. These results are seen in **FIG. 5**. The stem cells remained attached on all 5 coatings.

[0184] To quantify this effect, we performed cell adhesion experiments on all of the above-mentioned coatings at 1 hour and 1 day after plating (i.e., each of the five coatings, with and without Y-27632). **FIG. 6** shows the results for the 1-hour experiment for LN-521, LN-511, and Matrigel (MG) without Y-27632. The adhesion of human ES cells in O3 medium without any additives was roughly the same at this time point.

[0185] **FIG. 7** shows the results for the 1-day experiment for all of the coatings. The plates containing the Y-27632 inhibitor are labeled as "In&" along with the coating. At 1 day after plating, the stem cells adhered to LN-521 without additives 20 times better than to Matrigel without additives. In addition, the results showed that the adhesion of cells to LN-521 without additives was similar to the adhesion of cells on all dishes including the ROCK inhibitor. In other words, no ROCK inhibitor is necessary with LN-532 coating to obtain results similar to coatings that do contain the ROCK inhibitor.

[0186] We cultured human ES cells on human r-laminin-521. passaging them after complete dissociation into single cell suspension every 10-12 days in 1:25 to 1:30 ratios. The cells proliferated robustly with a stable and high rate for at least 9 passages (3 months). Moreover, after 3 passages (1 month), they underwent the same number of cell doublings as stem cells after 20 passages (100 days) cultured

using conventional methods. This is illustrated in **FIG. 8**, which shows the increase in the number of cells versus time. The cells on LN-521 increased at a much faster rate than on Matrigel. Thus, the new r-laminin-521 hES cell culture method was advantageous in terms of time and labor.

[0187] To confirm the identity of hES cells after several single cell suspension passages on human r-laminin-521 in O3 medium without any additives, we performed FACS analysis for Oct4, a marker of pluripotency. **FIG. 9** shows the results for cells grown on r-LN-521, while **FIG. 10** shows the results for cells grown on Matrigel. The percentage of positive cells is listed in parentheses. R-LN-521 had a much higher percentage of pluripotent cells.

[0188] In addition, the number of mRNA transcripts of the pluripotency markers Oct4 and Nanog was quantified. **FIG. 11** shows these results. For both markers, the cells on LN-521 showed higher numbers of transcripts.

[0189] Both methods showed that the new methods provides hES cells of the same or better quality than the current method, which uses Matrigel as a cell culture dish coating material and passaging of the cells in small clumps.

EXAMPLE 2

[0190] In this Example 2, expressed and purified recombinant LN-521 that was also expressed in pluripotent hES cells was studied for its effects on cultured hES and iPS cells. The results showed that LN-521, alone, strongly supports self-renewal of pluripotent hES and iPS cells, and, importantly, it allows survival of pluripotent stem cells following plating of trypsinized stem cells in single cell suspension on LN-521 at high dilutions. The effects of LN-521 were shown to be mediated by signaling via $\alpha 6\beta 1$ integrin through induction of hES cell migration and PI3K/Akt pathway activation. The results may be applied for an effective and even automated expansion method for large-scale production of pluripotent hES and iPS cells, as well as for development of new medium formulations for self-renewal of pluripotent stem cells. The new hES/hiPS cell culture method described here closely resembles standard cell culture methods, *e.g.* that are used to culture fibroblasts, and, therefore, it does not demand special skills. It consumes significantly less cell coating material per one cell division and is time efficient, thus providing significant

economical profits in comparison with the previous procedures for culturing hES/hiPS cells.

[0191] Human recombinant LN-521, which has previously not been available in pure form, was produced by cloning full-length laminin β2 cDNA and carrying out a triple-transfection of *HEK293* cells with the human laminin α5, β2 and γ1 chains. Human recombinant LN-111 and LN-121 were similarly generated following cloning of full-length α1 and β2 chain cDNAs and triple transfection of HEK293 cells with human α1, β1 and γ1 and α1, β2 and γ1 chain cDNAs, respectively. The purified LN-521, LN-111 and LN-121 proteins were shown to contain, respectively, pure α5, β2, and γ1 chains, α1, β1 and γ1 chains, as well as α1, β2 and γ1 chains, as shown by protein staining and Western blot analysis.

Methods

[0192] Human ES and iPS cell cultures.

[0193] Human ES cells of HS181 and HS401 were cultured on LN-521-coated culture dishes in O3 medium (described in Rodin et al., *Nature Biotechnol.*, vol. 28, pp. 611-615 (2010) with pH was adjusted to 7.35), mTeSR1 (STEMCELL Technologies) and chemically defined and xeno-free TeSR2 (STEMCELL Technologies) at 37°C, 5% CO_2. Initially, cells of the lines were transferred onto a LN-521 coating from a human feeder cell layer by careful scratching using a sterile knife with subsequent trypsinization, or the cells were trypsinized into single cell suspension from a LN-511 coating. The cells were provided once a day with fresh medium pre-warmed in an incubator for one hour, except for the first day after plating, when only a few drops of fresh medium were added. Cells were routinely passed once in 10-12 days by exposure to Trypsin/EDTA (GIBCO Invitrogen Corporation, Paisley, Scotland) for 5 minutes at 37°C, 5% CO_2. They were then gently pipetted to break into single-cell suspension and a Defined Trypsin Inhibitor (GIBCO Invitrogen) was added. The cell suspension was centrifuged at 25 rcf for 4 minutes, the supernatant was discarded, the cell pellet was resuspended in prewarmed O3 medium, and the cells were then passed through a 40 μm sieve. Subsequently, the cells were plated on new LN-521–coated dishes at a concentration of 30,000 cells/cm^2 in 1:25-1:30 split ratios. Control cells of the same

line were cultured on Matrigel (STEMCELL Technologies) and LN-511 in O3 medium as described previously.[9]

[0194] For defined and xeno-free cultures, TeSR2 (STEMCELL Technologies) medium and defined free from any animal derived component TrypLE™Select (GIBCO Invitrogen Corporation, Paisley, Scotland) enzyme were used. The cells were passed every 10-14 days by exposure to TrypLE™Select for 4-5 minutes at 37°C, 5% CO_2. Then, the enzyme was carefully aspirated and prewarmed TeSR2 medium was added. After that, the cells were gently pipetted to break into single-cell suspension, centrifuged, the supernatant was discarded, and the cell pellet was resuspended in prewarmed TeSR2. The cells were then passed through a 40 μm sieve and plated on a fresh LN-521 coated dish.

[0195] Tissue cell culture plates were coated overnight at 4°C with sterile solutions of the ECM proteins, such as human recombinant LN-521, all at a concentration of 30 μg/ml (5 $\mu g/cm^2$). Control plates were coated with Matrigel according to the STEMCELL Technologies' instructions. Prior to use, dishes were pre-warmed in an incubator for one hour after which prewarmed O3 medium was added without additional washing of the dish. After applying a fresh LN-521 solution to a new dish, the remaining LN-521 solution could be used at least once more. All the adhesion, survival, and inhibition of survival experiments were carried out using fresh coating materials.

[0196] The human iPS cells, ChiPSW line, were derived from lentivirally transduced human foreskin fibroblasts (HFF) with OCT-4/SOX2/NANOG/LIN28 reprogramming genes. The ChiPSW line had normal male karyotype, 46,XY, in repeated testing at different passages. Prior to cell culture and passaging experiments, the cells were first characterized in vitro for expression of pluripotency markers. Immunofluorescence study with antibodies against Oct3/4 (SC-5279), Nanog (SC-33759), TRA-1-60 (SC-21705) and SSEA4 (sc-21704) showed that the cells expressed all those markers of pluripotency. RT-PCR analysis confirmed that the cells lacked expression of the viral transgenes. The pluripotency of ChiPSW cells was confirmed by in vitro (embryoid bodies formation and immunofluorescence study) and in vivo (injection subcutaneously into SCID beige mice) experiments. Cells of ChiSW line could be further differentiated into beating cardiomyocytes. They were also capable of undergoing hematopoietic differentiation.

[0197] Before taken to the present feeder free cultures, the iPS cells were maintained and expanded over irradiated human foreskin fibroblasts. Knock-out serum (KSR, Invitrogen)-supplemented media was used for propagation, supplemented with 8 ng/ml of basic fibroblast growth factor (bFGF, R&D Systems). Cells were fed on a daily basis and weekly passaged using collagenase IV (1 mg/ml, Roche) or manual dissection when required.

[0198] *Prior to these experiments, the CutB1.2 cells were shown to express* pluripotency markers Oct4/Nanog/Sox2/TRA-1-60/TRA-1-81, to lack expression of the viral transgenes, and the pluripotency of the cells was confirmed both by *in vivo* and *in vitro* differentiation studies.

[0199] **Laminin-521 and other coating materials.**

[0200] *Human recombinant* LN-521, available from BioLamina, AB, Stockholm (www.biolamina.com), was produced in human embryonic kidney cells (HEK293; ATCC CRL-1573) sequentially transfected with full-length laminin γ1, β2 and α5 constructs essentially as described previously. For protein production, the HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with GlutaMax I for up to six days. The LN-521 molecules were affinity-purified using anti-FLAG matrix (Sigma), and then characterized using 3-8 % and 4-15 % gradient SDS-PAGE under reducing and nonreducing conditions. The proteins were visualized using Sypro Ruby (Bio-Rad) protein staining and immunostaining of the chains on polyvinylidene difluoride membranes. To further characterize the protein, Western blot analysis with antibodies against the laminin α5, β2 and γ1 chains was performed. Human recombinant laminin-111 and laminin-121 were produced similarly to LN-521 and shown to contain the correct chains of predicted molecular sizes by Western and SDS-PAGE. If not otherwise stated, the corresponding mouse laminin-111 (Invitrogen) was indicated as LN-111.

[0201] **Reagents and antibodies.**

[0202] InSolutionTM LY 294002 (a specific Akt inhibitor), InSolutionTM Wortmannin (a specific PI3K inhibitor), and InSolutionTM 98059 (a specific MEK1/Erk inhibitor) were purchased from Calbiochem. Antibodies to phospho-Akt (#4060), total-Akt (#9272), phospho-Erk (#9101), and total-Erk (#9102) were

obtained from Cell Signaling Technology. PathScan Phospho-Akt1 sandwich ELISA kit (#7160), PathScan total Akt1 sandwich ELISA kit (#7170), PathScan Phospho-Akt2 sandwich ELISA kit (#7048), and PathScan total Akt2 sandwich ELISA kit (#7046) were also purchased from Cell Signaling Technology. Antibodies to Calnexin (#ab10286) were obtained from Abcam. Function blocking antibodies to various integrin subunits, mouse isotype antibodies, and α-dystroglycan were purchased from Millipore. Since function blocking antibodies to αV (MAB1980) and α6 (MAB1378) were presented in a solution with sodium azide, before the inhibition of survival experiment all the antibodies to alpha integrin subunits were dialyzed thrice against O3 medium for 2 hours each time. Antibodies to Lutheran receptor and α-fetoprotein, as well as rat isotype control antibodies were obtained from R&D Systems. Antibodies to Oct4, Nanog, SSEA-4, smooth muscle actin and MAP-2 were purchased from Millipore.

[0203] Immunofluorescence.

[0204] For immunofluorescence studies, ES cells were cultured and fixed in 8-well slide chambers (BD Biosciences) or 96-well plate wells by 4 % paraformaldehyde, permeabilized by 0.1 % Triton-X and blocked by 10 % bovine fetal serum (GIBCO Invitrogen Corporation) in phosphate-saline buffer (PBS) containing 0.1 % Tween-20 (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) for one hour. Incubation with primary antibody was performed for 1.5 hours at room temperature. Incubation with secondary antibody and 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) was performed for 40 minutes. Between incubations, the specimens were washed with 0.1% Tween-20 in PBS buffer three to five times. Specimens were preserved in a fluorescence mounting medium (Dako, Glostrup, Denmark, http://www.dako.com), and observed under a fluorescence microscope (Leica, Heerbrugg, Switzerland, http://www.leica.com).

[0205] Real-time PCR quantification of different mRNAs.

[0206] Total RNA was isolated using Absolutely RNA Microprep Kit (Stratagene, La Jolla CA, www.stratagene.com) according to the manufacturer's instructions. cDNA was synthesized using 0.2 µg of total RNA in 20 µl reaction mixture, containing oligo(dT)12-18 primers and Superscript II reverse transcriptase (GIBCO

Invitrogen Corporation), according to the manufacturer's instructions. Real-time quantitative RT-PCR Taqman assays were performed using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). All reactions were done in quadruplicates with the use of a pre-developed gene expression assay mix (Applied Biosystems) containing primers and a probe for the mRNA of interest. Additional reactions for each experiment included pre-developed gene expression assay mix for GAPDH for normalizing the RNA input. All data was analyzed with 7300 System SDS Software v 1.4.

[0207] FACS analysis.

[0208] Cells were removed from the culture dish with Trypsin/EDTA, dissociated into single cell suspension and resuspended in ice-cold FACS buffer (2 % fetal bovine serum, 0.1 % sodium azid in Hank's buffer). Incubation with primary antibodies against SSEA-4 (from Millipore, Billerica, MA, http://www.millipore.com) was performed for one hour on ice. Then, the cells were washed three times with ice-cold FACS buffer. Subsequently, the cells were probed in FACS buffer with 1:400 dilution of Alexa Fluor anti-mouse secondary antibodies (GIBCO Invitrogen Corporation) for 30 minutes in the dark, and washed four times. Control cells were incubated with mouse immunoglobulins and, subsequently, with the secondary antibody as described above. Cells were analyzed on FACSCalibur Flow Cytometer (Becton Dickinson, San Jose, CA). Data were analyzed with the CellQuest software (Becton Dickinson).

[0209] Karyotyping.

[0210] Karyotyping of the cell lines was carried out using standard Q-banding techniques. Samples of cells were treated with colcemid KaryoMAX (0.1 μg/ml; Gibco Invitrogen Corporation) for up to 4 hours, followed by dissociation with Trypsin/EDTA solution (Gibco Invitrogen Corporation). The cells were pelleted via centrifugation and re-suspended in pre-warmed 0.0375 M KCl hypotonic solution and incubated for 10 minutes. Following centrifugation, the cells were resuspended in fixative (3:1 methanol:acetic acid). Metaphase spreads were prepared on glass microscope slides and G-banded by brief exposure to trypsin and stained with 4:1

Gurr's/Leishmann's stain (Sigma-Aldrich Co.). A minimum of 10 metaphase spreads were analyzed and additional 20 were counted.

[0211] Teratoma formation.

[0212] Teratoma formation experiments were performed by implantation of approximately 10^6 cells beneath the testicular capsule of a young (7-week-old) severe combined immunodeficiency (SCID) mouse. Three animals per each cell line were used. Teratoma growth was observed by weekly palpation, and the mice were sacrificed eight weeks after the implantation. The teratomas were fixed, and sections were stained with hematoxylin and eosin (HE) or with hematoxylin, eosin and PAS (HE-PAS). The presence of tissue components of all three embryonic germ line layers was demonstrated, as analyzed from the stained sections. All animal experiments were performed at the infection-free animal facility of the Karolinska University Hospital in accordance with ethical committee approval.

[0213] Embryoid body formation.

[0214] ES cells from LN-521 coated cell culture dishes were exposed to TrypLE™Select for one minute at 37°C, 5% CO_2, washes two times with a medium, broken into large pieces and cultured in suspension in low adhesion plates. The medium used for this was Knockout DMEM (GIBCO Invitrogen Corporation) supplemented with 2 mM L-glutamine, 20% fetal calf serum (GIBCO Invitrogen Corporation), 0.1 mM β-mercaptoethanol (GIBCO Invitrogen Corporation) and 1% non-essential amino acids (GIBCO Invitrogen Corporation). After 1-2 weeks in suspension, the embryoid bodies were transferred onto gelatin coated tissue cell culture 96-well plates (Sarstedt), cultured for 1-2 weeks, then fixed, stained with antibodies against markers of all three embryonic germ line layers (smooth muscle actin, MAP-2 and α-fetoprotein) and analyzed as described above for immunofluorescence.

[0215] Cell adhesion assay.

[0216] Briefly, 96-well plates were coated by extracellular matrix proteins as described above and blocked by O3 medium containing bovine serum albumin. The ES cells were plated at cell density of 50,000 cells/cm^2 onto extracellular matrix-

coated plates and left to adhere for 1 hour in a cell incubator. After that the plates were washed 3 times with the medium to remove the non-adherent cells, and then the adherent cells were fixed for 20 minutes by 5% glutaraldehyde, stained by 0.1% Crystal Violet. (Kebo Lab, Spanga, Sweden,http://www.kebolab.se). After one hour and 3 washes with water, Crystal Violet was extracted with 10% acetic acid and quantified by measuring optical density at 570 nm. All the experiments were performed in quadruplicate.

[0217] Cell survival and inhibition of survival assays.

[0218] The survival assay was performed as described for the cell adhesion assay above, except that the cells were left in the cell incubator for 24 hours. For inhibition of survival assay, the cells were kept in a medium with function blocking antibodies at the concentrations recommended by the manufacturer or pathway inhibitors at concentrations indicated in the text for 30 minutes, and then plated on the coated dishes. All the experiments were performed in quadruplicate.

[0219] Western blotting and ELISA.

[0220] HS 181 cells were trypsinized into single cell suspension as described above. For inhibition experiments, the cells were kept in O3 medium with blocking antibodies or pathway inhibitors for 30 minutes and then 450K cells were plated on 35 mm dishes precoated with the appropriate matrix coating. For other experiments, same number of cells was plated directly after trypsinization. In all cases the cells were allowed to spread for 1 hour at 37°C, 5% CO_2. After two washings in ice-cold PBS, the plates with cells were snap frozen in liquid nitrogen and stored at -80°C. To prepare samples for western blots and ELISA, the plates were slowly thawed and kept on ice with 100-150 µl of lysis buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.5% deoxycholate, 0.5% SDS, 1% Triton X-100, 1% Igepal, Complete™ (Roche) and Phospho-Stop™ (Roche)) on top. Then, the cells were scraped, pipetted and sheared through a 27G ¾" needle. After that, the cell pellets were clarified by centrifugation at 16,100 rcf for 15 minutes at 4°C. For western blots, 4-12% gradient gels were used for SDS electrophoresis and the proteins were transferred to PVDF membranes. The membrane was hybridized with the antibody of interest according to the manufacturer's instructions. Chemoluminescent HRP-substrate from

Amersham Biosciences was used for visualization. For the densitometry analysis the films were scanned at 2,400 dpi and analyzed by the ChemiImager5500 program (1D-Multi Line densitometry mode). For ELISA the samples were applied to the wells according to the manufacturer's instructions.

[0221] ***In vivo* imaging and migration assay.**

[0222] 24-well plates were coated by extracellular matrix proteins and blocked by O3 medium containing bovine serum albumin. The ES cells were plated at cell density of 30,000-40,000 cells/cm^2 onto extracellular matrix-coated plates and left to adhere for half an hour in a cell incubator. After that, the plate was transferred into high content imaging system Operetta (PerkinElmer) equipped with environmental control unit, which allowed to keep 37°C, 5% CO_2. For two movies that were made, the brightfield images were taken once in 15 minutes during 24 hours after plating using Harmony software (PerkinElmer), exported, and analyzed using ImageJ software (NIH, the US). For migration assay the images were taken every seven minutes during 18 hours after plating. Images acquired between the fifth and seventh hours after plating were analyzed using MTrackJ plug-in (University Medical Center, Rotterdam, The Netherlands). 100 attached cells on each coating were traced and mean distances from the current to the previous point of the track were calculated. Error bars show standard error of the mean s.e.m. (n=100).

[0223] **Statistics.** *Statistical significance was determined the by Student's two-*tailed t-test for unequal variances.

Discussion of Results for Example 2

[0224] Pluripotent hES cells express α1, α5, β1, β2 and γ1 laminin chains. To compare the adhesion and clonal survival of hES cells plated from single cell suspension to different coating substrata, hES cells growing as monolayers on LN-511 or as clusters on a feeder layer were trypsinized into single cell suspension in O3 medium and plated on cell culture dishes coated with Matrigel, LN-111, LN-511, *LN-521 or a mixture of LN-511 and LN-521, in the absence or presence of a ROCK* inhibitor (Y-27632) and analyzed after 24 hours.

[0225] The cells did not survive on Matrigel or LN-111, but they did so as single cells on human recombinant LN-511 and LN-521, as well as on the mixture of the two. However, the survival of cells on LN-521 was significantly higher than on LN-511 (compared in numbers below). Cells plated on human recombinant LN-111 or LN-121 failed to survive after 24 hours (data not shown), which demonstrates that the presence of the β2 chain in a laminin trimer, as such, is not sufficient to support the effect. In the presence of ROCK inhibitor Y-27632, the hES cells survived on all surfaces.

[0226] There were, however, clear differences with regard to cell shapes between cells growing on LN-521 in the absence or presence of ROCK inhibitor 24 hours after plating. In the absence of ROCK inhibitor 24 hours after plating, cells growing on LN-521 were round, while the cells growing in the presence of ROCK inhibitor adopted spindle or crescent like shape possibly caused by rearrangement of the actin cytoskeleton.

[0227] To test if LN-521 could be used as a cell coating material for long-term self-renewal of human pluripotent cells, HS181, HS401, H1 cells and human iPS ChiPSW and CutB1.2 cells were cultured on the protein in O3, mTeSR1 or TeSR2 media. Cells growing in O3 or TeSR1 media were passed in single cells suspension every 10-14 days at ratios of 1:20-1:30. Pluripotent hES cells proliferated at a stable rate similar or higher to that of cells grown on LN-511 or Matrigel when passed in small clumps. Thus, one passage on LN-521 yielded the same or higher number of cell divisions than that of control cells passed twice in clumps. HS181, HS401, and H1 cells proliferated for at least 24, 5 and 15 passages, respectively (9, 2 and 6 months) in an O3 medium. CutB1.2 iPS and ChiPSW cells have been cultured for 5 and 3 passages in mTeSR1, respectively. Interestingly, dissociated hES cells could be cultured on LN-521 under completely defined and xeno-free conditions, using TeSR2 medium and TrypLE Select enzyme. The plating efficacy after a passage was slightly lower than that of the cells in O3 or mTeSR1 media, and the dissociated cells were passed normally every 10-14 days in 1:15-1:20 ratios. H1 and HS401 cells have been cultured for 12 and 4 passages, respectively (5 and 1.5 months) in TeSR2.

[0228] The hES cells were usually plated in single cell suspension at 30,000 cells per 1 cm^2 of culture dish coated with LN-521, after which individual cells lacked any

direct contacts with each other. Eight hours after plating, the hES cells could be observed as single cells expressing pluripotency markers Oct4, Nanog, and Sox2. Twenty four hours after plating, the cells formed small monolayer colonies that eventually combined into large islands of monolayers covering most of the well.

[0229] Cells grown on LN-521 showed stable expression levels of pluripotency markers Oct4, Nanog, and SSEA4, which were similar to those in cells plated on Matrigel and passed as small clumps. To compare the level of spontaneous differentiation in LN-521 and Matrigel cultures, the amount of mRNAs for differentiation markers PAX6, SOX17 and SOX7 expressed in Matrigel cultures and in LN-521 cultures were compared after one (10 days) and 10 passages (4 months). Quantitative RT-PCR revealed similar or less levels of expression of all three markers of differentiation in LN-521 cultures independently on the passage number.

[0230] Karyotypes were confirmed to be normal for hES cell lines HS181 and H1 after 12 and 10 passages, respectively, on LN-521 in the O3 medium; and for H1 after 7 passages in TeSR2 medium. Histological examination of teratomas formed in SCID mice after injection of HS181 and H1 cells cultured for 13 and 12 passages, respectively, on LN-521 in O3 medium, and H1 cells cultured for 7 passages on LN-521 in TeSR2 revealed development of tissues containing all three germ lineages of the human embryo. Differentiation *in vitro* also revealed, that the cells in all three cases retained the competence to form embryoid bodies expressing markers of mesoderm (smooth-muscle actin), ectoderm (MAP-2) and endoderm (α-fetoprotein).

[0231] To quantify the efficacy of the coating substrata, adhesion after one hour and survival after 24 hours of dissociated hES cells on Matrigel, LN-111, LN-511, LN-521, and an equal mixture of LN-511 and LN-521 were studied. Interestingly, after one hour about the same 75-80% of cells had adhered to all the coatings, although the spreading of the cells was clearly better on LN-521 and LN-511 than on Matrigel or LN-111 (not shown). After 24 hours, almost no cells had survived on Matrigel or LN-111, and very few on LN-511. In contrast, the survival of hES cells on LN-521 was approximately 20 times higher than on Matrigel, and three times higher than on LN-511.

[0232] To qualitatively compare the effects of LN-521 and ROCK inhibitor (Y-27632), we plated cells from the same single cell suspension at the same plating density (40,000 cells per 1 cm^2) in a medium containing 10 μM of Y-27632 and

studied their survival on the different coatings. The untreated cells on LN-521 and Y-27632 treated cells on Matrigel showed similar survival rate 24 hours after plating. Interestingly, even Y-27632 treated human ES cells survived better on LN-521 than on Matrigel.

[0233] If exogenous bFGF was removed from the O3 medium, the cells still showed 20 times higher survival on LN-521 than on Matrigel. Moreover, hES cells survived on LN-521 24 hours after plating, even in a medium lacking all the growth factors of the TeSR1 formulation (bFGF, LiCl, γ-aminobutyric acid (GABA), pipecolic acid and TGFβ)[16], suggesting that the survival mechanism is independent of signaling induced by those factors.

[0234] An assay for inhibition of cell survival using function-blocking antibodies to potential receptors for LN-521 on the plasma membrane showed that antibodies to integrins α6, and to a slightly lesser extent to β1, inhibited survival of human ES cells on LN-521. Function-blocking antibodies to other tested integrin subunits, as well as to Lutheran receptor and α-dystroglycan, showed very little if any effects on human ES cell survival on LN-521.

[0235] Recently, it has been shown that ROCK inhibitors and blebbistatin act through abrogation of actin-myosin contractility, which is mediated by phosphorylation of the myosin light chain (MLC). To test if LN-521 had similar activity, levels of phosphorylated MLC were compared between the dissociated cells on Matrigel, LN-111, LN-511, and LN-521 one and six hours after plating (**FIG. 12** and **FIG. 13**). Interestingly, western blot showed that phosphorylation of MLC was even higher in the cells on LN-511 and LN-521 than that in the cells on Matrigel and LN-111. Since actin-myosin rearrangements are essential not only for contractions, but also for cell motility, we surmised that the cell migration could be caused by interaction between LN-521 and its integrin receptor α6β1. *In vivo* imaging of the cells on Matrigel and LN-521 revealed that the cells migrated much faster on the latter and survived by aggregation into small fast moving colonies. Migration of hES cells on the four coatings was also compared between the fifth and seventh hours after plating when the cells were still attached in all cases (**FIG. 14**). Motility of the hES cells on LN-521 was higher than that of the cells on other coatings and correlated with the ability to survive on them. Treatment with function blocking

antibody to integrin β1 significantly reduced motility (**FIG. 15**) and adhesion (data not shown) of the cells on LN-521.

[0236] An extensive body of data has shown that activation of the MEK1/Erk or PI3-kinase/Akt pathways by integrins can block anoikis. The effects of LY 294002 and PD 98059, specific inhibitors of Akt and MEK1, respectively, were examined to explore the potential role of these pathways in hES cell survival on LN-521. Blockade of Akt activation by LY 294002 was found to facilitate detachment and hES cell death, with no cells surviving 24 hours after plating on LN-521 (**FIG. 16**). In contrast, treatment with PD 98059 did not affect the survival that severely. hES cells treated with another PI3K/Akt specific inhibitor, Wortmannin, also failed to survive 24 hours after plating on LN-521, confirming that activation of PI3K/Akt was necessary for the cell survival on LN-521.

[0237] Since bFGF is known to be a potent activator of the MEK1/Erk pathway, the influence of which cannot be fully inhibited by PD 98059, we performed the same experiment in O3 medium without exogenous bFGF with the same result (**FIG. 17**). The efficacy of LY 294002 and PD 98059 treatment was confirmed by western blot analysis of the treated and control cells collected one hour after plating on LN-521 (**FIG. 18** and **FIG. 19**).

[0238] Western blot analysis of extracts of cells growing on Matrigel and LN-521 with antibodies to phospho-Akt showed that the PI3K/Akt pathway was active in both cases (data not shown). To determine the levels of Akt activation in the cells on different coating, ELISA was performed on cell lysates collected one hour after plating on Matrigel, LN-111, LN-511 and LN-521 when the cells still did not have direct contacts with each other (**FIG. 20** and **FIG. 21**). The level of Akt2 phosphorylation in the cells on different coatings correlated with survivability on them.

[0239] It has been demonstrated that α6 and β1 integrins are the most abundantly expressed integrin isoforms in human ES cells among alpha and beta subunits respectively. Integrin α6β1 shows a broad spectrum of specificity towards different laminins, but the binding affinity for LN-521 or LN-511 is higher than that for LN-111. Recently, it has been established that β2 laminins have higher affinity for integrins than the β1 laminins. Therefore, having the highest affinity for the integrin, LN-521 can provide the best anchorage for migration and can convey the highest dose of

signal via α6β1 integrin resulting in the best survivability of dissociated pluripotent hES cells, although *e.g.* LN-511 also can do it at a lesser extent.

[0240] The results of this work may facilitate culturing and expansion of pluripotent human stem cells in general, and even make automated expansion of such cells possible including cell aimed for clinical applications. The survival of dissociated hES cells on LN-521 appears to be dependent on migration and the cells can therefore not survive after plating at ultralow densities. The new hES cell culture method described here utilizes only a naturally occurring LN-521 adhesion protein that does not damage the cytoskeleton as ROCK inhibitor or blebbistatin treatment do. Thus, LN-521 most probably favors survival of the cells only with the correct integrin profile on the cell surface. The present results also showed that hES cells plated on LN-521 at relatively low densities of 20,000-30,000 cells per 1 cm^2 could survive and multiply at least as efficiently as in the other hES culture systems. The new method closely resembles standard cell culture procedures, e.g. culturing of fibroblasts, and, therefore, hES cell cultures on LN-521 do not demand specially trained personnel, which has been a major problem before since culturing of hES cells has been a technological challenge.

[0241] The widely used TeSR1 formulation for human pluripotent stem cell self-renewal was initally developed for use with Matrigel and it contains high doses of bFGF that mostly targets the MEK1/Erk pathway, which is not widely considered to have a beneficial effect for pluripotent hES cells. The present results can lead to development of new medium formulations utilizing benefits of LN-521 as coating material and specifically targeting pathways, which are important for human ES cell self-renewal.

[0242] In summary, the present work has demonstrated that LN-521, normally secreted by pluripotent hES, can as a sole coating material support self-renewal of human pluripotent stem cells in culture, similar to LN-511. Both laminins facilitated growth of the cells as homogenous monolayers *in vitro*. However, an important difference between the two laminins is that hES/hiPS cells could be trypsinized into single cell suspension, and plated and effectively expanded from single cells on LN-521, as opposed to manual splitting of cell clusters, as is currently required for expansion of hES/iPS cells grown on Matrigel or feeder cells. The results of this work

may facilitate culturing of pluripotent human stem cells and facilitate automated expansion of such cells.

EXAMPLE 3

[0243] Human embryonic stem cells were cultured upon a laminin-521 substrate in two different cell culture mediums. The two cell culture mediums differed in the amount of bFGF, 3.9 ng/ml and 100 ng/ml.

[0244] **FIG. 22** shows the growth curve of HS181 hES cells cultured in an O3 medium comprising 3.9 ng/mL of bFGF with a laminin-521 substrate after 5 passages (40 days) in solid black. The O3 medium was a variant of the commercially available chemically defined mTeSR1 medium with bovine serum albumin as the only animal derived component. The growth curve of HS181 hES cells cultured in an O3 medium comprising 100 ng/mL of bFGF with a laminin-521 substrate is shown in dashes. The cells were dissociated into single cell suspension for passaging. As seen here, the growth curve for the lower amount of bFGF was as good as or better than the higher amount of bFGF.

[0245] **FIG. 23** shows the relative amount of mRNA transcripts for pluripotency markers Oct4 and Nanog after 5 passages (40 days) for both media, which was obtained using real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. Again, similar amounts were obtained in both cell culture mediums, indicating that the stem cells in the lower amount of bFGF maintained pluripotency. Thus, a lower amount of bFGF can be used and still obtain good results, particularly in combination with the laminin-521 substrate.

EXAMPLE 4

[0246] In Examples 1 and 2, the dissociated stem cells survived mostly through *active motility on LN-521 and association into small effectively growing and migrating* monolayer islands. The cells plated at very low, cloning densities died through anoikis, a specialized from of programmed cell death. Normally, integrin-related signaling from extracellular matrix molecules, e.g. laminins, and cadherin-related cell-cell signaling, can prevent anoikis. The most abundant cadherin isoform on the human ES cell surface is epithelial-cadherin (E-Cadherin). The experiments in Examples 3 were performed to find out if a combination of LN-521 and E-Cadherin could protect human ES cells from anoikis and allow clonal survival of the cells.

[0247] hES cells were plated in mTeSR1 medium on different coatings at a density of 250 cells per cm^2 and monitored after 5 days in culture using an alkaline phosphatase staining kit. Neither laminin-521 nor E-Cadherin alone permitted efficient clonal survival of individualized hES cells.

[0248] Next, combinations of laminin-521 and E-Cadherin were tested to determine whether the combinations could sustain clonal survival of the cells. Fixed amounts of laminin-521 were used for the cell culture dish coating in combination with titrated E-Cadherin. Clonal survival of cells was achieved at different ratios of E-Cadherin to laminin-521 including from about 1:10 w/w to about 1:5 w/w.

[0249] Additional tests were performed to test the effects of various modifications to the mTeSR1 medium. A mixture of E-Cadherin to laminin-521 in a ratio of about 1:10 w/w was used to coat the plates. Unexpectedly, it was discovered that a 2x increase in albumin concentration significantly improved clonal survival of hES cells on the laminin-521/E-Cadherin matrix. The rate of individualized hES cell survival under these conditions was from 10 to 15% and was at least one order of magnitude higher than that of the cells on Matrigel, laminin-521, or E-Cadherin alone in both mTeSR1 medium and mTeSR1 medium with additional albumin. Time-lapse photography of the cells confirmed that laminin-521/E-Cadherin coatings facilitated cell survival through proliferation of the single cells, not through the aggregation of different cells. Laminin-521/E-Cadherin similarly sustained clonal survival of cells in completely chemically defined and xeno-free TeSR2 medium with the addition of recombinant human serum albumin (rHSA).

[0250] In additional testing, hES cell lines were derived. Twenty-four well tissue cell culture dish plates were washed twice with Dulbecco's Phosphate Buffered Saline. Next, the dish plates were coated for 2 hours at 37°C with sterile solution containing: 48 µL of 100 µg/mL laminin-521 (BioLamina AB, Stockholm); 6 µL of 82 µg/mL E-Cadherin (R@DSystems); and 300 µL of DPBS with calcium and magnesium (GIBCO) to produce a laminin-521/E-Cadherin matrix.

[0251] The donated embryos used to derive the hES cell lines were obtained from an accredited in vitro fertilization clinic after the consent of both partners and ethics approval were obtained. Only fresh or frozen embryos, which could not be used for infertility treatment, were used in the derivation procedures. One or two cells were isolated from an 8-cell stage embryo using a micropipette after making a small opening to the zona pellucida with a laser apparatus designed for this purpose. This

procedure has been accredited for clinical use in pre-implantation genetic diagnostics (PGD). The cells were placed on the laminin-521/E-Cadherin matrix in TeSR2 medium with additional rHSA. The cells successfully attached and started to proliferate. The parental embryos were allowed to grow to a blastocyst stage and frozen. The embryo culture was carried out using xeno-free standardized in vitro fertilization culture media in drops under oil at 37°C and 5% O_2/10% CO_2. After removal of the zona pellucida, the inner cell masses of five human blastocysts were mechanically isolated and plated in 4-well culture plates (Nunc) onto the laminin-521/E-Cadherin matrix. Following an initial 48 hours of culture, the culture medium was replaced on a daily basis. After 10 to 14 days, the outgrowths were mechanically isolated and replated onto laminin-521/E-Cadherin matrix. Mechanical passaging was used for the subsequent 2 to 3 passages after which colonies were passaged using TrypLE Select (GIBCO).

[0252] Using the laminin-521/E-Cadherin matrix described above with the mTeSR1 medium including additional bovine albumin, three new hES cell lines were derived from six cultured blastocysts. Four days after plating, the inner cell masses gave stem cell-like outgrowths. **FIG. 24** shows an early stage derivation of a new human embryonic stem HS841 cell line on laminin-521/E-Cadherin matrix. Morphologically typical human embryonic stem cells growing out from the inner cell mass of a day six blastocyst four days after mechanical isolation of the inner cell mass and plating on laminin-521/E-Cadherin matrix are shown.

[0253] Unexpectedly, the cell culture system and method gave stable, hES cell lines in 3 out of 6 embryos (50%), a derivation rate higher than that of standard methods.

[0254] The use of a laminin-521/E-Cadherin matrix and TeSR2 medium with additional rHSA, a completely chemically defined and xeno-free environment, yielded similar results in the derivation of new hES cell lines.

[0255] It will be appreciated that variants of the above-disclosed and other features and functions, or alternatives thereof, may be combined into many other different systems or applications. Various presently unforeseen or unanticipated alternatives, modifications, variations or improvements therein may be subsequently made by those skilled in the art which are also intended to be encompassed by the following claims.

CLAIMS:

1. A system for maintaining pluripotent stem cells, comprising:

a cell culture medium comprising from greater than zero to 3.9 ng/mL of basic fibroblast growth factor (bFGF); and

a substrate for providing support to the stem cells.

2. The system of claim 1, wherein the substrate contains laminin-521 or laminin-511.

3. The system of claim 2, wherein the substrate also contains a cadherin.

4. The system of claim 1, wherein the substrate comprises laminin-521 and wherein the amount of bFGF is 3.5 ng/mL or less.

5. The system of claim 1, wherein the cell culture medium comprises from 0.5 to 3.5 ng/mL of bFGF.

6. The system of claim 1, wherein the cell culture medium further comprises at least one additional growth factor, at least one trace mineral, and at least one lipid.

7. A cell culture medium that provides nutrition to pluripotent stem cells, comprising from greater than zero to 3.9 ng/mL of basic fibroblast growth factor (bFGF).

8. The cell culture medium of claim 7, comprising 3.5 ng/mL or less of bFGF.

9. The cell culture medium of claim 7, comprising from 0.5 to 3.5 ng/mL of bFGF.

10. The cell culture medium of claim 7, further comprising at least one inorganic salt.

11. The cell culture medium of claim 7, further comprising at least one trace mineral.

12. The cell culture medium of claim 7, further comprising at least one energy substrate.

13. The cell culture medium of claim 7, further comprising at least one lipid.

14. The cell culture medium of claim 7, further comprising at least one amino acid.

15. The cell culture medium of claim 7, further comprising at least one vitamin.

16. The cell culture medium of claim 7, further comprising at least one additional growth factor.

17. The cell culture medium of claim 7, further comprising at least one inorganic salt, at least one trace mineral, at least one energy substrate, at least one lipid, at least one amino acid, and at least one vitamin.

18. The cell culture medium of claim 7, wherein the cell culture medium does not contain any one of (1) albumin, (2) insulin or an insulin substitute, or (3) transferrin or a transferrin substitute.

19. The cell culture medium of claim 7, further comprising albumin, insulin, lithium chloride, GABA, TGF beta 1, pipecolic acid, L-glutamine, MEM non-essential amino acid solution, and DMEM/F12 solution.

20. The cell culture medium of claim 7, further comprising at least one additional growth factor, at least one trace mineral, and at least one lipid.

SEQUENCE LISTING

<110> Tryggvason, Karl
Sergey, Rodin

<120> Cell Culture Medium

<130> LCTI-200006WO02

<160> 8

<170> PatentIn version 3.5

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Gly Ala Pro His Tyr Val Ala Phe Tyr Ser Asn Ala Thr Gly Val
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Trp Leu Tyr Val Asp Asp Gln Leu Gln Gln Met Lys Pro His Arg
3215 3220 3225

Gly Pro Pro Pro Glu Leu Gln Pro Gln Pro Glu Gly Pro Pro Arg
3230 3235 3240

Leu Leu Leu Gly Gly Leu Pro Glu Ser Gly Thr Ile Tyr Asn Phe
3245 3250 3255

Ser Gly Cys Ile Ser Asn Val Phe Val Gln Arg Leu Leu Gly Pro
3260 3265 3270

Gln Arg Val Phe Asp Leu Gln Gln Asn Leu Gly Ser Val Asn Val
3275 3280 3285

Ser Thr Gly Cys Ala Pro Ala Leu Gln Ala Gln Thr Pro Gly Leu
3290 3295 3300

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Gly Pro Arg Gly Leu Gln Ala Thr Ala Arg Lys Ala Ser Arg Arg
    3305                3310                3315

Ser Arg Gln Pro Ala Arg His Pro Ala Cys Met Leu Pro Pro His
    3320                3325                3330

Leu Arg Thr Thr Arg Asp Ser Tyr Gln Phe Gly Gly Ser Leu Ser
    3335                3340                3345

Ser His Leu Glu Phe Val Gly Ile Leu Ala Arg His Arg Asn Trp
    3350                3355                3360

Pro Ser Leu Ser Met His Val Leu Pro Arg Ser Ser Arg Gly Leu
    3365                3370                3375

Leu Leu Phe Thr Ala Arg Leu Arg Pro Gly Ser Pro Ser Leu Ala
    3380                3385                3390

Leu Phe Leu Ser Asn Gly His Phe Val Ala Gln Met Glu Gly Leu
    3395                3400                3405

Gly Thr Arg Leu Arg Ala Gln Ser Arg Gln Arg Ser Arg Pro Gly
    3410                3415                3420

Arg Trp His Lys Val Ser Val Arg Trp Glu Lys Asn Arg Ile Leu
    3425                3430                3435

Leu Val Thr Asp Gly Ala Arg Ala Trp Ser Gln Glu Gly Pro His
    3440                3445                3450

Arg Gln His Gln Gly Ala Glu His Pro Gln Pro His Thr Leu Phe
    3455                3460                3465

Val Gly Gly Leu Pro Ala Ser Ser His Ser Ser Lys Leu Pro Val
    3470                3475                3480

Thr Val Gly Phe Ser Gly Cys Val Lys Arg Leu Arg Leu His Gly
    3485                3490                3495

Arg Pro Leu Gly Ala Pro Thr Arg Met Ala Gly Val Thr Pro Cys
    3500                3505                3510

Ile Leu Gly Pro Leu Glu Ala Gly Leu Phe Phe Pro Gly Ser Gly
    3515                3520                3525

Gly Val Ile Thr Leu Asp Leu Pro Gly Ala Thr Leu Pro Asp Val
    3530                3535                3540

Gly Leu Glu Leu Glu Val Arg Pro Leu Ala Val Thr Gly Leu Ile
    3545                3550                3555
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Phe His Leu Gly Gln Ala Arg Thr Pro Pro Tyr Leu Gln Leu Gln
3560 3565 3570

Val Thr Glu Lys Gln Val Leu Leu Arg Ala Asp Asp Gly Ala Gly
3575 3580 3585

Glu Phe Ser Thr Ser Val Thr Arg Pro Ser Val Leu Cys Asp Gly
3590 3595 3600

Gln Trp His Arg Leu Ala Val Met Lys Ser Gly Asn Val Leu Arg
3605 3610 3615

Leu Glu Val Asp Ala Gln Ser Asn His Thr Val Gly Pro Leu Leu
3620 3625 3630

Ala Ala Ala Ala Gly Ala Pro Ala Pro Leu Tyr Leu Gly Gly Leu
3635 3640 3645

Pro Glu Pro Met Ala Val Gln Pro Trp Pro Pro Ala Tyr Cys Gly
3650 3655 3660

Cys Met Arg Arg Leu Ala Val Asn Arg Ser Pro Val Ala Met Thr
3665 3670 3675

Arg Ser Val Glu Val His Gly Ala Val Gly Ala Ser Gly Cys Pro
3680 3685 3690

Ala Ala
3695

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Gln Ala Pro Ala Pro Asp Val Pro Gly Cys Ser Arg Gly Ser Cys Tyr
35 40 45

Pro Ala Thr Gly Asp Leu Leu Val Gly Arg Ala Asp Arg Leu Thr Ala
50 55 60

Ser Ser Thr Cys Gly Leu Asn Gly Pro Gln Pro Tyr Cys Ile Val Ser
65 70 75 80

His Leu Gln Asp Glu Lys Lys Cys Phe Leu Cys Asp Ser Arg Arg Pro

85 90 95

Phe Ser Ala Arg Asp Asn Pro His Ser His Arg Ile Gln Asn Val Val
100 105 110

Thr Ser Phe Ala Pro Gln Arg Arg Ala Ala Trp Trp Gln Ser Glu Asn
115 120 125

Gly Ile Pro Ala Val Thr Ile Gln Leu Asp Leu Glu Ala Glu Phe His
130 135 140

Phe Thr His Leu Ile Met Thr Phe Lys Thr Phe Arg Pro Ala Ala Met
145 150 155 160

Leu Val Glu Arg Ser Ala Asp Phe Gly Arg Thr Trp His Val Tyr Arg
165 170 175

Tyr Phe Ser Tyr Asp Cys Gly Ala Asp Phe Pro Gly Val Pro Leu Ala
180 185 190

Pro Pro Arg His Trp Asp Asp Val Val Cys Glu Ser Arg Tyr Ser Glu
195 200 205

Ile Glu Pro Ser Thr Glu Gly Glu Val Ile Tyr Arg Val Leu Asp Pro
210 215 220

Ala Ile Pro Ile Pro Asp Pro Tyr Ser Ser Arg Ile Gln Asn Leu Leu
225 230 235 240

Lys Ile Thr Asn Leu Arg Val Asn Leu Thr Arg Leu His Thr Leu Gly
245 250 255

Asp Asn Leu Leu Asp Pro Arg Arg Glu Ile Arg Glu Lys Tyr Tyr Tyr
260 265 270

Ala Leu Tyr Glu Leu Val Val Arg Gly Asn Cys Phe Cys Tyr Gly His
275 280 285

Ala Ser Glu Cys Ala Pro Ala Pro Gly Ala Pro Ala His Ala Glu Gly
290 295 300

Met Val His Gly Ala Cys Ile Cys Lys His Asn Thr Arg Gly Leu Asn
305 310 315 320

Cys Glu Gln Cys Gln Asp Phe Tyr Arg Asp Leu Pro Trp Arg Pro Ala
325 330 335

Glu Asp Gly His Ser His Ala Cys Arg Lys Cys Glu Cys His Gly His
340 345 350

Thr His Ser Cys His Phe Asp Met Ala Val Tyr Leu Ala Ser Gly Asn

355 360 365

Val Ser Gly Gly Val Cys Asp Gly Cys Gln His Asn Thr Ala Gly Arg
370 375 380

His Cys Glu Leu Cys Arg Pro Phe Phe Tyr Arg Asp Pro Thr Lys Asp
385 390 395 400

Leu Arg Asp Pro Ala Val Cys Arg Ser Cys Asp Cys Asp Pro Met Gly
405 410 415

Ser Gln Asp Gly Gly Arg Cys Asp Ser His Asp Asp Pro Ala Leu Gly
420 425 430

Leu Val Ser Gly Gln Cys Arg Cys Lys Glu His Val Val Gly Thr Arg
435 440 445

Cys Gln Gln Cys Arg Asp Gly Phe Phe Gly Leu Ser Ile Ser Asp Arg
450 455 460

Leu Gly Cys Arg Arg Cys Gln Cys Asn Ala Arg Gly Thr Val Pro Gly
465 470 475 480

Ser Thr Pro Cys Asp Pro Asn Ser Gly Ser Cys Tyr Cys Lys Arg Leu
485 490 495

Val Thr Gly Arg Gly Cys Asp Arg Cys Leu Pro Gly His Trp Gly Leu
500 505 510

Ser His Asp Leu Leu Gly Cys Arg Pro Cys Asp Cys Asp Val Gly Gly
515 520 525

Ala Leu Asp Pro Gln Cys Asp Glu Gly Thr Gly Gln Cys His Cys Arg
530 535 540

Gln His Met Val Gly Arg Arg Cys Glu Gln Val Gln Pro Gly Tyr Phe
545 550 555 560

Arg Pro Phe Leu Asp His Leu Ile Trp Glu Ala Glu Asp Thr Arg Gly
565 570 575

Gln Val Leu Asp Val Val Glu Arg Leu Val Thr Pro Gly Glu Thr Pro
580 585 590

Ser Trp Thr Gly Ser Gly Phe Val Arg Leu Gln Glu Gly Gln Thr Leu
595 600 605

Glu Phe Leu Val Ala Ser Val Pro Lys Ala Met Asp Tyr Asp Leu Leu
610 615 620

Leu Arg Leu Glu Pro Gln Val Pro Glu Gln Trp Ala Glu Leu Glu Leu

625 630 635 640

Ile Val Gln Arg Pro Gly Pro Val Pro Ala His Ser Leu Cys Gly His
645 650 655

Leu Val Pro Lys Asp Asp Arg Ile Gln Gly Thr Leu Gln Pro His Ala
660 665 670

Arg Tyr Leu Ile Phe Pro Asn Pro Val Cys Leu Glu Pro Gly Ile Ser
675 680 685

Tyr Lys Leu His Leu Lys Leu Val Arg Thr Gly Gly Ser Ala Gln Pro
690 695 700

Glu Thr Pro Tyr Ser Gly Pro Gly Leu Leu Ile Asp Ser Leu Val Leu
705 710 715 720

Leu Pro Arg Val Leu Val Leu Glu Met Phe Ser Gly Gly Asp Ala Ala
725 730 735

Ala Leu Glu Arg Gln Ala Thr Phe Glu Arg Tyr Gln Cys His Glu Glu
740 745 750

Gly Leu Val Pro Ser Lys Thr Ser Pro Ser Glu Ala Cys Ala Pro Leu
755 760 765

Leu Ile Ser Leu Ser Thr Leu Ile Tyr Asn Gly Ala Leu Pro Cys Gln
770 775 780

Cys Asn Pro Gln Gly Ser Leu Ser Ser Glu Cys Asn Pro His Gly Gly
785 790 795 800

Gln Cys Leu Cys Lys Pro Gly Val Val Gly Arg Arg Cys Asp Leu Cys
805 810 815

Ala Pro Gly Tyr Tyr Gly Phe Gly Pro Thr Gly Cys Gln Ala Cys Gln
820 825 830

Cys Ser His Glu Gly Ala Leu Ser Ser Leu Cys Glu Lys Thr Ser Gly
835 840 845

Gln Cys Leu Cys Arg Thr Gly Ala Phe Gly Leu Arg Cys Asp Arg Cys
850 855 860

Gln Arg Gly Gln Trp Gly Phe Pro Ser Cys Arg Pro Cys Val Cys Asn
865 870 875 880

Gly His Ala Asp Glu Cys Asn Thr His Thr Gly Ala Cys Leu Gly Cys
885 890 895

Arg Asp His Thr Gly Gly Glu His Cys Glu Arg Cys Ile Ala Gly Phe

900 905 910

His Gly Asp Pro Arg Leu Pro Tyr Gly Gly Gln Cys Arg Pro Cys Pro
915 920 925

Cys Pro Glu Gly Pro Gly Ser Gln Arg His Phe Ala Thr Ser Cys His
930 935 940

Gln Asp Glu Tyr Ser Gln Gln Ile Val Cys His Cys Arg Ala Gly Tyr
945 950 955 960

Thr Gly Leu Arg Cys Glu Ala Cys Ala Pro Gly His Phe Gly Asp Pro
965 970 975

Ser Arg Pro Gly Gly Arg Cys Gln Leu Cys Glu Cys Ser Gly Asn Ile
980 985 990

Asp Pro Met Asp Pro Asp Ala Cys Asp Pro His Thr Gly Gln Cys Leu
995 1000 1005

Arg Cys Leu His His Thr Glu Gly Pro His Cys Ala His Cys Lys
1010 1015 1020

Pro Gly Phe His Gly Gln Ala Ala Arg Gln Ser Cys His Arg Cys
1025 1030 1035

Thr Cys Asn Leu Leu Gly Thr Asn Pro Gln Gln Cys Pro Ser Pro
1040 1045 1050

Asp Gln Cys His Cys Asp Pro Ser Ser Gly Gln Cys Pro Cys Leu
1055 1060 1065

Pro Asn Val Gln Gly Pro Ser Cys Asp Arg Cys Ala Pro Asn Phe
1070 1075 1080

Trp Asn Leu Thr Ser Gly His Gly Cys Gln Pro Cys Ala Cys His
1085 1090 1095

Pro Ser Arg Ala Arg Gly Pro Thr Cys Asn Glu Phe Thr Gly Gln
1100 1105 1110

Cys His Cys Arg Ala Gly Phe Gly Gly Arg Thr Cys Ser Glu Cys
1115 1120 1125

Gln Glu Leu His Trp Gly Asp Pro Gly Leu Gln Cys His Ala Cys
1130 1135 1140

Asp Cys Asp Ser Arg Gly Ile Asp Thr Pro Gln Cys His Arg Phe
1145 1150 1155

Thr Gly His Cys Ser Cys Arg Pro Gly Val Ser Gly Val Arg Cys

1160 1165 1170

Asp Gln Cys Ala Arg Gly Phe Ser Gly Ile Phe Pro Ala Cys His
1175 1180 1185

Pro Cys His Ala Cys Phe Gly Asp Trp Asp Arg Val Val Gln Asp
1190 1195 1200

Leu Ala Ala Arg Thr Gln Arg Leu Glu Gln Arg Ala Gln Glu Leu
1205 1210 1215

Gln Gln Thr Gly Val Leu Gly Ala Phe Glu Ser Ser Phe Trp His
1220 1225 1230

Met Gln Glu Lys Leu Gly Ile Val Gln Gly Ile Val Gly Ala Arg
1235 1240 1245

Asn Thr Ser Ala Ala Ser Thr Ala Gln Leu Val Glu Ala Thr Glu
1250 1255 1260

Glu Leu Arg Arg Glu Ile Gly Glu Ala Thr Glu His Leu Thr Gln
1265 1270 1275

Leu Glu Ala Asp Leu Thr Asp Val Gln Asp Glu Asn Phe Asn Ala
1280 1285 1290

Asn His Ala Leu Ser Gly Leu Glu Arg Asp Arg Leu Ala Leu Asn
1295 1300 1305

Leu Thr Leu Arg Gln Leu Asp Gln His Leu Asp Leu Leu Lys His
1310 1315 1320

Ser Asn Phe Leu Gly Ala Tyr Asp Ser Ile Arg His Ala His Ser
1325 1330 1335

Gln Ser Ala Glu Ala Glu Arg Arg Ala Asn Thr Ser Ala Leu Ala
1340 1345 1350

Val Pro Ser Pro Val Ser Asn Ser Ala Ser Ala Arg His Arg Thr
1355 1360 1365

Glu Ala Leu Met Asp Ala Gln Lys Glu Asp Phe Asn Ser Lys His
1370 1375 1380

Met Ala Asn Gln Arg Ala Leu Gly Lys Leu Ser Ala His Thr His
1385 1390 1395

Thr Leu Ser Leu Thr Asp Ile Asn Glu Leu Val Cys Gly Ala Pro
1400 1405 1410

Gly Asp Ala Pro Cys Ala Thr Ser Pro Cys Gly Gly Ala Gly Cys

1415 1420 1425

Arg Asp Glu Asp Gly Gln Pro Arg Cys Gly Gly Leu Ser Cys Asn
1430 1435 1440

Gly Ala Ala Ala Thr Ala Asp Leu Ala Leu Gly Arg Ala Arg His
1445 1450 1455

Thr Gln Ala Glu Leu Gln Arg Ala Leu Ala Glu Gly Gly Ser Ile
1460 1465 1470

Leu Ser Arg Val Ala Glu Thr Arg Arg Gln Ala Ser Glu Ala Gln
1475 1480 1485

Gln Arg Ala Gln Ala Ala Leu Asp Lys Ala Asn Ala Ser Arg Gly
1490 1495 1500

Gln Val Glu Gln Ala Asn Gln Glu Leu Gln Glu Leu Ile Gln Ser
1505 1510 1515

Val Lys Asp Phe Leu Asn Gln Glu Gly Ala Asp Pro Asp Ser Ile
1520 1525 1530

Glu Met Val Ala Thr Arg Val Leu Glu Leu Ser Ile Pro Ala Ser
1535 1540 1545

Ala Glu Gln Ile Gln His Leu Ala Gly Ala Ile Ala Glu Arg Val
1550 1555 1560

Arg Ser Leu Ala Asp Val Asp Ala Ile Leu Ala Arg Thr Val Gly
1565 1570 1575

Asp Val Arg Arg Ala Glu Gln Leu Leu Gln Asp Ala Arg Arg Ala
1580 1585 1590

Arg Ser Trp Ala Glu Asp Glu Lys Gln Lys Ala Glu Thr Val Gln
1595 1600 1605

Ala Ala Leu Glu Glu Ala Gln Arg Ala Gln Gly Ile Ala Gln Gly
1610 1615 1620

Ala Ile Arg Gly Ala Val Ala Asp Thr Arg Asp Thr Glu Gln Thr
1625 1630 1635

Leu Tyr Gln Val Gln Glu Arg Met Ala Gly Ala Glu Arg Ala Leu
1640 1645 1650

Ser Ser Ala Gly Glu Arg Ala Arg Gln Leu Asp Ala Leu Leu Glu
1655 1660 1665

Ala Leu Lys Leu Lys Arg Ala Gly Asn Ser Leu Ala Ala Ser Thr

1670 1675 1680

Ala Glu Glu Thr Ala Gly Ser Ala Gln Gly Arg Ala Gln Glu Ala
1685 1690 1695

Glu Gln Leu Leu Arg Gly Pro Leu Gly Asp Gln Tyr Gln Thr Val
1700 1705 1710

Lys Ala Leu Ala Glu Arg Lys Ala Gln Gly Val Leu Ala Ala Gln
1715 1720 1725

Ala Arg Ala Glu Gln Leu Arg Asp Glu Ala Arg Asp Leu Leu Gln
1730 1735 1740

Ala Ala Gln Asp Lys Leu Gln Arg Leu Gln Glu Leu Glu Gly Thr
1745 1750 1755

Tyr Glu Glu Asn Glu Arg Ala Leu Glu Ser Lys Ala Ala Gln Leu
1760 1765 1770

Asp Gly Leu Glu Ala Arg Met Arg Ser Val Leu Gln Ala Ile Asn
1775 1780 1785

Leu Gln Val Gln Ile Tyr Asn Thr Cys Gln Lys Ser Ser Trp Pro
1790 1795 1800

Gly Arg Ala Pro Asn Lys Pro Val
1805 1810

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Ala Gln Ala Ala Met Asp Glu Cys Thr Asp Glu Gly Gly Arg Pro Gln
35 40 45

Arg Cys Met Pro Glu Phe Val Asn Ala Ala Phe Asn Val Thr Val Val
50 55 60

Ala Thr Asn Thr Cys Gly Thr Pro Pro Glu Glu Tyr Cys Val Gln Thr
65 70 75 80

Gly Val Thr Gly Val Thr Lys Ser Cys His Leu Cys Asp Ala Gly Gln
85 90 95

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Pro His Leu Gln His Gly Ala Ala Phe Leu Thr Asp Tyr Asn Asn Gln
            100                 105                 110

Ala Asp Thr Thr Trp Trp Gln Ser Gln Thr Met Leu Ala Gly Val Gln
        115                 120                 125

Tyr Pro Ser Ser Ile Asn Leu Thr Leu His Leu Gly Lys Ala Phe Asp
    130                 135                 140

Ile Thr Tyr Val Arg Leu Lys Phe His Thr Ser Arg Pro Glu Ser Phe
145                 150                 155                 160

Ala Ile Tyr Lys Arg Thr Arg Glu Asp Gly Pro Trp Ile Pro Tyr Gln
                165                 170                 175

Tyr Tyr Ser Gly Ser Cys Glu Asn Thr Tyr Ser Lys Ala Asn Arg Gly
            180                 185                 190

Phe Ile Arg Thr Gly Gly Asp Glu Gln Gln Ala Leu Cys Thr Asp Glu
        195                 200                 205

Phe Ser Asp Ile Ser Pro Leu Thr Gly Gly Asn Val Ala Phe Ser Thr
    210                 215                 220

Leu Glu Gly Arg Pro Ser Ala Tyr Asn Phe Asp Asn Ser Pro Val Leu
225                 230                 235                 240

Gln Glu Trp Val Thr Ala Thr Asp Ile Arg Val Thr Leu Asn Arg Leu
                245                 250                 255

Asn Thr Phe Gly Asp Glu Val Phe Asn Asp Pro Lys Val Leu Lys Ser
            260                 265                 270

Tyr Tyr Tyr Ala Ile Ser Asp Phe Ala Val Gly Gly Arg Cys Lys Cys
        275                 280                 285

Asn Gly His Ala Ser Glu Cys Met Lys Asn Glu Phe Asp Lys Leu Val
    290                 295                 300

Cys Asn Cys Lys His Asn Thr Tyr Gly Val Asp Cys Glu Lys Cys Leu
305                 310                 315                 320

Pro Phe Phe Asn Asp Arg Pro Trp Arg Arg Ala Thr Ala Glu Ser Ala
                325                 330                 335

Ser Glu Cys Leu Pro Cys Asp Cys Asn Gly Arg Ser Gln Glu Cys Tyr
            340                 345                 350

Phe Asp Pro Glu Leu Tyr Arg Ser Thr Gly His Gly Gly His Cys Thr
        355                 360                 365
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Asn Cys Gln Asp Asn Thr Asp Gly Ala His Cys Glu Arg Cys Arg Glu
    370                 375                 380

Asn Phe Phe Arg Leu Gly Asn Asn Glu Ala Cys Ser Ser Cys His Cys
385                 390                 395                 400

Ser Pro Val Gly Ser Leu Ser Thr Gln Cys Asp Ser Tyr Gly Arg Cys
                405                 410                 415

Ser Cys Lys Pro Gly Val Met Gly Asp Lys Cys Asp Arg Cys Gln Pro
            420                 425                 430

Gly Phe His Ser Leu Thr Glu Ala Gly Cys Arg Pro Cys Ser Cys Asp
        435                 440                 445

Pro Ser Gly Ser Ile Asp Glu Cys Asn Ile Glu Thr Gly Arg Cys Val
    450                 455                 460

Cys Lys Asp Asn Val Glu Gly Phe Asn Cys Glu Arg Cys Lys Pro Gly
465                 470                 475                 480

Phe Phe Asn Leu Glu Ser Ser Asn Pro Arg Gly Cys Thr Pro Cys Phe
                485                 490                 495

Cys Phe Gly His Ser Ser Val Cys Thr Asn Ala Val Gly Tyr Ser Val
            500                 505                 510

Tyr Ser Ile Ser Ser Thr Phe Gln Ile Asp Glu Asp Gly Trp Arg Ala
        515                 520                 525

Glu Gln Arg Asp Gly Ser Glu Ala Ser Leu Glu Trp Ser Ser Glu Arg
    530                 535                 540

Gln Asp Ile Ala Val Ile Ser Asp Ser Tyr Phe Pro Arg Tyr Phe Ile
545                 550                 555                 560

Ala Pro Ala Lys Phe Leu Gly Lys Gln Val Leu Ser Tyr Gly Gln Asn
                565                 570                 575

Leu Ser Phe Ser Phe Arg Val Asp Arg Arg Asp Thr Arg Leu Ser Ala
            580                 585                 590

Glu Asp Leu Val Leu Glu Gly Ala Gly Leu Arg Val Ser Val Pro Leu
        595                 600                 605

Ile Ala Gln Gly Asn Ser Tyr Pro Ser Glu Thr Thr Val Lys Tyr Val
    610                 615                 620

Phe Arg Leu His Glu Ala Thr Asp Tyr Pro Trp Arg Pro Ala Leu Thr
625                 630                 635                 640
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Pro Phe Glu Phe Gln Lys Leu Leu Asn Asn Leu Thr Ser Ile Lys Ile
645 650 655

Arg Gly Thr Tyr Ser Glu Arg Ser Ala Gly Tyr Leu Asp Asp Val Thr
660 665 670

Leu Ala Ser Ala Arg Pro Gly Pro Gly Val Pro Ala Thr Trp Val Glu
675 680 685

Ser Cys Thr Cys Pro Val Gly Tyr Gly Gly Gln Phe Cys Glu Met Cys
690 695 700

Leu Ser Gly Tyr Arg Arg Glu Thr Pro Asn Leu Gly Pro Tyr Ser Pro
705 710 715 720

Cys Val Leu Cys Ala Cys Asn Gly His Ser Glu Thr Cys Asp Pro Glu
725 730 735

Thr Gly Val Cys Asn Cys Arg Asp Asn Thr Ala Gly Pro His Cys Glu
740 745 750

Lys Cys Ser Asp Gly Tyr Tyr Gly Asp Ser Thr Ala Gly Thr Ser Ser
755 760 765

Asp Cys Gln Pro Cys Pro Cys Pro Gly Gly Ser Ser Cys Ala Val Val
770 775 780

Pro Lys Thr Lys Glu Val Val Cys Thr Asn Cys Pro Thr Gly Thr Thr
785 790 795 800

Gly Lys Arg Cys Glu Leu Cys Asp Asp Gly Tyr Phe Gly Asp Pro Leu
805 810 815

Gly Arg Asn Gly Pro Val Arg Leu Cys Arg Leu Cys Gln Cys Ser Asp
820 825 830

Asn Ile Asp Pro Asn Ala Val Gly Asn Cys Asn Arg Leu Thr Gly Glu
835 840 845

Cys Leu Lys Cys Ile Tyr Asn Thr Ala Gly Phe Tyr Cys Asp Arg Cys
850 855 860

Lys Asp Gly Phe Phe Gly Asn Pro Leu Ala Pro Asn Pro Ala Asp Lys
865 870 875 880

Cys Lys Ala Cys Asn Cys Asn Leu Tyr Gly Thr Met Lys Gln Gln Ser
885 890 895

Ser Cys Asn Pro Val Thr Gly Gln Cys Glu Cys Leu Pro His Val Thr
900 905 910

Gly Gln Asp Cys Gly Ala Cys Asp Pro Gly Phe Tyr Asn Leu Gln Ser
915 920 925

Gly Gln Gly Cys Glu Arg Cys Asp Cys His Ala Leu Gly Ser Thr Asn
930 935 940

Gly Gln Cys Asp Ile Arg Thr Gly Gln Cys Glu Cys Gln Pro Gly Ile
945 950 955 960

Thr Gly Gln His Cys Glu Arg Cys Glu Val Asn His Phe Gly Phe Gly
965 970 975

Pro Glu Gly Cys Lys Pro Cys Asp Cys His Pro Glu Gly Ser Leu Ser
980 985 990

Leu Gln Cys Lys Asp Asp Gly Arg Cys Glu Cys Arg Glu Gly Phe Val
995 1000 1005

Gly Asn Arg Cys Asp Gln Cys Glu Glu Asn Tyr Phe Tyr Asn Arg
1010 1015 1020

Ser Trp Pro Gly Cys Gln Glu Cys Pro Ala Cys Tyr Arg Leu Val
1025 1030 1035

Lys Asp Lys Val Ala Asp His Arg Val Lys Leu Gln Glu Leu Glu
1040 1045 1050

Ser Leu Ile Ala Asn Leu Gly Thr Gly Asp Glu Met Val Thr Asp
1055 1060 1065

Gln Ala Phe Glu Asp Arg Leu Lys Glu Ala Glu Arg Glu Val Met
1070 1075 1080

Asp Leu Leu Arg Glu Ala Gln Asp Val Lys Asp Val Asp Gln Asn
1085 1090 1095

Leu Met Asp Arg Leu Gln Arg Val Asn Asn Thr Leu Ser Ser Gln
1100 1105 1110

Ile Ser Arg Leu Gln Asn Ile Arg Asn Thr Ile Glu Glu Thr Gly
1115 1120 1125

Asn Leu Ala Glu Gln Ala Arg Ala His Val Glu Asn Thr Glu Arg
1130 1135 1140

Leu Ile Glu Ile Ala Ser Arg Glu Leu Glu Lys Ala Lys Val Ala
1145 1150 1155

Ala Ala Asn Val Ser Val Thr Gln Pro Glu Ser Thr Gly Asp Pro
1160 1165 1170

Asn Asn Met Thr Leu Leu Ala Glu Glu Ala Arg Lys Leu Ala Glu
1175 1180 1185

Arg His Lys Gln Glu Ala Asp Asp Ile Val Arg Val Ala Lys Thr
1190 1195 1200

Ala Asn Asp Thr Ser Thr Glu Ala Tyr Asn Leu Leu Leu Arg Thr
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Leu Ala Gly Glu Asn Gln Thr Ala Phe Glu Ile Glu Glu Leu Asn
1220 1225 1230

Arg Lys Tyr Glu Gln Ala Lys Asn Ile Ser Gln Asp Leu Glu Lys
1235 1240 1245

Gln Ala Ala Arg Val His Glu Glu Ala Lys Arg Ala Gly Asp Lys
1250 1255 1260

Ala Val Glu Ile Tyr Ala Ser Val Ala Gln Leu Ser Pro Leu Asp
1265 1270 1275

Ser Glu Thr Leu Glu Asn Glu Ala Asn Asn Ile Lys Met Glu Ala
1280 1285 1290

Glu Asn Leu Glu Gln Leu Ile Asp Gln Lys Leu Lys Asp Tyr Glu
1295 1300 1305

Asp Leu Arg Glu Asp Met Arg Gly Lys Glu Leu Glu Val Lys Asn
1310 1315 1320

Leu Leu Glu Lys Gly Lys Thr Glu Gln Gln Thr Ala Asp Gln Leu
1325 1330 1335

Leu Ala Arg Ala Asp Ala Ala Lys Ala Leu Ala Glu Glu Ala Ala
1340 1345 1350

Lys Lys Gly Arg Asp Thr Leu Gln Glu Ala Asn Asp Ile Leu Asn
1355 1360 1365

Asn Leu Lys Asp Phe Asp Arg Arg Val Asn Asp Asn Lys Thr Ala
1370 1375 1380

Ala Glu Glu Ala Leu Arg Lys Ile Pro Ala Ile Asn Gln Thr Ile
1385 1390 1395

Thr Glu Ala Asn Glu Lys Thr Arg Glu Ala Gln Gln Ala Leu Gly
1400 1405 1410

Ser Ala Ala Ala Asp Ala Thr Glu Ala Lys Asn Lys Ala His Glu
1415 1420 1425

Ala Glu Arg Ile Ala Ser Ala Val Gln Lys Asn Ala Thr Ser Thr
1430 1435 1440

Lys Ala Glu Ala Glu Arg Thr Phe Ala Glu Val Thr Asp Leu Asp
1445 1450 1455

Asn Glu Val Asn Asn Met Leu Lys Gln Leu Gln Glu Ala Glu Lys
1460 1465 1470

Glu Leu Lys Arg Lys Gln Asp Asp Ala Asp Gln Asp Met Met Met
1475 1480 1485

Ala Gly Met Ala Ser Gln Ala Ala Gln Glu Ala Glu Ile Asn Ala
1490 1495 1500

Arg Lys Ala Lys Asn Ser Val Thr Ser Leu Leu Ser Ile Ile Asn
1505 1510 1515

Asp Leu Leu Glu Gln Leu Gly Gln Leu Asp Thr Val Asp Leu Asn
1520 1525 1530

Lys Leu Asn Glu Ile Glu Gly Thr Leu Asn Lys Ala Lys Asp Glu
1535 1540 1545

Met Lys Val Ser Asp Leu Asp Arg Lys Val Ser Asp Leu Glu Asn
1550 1555 1560

Glu Ala Lys Lys Gln Glu Ala Ala Ile Met Asp Tyr Asn Arg Asp
1565 1570 1575

Ile Glu Glu Ile Met Lys Asp Ile Arg Asn Leu Glu Asp Ile Arg
1580 1585 1590

Lys Thr Leu Pro Ser Gly Cys Phe Asn Thr Pro Ser Ile Glu Lys
1595 1600 1605

Pro

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ggctgttcca ggggaagctg ctaccccgcc acgggcgacc tgctggtggg ccgagctgac 180

agactgactg cctcatccac ttgtggcctg aatggccccc agccctactg catcgtcagt 240

cacctgcagg acgaaaagaa gtgcttcctt tgtgactccc ggcgcccctt ctctgctaga 300

gacaacccac acagccatcg catccagaat gtagtcacca gctttgcacc acagcggcgg 360

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gctgagtttc atttcacaca cctcattatg accttcaaga catttcgccc tgctgccatg 480

ctggtggaac gctcagcaga ctttggccgc acctggcatg tgtaccgata tttctcctat 540

gactgtgggg ctgacttccc aggagtccca ctagcacccc cacggcactg ggatgatgta 600

gtctgtgagt cccgctactc agagattgag ccatccactg aaggcgaggt catctatcgt 660

gtgctggacc ctgccatccc tatcccagac ccctacagct cacggattca gaacctgttg 720

aagatcacca acctacgggt gaacctgact cgtctacaca cgttgggaga caacctactc 780

gacccacgga gggagatccg agagaagtac tactatgccc tctatgagct ggttgtacgt 840

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catgctgagg gcatggtgca cggagcttgc atctgcaaac acaacacacg tggcctcaac 960

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