NOVEL METHODS AND CULTURE MEDIA FOR CULTURING PLURIPOTENT STEM CELLS

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to a method of culturing pluripotent stem cells in a suspension culture as single cells devoid of clumps, and to isolated populations of pluripotent stem cells generated thereby, and, more particularly, but not exclusively, to novel culture media which can maintain pluripotent stem cells in an undifferentiated state, and to methods of culturing the pluripotent stem cells in two-dimensional or three-dimensional culture systems while maintaining the cells in a proliferative, pluripotent and undifferentiated state.

The exceptional differentiation potential of human embryonic stem cells (hESCs) underlines them as one of the best models to study early human development, lineage commitment, differentiation processes and to be used for industrial purposes and cell-based therapy.

Induced pluripotent (iPS) cells are somatic cells which are re-programmed to ESC-like cells capable of differentiation into representative tissues of the three embryonic germ layers both *in vitro* and *in vivo*. Mouse or human iPS cells were generated by over expression of four transcription factors, c-Myc, Oct4, KLF4 and Sox2 in somatic cells. The iPS cells were shown to form the same colony morphology as ESCs and to express some typical ESCs markers such as *Myb*, *Kit*, *Gdf3* and *Zic3*, but less prominently markers such as *Dnmt3a*, *Dnmt3b*, *Utf1*, *Tcl1* and the *LIF* receptor gene, confirming that iPS cells are similar but not identical to ES cells [Takahashi and Yamanaka, 2006; Takahashi et al, 2007; Meissner et al, 2007; Okita et al, 2007]. Yu Junying et al. (Science 318:1917-1920, 2007) found a common gene expression pattern to fibroblasts-derived iPS cells and hESCs.

Further studies revealed that iPS cells could be obtained by transforming somatic cells with Oct4, Sox2, Nanog and Lin28 while omitting the use of the oncogene C-Myc [Yu J., et al, 2007, Science, 318: 1917-1920; Nakagawa et al, 2008]. Improvements of iPS cells derivation methods include the use of plasmids instead of viral vectors or derivation without any integration to the genome, which might simplify the future use of iPS cells for clinical applications [Yu J, et al., Science. 2009, 324: 797-801].

The currently available iPS cells are those derived from embryonic fibroblasts [Takahashi and Yamanaka, 2006; Meissner et al, 2007], fibroblasts formed from hESCs [Park et al, 2008], Fetal fibroblasts [Yu et al, 2007; Park et al, 2008], foreskin fibroblast [Yu et al, 2007; Park et al, 2008], adult dermal and skin tissues [Hanna et al, 2007; Lowry et al, 2008], b-lymphocytes [Hanna et al 2007] and adult liver and stomach cells [Aoi et al, 2008].

Similarly to hESCs, iPS cells are traditionally cultured with a supportive layer in 2D culture, which allows their continuous growth in the undifferentiated state. For example, iPS cells were cultured on feeder-layers consisting of inactivated mouse embryonic fibroblasts (MEF) or foreskin fibroblasts [Takahashi and Yamanaka 2006, Meisnner at al 2007] in the presence of a medium supplemented with fetal bovine serum (FBS). Further improvements of the culturing methods include culturing iPS cells on MEF feeder layers in the presence of a more defined culture medium containing serum replacement and 10 ng/ml of basic fibroblasts growth factor (bFGF) (Park et al., 2008). However, for clinical applications (e.g., cell-based therapy) or industrial purposes, the iPS cells should be cultured in a defined, xeno-free (e.g., animal-free) and a scalable culture system with controlled processes.

PCT Publication No. WO2007/026353 discloses a well-defined, xeno-free culture media which comprise a TGF-beta isoform or the chimera formed between IL6 and the soluble IL6 receptor (IL6RIL6 hereinafter) for maintaining human embryonic stem cells, in an undifferentiated state in a two-dimensional culture system.

U.S. Patent Application No. 20050233446 discloses a defined medium which comprises bFGF, insulin and ascorbic acid for maintaining hESCs when cultured on Matrigel™ in an undifferentiated state.

Ludwig TE., et al., 2006 (Nature Biotechnology, 24: 185-7) discloses the TeSR1 defined medium for culturing hESCs on a matrix composed of Collagen IV, fibronectin, laminin and virtonectin.

U.S. Patent Application No. 20090029462 discloses methods of expanding pluripotent stem cells in suspension using microcarriers or cell encapsulation.

PCT Publication No. WO/2008/015682 discloses a method of expanding and maintaining human embryonic stem cells in a suspension culture under culturing conditions devoid of substrate adherence.

U.S. Patent Application No. 20070155013 discloses a method of growing pluripotent stem cells in suspension using a carrier which adheres to the pluripotent stem cells.

U.S. Patent Application No. 20080241919 (Parsons et al.) discloses a method of culturing pluripotent stem cells in a suspension culture in a medium which comprises bFGF, insulin and ascorbic acid in a cell culture vessel that includes a cell-free matrix.

U.S. Patent Application No. 20080159994 (Mantalaris et al.) discloses a method of culturing pluripotent ES cells encapsulated within alginate beads in a three-dimensional culture in a medium which comprises serum replacement and bFGF.

U.S. Patent Application No. 20070264713 discloses a method of culturing undifferentiated stem cells in suspension on microcarriers in vessels using a conditioned medium.

PCT Publication No. WO2006/040763 discloses isolated primate embryonic cells which are derived from extended blastocysts (e.g., from at least nine days post fertilization) and methods generated and using same.

Additional background art includes U.S. Patent application 20090130759; Stankoff B., et al., J. Neuroscience 22: 9221-9227, 2002; Ernst M., et al., Journal of Biological Chemistry, 271: 30136-30143, 1996; Roeb E, et al., Hepatology, 1993, 18:1437-42; U.S. Patent application 20040235160; Pera M.F., et al. 2000. Journal of Cell Science 113, 5-10. Human embryonic stem cells. Commentary.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided an isolated population of human pluripotent stem cells comprising at least 50% human pluripotent stem cells characterized by an $OCT4^{+}/TRA1\text{-}60^{-}/TRA1\text{-}81^{-}/SSEA1^{+}/SSEA4^{-}$ expression signature, wherein the human pluripotent stem cells are capable of differentiating into the endoderm, ectoderm and mesoderm embryonic germ layers.

According to an aspect of some embodiments of the present invention there is provided a method of expanding and maintaining pluripotent stem cells (PSCs) in an undifferentiated state, the method comprising: (a) passaging the PSCs in a suspension culture by mechanical dissociation of PSC clumps to single cells for at least 2 and no

more than 10 passages, to thereby obtain a suspension culture of PSCs devoid of clumps, and; (b) passaging the suspension culture of PSCs devoid of the clumps without dissociation of the clumps, thereby expanding and maintaining the PSCs in the undifferentiated state.

According to some embodiments of the invention, the method further comprising culturing the PSCs under conditions which allow expansion of the pluripotent stem cells in the undifferentiated state.

According to an aspect of some embodiments of the present invention there is provided a method of deriving an embryonic stem cell line, the method comprising: (a) obtaining embryonic stem cells (ESCs) from a pre-implantation stage blastocyst, post-implantation stage blastocyst and/or a genital tissue of a fetus; and (b) passaging the ESCs in a suspension culture by mechanical dissociation of ESC clumps to single cells for at least 2 and no more than 10 passages, to thereby obtain a suspension culture of ESCs devoid of clumps, and; (c) passaging the suspension culture of ESCs devoid of the clumps without dissociation of the clumps, thereby deriving the embryonic stem cell line.

According to some embodiments of the invention, the method further comprising culturing the ESCs under conditions which allow expansion of the embryonic single stem cells in the undifferentiated state.

According to some embodiments of the invention, the passaging is performed under conditions devoid of an enzymatic dissociation.

According to an aspect of some embodiments of the present invention there is provided a method of cloning a pluripotent stem cell, comprising: culturing a single pluripotent stem cell obtained according to the method of some embodiments of the invention, or a single embryonic stem cell obtained according to the method of some embodiments of the invention, in a suspension culture under conditions which allow expansion of the single pluripotent stem cell or of the single embryonic stem cell, respectively, in the undifferentiated state, thereby expanding the single pluripotent stem cell or the embryonic stem cell, respectively, into a clonal culture, thereby cloning the pluripotent stem cell.

According to some embodiments of the invention, the culturing is effected without dissociating cell clumps.

According to an aspect of some embodiments of the present invention there is provided a method of generating lineage-specific cells from pluripotent stem cells, the method comprising: (a) culturing the pluripotent stem cells in a suspension culture according to the method of some embodiments of the invention to thereby obtain expanded, undifferentiated pluripotent stem cells devoid of clumps; and (b) subjecting the expanded, undifferentiated pluripotent stem cells devoid of clumps to culturing conditions suitable for differentiating and/or expanding lineage specific cells, thereby generating the lineage-specific cells from the pluripotent stem cells.

According to an aspect of some embodiments of the present invention there is provided a method of generating embryoid bodies from pluripotent stem cells, the method comprising: (a) culturing the pluripotent stem cells in a suspension culture according to the method of some embodiments of the invention to thereby obtain expanded, undifferentiated pluripotent stem cells devoid of clumps; and (b) subjecting the expanded, undifferentiated pluripotent stem cells devoid of clumps to culturing conditions suitable for differentiating the pluripotent stem cells to embryoid bodies; thereby generating the embryoid bodies from the pluripotent single cells.

According to an aspect of some embodiments of the present invention there is provided a method of generating lineage-specific cells from pluripotent stem cells, the method comprising: (a) culturing the pluripotent stem cells in a suspension culture according to the method of some embodiments of the invention, to thereby obtain expanded, undifferentiated pluripotent stem cells devoid of clumps; (b) subjecting the expanded, undifferentiated pluripotent stem cells devoid of clumps to culturing conditions suitable for differentiating the pluripotent stem cells to embryoid bodies; and (c) subjecting cells of the embryoid bodies to culturing conditions suitable for differentiating and/or expanding lineage specific cells; thereby generating the lineage-specific cells from the pluripotent stem cells.

According to some embodiments of the invention, the suspension culture devoid of clumps comprises single cells or small clusters, each of the clusters comprising no more than about 200 pluripotent stem cells.

According to some embodiments of the invention, the culturing is effected under culturing conditions devoid of substrate adherence.

According to some embodiments of the invention, the culturing conditions being devoid of a Rho-associated kinase (ROCK) inhibitor.

According to some embodiments of the invention, the pluripotent stem cells are human pluripotent stem cells.

According to some embodiments of the invention, the human pluripotent stem cells are embryonic stem cells.

According to some embodiments of the invention, the human pluripotent stem cells are induced pluripotent stem cells.

According to an aspect of some embodiments of the present invention there is provided an isolated population of pluripotent stem cells devoid of cell clumps generated according to the method of some embodiments of the invention and being capable of differentiating into the endoderm, ectoderm and mesoderm embryonic germ layers.

According to an aspect of some embodiments of the present invention there is provided a method of generating a mesenchymal stem cell in a suspension culture, comprising culturing the pluripotent stem cells of some embodiments of the invention in a suspension culture under conditions suitable for differentiation of pluripotent stem cells to mesenchymal stem cells, thereby generating the mesenchymal stem cell in the suspension culture.

According to an aspect of some embodiments of the present invention there is provided an isolated population of mesenchymal stem cells (MSCs) in a suspension culture generated by the method of some embodiments of the invention.

According to some embodiments of the invention, at least 40% of the cells are characterized by a CD73+/CD31-/CD105+ expression signature.

According to some embodiments of the invention, the MSCs are capable of differentiation in a suspension culture into a cell lineage selected from the group consisting of an adipogenic lineage, an osteoblastic lineage, and a chrondrogenic lineage.

According to an aspect of some embodiments of the present invention there is provided a method of generating a neuronal progenitor cell in a suspension culture, comprising culturing the pluripotent stem cells of some embodiments of the invention in a suspension culture under conditions suitable for differentiation of neuronal progenitor cell, thereby generating the neuronal progenitor cell in the suspension culture.

According to an aspect of some embodiments of the present invention there is provided an isolated population of neuronal progenitor cells in a suspension culture generated by the method of some embodiments of the invention.

According to an aspect of some embodiments of the invention, there is provided a method of generating an endodermal cell in a suspension culture, comprising culturing the pluripotent stem cells of some embodiments of the invention in a suspension culture under conditions suitable for differentiation of the pluripotent stem cells to endodermal cells, thereby generating the endodermal cell in the suspension culture.

According to an aspect of some embodiments of the invention, there is provided an isolated population of endodermal cells in a suspension culture generated by the method of some embodiments of the invention.

According to an aspect of some embodiments of the present invention there is provided a culture medium comprising interleukin 11 (IL11) and Ciliay Neurotrophic Factor (CNTF).

According to an aspect of some embodiments of the present invention there is provided a culture medium comprising basic fibroblast growth factor (bFGF) at a concentration of at least 50 ng/ml and an IL6RIL6 chimera.

According to an aspect of some embodiments of the present invention there is provided a culture medium comprising an animal contaminant-free serum replacement and an IL6RIL6 chimera.

According to an aspect of some embodiments of the present invention there is provided a cell culture comprising pluripotent stem cells and the culture medium of some embodiments of the invention.

According to an aspect of some embodiments of the present invention there is provided a culture system comprising a matrix and the culture medium of some embodiments of the invention.

According to an aspect of some embodiments of the present invention there is provided a cell culture comprising pluripotent stem cells and a serum-free culture medium, the culture medium comprising a soluble interleukin 6 receptor (sIL6R) and interleukin 6 (IL6), wherein a concentration of the sIL6R is at least 5 ng/ml, and wherein a concentration of the IL6 is at least 3 ng/ml.

According to an aspect of some embodiments of the present invention there is provided a cell culture comprising pluripotent stem cells and a culture medium which comprises interleukin 11 (IL11) and oncostatin.

According to an aspect of some embodiments of the present invention there is provided a method of expanding and maintaining pluripotent stem cells in an undifferentiated state, the method comprising culturing the pluripotent stem cells in the culture medium of some embodiments of the invention, thereby expanding and maintaining the pluripotent stem cells in the undifferentiated state.

According to an aspect of some embodiments of the present invention there is provided a method of generating lineage-specific cells from pluripotent stem cells, the method comprising: (a) culturing the pluripotent stem cells according to the method of some embodiments of the invention, to thereby obtain expanded, undifferentiated stem cells; (b) subjecting the expanded, undifferentiated stem cells to culturing conditions suitable for differentiating and/or expanding lineage specific cells; thereby generating the lineage-specific cells from the pluripotent stem cells.

According to an aspect of some embodiments of the present invention there is provided a cell culture comprising a population of pluripotent stem cells generated according to the method of some embodiments of the invention, the population comprises at least 1000 pluripotent stem cells per milliliter of medium.

According to an aspect of some embodiments of the present invention there is provided a use of the cell culture of some embodiments of the invention for cell based therapy.

According to an aspect of some embodiments of the present invention there is provided a use of the cell culture of some embodiments of the invention for drug screening.

According to an aspect of some embodiments of the present invention there is provided a use of the cell culture of some embodiments of the invention for production of a vaccine.

According to an aspect of some embodiments of the present invention there is provided a use of the cell culture of some embodiments of the invention for production of proteins.

According to some embodiments of the invention, the IL11 is provided at a concentration of at least 0.1 ng/ml.

According to some embodiments of the invention, the CNTF is provided at a concentration of at least 0.1 ng/ml.

According to some embodiments of the invention, the IL11 is provided at a concentration of 1 ng/ml.

According to some embodiments of the invention, the CNTF is provided at a concentration of 1 ng/ml.

According to some embodiments of the invention, the concentration of the bFGF is selected from the range of between 50 ng/ml to 150 ng/ml.

According to some embodiments of the invention, the IL6RIL6 chimera is provided at a concentration of at least 50 ng/ml.

According to some embodiments of the invention, the IL6RIL6 chimera is provided at a concentration of at least 50 ng/ml.

According to some embodiments of the invention, the culture medium further comprising serum replacement.

According to some embodiments of the invention, the serum replacement is provided at a concentration of at least 10%.

According to some embodiments of the invention, the serum replacement is devoid of animal contaminants.

According to some embodiments of the invention, the IL6RIL6 chimera is provided at a concentration of 50-150 ng/ml.

According to some embodiments of the invention, the IL6RIL6 chimera is provided at a concentration of 50-150 pg/ml.

According to some embodiments of the invention, the culture medium further comprising basic fibroblast growth factor (bFGF).

According to some embodiments of the invention, the bFGF is provided at a concentration of at least 4 ng/ml.

According to some embodiments of the invention, the culture medium further comprising ascorbic acid.

According to some embodiments of the invention, the ascorbic acid is provided at a concentration of 25-100 μg/ml.

According to some embodiments of the invention, the bFGF is provided at a concentration of 100 ng/ml and the IL6RIL6 is provided at a concentration of 100 ng/ml.

According to some embodiments of the invention, the bFGF is provided at a concentration of 100 ng/ml and the IL6RIL6 is provided at a concentration of 100 pg/ml.

According to some embodiments of the invention, the culture medium further comprising TGFβ.

According to some embodiments of the invention, the TGFβ comprises TGFβ1.

According to some embodiments of the invention, the TGFβ comprises TGFβ3.

According to some embodiments of the invention, the culture medium is serum-free.

According to some embodiments of the invention, the culture medium is devoid of animal contaminants.

According to some embodiments of the invention, expanding and maintaining the pluripotent stem cells in the undifferentiated state is effected in a suspension culture.

According to some embodiments of the invention, the culturing is effected under conditions comprising a static suspension culture.

According to some embodiments of the invention, the culturing is effected under conditions comprising a dynamic suspension culture.

According to some embodiments of the invention, the culturing is effected under conditions which enable expansion of the pluripotent stem cells as single cells.

According to some embodiments of the invention, the culturing is effected under conditions devoid of enzymatic dissociation of cell clusters.

According to some embodiments of the invention, the expanding and maintaining the pluripotent stem cells in the undifferentiated state is effected in a two-dimensional culture system.

According to some embodiments of the invention, the two-dimensional culture system comprises a matrix and the culture medium.

According to some embodiments of the invention, the pluripotent stem cells comprise embryonic stem cells.

According to some embodiments of the invention, the pluripotent stem cells comprise induced pluripotent stem (iPS) cells.

According to some embodiments of the invention, the embryonic stem cells are human embryonic stem cells.

According to some embodiments of the invention, the induced pluripotent stem cells are human induced pluripotent stem cells.

According to some embodiments of the invention, the culture medium is capable of expanding the pluripotent stem cells in an undifferentiated state.

According to some embodiments of the invention, at least 85% of the pluripotent stem cells are in an undifferentiated state.

According to some embodiments of the invention, the culture conditions comprise a culture medium which comprises interleukin 11 (IL11) and Ciliay Neurotrophic Factor (CNTF).

According to some embodiments of the invention, the culture conditions comprise a culture medium which comprises basic fibroblast growth factor (bFGF) at a concentration of at least 50 ng/ml and an IL6RIL6 chimera.

According to some embodiments of the invention, the culture conditions comprise a culture medium which comprises an animal contaminant-free serum replacement and an IL6RIL6 chimera.

According to some embodiments of the invention, the culture conditions comprise a serum-free culture medium which comprises a soluble interleukin 6 receptor (sIL6R) and interleukin 6 (IL6), wherein a concentration of the sIL6R is at least 5 ng/ml, and wherein a concentration of the IL6 is at least 3 ng/ml.

According to some embodiments of the invention, the culture conditions comprise a culture medium which comprises interleukin 11 (IL11) and oncostatin.

According to an aspect of some embodiments of the present invention there is provided a culture medium comprising serum and serum replacement.

According to some embodiments of the invention, the serum replacement is provided at a concentration of about 10%.

According to some embodiments of the invention, the serum is provided at a concentration of 10%.

According to some embodiments of the invention, the culture medium which comprises serum and serum replacement is devoid of bFGF.

According to some embodiments of the invention, the culture medium which comprises serum and serum replacement is devoid of the IL6RIL6 chimera.

According to some embodiments of the invention, the culture medium which comprises serum and serum replacement further comprises L-glutamine, β–mercaptoethanol, and non-essential amino acid stock.

According to some embodiments of the invention, the culture medium which comprises serum and serum replacement consists of 80% DMEM/F12, 10% knockout serum replacement (SR), 10% FBS, 2 mM L-glutamine, 0.1 mM β–mercaptoethanol, 1% non-essential amino acid stock.

According to some embodiments of the invention, the culture medium which comprises serum and serum replacement is suitable for differentiation in suspension of pluripotent stem cells into mesenchymal stem cells.

According to some embodiments of the invention, the conditions suitable for differentiation of the pluripotent stem cells to the mesenchymal stem cells comprise a culture medium which comprises serum and serum replacement.

According to some embodiments of the invention, the method further comprising shipping the pluripotent stem cells of some embodiments of the invention as non-frozen living cells.

According to some embodiments of the invention, the pluripotent stem cells remain viable, proliferative and undifferentiated following shipping the cells as non-frozen living cells.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

Implementation of the method and/or system of embodiments of the invention can involve performing or completing selected tasks manually, automatically, or a combination thereof. Moreover, according to actual instrumentation and equipment of embodiments of the method and/or system of the invention, several selected tasks could

be implemented by hardware, by software or by firmware or by a combination thereof using an operating system.

For example, hardware for performing selected tasks according to embodiments of the invention could be implemented as a chip or a circuit. As software, selected tasks according to embodiments of the invention could be implemented as a plurality of software instructions being executed by a computer using any suitable operating system. In an exemplary embodiment of the invention, one or more tasks according to exemplary embodiments of method and/or system as described herein are performed by a data processor, such as a computing platform for executing a plurality of instructions. Optionally, the data processor includes a volatile memory for storing instructions and/or data and/or a non-volatile storage, for example, a magnetic hard-disk and/or removable media, for storing instructions and/or data. Optionally, a network connection is provided as well. A display and/or a user input device such as a keyboard or mouse are optionally provided as well.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-C are photographs of I3 ESCs, which were grown in a suspension culture according to some embodiments of the invention, following shipment of living cells over the Atlantic Ocean (from Israel to Baltimore USA) which lasted for four days. I3 cells were cultured in suspension for at least 20 passages before they were shipped. Figures 1A and 1B - Morphology of I3, 3 days (Figure 1A) and 1 day (Figure 1B) after arrival and re-plating in suspension using CM100Fp culture medium. The cells demonstrate typical sphere morphology consisting undifferentiated cells. Figure 1C - Morphology of I3, 3 days after arrival and re-plating with MEFs. The cells demonstrate ESCs typical colony morphology

FIGs. 2A-D are fluorescent images of I3.2 hESCs stained with antibodies to various markers of pluripotency (immunofluorescence staining). Cells cultured in the novel medium of some embodiments of the invention (e.g., the CMTeSR2 medium in this case) were tested for their pluripotency using the typical markers Oct4 (Figure 2A), SSEA4 (Figure 2B), Tra-160 (Figure 2C) and TRA-1-81 (Figure 2D). In this example I3.2 at passage p19+83 (*i.e.*, the I3.2 clonal cell line was derived from I3 cell line at passage 19, and the cells for analysis were at passage 83 following isolation of the clone) were cultured with CMTESR2 medium for 5 passages in suspension and then re-cultured on MEF. The cells were found positive for all tested markers.

FIGs. 3A-C are photographs of the I3.2 ESC line depicting morphology of cells cultured in suspension using the novel culture medium of some embodiments of the invention. Figure 3A - I3.2 at passage p19+87 (*i.e.*, 87 passages following isolation of clone) were cultured in suspension using cmTeSR2 for 26 passages, and then were re-plated with MEFs demonstrating typical ESCs colony morphology. Figure 3B - J3 cells [delayed (extended) blastocyst cell line] at passage 80 (p80), cultured for 2 passages using NCM100 medium in suspension demonstrating typical sphere morphology of undifferentiated cells. Figure 3C - H9.2 cells at p29+48 (*i.e.*, H9 cell line at passage 29 was subject to single cell cloning and the resulting clonal hESC line at passage 48 following isolation was used) cultured for 5 passages using ILCNTF medium in suspension demonstrating typical sphere morphology of undifferentiated cells.

FIGs. 4A-B are photographs of the H9 hESC line (Figure 4A) and the human C2 iPS cell line (Figure 4B) depicting the single cells in the suspension culture. Figure 4A - H9 at p53 (passage 53) cultured using CMrb100Fp medium for 9 passages in suspension as single cells in a static culture. Figure 4B - C2 iPS cells cultured for 1.5 months in a spinner flask (a dynamic culture) as single cells using CM100Fp medium. The cells were stained with trepan blue. Dead cells are stained with blue. These results demonstrate that pluripotent stem cells cultured in a suspension culture according to some embodiments of the invention adopt the single cell growth pattern.

FIGs. 5A-C are microscopy photographs depicting pluripotent stem cells cultured in suspension under dynamic conditions using a Controlled Wave-bioreactor (Biostat® Cultibag RM, Sartorius North America, Edgewood, New York, USA). Induced pluripotent stem cell line C2 was cultured in controlled wave- bioreactor for

five days as single cells (Figure 5A) or as small spheres of up to 200 μM (Figure 5B). Figure 5C – Cells grown in suspension as single cells were re-cultured on MEFs (Oct-4 staining). Living cells numbers increased in 64 folds while maintaining iPS cells features such as Oct4 expression (Figure 5C).

FIGs. 6A-C are microscopy photographs depicting pluripotent stem cells after freeze/thaw and shears of single cells cultured in suspension. C2 cell line (iPS from foreskin fibroblasts, at passage 89 from derivation, of which the cells were cultured for 48 passages in a suspension culture in the presence of the cmrb100p culture medium) were frozen using the following freezing solutions: 90% serum replacement (SR) and 10% DMSO (Figure 6A); 20% SR, 20% feral bovine serum (FBS) and 10% DMSO (Figure 6B); and Serum free freezing solution from Biological Industries (Beit HaEmek, Israel) (Figure 6C). After being frozen for 5 days in liquid nitrogen the cells were thawed and re-cultured in a suspension culture. Shown are the cells after freeze/thaw and re-culture in a suspension culture. Note that more than 70% of the cells survived the procedure and recovered directly to suspension culture.

FIGs. 7A-C are images of immunofluorescence staining demonstrating directed differentiation of pluripotent stem cells into cells from the nerve lineage. I6 cultured in suspension for more than 40 passages were induced to differentiation by addition of Retinoic acid and were stained for typical nerve markers: Nestin (Figure 7A), β-tubulin (Figure 7B) and Ploysialylated (PSA) Neural Cell Adhesion Molecule (NCAM) (Figure 7C). The specific markers are stained with red and the blue staining represents DAPI staining.

FIGs. 8A-B are FACS analyses demonstrating differentiation of the pluripotent stem cells into the nerve lineage. Figure 8A – FACS analysis using the NCM FITC antibody, showing that 68% of the cells are positive for NCAM; Figure 8B – FACS analysis, isotype control, using NCAM IgG.

FIGs. 9A-G is a histogram (Figure 9A) and gel images (Figures 9B-G) depicting the results of a semi quantitated RT-PCR analysis with nerve-specific markers. RT-PCR analysis was performed on cells cultured in suspension and induced to nerve cell lineage by retinoic acid and on cells cultured in suspension as undifferentiated. RT-PCR primers of the OCT-4 (Figure 9B), PAX6 (Figure 9C), Heavy chain neural filament (HNF) (Figure 9D), Nestin (Figure 9E), and LIM homeobox 2 (LHX2) (Figure 9F), and

GAPDH (control gene, Figure 9G) genes are described in Table 1 in the Examples section which follows. The results represent average of three independent experiments. Lanes 1-3 are from three different biological repeats, and lane 4 are undifferentiated cells of I6 cultured in suspension for 40 passages.

FIGs. 10A-B are immuno-fluorescence images depicting induction of pluripotent stem cells to cells of the endodermal lineage. Cells from C2 cell line induced to differentiate to endodermal lineage. 10 days post the differentiation induction cells were stained for PDX1 marker (transcription factor related to β–cells) (Figure 10A, green) and for DAPI (nucleus staining) (Figure 10B, blue).

FIGs. 11A-B are two representative images depicting morphology of hESC colonies after re-plating on MEFs. CL1 (13E1) cells which were cultured for 17 passages in suspension as single cells were re-plated on MEFs and photographed using a phase contrast. Note that when re-plated on feeder cells (MEFs) the cells form colonies characterized by typical morphology of pluripotent cells with spaces between cells, clear borders and high nucleus to cytoplasm ratio.

FIGs. 12A-J are histograms depicting FACS analyses of pluripotent markers. Human ESCs were grown on two-dimensional (2-D) MEFs (Figures 12A-B), in a suspension culture as cell clumps (Figures 12C-D, 12G-H) or in a suspension culture as single cells devoid of cell clumps (Figures 12E-F, 12I-J) and the expression of the TRA1-60, TRA1-81, SSEA1 and SSEA4 markers was assayed by FACS. Figure 12A - H14 cells cultured in 2D, sorted by a TRA1-60 antibody (blue curve). Note that 74.9% of the cells are TRA1-60-positive; Figure 12B - H14 cells cultured in 2D, sorted by a TRA1-81 antibody (blue curve). Note that 71.2% of the cells are TRA1-81-positive; Figure 12C - I3 cells cultured in suspension as cell clumps for more than 10 passages, sorted by a TRA1-60 antibody (blue curve). Note that 94.6% of the cells are TRA1-60-positive; Figure 12D - I3 cells cultured in suspension as cell clumps for more than 10 passages, sorted by a TRA1-81 antibody (blue curve). Note that 93% of the cells are TRA1-81-positive; Figure 12E - H14 cells cultured in suspension as single cells for more than 10 passages, sorted by a TRA1-60 antibody (blue curve). Note that only 0.65% of the cells are TRA1-60-positive; Figure 12F - H14 cells cultured in suspension as single cells for more than 10 passages, sorted by a TRA1-81 antibody (blue curve). Note that only 0.7% of the cells are TRA1-81-positive; Figure 12G - I3 cells cultured in

suspension as cell clumps for more than 10 passages, sorted by a SSEA1 antibody (blue curve). Note that 11.1% of the cells are SSEA1-positive; Figure 12H - I3 cells cultured in suspension as cell clumps for more than 10 passages, sorted by an SSEA4 antibody (grey curve). Note that 98.4% of the cells are SSEA4-positive; Figure 12I - H7 cells cultured in suspension as single cells for more than 10 passages, sorted by an SSEA1 antibody (blue curve). Note that 78.5% of the cells are SSEA1-positive; Figure 12J - H7 cells cultured in suspension as single cells for more than 10 passages, sorted by an SSEA4-antibody (blue curve). Note only 5.43% of the cells are SSEA4-positive. The red curve in each of Figures 12A-G and 12I-J represents a negative control.

FIGs. 13A-B are histograms depicting RT-PCR analyses. Shown is the average fold change (three repeats from each) in gene expression by real time PCR for the H7 and CL1 pluripotent stem cells. The average fold change was calculated in comparison to the expression level of the indicated genes in the H7 and CL1 pluripotent stem cells when cultured on MEFs (designated as "1"). Figure 13A – Shown are the results for Sox2, Rex1, Nanog and Oct4 pluripotency genes; Figure 13B – Shown are the results for FBLN5, CTNNB1, PLXNA2, EGFR, ITGA7, IGTA6, ITGA2, CLDN18, CLDN6, CDH2, CDH1 and FN1 adhesion molecule genes. Blue bars = single cells (SC) cultured in suspension for more than 10 passages; Red bars = cell clumps (Cl) cultured in suspension for more than 10 passages; Green bars = pluripotent stem cells cultured on mouse embryonic fibroblasts (MEFs) in a standard 2-D culture. Note the slight decrease in Nanog expression in the pluripotent single stem cells as compared to the pluripotent stem cells cultured on MEFs, while the expression of Oct4 was increased in cells cultured as single cells as compared to the same cells when cultured on MEFs or as cell clumps in a suspension culture.

FIG. 14 is an image depicting cloning efficiency of hESCs which were cultured in a suspension culture as single cells. Single cell clones were formed by plating single cells of the H7 hESC line which were cultured in a suspension culture as single cells devoid of cell clumps. Each cell was plated in a single well of a low adhesion 96- well plate and cultured in suspension. Note that the cloning efficiency of the hESCs cultured in a suspension culture as single cells is 95%.

FIG. 15 is an image depicting the thawing efficiency of hESCs cultured in a suspension culture as single cells. Human ESCs cultured as single cells in a suspension

culture were frozen using standard freezing solutions, and then were thawed in a suspension culture. The cells recovered well with at least 80% cells surviving.

FIGs. 16A-B are images depicting genetic manipulation of hESCs cultured in a suspension culture as single cells. Human ESCs cultured in a suspension culture as single cells were subjected to electroporation with a nucleic acid construct including the GFP gene under the CMV promoter. Figure 16A – a phase contrast image of the cells after genetic manipulation. Note that most of the cells (at least 90%) survived the electroporation procedure; Figure 16B – a fluorescent microscopy image of the cells after genetic manipulation. The green signals correspond to cells expressing the recombinant construct (GFP under the transcriptional regulation of the CMV promoter).

FIGs. 17A-C are microscopic images depicting the differentiation of human ESCs cultured in a suspension culture as single cells into neural progenitors (NP). Human ESCs cultured in suspension as single cells were induced to differentiate into the neuronal cell lineage. Figure 17A – astrocytes, GFAP (Red); Figure 17B – Oligodendrocytes, O4 (green); Figure 17C – neurons, β-Tubulin (green) and Nestin (red).

FIGs. 18A-C are histograms depicting FACS analyses of MSCs which were isolated by differentiation of hESCs grown in suspension culture as single cells. Figure 18A – MSCs derived from the J3 hESC line grown in animal-free medium, sorted by a CD73 antibody (blue curve). Note that 82.5% are CD73-positive; Figure 18B - MSCs derived from the J3 hESC line grown in animal-free medium, sorted by a CD31 antibody (blue curve). Note that only 4.83% are CD31-positive; Figure 18C - MSCs derived from the J3 hESC line grown in a serum-containing medium, sorted by a CD105 antibody (blue curve). Note that 99.3% are CD105-positive.

FIGs. 19A-D are images depicting differentiation of hESCs which are cultured in suspension as single cells into MSCs. Single cells cultured in suspension as single cells can differentiate both in suspension and in 2D to potent MSCs. Figures 19A-B – phase contrast images of MSCs differentiated from human ESCs which were cultured in suspension as single cells. The hESCs were re-plated in a suspension culture and differentiated into MSCs having typical MSCs morphology. Figure 19A – CL1 cells were differentiated in Fy enriched medium; Figure 19B - CL1 cells were differentiated in MeSusII medium; Figure 19C – Alizarin red staining of differentiated MSCs (which

were formed by differentiation of the hESCs grown in suspension as single cells) into the bone lineage. Figure 19D - Oil red staining of differentiated MSCs (which were formed by differentiation of the hESCs grown in suspension as single cells) into adipocytes.

FIGs. 20A-B are images depicting differentiation of hESCs which are cultured in suspension as single cells into the endoderm germ layer. C2 cells were cultured for more than 10 passages as single cells in suspension. For endoderm differentiation, the bFGF and the IL6RIL6 chimera were removed from the culture medium and activin A in concentration of 10 ng/ml was added for 48 hours in a suspension culture. 10 days after exposure to activin A, the cells were plated on Matrigel or HFF matrix and were stained for PDX1 expression using the anti-PDX1 antibody (R&D Biosystems). Figure 20A - DAPI staining (nuclear staining) (blue); Figure 20B - PDX1 (red). Note that all cells which are stained by DAPI (nuclear staining) are also stained with PDX1.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to novel methods and culture media which can maintain pluripotent stem cells in an undifferentiated state, novel pluripotent stem cells which are cultured in suspension as single stem cells devoid of cell clumps, and, more particularly, but not exclusively, to methods of culturing the pluripotent stem cells in two-dimensional or three-dimensional culture systems while maintaining the pluripotent stem cells in a proliferative, pluripotent and undifferentiated state.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

The present inventors have uncovered following laborious experimentations defined culture media, which are serum-free and devoid of animal contaminants and which can maintain pluripotent stem cells such as human iPS and ESCs in an undifferentiated state in the absence of feeder cell support while preserving their pluripotent potential to differentiae into all three embryonic germ layers.

Thus, as shown in the Examples section which follows, hESCs and iPS cells (e.g., derived from adult or foreskin fibroblast) were cultured in an undifferentiated state on either two-dimensional or three-dimensional culture systems in the presence of serum-free and defined culture media (e.g., yFIL25, CMrb100F, CMrb100Fp, ILCNTF) as well as in the presence of well-defined culture media which comprise an animal contaminant-free serum replacement (e.g., NCM100F, NCM100Fp, NCMrb100F, NCMrb100Fp, NILCNTF, CmHA13, CmHA13p) which are suitable for use in clinical /therapeutic applications since human pluripotent stem cells cultured therein are completely devoid of animal contaminants. Furthermore, as shown in Example 4 of the Examples section which follows, the pluripotent stem cells cultured in suspension can remain viable, proliferative and pluripotent while being transferred across countries as living cells. While in culture, the pluripotent stem cells exhibit an undifferentiated morphology, and molecular characteristics which is typical to iPS or hESCs including normal karyotype, expression of markers of pluripotency (e.g., Oct4, SSEA4, TRA-1-81, TRA-1-60), and ability to differentiate into all three embryonic germ layers both *in vitro* (by formation of embryoid bodies after at least 10) and *in vivo* (by formation of teratomas after at least 20 passages). In addition, as shown in Figures 7-10 and described in Example 7 of the Examples section which follows, the pluripotent stem cells were used to generate lineages specific cells of the neuronal, endoderemal and mesodermal cell lineages.

In addition, the present inventors have uncovered culturing conditions suitable for maintaining undifferentiated, pluripotent stem cells in a suspension culture as single cells devoid of cell clumps, and isolated a novel population of human pluripotent stem cells which are cultured in a suspension culture as single cells.

Thus, as described in Example 3 of the Examples section which follows, the present inventors cultured pluripotent stem cells (e.g., hESC and human iPS cells) in a suspension culture by mechanically passaging the cells (e.g., using a pipette) without the use of trypsin or ROCK inhibitor. After about 3-7 passages of mechanically separating cell clumps to single cells, the pluripotent stem cells adopted a single cell mode of expansion, which required no further mechanical separation for culture passaging, thus allowing mass production of these cells. When the suspension culture which was cultured as single cells was re-plated on MEFs, the cells formed colonies with typical

morphology of pluripotent stem cells (Figures 11A-B). As is further described in Example 8 of the Examples section which follows, the human pluripotent stem cells which were cultured in a suspension culture as single cells exhibit a more naïve pattern of gene expression as compared to human ESCs cultured on MEFs or as compared to hESCs which are cultured in a suspension culture as cell clumps. Thus, the isolated population of pluripotent stem cells which are cultured in suspension as single cells devoid of cell clumps exhibit an $SSEA4^-/TRA1\text{-}60^-/TRA1\text{-}81^-/SSEA1^+$ expression signature (Figures 12E, 12F, 12I and 12J; Table 3), which is different from the typical $SSEA4^+/TRA1^-60^+/TRA1\text{-}81^+/SSEA1^-$ expression signature of human ESCs cultured on MEFs or in a suspension culture as cell clumps (Figures 12A, 12B, 12C, 12D, 12G, 12H; Table 3). In contrast, the pluripotent stem cells, which were cultured in a suspension culture as single cells, exhibit increased levels of OCT-4, a marker of pluripotency, as compared to hESCs cultured on MEFs (2-D) or to hESCs cultured in a suspension culture as cell clumps (Example 8, Figure 13A). In addition, the pluripotent stem cells which were cultured in suspension as single cells were found to exhibit an increased cloning efficiency (e.g., about 95% efficiency for hESCs) as compared to pluripotent stem cells cultured on 2-D (e.g., between 4-18%, depending on the use of ROCK inhibitor) (Example 9, Table 4), increased survival to freezing and thawing cycles (Example 9, Figure 15), and higher survival to and efficiency of genetic manipulation (Example 9, Figures 16A-B). The pluripotent stem cells which were cultured in suspension as single cells were shown capable of differentiation to all three embryonic germ layers, *i.e.*, the ectoderm germ layer, by forming neuronal progenitor cells expressing GFAP (Glial fibrillary acidic protein), a marker of astrocytes, O4, a marker of oligodendrocytes, and β-Tubulin and Nestin, markers of neurons (Example 10, Figures 17A-C); the mesoderm germ layer, by forming mesenchymal stem cells expressing CD73 and CD105 (Example 11, Figures 18A and 18C) and not-expressing CD31 (Example 11, Figure 18B); and the endoderm germ layer, by forming endodermal cells which express PDX1 (Example 12, Figures 20A-B). In addition, the present inventors have demonstrated for the first time, the *in vitro* differentiation in a suspension culture of pluripotent stem cells into mesenchymal stem cells (Example 11). These MSCs were capable of differentiation into an adipogenic cell lineage (Example 11, Figure 19D), an osteogenic cell lineage (Example 11, Figure 19C), and a chondrogenic

cell lineage (Example 11, and data not shown). Altogether, the novel pluripotent stem cells identified herein can be used as an unlimited source of pluripotent, undifferentiated stem cells for various cell based therapy, drug screening, production of a vaccine and/or production of proteins.

Thus, according to an aspect of some embodiments of the invention there is provided a method of expanding and maintaining pluripotent stem cells (PSCs) in an undifferentiated state, the method comprising: (a) passaging the PSCs in a suspension culture by mechanical dissociation of PSC clumps to single cells for at least 2 and no more than 10 passages, to thereby obtain a suspension culture of PSCs devoid of clumps, and; (b) passaging the suspension culture of PSCs devoid of the clumps without dissociation of the clumps, thereby expanding and maintaining the PSCs in the undifferentiated state.

According to some embodiments of the invention, passaging the PSCs in a suspension culture by mechanical dissociation of PSC clumps to single cells is effected for at least 2 and no more than 9 passages, for at least 2 and no more than 8 passages, for at least 2 and no more than 7 passages, for at least 2 and no more than 6 passages, for at least 2 and no more than 5 passages, for at least 2 and no more than 4 passages, for at least 3 and no more than 9 passages, for at least 3 and no more than 8 passages, for at least 3 and no more than 7 passages, for at least 3 and no more than 6 passages, for at least 3 and no more than 5 passages.

According to some embodiments of the invention, the method further comprising culturing the PSCs under conditions which allow expansion of the pluripotent stem cells in the undifferentiated state.

As used herein the phrase "pluripotent stem cells" refers to cells which are capable of differentiating into cells of all three embryonic germ layers (*i.e.*, endoderm, ectoderm and mesoderm). The phrase "pluripotent stem cells" may read on embryonic stem cells (ESCs) and/or induced pluripotent stem cells (iPS cells).

The phrase "embryonic stem cells" as used herein refers to cells which are obtained from the embryonic tissue formed after gestation (e.g., blastocyst) before implantation (*i.e.*, a pre-implantation blastocyst); extended blastocyst cells (EBCs) which are obtained from a post-implantation/pre-gastrulation stage blastocyst (see WO2006/040763]; and/or embryonic germ (EG) cells which are obtained from the

genital tissue of a fetus any time during gestation, preferably before 10 weeks of gestation.

According to some embodiments of the invention, the pluripotent stem cells of the invention are embryonic stem cells, such as from a human or primate (e.g., monkey) origin.

The embryonic stem cells of the invention can be obtained using well-known cell-culture methods. For example, human embryonic stem cells can be isolated from human blastocysts. Human blastocysts are typically obtained from human *in vivo* preimplantation embryos or from *in vitro* fertilized (IVF) embryos. Alternatively, a single cell human embryo can be expanded to the blastocyst stage. For the isolation of human ES cells the zona pellucida is removed from the blastocyst and the inner cell mass (ICM) is isolated by immunosurgery, in which the trophectoderm cells are lysed and removed from the intact ICM by gentle pipetting. The ICM is then plated in a tissue culture flask containing the appropriate medium which enables its outgrowth. Following 9 to 15 days, the ICM derived outgrowth is dissociated into clumps either by a mechanical dissociation or by an enzymatic degradation and the cells are then re-plated on a fresh tissue culture medium. Colonies demonstrating undifferentiated morphology are individually selected by micropipette, mechanically dissociated into clumps, and re-plated. Resulting ES cells are then routinely split every 4-7 days. For further details on methods of preparation human ES cells see Thomson et al., [U.S. Pat. No. 5,843,780; Science 282: 1145, 1998; Curr. Top. Dev. Biol. 38: 133, 1998; Proc. Natl. Acad. Sci. USA 92: 7844, 1995]; Bongso et al., [Hum Reprod 4: 706, 1989]; and Gardner et al., [Fertil. Steril. 69: 84, 1998].

It will be appreciated that commercially available stem cells can also be used with this aspect of the present invention. Human ES cells can be purchased from the NIH human embryonic stem cells registry (www://escr.nih.gov). Non-limiting examples of commercially available embryonic stem cell lines are BG01, BG02, BG03, BG04, CY12, CY30, CY92, CY10, TE03, TE04 and TE06.

Extended blastocyst cells (EBCs) can be obtained from a blastocyst of at least nine days post fertilization at a stage prior to gastrulation. Prior to culturing the blastocyst, the zona pellucida is digested [for example by Tyrode's acidic solution (Sigma Aldrich, St Louis, MO, USA)] so as to expose the inner cell mass. The

blastocysts are then cultured as whole embryos for at least nine and no more than fourteen days post fertilization (*i.e.*, prior to the gastrulation event) *in vitro* using standard embryonic stem cell culturing methods.

Embryonic germ (EG) cells are prepared from the primordial germ cells obtained from fetuses of about 8-11 weeks of gestation (in the case of a human fetus) using laboratory techniques known to anyone skilled in the arts. The genital ridges are dissociated and cut into small chunks which are thereafter disaggregated into cells by mechanical dissociation. The EG cells are then grown in tissue culture flasks with the appropriate medium. The cells are cultured with daily replacement of medium until a cell morphology consistent with EG cells is observed, typically after 7-30 days or 1-4 passages. For additional details on methods of preparation human EG cells see Shamblott et al., [Proc. Natl. Acad. Sci. USA 95: 13726, 1998] and U.S. Pat. No. 6,090,622.

The phrase "induced pluripotent stem (iPS) cell" (or embryonic-like stem cell) as used herein refers to a proliferative and pluripotent stem cell which is obtained by de-differentiation of a somatic cell (e.g., an adult somatic cell).

According to some embodiments of the invention, the iPS cell is characterized by a proliferative capacity which is similar to that of ESCs and thus can be maintained and expanded in culture for an almost unlimited time.

IPS cells can be endowed with pluripotency by genetic manipulation which re-program the cell to acquire embryonic stem cells characteristics. For example, the iPS cells of the invention can be generated from somatic cells by induction of expression of Oct-4, Sox2, Kfl4 and c-Myc in a somatic cell essentially as described in Takahashi and Yamanaka, 2006, Takahashi et al, 2007, Meissner et al, 2007, and Okita K., et al, 2007, Nature 448: 313-318). Additionally or alternatively, the iPS cells of the invention can be generated from somatic cells by induction of expression of Oct4, Sox2, Nanog and Lin28 essentially as described in Yu et al, 2007, and Nakagawa et al, 2008. It should be noted that the genetic manipulation (re-programming) of the somatic cells can be performed using any known method such as using plasmids or viral vectors, or by derivation without any integration to the genome [Yu J, et al., Science. 2009, 324: 797-801].

The iPS cells of the invention can be obtained by inducing de-differentiation of embryonic fibroblasts [Takahashi and Yamanaka, 2006; Meissner et al, 2007], fibroblasts formed from hESCs [Park et al, 2008], Fetal fibroblasts [Yu et al, 2007; Park et al, 2008], foreskin fibroblast [Yu et al, 2007; Park et al, 2008], adult dermal and skin tissues [Hanna et al, 2007; Lowry et al, 2008], b-lymphocytes [Hanna et al 2007] and adult liver and stomach cells [Aoi et al, 2008].

IPS cell lines are also available via cell banks such as the WiCell bank. Non-limiting examples of commercially available iPS cell lines include the iPS foreskin clone 1 [WiCell Catalogue No. iPS(foreskin)-1-DL-1], the iPSIMR90 clone 1 [WiCell Catalogue No. iPS(IMR90)-1-DL-1], and the iPSIMR90 clone 4 [WiCell Catalogue No. iPS(IMR90)-4-DL-1].

According to some embodiments of the invention, the induced pluripotent stem cells are human induced pluripotent stem cells.

As used herein the term "expanding" refers to increasing the number of pluripotent stem cells over the culturing period (by at least about 5 %, 10 %, 15 %, 20 %, 30 %, 50 %, 100 %, 200 %, 500 %, 1000 %, and more). It will be appreciated that the number of pluripotent stem cells, which can be obtained from a single pluripotent stem cell, depends on the proliferation capacity of the pluripotent stem cell. The proliferation capacity of a pluripotent stem cell can be calculated by the doubling time of the cell (*i.e.*, the time needed for a cell to undergo a mitotic division in the culture) and the period the pluripotent stem cell culture can be maintained in the undifferentiated state (which is equivalent to the number of passages multiplied by the days between each passage).

According to some embodiments of the invention, the method of some embodiments of the invention enables the expansion of a single pluripotent stem cell (e.g., hESC or human iPS cell) by at least 8 folds in 5 days, e.g., at least 16 folds in 5 days, e.g., at least 32 folds in 5 days, e.g., at least 64 folds in 5 days.

According to some embodiments of the invention, the method of some embodiments of the invention enables the expansion of a single pluripotent stem cell (e.g., hESC or human iPS cell) or a small cluster of 2-100 cells by at least 2^8, e.g., 2^{10}, e.g., 2^{14}, e.g., 2^{16}, e.g., 2^{18}, e.g., 2^{20} folds within about one month.

As used herein the term "clump" refers to a cluster of cells which adhere to each other in suspension.

According to some embodiments of the invention, the cell clump remains intact when the medium of the suspension culture is changed (e.g., increased, decreased or replaced) without employing any mechanical or enzymatic dissociation of the clumps.

According to some embodiments of the invention, each of the pluripotent stem cell clumps comprises at least about 200 cells (e.g., about 200), e.g., at least about 500 cells (e.g., about 500), at least about 600 cells (e.g., about 600), at least about 700 cells (e.g., about 700), at least about 800 cells (e.g., about 800), at least about 900 cells (e.g., about 900), at least about 1000 cells (e.g., about 1000), at least about 1100 cells (e.g., about 1100), at least about 1200 cells (e.g., about 1200), at least about 1300 cells (e.g., about 1300), at least about 1400 cells (e.g., about 1400), at least about 1500 cells (e.g., about 1500), at least about $5x10^3$ cells (e.g., about $5x10^3$), at least about $1x10^4$ cells (e.g., about $1x10^4$), at least about $5x10^4$ cells (e.g., about $5x10^4$), at least about $1x10^5$ cells (e.g., about $1x10^5$), or more.

As used herein the term "passaging" as used herein refers to splitting the cells in the culture vessel to 2 or more culture vessels, typically including addition of fresh medium. Passaging is typically done when the cells reach a certain density in culture.

According to some embodiments of the invention, passaging of a cell culture seeded at a concentration of about $1x10^6$ cells per milliliter under static three-dimensional culture system is done when the cells' concentration increases to about 2 or 3 folds (e.g., at a concentration of about $2x10^6$-$3x10^6$ cells/ml), but no more than up to about 4 folds (e.g., at a concentration about $4x10^6$ cells/ml).

According to some embodiments of the invention, passaging of a cell culture seeded at a concentration of about $1x10^6$ cells per milliliter under dynamic three-dimensional culture system is done when the cells' concentration increases about 20-40 folds (e.g., at a concentration of about $20x10^6$-$40x10^6$ cells/ml), but no more than up to about 50 folds (e.g., at a concentration of about $50x10^6$ cells/ml).

According to some embodiments of the invention, the passaging does not necessarily require dissociation of the cell clumps in the cell culture.

As used herein the phrase "mechanical dissociation" refers to separating the pluripotent stem cell clumps to single cells by employing a physical force rather than an enzymatic activity.

As used herein the phrase "single cells" refers to the state in which the pluripotent stem cells do not form cell clusters, each cluster comprising more than about 200 pluripotent stem cells, in the suspension culture.

According to some embodiments of the invention, the pluripotent stem cells do not form cell clusters, each cluster comprising more than about 150, about 100, about 90, about 80, about 70, about 60, about 50, about 40, about 30, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 3, about 2, or about 1 pluripotent stem cell, in the suspension culture.

According to some embodiments of the invention, each of the plurality of the pluripotent stem cells does not adhere to another pluripotent stem cell while in the suspension culture.

For mechanical dissociation, a pellet of pluripotent stem cells (which may be achieved by centrifugation of the cells) or an isolated pluripotent stem cells clump can be dissociated by pipetting the cells up and down in a small amount of medium (e.g., 0.2-1ml). For example, pipetting can be performed for several times (e.g., between 3-20 times) using a tip of a 200 μl or 1000 μl pipette.

Additionally or alternatively, mechanical dissociation of large pluripotent stem cells clumps can be performed using a device designed to break the clumps to a predetermined size. Such a device can be obtained from CellArtis Goteborg, Sweden. Additionally or alternatively, mechanical dissociation can be manually performed using a needle such as a 27g needle (BD Microlance, Drogheda, Ireland) while viewing the clumps under an inverted microscope.

According to some embodiments of the invention, passaging is effected under conditions devoid of enzymatic dissociation.

According to some embodiments of the invention, culturing in suspension is effected under conditions devoid of enzymatic dissociation of cell clusters/clumps.

According to some embodiments of the invention, the culturing conditions are devoid of using an anti-apoptotic agent.

According to some embodiments of the invention, the culturing conditions are devoid of using a Rho-associated kinase (ROCK) inhibitor.

According to some embodiments of the invention, culturing is effected for at least one passage, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 passages in an undifferentiated pluripotent state.

The present inventors have uncovered that when the pluripotent stem cells in a suspension culture are mechanically passaged without enzymatic dissociation of cell clusters for at least about 2 and no more than about 10 passages, the pluripotent stem cells adopt the single cell mode of cell growth (*i.e.,* they are expanded as single cells and not as cell clumps). Thus, as described in Example 3 of the Examples section which follows, cells cultured in suspension while being passaged by only mechanical dissociation of cell clusters for the first 2-10 passages adopted the single cell mode of expansion and grew without the need of further dissociation of cell clusters for at least about 15, 20 or 25 additional passages.

It should be noted that while the cells are cultured as single cells, they still need to be diluted when the concentration of cells exceeds about $1x10^6$ cells per milliliter (e.g., $5x10^6$ cells per 5 ml of Petri dish).

As used herein the phrase "suspension culture" refers to a culture in which the pluripotent stem cells are suspended in a medium rather than adhering to a surface.

It should be noted that some protocols of culturing pluripotent stem cells such as hESCs and iPS cells include microencapsulation of the cells inside a semipermeable hydrogel membrane, which allows the exchange of nutrients, gases, and metabolic products with the bulk medium surrounding the capsule (for details see e.g., U.S. Patent Application No. 20090029462 to Beardsley et al.).

According to some embodiments of the invention, the pluripotent stem cells cultured in the suspension culture are devoid of cell encapsulation.

According to some embodiments of the invention, the conditions for culturing the pluripotent stem cells in suspension are devoid of substrate adherence, *e.g.,* without adherence to an external substrate such as components of extracellular matrix, a glass microcarrier or beads.

According to some embodiments of the invention, the culture medium and/or the conditions for culturing the pluripotent stem cells in suspension are devoid of a protein carrier.

As used herein the phrase "protein carrier" refers to a protein which acts in the transfer of proteins or nutrients (e.g., minerals such as zinc) to the cells in the culture. Such protein carriers can be, for example, albumin (e.g., bovine serum albumin), Albumax (lipid enriched albumin) or plasmanate (human plasma isolated proteins). Since these carriers are derived from either human or animal sources their use in hESCs of human iPS cell cultures is limited by batch-specific variations and/or exposure to pathogens. Thus, a culture medium which is devoid of a protein carrier (e.g., albumin) is highly advantageous since it enables a truly defined medium that can be manufacture from recombinant or synthetic materials.

Culturing in a suspension culture according to the method of some embodiments of the invention is effected by plating the pluripotent stem cells in a culture vessel at a cell density which promotes cell survival and proliferation but limits differentiation. Typically, a plating density (or a seeding density) of between about 1 x 10^3 per ml to about 2 x 10^6 cells per ml is used. When a bioreactor is used, the concentration of cells seeded in the bioreactor can be from about 1x10^4 to about 10^6 cells per ml. It will be appreciated that although single-cell suspensions of stem cells are usually seeded, small clusters such as 10-200 cells may also be used.

In order to provide the pluripotent stem cells with sufficient and constant supply of nutrients and growth factors while in the suspension culture, the culture medium can be replaced on a daily basis, or, at a pre-determined schedule such as every 2-3 days. For example, replacement of the culture medium can be performed by subjecting the pluripotent stem cells suspension culture to centrifugation for about 3 minutes at 80 g, and resuspension of the formed pluripotent stem cells pellet in a fresh medium. Additionally or alternatively, a culture system in which the culture medium is subject to constant filtration or dialysis so as to provide a constant supply of nutrients or growth factors to the pluripotent stem cells may be employed.

The culture vessel used for culturing the pluripotent stem cells in suspension according to the method of some embodiments of the invention can be any tissue culture vessel (e.g., with a purity grade suitable for culturing pluripotent stem cells)

having an internal surface designed such that pluripotent stem cells cultured therein are unable to adhere or attach to such a surface (e.g., non-tissue culture treated cells, to prevent attachment or adherence to the surface). Preferably, in order to obtain a scalable culture, culturing according to some embodiments of the invention is effected using a controlled culturing system (preferably a computer-controlled culturing system) in which culture parameters such as temperature, agitation, pH, and pO_2 is automatically performed using a suitable device. Once the culture parameters are recorded, the system is set for automatic adjustment of culture parameters as needed for pluripotent stem cells expansion.

According to some embodiments of the invention, culturing is effected under conditions comprising a static (i.e., non-dynamic) suspension culture.

For non-dynamic culturing of pluripotent stem cells, the pluripotent stem cells can be cultured in uncoated 58 mm Petri dishes (Greiner, Frickenhausen, Germany). For example, to initiate a suspension culture on 58 mm Petri dishes the pluripotent stem cells are seeded at a cell density of $1 \times 10^6 - 5 \times 10^6$ cells/dish.

While in the non-dynamic suspension culture, the pluripotent stem cells can be passaged every 5-7 days by dissociating the cell clumps as described above and splitting the culture into additional culture vessels in a ratio of about 1:2-1:4.

According to some embodiments of the invention, culturing is effected under conditions comprising a dynamic suspension culture (e.g., using a Wave reactor or stirred reactor).

For dynamic culturing of pluripotent stem cells, the pluripotent stem cells can be cultured in spinner flasks [e.g., of 200 ml to 1000 ml, for example 250 ml which can be obtained from CellSpin of Integra Biosciences, Fernwald, Germany; of 100 ml which can be obtained from Bellco, Vineland, NJ; or in 125 ml Erlenmeyer (Corning Incorporated, Corning NY, USA)] which can be connected to a control unit and thus present a controlled culturing system. The culture vessel (e.g., a spinner flask, an Erlenmeyer) is shaken continuously. According to some embodiments of the invention the culture vessels are shaken at 40-110 rounds per minute (rpm) using magnetic plate, and placed in the incubator. Additionally or alternatively, the culture vessel can be shaken using a shaker (S3.02.10L, ELMI ltd, Riga, Latvia). According to some embodiments of the invention the culture medium is changed every 1-3 days, e.g., every

day. Other suitable controlled-bioreactors which stir the medium by an impeller and can be used for dynamic culturing of the pluripotent stem cells in the culture medium according to some embodiments of the invention include the Biostat®Aplus cell culture (Sartorius North America, Edgewood, New York, USA), Cell Optimizer controlled bioreactor (Wheaton Science Products, Millville, NJ, USA) equipped with Cell Lift impeller (Infors HT, Rittergasse, Switzerland), Informs HT Multifors stirred reactor (Informs GA, CH-4103 Bottmingen Switzerland).

Additionally or alternatively, dynamic culturing of pluripotent stem cells can be achieved using a controlled bioreactor in which the dynamics of the cells is achieved by a wave-like motion, such as the Biostat® Cultibag RM (Sartorius North America, Edgewood, New York, USA) (2 litter bag with 1 litter). The reactor parameters may include a speed of tilting: 10-16 rounds per minute (rpm); angle 7°; Temperature: 37°C, PH: 7-7.4, O_2 concentration: 50%. Another suitable bioreactor is the WavePod system 20/50 EH5 Wave Bioreactor (GE Healthcare, USA), which while using the same parameters enables increase in 70 folds during 12 days. Additional suitable bioreactor is the 55 ml RWV/STLV bioreactor which allows minimum shear forces within the reactor (Synthecon Incorporated, Houston, TX, USA).

For example, to initiate a suspension culture under dynamic conditions, the pluripotent stem cells are seeded at a concentration of about 10^4-10^6 cells/ml.

While in the dynamic suspension culture, the pluripotent stem cells can be passaged every 5-7 days by dissociating the cell clumps as described above. Since the bioreactors have a large capacity, the cell culture needs no further splitting into additional culture vessels and only addition and/or replacement of medium with a fresh medium can be performed every 3-10 days.

The teachings of the invention can be used for deriving a pluripotent stem cell line.

The term "deriving" as used herein refers to generating an embryonic stem cell line or an induced pluripotent stem cell line from at least one embryonic stem or induced pluripotent cell.

As used herein the phrase "embryonic stem cell line" refers to embryonic stem cells which are derived from a single or a group of embryonic stem cells of a single organism (e.g., a single human blastocyst), and which are characterized by the ability to

proliferate in culture while maintaining the undifferentiated state and the pluripotent capacity.

As used herein the phrase "induced pluripotent stem cell line" refers to induced pluripotent stem cells which are derived from a single or a group of induced pluripotent stem cells of a single organism), and which are characterized by the ability to proliferate in culture while maintaining the undifferentiated state and the pluripotent capacity.

According to an aspect of some embodiments of the invention there is provided a method of deriving an embryonic stem cell line, the method comprising: (a) obtaining embryonic stem cells (ESCs) from a pre-implantation stage blastocyst, post-implantation stage blastocyst and/or a genital tissue of a fetus; and (b) passaging the ESCs in a suspension culture by mechanical dissociation of ESC clumps to single cells for at least 2 and no more than 10 passages, to thereby obtain a suspension culture of ESCs devoid of clumps, and; (c) passaging the suspension culture of ESCs devoid of the clumps without dissociation of the clumps, thereby deriving the embryonic stem cell line.

Obtaining an embryonic stem cell from a pre-implantation stage blastocyst, post-implantation stage blastocyst and/or a genital tissue of a fetus can be performed using methods known in the art and as described hereinabove.

According to an aspect of some embodiments of the invention, the method of deriving the embryonic stem cell line further comprising culturing the ESCs under conditions which allow expansion of the embryonic single stem cells in the undifferentiated state.

According to an aspect of some embodiments of the invention there is provided a method of deriving an induced pluripotent stem cell (iPS cell) line, the method comprising: inducing a somatic cell to a pluripotent stem cell; and expanding and maintaining the induced pluripotent stem cells in an undifferentiated state according to the method of some embodiments of the invention (e.g., as described hereinabove and in the Examples section which follows), thereby deriving the induced pluripotent stem cell (iPS cell) line.

As mentioned above and described in Table 4 and Example 9 of the Examples section which follows, the cloning efficiency of the pluripotent stem cells which are cultured in suspension as single cells is significantly higher than that of the same cells

when cultured on a 2-dimensional culture system (e.g., on MEFs), without the use of an anti-apoptotic agent such as the ROCK inhibitor.

According to an aspect of some embodiments of the invention there is provided a method of cloning pluripotent stem cells. The method is effected by culturing a single pluripotent stem cell (*i.e.,* one cell) obtained according to the method of some embodiments of the invention, or a single embryonic stem cell (*i.e.,* one cell) obtained according to the method of some embodiments of the invention, in a suspension culture under conditions which allow expansion of the single pluripotent stem cell or of the single embryonic stem cell in the undifferentiated state, thereby expanding the single pluripotent stem cell or the embryonic stem cell into a clonal culture, thereby cloning the pluripotent stem cells.

According to some embodiments of the invention, culturing the single cell suspension culture is performed without dissociating the clumps.

As described in Example 9 of the Examples section which follows, pluripotent stem cells which are cultured as single cells in a suspension culture have a higher tolerance to a freezing-thawing cycle (e.g., about 80% survival) as compared to when the same cells are cultured on 2-D (e.g., on MEFs, up to 50%) under identical assay conditions.

According to some embodiments of the invention, the pluripotent stem cells, which are cultured as single cells in a suspension culture, can be subject to at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten cycles (e.g., up to 10 cycles) of freeze/thaw without hampering the proliferative capacity of the cells in the undifferentiated state while preserving their pluripotent capacity.

As described in Example 8 of the Examples section which follows, pluripotent stem cells which are cultured according to the method of some embodiments of the invention as single cells in a suspension culture exhibit a unique expression pattern, which is slightly different from that of hESCs, but which is similar to the expression pattern of mouse ESCs ($TRA1\text{-}60^{-}/TRA1\text{-}81^{-}/SSEA1^{+}/SSEA4^{-}$; see Pera M.F., et al. 2000. Journal of Cell Science 113, 5-10. Human embryonic stem cells. Commentary). Thus, as shown in Table 3 and in Figure 13A, pluripotent stem cells which are cultured in a suspension culture as single cells (devoid of cell clumps) express OCT4, a marker of

pluripotency, at a significantly higher level (e.g., about 8 folds higher RNA levels) as compared to the level of OCT4 RNA in pluripotent stem cells cultured on MEFs, or as compared to the level of OCT4 RNA in pluripotent stem cells which are cultured in a suspension culture as cell clumps (e.g., with clumps having more than about 200-1x10^5 cells per clump).

Cells cultured according to the method of some embodiments of the invention can be further isolated.

Thus, according to an aspect of some embodiments of the invention there is provided an isolated population of pluripotent stem cells generated according to the method of some embodiments of the invention and being capable of differentiating into the endoderm, ectoderm and mesoderm embryonic germ layers.

As shown in Figures 12A-J and described in Example 8 of the Examples section which follows, the pluripotent stem cells which were cultured in suspension as single cells do not express TRA1-60, TRA1-81 or SSEA-4, but do express SSEA1.

Thus, according to an aspect of some embodiments of the invention there is provided an isolated population of human pluripotent stem cells comprising at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 65%, at least about 70% (e.g., 70%), at least about 75% (e.g., 75%), at least about 80% (e.g., 80%), at least about 81% (e.g., 81%), at least about 82% (e.g., 82%), at least about 83% (e.g., 83%), at least about 84% (e.g., 84%), at least about 85% (e.g., 85%), at least about 86% (e.g., 86%), at least about 87% (e.g., 87%), at least about 88% (e.g., 88%), at least about 89% (e.g., 89%), at least about 90% (e.g., 90%), at least about 91% (e.g., 91%), at least about 92% (e.g., 92%), at least about 93% (e.g., 93%), at least about 94% (e.g., 94%), at least about 95% (e.g., 95%), at least about 96% (e.g., 96%), at least about 97% (e.g., 97%), at least about 98% (e.g., 98%), at least about 99% (e.g., 99%), e.g., 100% of human pluripotent stem cells characterized by an $OCT4^+/TRA1\text{-}60^-/TRA1\text{-}81^-/SSEA1^+/SSEA4^-$ expression signature, wherein the human pluripotent stem cells are capable of differentiating into the endoderm, ectoderm and mesoderm embryonic germ layers.

According to some embodiments of the invention, the isolated cell population comprises cells expressing Rex1, Sox2, EGFR, TGA7, TGA6, ITGA2, CTNNB1, CDH1 at a comparable level (within the same order of magnitude) as hESCs cultured on

MEFs; and cells expressing significantly higher levels of FBLN5 and PLXNA2 as compared to hESCs cultured on MEFs under identical assay conditions.

As described in Examples 1 and 2 of the Examples section which follows, the present inventors have uncovered novel culture media which can be used to maintain and expand pluripotent stem cells in a proliferative and undifferentiated state.

According to an aspect of some embodiments of the invention, there is provided a defined culture medium suitable for maintaining and expanding pluripotent stem cells in a proliferative, pluripotent and undifferentiated state in the absence of feeder-cell support, under two-dimensional or three-dimensional culture systems.

As used herein the phrase "culture medium" refers to a liquid substance used to support the growth of pluripotent stem cells and maintain them in an undifferentiated state. The culture medium used by the invention according to some embodiments can be a water-based medium which includes a combination of substances such as salts, nutrients, minerals, vitamins, amino acids, nucleic acids, proteins such as cytokines, growth factors and hormones, all of which are needed for cell proliferation and are capable of maintaining the pluripotent stem cells in an undifferentiated state. For example, a culture medium according to an aspect of some embodiments of the invention can be a synthetic tissue culture medium such as the Ko-DMEM (Gibco-Invitrogen Corporation products, Grand Island, NY, USA), DMEM/F12 (Biological Industries, Biet HaEmek, Israel), Mab ADCB medium (HyClone, Utah, USA), Nutristem™ (Biological Industries, Beit HaEmek, Israel; also known as Stemedia™ NutriStem™ XF/FF Culture Medium, STEMGENT, USA), TeSR™ (StemCell Technologies) and TeSR2™ (StemCell Technologies) supplemented with the necessary additives as is further described hereinunder.

According to some embodiments of the invention, the culture medium comprising DMEM/F12 at a concentration range of 80-90%, e.g., about 85%.

According to some embodiments of the invention, the culture medium is serum free.

As used herein the phrase "serum-free" refers to being devoid of a human or an animal serum.

It should be noted that the function of serum in culturing protocols is to provide the cultured cells with an environment similar to that present *in vivo* (*i.e.*, within the

organism from which the cells are derived, e.g., a blastocyst of an embryo). However, the use of serum, which is derived from either an animal source (e.g., bovine serum) or a human source (human serum), is limited by the significant variations in serum components between the donor individuals (from which the serum is obtained) and the risk of having xeno contaminants (in case of an animal serum is used).

According to some embodiments of the invention, the serum-free culture medium does not comprise serum or portions thereof.

According to some embodiments of the invention, the serum-free culture medium of the invention is devoid of serum albumin (e.g., albumin which is purified from human serum or animal serum).

According to some embodiments of the invention the culture medium comprises serum replacement.

As used herein the phrase "serum replacement" refers to a defined formulation, which substitutes the function of serum by providing pluripotent stem cells with components needed for growth and viability.

Various serum replacement formulations are known in the art and are commercially available.

For example, GIBCO™ Knockout™ Serum Replacement (Gibco-Invitrogen Corporation, Grand Island, NY USA, Catalogue No. 10828028) is a defined serum-free formulation optimized to grow and maintain undifferentiated ES cells in culture. It should be noted that the formulation of GIBCO™ Knockout™ Serum Replacement includes Albumax (Bovine serum albumin enriched with lipids) which is from an animal source (International Patent Publication No. WO 98/30679 to Price, P.J. et al). However, a recent publication by Crook et al., 2007 (Crook JM., et al., 2007, Cell Stem Cell, 1: 490-494) describes six clinical-grade hESC lines generated using FDA-approved clinical grade foreskin fibroblasts in cGMP-manufactured Knockout™ Serum Replacement (Invitrogen Corporation, USA, Catalogue No. 04-0095).

Another commercially available serum replacement is the B27 supplement without vitamin A which is available from Gibco-Invitrogen, Corporation, Grand Island, NY USA, Catalogue No. 12587-010. The B27 supplement is a serum-free formulation which includes d-biotin, fatty acid free fraction V bovine serum albumin (BSA), catalase, L-carnitine HCl, corticosterone, ethanolamine HCl, D-galactose (Anhyd.),

glutathione (reduced), recombinant human insulin, linoleic acid, linolenic acid, progesterone, putrescine-2-HCl, sodium selenite, superoxide dismutase, T-3/albumin complex, DL alpha-tocopherol and DL alpha tocopherol acetate. However, the use of B27 supplement is limited since it includes albumin from an animal source.

According to some embodiments of the invention, the serum replacement is devoid of (completely free of) animal contaminants. Such contaminants can be pathogens which can infect human cells, cellular components or a-cellular components (e.g., fluid) of animals.

It should be noted that when an animal-contaminant-free serum replacement is used to culture human cells, then the serum replacement is referred to as being "xeno-free".

The term "xeno" is a prefix based on the Greek word "Xenos", *i.e.*, a stranger. As used herein the phrase "xeno-free" refers to being devoid of any components/contaminants which are derived from a xenos (*i.e.*, not the same, a foreigner) species.

For example, a xeno-free serum replacement for use with human cells (i.e., an animal contaminant-free serum replacement) can include a combination of insulin, transferrin and selenium. Additionally or alternatively, a xeno-free serum replacement can include human or recombinantly produced albumin, transferrin and insulin.

Non-limiting examples of commercially available xeno-free serum replacement compositions include the premix of ITS (Insulin, Transferrin and Selenium) available from Invitrogen corporation (ITS, Invitrogen, Catalogue No. 51500-056); Serum replacement 3 (SR3; Sigma, Catalogue No. S2640) which includes human serum albumin, human transferring and human recombinant insulin and does not contain growth factors, steroid hormones, glucocorticoids, cell adhesion factors, detectable Ig and mitogens; KnockOut™ SR XenoFree [Catalogue numbers A10992-01, A10992-02, part Nos. 12618-012 or 12618-013, Invitrogen GIBCO] which contains only human-derived or human recombinant proteins.

According to some embodiments of the invention, the ITS (Invitrogen corporation) or SR3 (Sigma) xeno-free serum replacement formulations are diluted in a 1 to 100 ratio in order to reach a x1 working concentration.

According to some embodiments of the invention, the concentration of the serum replacement [e.g., KnockOut™ SR XenoFree (Invitrogen)] in the culture medium is in the range of from about 1 % [volume/volume (v/v)] to about 50 % (v/v), e.g., from about 5 % (v/v) to about 40 % (v/v), e.g., from about 5 % (v/v) to about 30 % (v/v), e.g., from about 10 % (v/v) to about 30 % (v/v), e.g., from about 10 % (v/v) to about 25 % (v/v), e.g., from about 10 % (v/v) to about 20 % (v/v), e.g., about 10 % (v/v), e.g., about 15 % (v/v), e.g., about 20 % (v/v), e.g., about 30 % (v/v).

According to some embodiments of the invention the culture medium is capable of maintaining the pluripotent stem cell in a proliferative, pluripotent and undifferentiated state for at least 5 passages, at least 10 passages, at least 15 passages, at least 20 passages, at least 25 passages, at least 30 passages, at least 35 passages, at least 40 passages, at least 45 passages, at least 50 passages (e.g., at least 25, 50, 75, 100, or 250 days in culture).

According to some embodiments of the invention the culture medium is capable of expanding the pluripotent stem cells in an undifferentiated state.

For example, as described in Example 1 of the Examples section which follows, the hESCs or human iPS cells could be maintained in the undifferentiated state for at least 20 passages on a two-dimensional culture system, or for at least 50 passages on a three-dimensional culture system when cultured in suspension. Given that each passage occurs every 5-7 days (e.g., 144 hours), and an observed doubling time of about 25-36 hours, a single hESC or human iPS cell cultured under these conditions could be expanded to give rise to 2^4 - 2^5cells (within 6 days). It should be noted that when cultured in a controlled bioreactor, the expansion capacity of the pluripotent stem cells increases to about 64 fold within 5 days. Thus, within a month of culturing (i.e., 720 hours), a single pluripotent stem cells can be expanded up to 2^{20} ($1x10^6$) hESCs or human iPS cells.

The present inventors have uncovered that the combination of growth factors interleukin 11 (IL11) and Ciliay Neurotrophic Factor (CNTF); or interleukin 11 (IL11) and oncostatin can be used to support the growth and expansion of pluripotent stem cells in a proliferative, undifferentiated, pluripotent state.

According to an aspect of some embodiments of the invention, there is provided a culture medium comprising interleukin 11 (IL11) and Ciliay Neurotrophic Factor (CNTF); or interleukin 11 and oncostatin.

As used herein the term "interleukin 11" refers to a protein member of the gp130 family of cytokines, also known as AGIF and IL-11. Interleukin 11 [e.g., the human IL-11 polypeptide GenBank Accession No. NP_000632.1 (SEQ ID NO:32); human IL-11 polynucleotide GenBank Accession No. NM_000641.2 (SEQ ID NO:33)] can be obtained from various commercial sources such as R&D Systems or PeproTech.

As used herein the term "Ciliay Neurotrophic Factor" (also known as HCNTF; CNTF) refers to a polypeptide hormone whose actions appear to be restricted to the nervous system where it promotes neurotransmitter synthesis and neurite outgrowth in certain neuronal populations. The protein is a potent survival factor for neurons and oligodendrocytes and may be relevant in reducing tissue destruction during inflammatory attacks. CNTF [e.g., the human CNTF polypeptide GenBank Accession No. NP_000605.1 (SEQ ID NO:34); human CNTF polynucleotide GenBank Accession No. NM_000614 (SEQ ID NO:35)] can be obtained from various commercial sources such as R&D Systems or PeproTech.

As used herein the term "oncostatin" (also known as OSM oncostatin M, OSM) refers to a polypeptide member of a cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor, and interleukin 6. Oncostatin [e.g., the human oncostatin polypeptide GenBank Accession NO. NP_065391.1 (SEQ ID NO:36, or P13725 (SEQ ID NO:37); human polynucleotide GenBank Accession No. NM_020530.3 (SEQ ID NO:38)] can be obtained from various commercial sources such as R&D Systems (e.g., R&D Systems Catalogue Number 295-OM-010).

According to some embodiments of the invention, the culture medium is devoid of a Glycogen Synthase Kinase 3 (GSK3) inhibitor

Non-limiting examples of GSK3 inhibitors include inhibitors of GSK-alpha or GSK-beta such as CHIR 98014, CHIR 99021, AR-AO144-18, SB216763 and SB415286. Examples of GSK3 inhibitors are described in Bennett C, et al, J. Biological Chemistry, vol. 277, no. 34, Aug. 23, 2002, pp 30998-31004; and in Ring D B, et al, Diabetes, vol. 52, March 2003, pp 588-595, each of which is fully incorporated herein by reference.

According to some embodiments of the invention, the IL11 is provided at a concentration of at least about 0.1 ng/ml and no more than about 10 ng/ml, e.g., at a concentration of at least about 0.2 ng/ml, e.g., at least about 0.3 ng/ml, e.g., at least about 0.4 ng/ml, e.g., at least about 0.5 ng/ml, e.g., at least about 0.6 ng/ml, e.g., at least about 0.7 ng/ml, e.g., at least about 0.8 ng/ml, e.g., at least about 0.9 ng/ml, e.g., at least about 1 ng/ml, e.g., about 1 ng/ml.

According to some embodiments of the invention, the IL11 is provided at a concentration of between about 0.5 ng/ml to about 5 ng/ml.

According to some embodiments of the invention, the CNTF is provided at a concentration of at least 0.1 ng/ml and no more than about 10 ng/ml, e.g., at a concentration of at least about 0.2 ng/ml, e.g., at least about 0.3 ng/ml, e.g., at least about 0.4 ng/ml, e.g., at least about 0.5 ng/ml, e.g., at least about 0.6 ng/ml, e.g., at least about 0.7 ng/ml, e.g., at least about 0.8 ng/ml, e.g., at least about 0.9 ng/ml, e.g., at least about 1 ng/ml, e.g., about 1 ng/ml.

According to some embodiments of the invention, the CNTF is provided at a concentration of between about 0.5 ng/ml to about 5 ng/ml.

According to some embodiments of the invention, the oncostatin is provided at a concentration of at least 0.1 ng/ml and no more than about 10 ng/ml, e.g., at a concentration of at least about 0.2 ng/ml, e.g., at least about 0.3 ng/ml, e.g., at least about 0.4 ng/ml, e.g., at least about 0.5 ng/ml, e.g., at least about 0.6 ng/ml, e.g., at least about 0.7 ng/ml, e.g., at least about 0.8 ng/ml, e.g., at least about 0.9 ng/ml, e.g., at least about 1 ng/ml, e.g., about 1 ng/ml.

According to some embodiments of the invention, the oncostatin is provided at a concentration of between about 0.5 ng/ml to about 5 ng/ml.

According to some embodiments of the invention, the medium which comprises IL11 and CNTF; or IL11 and oncostatin further comprises serum replacement (e.g., an animal contaminant-free serum replacement) at a concentration between about 10% to about 20%, e.g., about 15%.

According to some embodiments of the invention, the culture medium which comprises IL11 and CNTF; or IL11 and oncostatin further comprises basic fibroblast growth factor (bFGF).

Basic fibroblast growth factor (also known as bFGF, FGF2 or FGF-β) is a member of the fibroblast growth factor family. BFGF [(e.g., human bFGF polypeptide GenBank Accession No. NP_001997.5 (SEQ ID NO:39); human bFGF polynucleotide GenBank Accession No. NM_002006.4 (SEQ ID NO:40) can be obtained from various commercial sources such as Cell Sciences®, Canton, MA, USA (e.g., Catalogue numbers CRF001A and CRF001B), Invitrogen Corporation products, Grand Island NY, USA (e.g., Catalogue numbers: PHG0261, PHG0263, PHG0266 and PHG0264), ProSpec-Tany TechnoGene Ltd. Rehovot, Israel (e.g., Catalogue number: CYT-218), and Sigma, St Louis, MO, USA (e.g., catalogue number: F0291).

The concentration of bFGF in the culture medium which comprises IL11 and CNTF; or IL11 and oncostatin can be at least about 4 ng/ml and no more than 100 ng/ml, e.g., at least about 5 ng/ml, e.g., at least about 6 ng/ml, e.g., at least about 7 ng/ml, e.g., at least about 8 ng/ml, e.g., at least about 9 ng/ml, e.g., at least about 10 ng/ml.

Non-limiting examples of culture media which comprise the IL11 and CNTF include the ILCNTF, NILCNTF media described in the Examples section which follows, which were shown capable of supporting the growth of hESCs and iPS cells in a proliferative, pluripotent and undifferentiated state for at least 12 passages in a two-dimensional culture system and for at least 10 in a suspension culture.

The present inventors have uncovered that the IL6RIL6 chimera can be used in culture media which are completely devoid of animal contaminants in order to support the growth of human pluripotent stem cells in an undifferentiated state.

Thus, according to an aspect of some embodiments of the invention there is provided a culture medium comprising an animal contaminant-free serum replacement and an IL6RIL6 chimera.

As used herein the phrase "IL6RIL6 chimera" refers to a chimeric polypeptide which comprises the soluble portion of interleukin-6 receptor [IL-6-R, e.g., the human IL-6-R as set forth by GenBank Accession No. AAH89410; SEQ ID NO:41; e.g., a portion of the soluble IL6 receptors as set forth by amino acids 112-355 (SEQ ID NO:42) of GenBank Accession No. AAH89410] and the interleukin-6 (IL6; e.g., human IL-6 as set forth by GenBank Accession No. CAG29292; SEQ ID NO:43) or a biologically active fraction thereof (e.g., a receptor binding domain).

It should be noted that when constructing the IL6RIL6 chimera the two functional portions (*i.e.*, the IL6 and its receptor) can be directly fused (e.g., attached or translationally fused, *i.e.*, encoded by a single open reading frame) to each other or conjugated (attached or translationally fused) via a suitable linker (e.g., a polypeptide linker). According to some embodiments of the invention, the IL6RIL6 chimeric polypeptide exhibits a similar amount and pattern of glycosylation as the naturally occurring IL6 and IL6 receptor. For example, a suitable IL6RIL6 chimera is as set forth in SEQ ID NO:19 and in Figure 11 of WO 99/02552 to Revel M., et al., which is fully incorporated herein by reference.

It should be noted that once the serum replacement is completely devoid of animal contaminants, the additional culture medium ingredients can be also selected devoid of animal contaminants (e.g., synthetic, recombinant or purified from human sources) such that the entire culture medium is devoid of animal contaminant and can be used as a xeno-free medium for culturing human pluripotent stem cells, suitable for clinical/therapeutic purposes.

The present inventors have uncovered that the IL6RIL6 chimera can be provided at either a high concentration, i.e., between 50-150 ng/ml or at a low concentration, i.e., between 50- 150 pg/ml while still maintaining the ability of the medium to support the growth of pluripotent stem cells in an undifferentiated state.

According to some embodiments of the invention, the concentration of the IL6RIL6 chimera is at least about 50 ng/ml and no more than about 350 ng/ml, e.g., between about 50-200 ng/ml, e.g., is in the range from about 55 ng/ml to about 195 ng/ml, e.g., from about 60 ng/ml to about 190 ng/ml, e.g., from about 65 ng/ml to about 185 ng/ml, e.g., from about 70 ng/ml to about 180 ng/ml, e.g., from about 75 ng/ml to about 175 ng/ml, e.g., from about 80 ng/ml to about 170 ng/ml, e.g., from about 85 ng/ml to about 165 ng/ml, e.g., from about 90 ng/ml to about 150 ng/ml, e.g., from about 90 ng/ml to about 140 ng/ml, e.g., from about 90 ng/ml to about 130 ng/ml, e.g., from about 90 ng/ml to about 120 ng/ml, e.g., from about 90 ng/ml to about 110 ng/ml, e.g., from about 95 ng/ml to about 105 ng/ml, e.g., from about 98 ng/ml to about 102 ng/ml, e.g., about 100 ng/ml of the IL6RIL6 chimera.

Non-limiting examples of animal contaminant-free culture media which comprise between about 50-200 ng/ml of the IL6RIL6 chimera include the cmTeSR2, NCMrb100F, NCM100F, cmV5b, and cmHA13.

According to some embodiments of the invention, the concentration of the IL6RIL6 chimera is at least 50 pg/ml and no more than about 150 pg/ml, e.g., between about 50-200 pg/ml, e.g., in the range from about 55 pg/ml to about 195 pg/ml, e.g., from about 60 pg/ml to about 190 pg/ml, e.g., from about 65 pg/ml to about 185 pg/ml, e.g., from about 70 pg/ml to about 180 pg/ml, e.g., from about 75 pg/ml to about 175 pg/ml, e.g., from about 80 pg/ml to about 170 pg/ml, e.g., from about 85 pg/ml to about 165 pg/ml, e.g., from about 90 pg/ml to about 150 pg/ml, e.g., from about 90 pg/ml to about 140 pg/ml, e.g., from about 90 pg/ml to about 130 pg/ml, e.g., from about 90 pg/ml to about 120 pg/ml, e.g., from about 90 pg/ml to about 110 pg/ml, e.g., from about 95 pg/ml to about 105 pg/ml, e.g., from about 98 pg/ml to about 102 pg/ml, e.g., about 100 pg/ml of the IL6RIL6 chimera.

Non-limiting examples of xeno-free culture media which comprise between about 50-200 pg/ml of the IL6RIL6 chimera include the cmTeSR2p, NCMrb100Fp, NCM100Fp, cmV5bp, and cmHA13p.

For example the IL6RIL6 chimera can be added to the TeSR™2 Animal Protein-Free Medium (StemCell Technologies, Catalog #05860/05880) culture medium. The TeSR™2 medium is a complete, animal protein-free, serum-free, defined formulation which contains recombinant human basic fibroblast growth factor (rhbFGF) and recombinant human transforming growth factor β (rhTGFβ).

According to some embodiments of the invention, the animal contaminant-free culture medium which comprises the IL6RIL6 chimera further comprises bFGF.

BFGF can be provided at either a low concentration (e.g., between about 4-20 ng/ml) or at a high concentration (e.g., between 50-150 ng/ml).

According to some embodiments of the invention, the culture medium which comprises an animal contaminant-free serum replacement and the IL6RIL6 chimera, further comprises bFGF at a concentration of at least about 4 ng/ml, e.g., at least about 5 ng/ml, e.g., at least about 6 ng/ml, e.g., at least about 7 ng/ml, e.g., at least about 8 ng/ml, e.g., at least about 9 ng/ml, e.g., at least about 10 ng/ml, e.g., at least about 15

ng/ml, e.g., at least about 20 ng/ml. Non-limiting examples of such culture media include the cmV5b, NCM100Fp, NCM100F and cmV5bp.

According to some embodiments of the invention, the culture medium which comprises an animal contaminant-free serum replacement and the IL6RIL6 chimera, further comprises bFGF at a concentration of at least about 50 ng/ml to about 1 μg, e.g., from about 60 ng/ml to about 1 μg/ml, e.g., from about 70 ng/ml to about 500 ng/ml, e.g., from about 80 ng/ml to about 500 ng/ml, e.g., from about 90 ng/ml to about 250 ng/ml, e.g., from about 50 ng/ml to about 200 ng/ml, e.g., from about 50 ng/ml to about 150 ng/ml, e.g., about 60 ng/ml, e.g., about 70 ng/ml, e.g., about 80 ng/ml, e.g., about 90 ng/ml, e.g., about 50 ng/ml, e.g., about 60 ng/ml, e.g., about 70 ng/ml, e.g., about 80 ng/ml, e.g., about 100 ng/ml, e.g., about 110 ng/ml, e.g., about 120 ng/ml, e.g., about 130 ng/ml, e.g., about 140 ng/ml, e.g., about 150 ng/ml. Non-limiting examples of such culture media include the NCMrb100F and NCMrb100Fp, cmTeSR2, and cmTeSR2p.

According to some embodiments of the invention, the animal contaminant-free culture medium which comprises the IL6RIL6 chimera further comprising ascorbic acid.

Ascorbic acid (also known as vitamin C) is a sugar acid ($C_6H_8O_6$; molecular weight 176.12 grams/mole) with antioxidant properties. The ascorbic acid used by the culture medium of some embodiments of the invention can be a natural ascorbic acid, a synthetic ascorbic acid, an ascorbic acid salt (e.g., sodium ascorbate, calcium ascorbate, potassium ascorbate), an ester form of ascorbic acid (e.g., ascorbyl palmitate, ascorbyl stearate), a functional derivative thereof (a molecule derived from ascorbic acid which exhibits the same activity/function when used in the culture medium of the invention), or an analogue thereof (e.g., a functional equivalent of ascorbic acid which exhibits an activity analogous to that observed for ascorbic acid when used in the culture medium of the invention). Non-limiting examples of ascorbic acid formulations which can be used in the culture medium of some embodiments of the invention include L-ascorbic acid and ascorbic acid 3-phosphate.

Ascorbic acid can be obtained from various manufacturers such as Sigma, St Louis, MO, USA (e.g., Catalogue numbers: A2218, A5960, A7506, A0278, A4403, A4544, A2174, A2343, 95209, 33034, 05878, 95210, 95212, 47863, 01-6730, 01-6739, 255564, A92902, W210901).

According to some embodiments of the invention, the concentration of ascorbic acid in the animal contaminant-free culture medium which comprises the IL6RIL6 chimera is between about 25-200 μg/ml, e.g., between 25-150 μg/ml, e.g., between 30-150 μg/ml, e.g., between about 40-120 μg/ml, e.g., between about 40-100 μg/ml, e.g., between about 40-80 μg/ml, e.g., between about 40-60 μg/ml, e.g., about 50 μg/ml. Non-limiting examples of such culture media include the cmHA13p and cmHA13 media described in the Examples section which follows.

According to some embodiments of the invention, the animal contaminant-free culture medium which comprises the IL6RIL6 chimera further comprises a transforming growth factor beta (TGFβ) isoform.

As used herein the phrase "transforming growth factor beta (TGFβ)" refers to any isoform of the transforming growth factor beta (β), which functions through the same receptor signaling system in the control of proliferation, differentiation, and other functions in many cell types. TGFβ acts in inducing transformation and also acts as a negative autocrine growth factor.

According to some embodiments of the invention the term TGFβ refers to $TGF\beta_1$ [Human TGFβ1 mRNA sequence GenBank Accession NO. NM_000660.4 (SEQ ID NO:44), polypeptide sequence GenBank Accession No. NP_000651.3 (SEQ ID NO:45)], $TGF\beta_2$ [human TGFβ2 mRNA sequence GenBank Accession NO. NM_001135599.1 isoform 1 (SEQ ID NO:46), or GenBank Accession NO. NM_003238.2 isoform 2 (SEQ ID NO:47); polypeptide sequence GenBank Accession No. NP_001129071.1 isoform 2 (SEQ ID NO:48) or GenBank Accession NO. NP_003229.1 isoform 2 (SEQ ID NO:49] or $TGF\beta_3$ [human TGFβ3 mRNA sequence GenBank Accession NO. NM_003239.2 (SEQ ID NO:50), polypeptide sequence GenBank Accession No. NP_003230.1 (SEQ ID NO:51)]. The TGFβ isoforms can be obtained from various commercial sources such as R&D Systems Minneapolis MN, USA, and Sigma, St Louis, MO, USA.

According to some embodiments of the invention, the TGFβ which is included in the culture medium is TGFβ1.

According to some embodiments of the invention, the concentration of $TGF\beta_1$ in the culture medium is in the range of about 0.05 ng/ml to about 1 μg/ml, e.g., from 0.1 ng/ml to about 1 μg/ml, e.g., from about of about 0.5 ng/ml to about 100 ng/ml.

According to some embodiments of the invention, the concentration of TGFβ1 in the culture medium is at least about 0.5 ng/ml, e.g., at least about 0.6 ng/ml, e.g., at least about 0.8 ng/ml, e.g., at least about 0.9 ng/ml, e.g., at least about 1 ng/ml, e.g., at least about 1.2 ng/ml, e.g., at least about 1.4 ng/ml, e.g., at least about 1.6 ng/ml, e.g., at least about 1.8 ng/ml, e.g., about 2 ng/ml.

Non-limiting examples of an animal contaminant-free culture medium which comprises the IL6RIL6 chimera, bFGF and TGFβ1 is the cmV5b, cmV5bp, cmTeSR2 and cmTeSR2p which are described in the Examples section which follows.

According to some embodiments of the invention, the TGFβ which is included in the culture medium is TGFβ3.

According to some embodiments of the invention, the concentration of $TGF\beta_3$ in the culture medium is in the range of about 0.05 ng/ml to about 1 μg/ml, e.g., from 0.1 ng/ml to about 1 μg/ml, e.g., from about of about 0.5 ng/ml to about 100 ng/ml.

According to some embodiments of the invention, the concentration of $TGF\beta_3$ in the culture medium is at least about 0.5 ng/ml, e.g., at least about 0.6 ng/ml, e.g., at least about 0.8 ng/ml, e.g., at least about 0.9 ng/ml, e.g., at least about 1 ng/ml, e.g., at least about 1.2 ng/ml, e.g., at least about 1.4 ng/ml, e.g., at least about 1.6 ng/ml, e.g., at least about 1.8 ng/ml, e.g., about 2 ng/ml.

According to an aspect of some embodiments of the invention, there is provided culture medium comprises bFGF at a concentration of at least about 50 ng/ml (e.g., between 50-200 ng/ml) and an IL6RIL6 chimera at either a high concentration (e.g., between 50-200 ng/ml) or low concentration (e.g., between 50-200 pg/ml). Non-limiting examples of such culture media include the CMrb100F, CMrb100Fp, NCMrb100F and NCMrb100Fp culture media which were shown capable of maintaining hESCs and iPS cells in a proliferative, pluripotent and undifferentiated state for at least 5 passages in a two-dimensional culture system, and for at least 15 passages in a three-dimensional culture system.

The present inventors have uncovered that a culture medium which comprises high concentrations of a soluble interleukin 6 receptor (sIL6R) and interleukin 6 (IL6)

can be used to support the growth of pluripotent stem cells in a proliferative, undifferentiated and pluripotent state.

Thus, according to an aspect of some embodiments of the invention there is provided a culture medium which comprises sIL6R and IL6, wherein a concentration of the sIL6R is at least about 5 ng/ml, and wherein a concentration of the IL6 is at least about 3 ng/ml.

According to some embodiments of the invention, the concentration of sIL6 is at least about 5 ng/ml, e.g., at least about 6 ng/ml, at least about 7 ng/ml, at least about 8 ng/ml, at least about 9 ng/ml, at least about 10 ng/ml, at least about 15 ng/ml, at least about 20 ng/ml, at least about 25 ng/ml, e.g., in the range of between 10 ng/ml to between 50 ng/ml, e.g., between 20-40 ng/ml, e.g., about 25 ng/ml.

According to some embodiments of the invention, the concentration of IL6 is at least about 3 ng/ml, e.g., at least about 4 ng/ml, at least about 5 ng/ml, at least about 6 ng/ml, at least about 7 ng/ml, at least about 8 ng/ml, at least about 9 ng/ml, at least about 10 ng/ml, at least about 15 ng/ml, at least about 20 ng/ml, at least about 25 ng/ml, e.g., in the range of between 10 ng/ml to between 50 ng/ml, e.g., between 20-40 ng/ml, e.g., about 25 ng/ml.

According to some embodiments of the invention, the medium which comprises sIL6 and IL6 further includes bFGF at a concentration of at least about 4 ng/ml and no more than 100 ng/ml, e.g., at least about 5 ng/ml, e.g., at least about 6 ng/ml, e.g., at least about 7 ng/ml, e.g., at least about 8 ng/ml, e.g., at least about 9 ng/ml, e.g., at least about 10 ng/ml.

According to some embodiments of the invention, the medium which comprises sIL6 and IL6 further includes serum replacement at a concentration of between 10-30%, e.g., about 15%. It should be noted that the concentration of serum replacement can vary depending on the type of serum replacement used.

Non-limiting examples of culture media which comprise sIL6 and IL6 include the yFIL25 medium described in the Examples section which follows.

According to some embodiments of the invention, the culture medium further comprises insulin. Insulin can be obtained from Invitrogen Carlsbad CA, Sigma, St Louis, MO, USA.

The concentration of insulin in the culture medium can be between 0.0001-1 grams/litter (e.g., between about 0.001 μg/μl to about 0.1 μg/μl, e.g., between about 0.005 μg/μl to about 0.05 μg/μl, e.g., about 0.01 μg/μl).

According to some embodiments of the invention, the culture medium further comprises albumin. Albumin can be obtained from Sigma, St Louis, MO, USA.

The concentration of albumin in the culture medium can be between about 0.1% to about 5%.

According to some embodiments of the invention, the culture medium further comprises transferrin. Transferrin can be obtained from Invitrogen Carlsbad CA, Sigma, St Louis, MO, USA.

According to some embodiments of the invention, the culture medium further comprises a lipid mixture.

As used herein the phrase "lipid mixture" refers to a defined (e.g., chemically defined) lipid composition needed for culturing the pluripotent stem cells. It should be noted that the lipid mixture is usually added to a culture medium which is devoid of serum or serum replacement and thus substitutes the lipids which are usually added to formulations of serum or serum replacement.

A non-limiting example of a commercially available lipid mixture, which can be used in the culture medium of some embodiments of the invention, include the Chemically Define Lipid Concentrate available from Invitrogen (Catalogue No. 11905-031).

According to some embodiments of the invention, the concentration of the lipid mixture in the culture medium is from about 0.5 % [volume/volume (v/v)] to about 3 % v/v, e.g., from about 0.5 % v/v to about 2 % v/v, e.g., from about 0.5 % v/v to about 1 % v/v, e.g., about 1 % v/v.

According to some embodiments of the invention, the culture medium further comprises sodium bicarbonate. Sodium bicarbonate can be obtained from Biological Industries, Beit HaEmek, Israel.

According to some embodiments of the invention, the concentration of sodium bicarbonate in the culture medium is from about 5 % to about 10 %, e.g., from about 6 % to about 9 %, e.g., from about 7 % to about 8 %, e.g., about 7.5 %.

According to some embodiments of the invention, the culture medium further comprising L-glutamine. The concentration of L-glutamine in the culture medium can be from about 0.5 millimolar (mM) to about 10 mM, e.g., about 1-5 mM, e.g., 2 mM.

According to some embodiments of the invention, the culture medium further comprising non-essential amino acid. Non-essential amino acids can be obtained as a stock of 10 mM from various suppliers such as Invitrogen Corporation products, Grand Island NY, USA. The concentration of the non-essential amino acid in the culture medium can be from about 0.1-10 %, e.g., about 0.2-5%, e.g., about 0.5-2%, e.g., about 1%.

According to some embodiments of the invention, the culture medium further comprising a reducing agent such as beta–mercaptoethanol (β-mercaptoethanol), at a concentration range between about 0.01-1 mM, e.g., 0.1 mM.

As mentioned, any of the proteinaceous factors used in the culture medium of the present invention (e.g., the interleukin 11, CNTF, oncostatin, bFGF, IL6RIL6 chimera, TGFβ1, $TGF\beta_3$, insulin, albumin, transferrin) can be recombinantly expressed or biochemically synthesized. In addition, naturally occurring proteinaceous factors such as bFGF and TGFβ can be purified from biological samples (e.g., from human serum, cell cultures) using methods well known in the art. It should be noted that for the preparation of an animal contaminant-free culture medium the proteinaceous factor is preferably purified from a human source or is recombinantly expressed.

Biochemical synthesis of the proteinaceous factors of the present invention (e.g., the IL6RIL6 chimera) can be performed using standard solid phase techniques. These methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation and classical solution synthesis.

Recombinant expression of the proteinaceous factors of the present invention can be generated using recombinant techniques such as described by Bitter et al., (1987) Methods in Enzymol. 153:516-544, Studier et al. (1990) Methods in Enzymol. 185:60-89, Brisson et al. (1984) Nature 310:511-514, Takamatsu et al. (1987) EMBO J. 6:307-311, Coruzzi et al. (1984) EMBO J. 3:1671-1680, Brogli et al., (1984) Science 224:838-843, Gurley et al. (1986) Mol. Cell. Biol. 6:559-565 and Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463. Specifically, the IL6RIL6 chimera can be generated as described in PCT publication

WO 99/02552 to Revel M., et al. and Chebath J, et al., 1997, which are fully incorporated herein by reference.

Thus, according to an aspect of some embodiments of the invention there is provided a cell culture which comprises the pluripotent stem cells of some embodiments of the invention (e.g., the hESCs or iPSCs which are cultured in suspension as single cells devoid of cell clumps; the hESCs or iPSCs which are cultured in suspension as cell clumps; the pluripotent stem cells cultured on 2-dimensional culture systems; and the like) and the culture medium of some embodiments of the invention.

According to some embodiments of the invention cell culture is feeder cells free (devoid of feeder cell support).

The phrase "feeder cell support" as used herein refers to the ability of a feeder cell (e.g., fibroblasts) to maintain pluripotent stem cells in a proliferative and undifferentiated state when the pluripotent stem cells are co-cultured on the feeder cells or when the pluripotent stem cells are cultured on a matrix (e.g., an extracellular matrix, a synthetic matrix) in the presence of a conditioned medium generated by the feeder cells. The support of the feeder cells depends on the structure of the feeder cells while in culture (e.g., the three dimensional matrix formed by culturing the feeder cells in a tissue culture plate), function of the feeder cells (e.g., the secretion of growth factors, nutrients and hormones by the feeder cells, the growth rate of the feeder cells, the expansion ability of the feeder cells before senescence) and/or the attachment of the pluripotent stem cells to the feeder cell layer(s).

The phrase "absence of feeder cell support" as used herein refers to a culture medium and/or a cell culture being devoid of feeder cells and/or a conditioned medium generated thereby.

According to some embodiments of the invention the pluripotent stem cells which are included in the cell culture of some embodiments of the invention exhibit a stable karyotype (chromosomal stability) during the culturing period, e.g., for at least 2 passages, e.g., at least 4 passages, e.g., at least 8 passages, e.g., at least 15 passages, e.g., at least 20 passages, e.g., at least 25 passages, e.g., at least 30 passages, e.g., at least 35 passages, e.g., at least 40 passages, e.g., at least 45 passages, e.g., at least 50 passages.

According to some embodiments of the invention, the cell culture of the invention exhibits a doubling time of at least 20 hours, e.g., a doubling time which is between 20 to 40 hours (e.g., about 36 hours), thus representing a non-tumorigenic, genetically stable pluripotent stem cells (e.g., hESCs and iPS cells).

According to some embodiments of the invention, the cell culture of the invention is characterized by at least 40 %, at least 50 %, at least 60 %, e.g., at least 70 %, e.g., at least 80 %, e.g., at least 85 %, e.g., at least 90 %, e.g., at least 95 % of undifferentiated pluripotent stem cells.

The cell culture of some embodiments of the invention comprises at least 1000 pluripotent and undifferentiated stem cells per milliliter (ml) of culture medium. It should be noted that for several applications such as for single cell cloning of the pluripotent stem cells, the concentration of cells can be about 1 cell per 100-200 μl of medium, each cell is placed (seeded) in a separate dish, preferably a dish which is not coated (e.g., a non-culture treated dish), to prevent adhesion of the cell to the dish.

The differentiation or undifferentiation state of the pluripotent stem cells cultured on 2-D, or in suspension as cell clumps can be determined using known methods (e.g., as described in Thomson et al, 1998). For example, the differentiation state can be determined using various approaches including, for example, morphological evaluation (e.g., as shown in Figures 1A-C and 3A-C) and/or detection of the expression pattern of typical markers of the undifferentiated state using immunological techniques such as flow cytometry for membrane-bound markers, immunohistochemistry or immunofluorescence for extracellular and intracellular markers and enzymatic immunoassay, for secreted molecular markers. For example, immunofluorescence employed on hESCs or human iPS cells cultured in the culture medium according to some embodiments of the invention revealed the expression of Oct4, stage-specific embryonic antigen (SSEA) 4, the tumor-rejecting antigen (TRA)-1-60 and TRA-1-81 (e.g., Figures 2A-D). Additionally, the level of transcripts of specific undifferentiation markers (e.g., Oct 4, Nanog, Sox2, Rex1, Cx43, FGF4) or differentiation markers (e.g., albumin, glucagons, α-cardiac actin, β-globulin, Flk1, AC133 and neurofilament) can be detected using RNA-based techniques such as RT-PCR analysis and/or cDNA microarray analysis.

Determination of ES cell differentiation can also be effected via measurements of alkaline phosphatase activity. Undifferentiated human ES cells have alkaline phosphatase activity which can be detected by fixing the cells with 4 % paraformaldehyde and developing with the Vector Red substrate kit according to manufacturer's instructions (Vector Laboratories, Burlingame, California, USA).

According to some embodiments of the invention, the cell culture comprises pluripotent stem cells and a xeno-free medium, thus the medium does not contain any contaminants from a species other than that of the pluripotent stem cells. For example, when the cell culture comprises human pluripotent stem cells then the medium is devoid of animal contaminants. Similarly, when the cell culture comprises primate pluripotent stem cells (e.g., monkey) the culture medium is devoid of other animals or human contaminants.

According to an aspect of some embodiments of the invention, there is provided a method of expanding and maintaining pluripotent stem cells in a pluripotent and undifferentiated state.

According to some embodiments of the invention, the method of expanding and maintaining pluripotent stem cells in an undifferentiated state is effected by culturing the pluripotent stem cells in any of the novel culture media of the invention (described herein).

According to some embodiments of the invention, expanding and maintaining the pluripotent stem cells in the undifferentiated state is effected in a suspension culture.

According to some embodiments of the invention, culturing of the pluripotent stem cells in a suspension culture is effected in a serum-free, and feeder cell-free culture medium.

Since large clusters of pluripotent stem cells may cause cell differentiation, measures are taken to avoid large pluripotent stem cells aggregates. According to some embodiments of the invention, the formed pluripotent stem cells clumps are dissociated every 5-7 days and the single cells or small clumps of cells are either split into additional culture vessels (*i.e.*, passaged) or remained in the same culture vessel yet with additional culture medium.

According to some embodiments of the invention, culturing is effected under conditions which enable expansion of the pluripotent stem cells as single cells.

As described hereinabove, passaging of the pluripotent stem cells can be effected using mechanical dissociation of cell clumps.

Additionally and/or alternatively, passaging of pluripotent stem cells in a suspension culture can be performed using an enzymatic digestion with or without a subsequent mechanical dissociation.

Enzymatic digestion of pluripotent stem cells clump(s) can be performed by subjecting the clump(s) to an enzyme such as type IV Collagenase (Worthington biochemical corporation, Lakewood, NJ, USA) and/or Dispase (Invitrogen Corporation products, Grand Island NY, USA). The time of incubation with the enzyme depends on the size of cell clumps present in the suspension culture. Typically, when pluripotent stem cells cell clumps are dissociated every 5-7 days while in the suspension culture, incubation of 20-60 minutes with 1.5 mg/ml type IV Collagenase results in small cell clumps which can be further cultured in the undifferentiated state. Alternatively, pluripotent stem cells clumps can be subjected to incubation of about 25 minutes with 1.5 mg/ml type IV Collagenase followed by five minutes incubation with 1 mg/ml Dispase. It should be noted that passaging of human ESCs with trypsin may result in chromosomal instability and abnormalities (see for example, Mitalipova MM., et al., Nature Biotechnology, 23: 19-20, 2005 and Cowan CA et al., N. Engl. J. of Med. 350: 1353-1356, 2004). According to some embodiments of the invention, passaging hESC or iPS cell with trypsin should be avoided.

According to some embodiments of the invention, following enzymatic or mechanical dissociation of the large cell clumps, the dissociated pluripotent stem cells clumps are further broken to small clumps using 200 μl Gilson pipette tips (e.g., by pipetting up and down the cells).

According to some embodiments of the invention, the method of expanding and maintaining the pluripotent stem cells in the undifferentiated state is effected in a two-dimensional culture system.

The two-dimensional culture system may comprise a matrix or feeder-cell layer.

For example, culturing on a two-dimensional culture system can be performed by plating the pluripotent stem cells onto a matrix or a feeder cell layer in a cell density which promotes cell survival and proliferation but limits differentiation. Typically, a

plating density of between about 15,000 cells/cm^2 and about 3,000,000 cells/cm^2 is used.

It will be appreciated that although single-cell suspensions of pluripotent stem cells are usually seeded, small clusters may also be used. To this end, enzymatic digestion (such as with type IV collagenase) utilized for cluster disruption (see "General Materials and Experimental Methods" in the Examples section which follows) is terminated before stem cells become completely dispersed and the cells are triturated with a pipette such that clumps *(i.e.,* 10-200 cells) are formed. However, measures are taken to avoid large clusters which may cause cell differentiation.

According to some embodiments of the invention, the culture system comprises a matrix and the culture medium of some embodiments of the invention.

As used herein, the term "matrix" refers to any substance to which the pluripotent stem cells can adhere and which therefore can substitute the cell attachment function of feeder cells. Such a matrix typically contains extracellular components to which the pluripotent stem cells can attach and thus it provides a suitable culture substrate.

According to some embodiments of the invention the matrix comprises an extracellular matrix.

The extracellular matrix can be composed of components derived from basement membrane or extracellular matrix components that form part of adhesion molecule receptor-ligand couplings. MATRIGEL® (Becton Dickinson, USA) is one example of a commercially available matrix which is suitable for use with the present invention. MATRIGEL® is a soluble preparation from Engelbreth-Holm-Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane; MATRIGEL® is also available as a growth factor reduced preparation. Other extracellular matrix components and component mixtures which are suitable for use with the present invention include foreskin matrix, laminin matrix, fibronectin matrix, proteoglycan matrix, entactin matrix, heparan sulfate matrix, collagen matrix and the like, alone or in various combinations thereof.

According to some embodiments of the invention the matrix is devoid of animal contaminant (a xeno-free matrix for culturing human pluripotent stem cells).

In cases where complete animal-free culturing conditions are desired, the matrix is preferably derived from a human source or synthesized using recombinant techniques such as described hereinabove. Such matrices include, for example, human-derived fibronectin, recombinant fibronectin, human-derived laminin, foreskin fibroblast matrix or a synthetic fibronectin matrix. Human derived fibronectin can be from plasma fibronectin or cellular fibronectin, both of which can be obtained from Sigma, St. Louis, MO, USA. Human derived laminin and foreskin fibroblast matrix can be obtained from Sigma, St. Louis, MO, USA. A synthetic fibronectin matrix can be obtained from Sigma, St. Louis, MO, USA.

In case a feeder cell layer is desired, human pluripotent stem cells can be cultured on a human foreskin fibroblasts feeder cell layer.

The present inventors have uncovered that pluripotent stem cells can be shipped as living, non-frozen cells and still remain viable, undifferentiated and pluripotent.

According to some embodiments of the invention, the cells remain viable, undifferentiated and pluripotent following shipment (via air or over-sea) which lasts at least 4 days.

The present inventors have uncovered that the novel culture media of the invention can be used to derive new pluripotent stem cell lines.

According to some embodiments of the invention, the pluripotent stem cell line is an embryonic stem cell line, and the method of deriving the embryonic stem cell line is effected by: (a) obtaining an embryonic stem cell from a pre-implantation stage blastocyst, post-implantation stage blastocyst and/or a genital tissue of a fetus; and (b) culturing the embryonic stem cell in the culture medium of some embodiments of the invention, thereby deriving the embryonic stem cell line.

According to some embodiments of the invention, the pluripotent stem cell line is an induced pluripotent stem cell (iPS cell) line, and the method of deriving the iPS cell line is effected by: (a) inducing a somatic cell to a pluripotent stem cell; and (b) culturing the pluripotent stem cell in the culture medium of some embodiments of the invention, thereby deriving the induced pluripotent stem cell line.

Once obtained the ESCs of iPS cells are further cultured in any of the culture media described hereinabove which allow expansion of the pluripotent stem cells in the undifferentiated state, essentially as described hereinabove.

It will be appreciated that an established pluripotent stem cell line (e.g., embryonic stem cell line or induced pluripotent stem cell line) can be subject to freeze/thaw cycles without hampering the proliferative capacity of the cells in the undifferentiated state while preserving their pluripotent capacity. For example, as is shown in Figures 6A-C and described in Example 6 of the Examples section which follows, using serum replacement (from 10% to 95%) and dimethyl sulfoxide (DMSO; from 5% to 10%) hESCs or human iPS cells were successfully frozen and thawed and more than 70% of the cells survived and directly recovered to the suspension culture.

It should be noted that any of the novel culture media described hereinabove can be used to culture, maintain and expand pluripotent, undifferentiated stem cells in a suspension culture as single cells devoid of cell clumps.

According to some embodiments of the invention, the culture conditions for expanding and maintaining pluripotent stem cells in an undifferentiated state in a suspension culture as single cells devoid of cell clumps comprise the culture medium which comprises interleukin 11 (IL11) and Ciliay Neurotrophic Factor (CNTF).

According to some embodiments of the invention, the culture conditions for expanding and maintaining pluripotent stem cells in an undifferentiated state in a suspension culture as single cells devoid of cell clumps comprise the culture medium which comprises basic fibroblast growth factor (bFGF) at a concentration of at least 50 ng/ml and an IL6RIL6 chimera.

According to some embodiments of the invention, the culture conditions for expanding and maintaining pluripotent stem cells in an undifferentiated state in a suspension culture as single cells devoid of cell clumps comprise the culture medium which comprises an animal contaminant-free serum replacement and an IL6RIL6 chimera.

According to some embodiments of the invention, the culture conditions for expanding and maintaining pluripotent stem cells in an undifferentiated state in a suspension culture as single cells devoid of cell clumps comprise the serum-free culture medium which comprises a soluble interleukin 6 receptor (sIL6R) and interleukin 6 (IL6), wherein a concentration of the sIL6R is at least 5 ng/ml, and wherein a concentration of the IL6 is at least 3 ng/ml

According to some embodiments of the invention, the culture conditions for expanding and maintaining pluripotent stem cells in an undifferentiated state in a suspension culture as single cells devoid of cell clumps comprise the culture medium which comprises interleukin 11 (IL11) and oncostatin.

Following is a non-limiting description of methods for production of differentiated cell lineages from the pluripotent stem cells of some embodiments of the invention.

As described in Example 2 of the Examples section which follows, hESCs and human iPS cells which were expanded and maintained in any of the culture media described hereinabove are pluripotent (*i.e.*, capable of differentiating into all cell types of the three embryonic germ layers, the ectoderm, the endoderm and the mesoderm) as evidenced *in vitro* (by the formation of EBs) and *in vivo* (by the formation of teratomas) after a prolonged culture period (e.g., of at least 10 or 30 passages) in the two-dimensional (e.g., feeder-free matrices) or three-dimensional (e.g., static or dynamic suspension cultures) culture systems.

Thus, hESCs or human iPS cells cultured according to the teachings of the present invention can be used as a source for generating differentiated, lineage-specific cells. Such cells can be obtained directly from the pluripotent stem cells by subjecting the ESCs to various differentiation signals (e.g., cytokines, hormones, growth factors) or indirectly, via the formation of embryoid bodies and the subsequent differentiation of cells of the EBs to lineage-specific cells.

Thus, according to an aspect of the some embodiments of the invention there is provided a method of generating embryoid bodies from pluripotent stem cells. The method is effected by (a) culturing the pluripotent stem cells of some embodiments of the invention according to the method of some embodiment of the invention to thereby obtain expanded, undifferentiated pluripotent stem cells; and (b) subjecting the expanded, undifferentiated pluripotent stem cells to culturing conditions suitable for differentiating the stem cells to embryoid bodies, thereby generating the embryoid bodies from the pluripotent stem cells.

As used herein the phrase "embryoid bodies" refers to morphological structures comprised of a population of ESCs, extended blastocyst cells (EBCs), embryonic germ cells (EGCs) and/or induced pluripotent stem cells which have undergone

differentiation. EBs formation initiates following the removal of differentiation blocking factors from the pluripotent stem cell cultures. In the first step of EBs formation, the pluripotent stem cells proliferate into small masses of cells which then proceed with differentiation. In the first phase of differentiation, following 1-4 days in culture for either human ESCs or human iPS cells, a layer of endodermal cells is formed on the outer layer of the small mass, resulting in "simple EBs". In the second phase, following 3-20 days post-differentiation, "complex EBs" are formed. Complex EBs are characterized by extensive differentiation of ectodermal and mesodermal cells and derivative tissues.

Thus, the method according to some embodiments of the invention involves the culturing of the pluripotent stem cells of some embodiments of the invention in any of the culture media described hereinabove (e.g., in suspension as cell clumps or as single cells devoid of cell clumps, or in a 2-dimensional culture system) in order to obtain expanded, undifferentiated pluripotent stem cells and then subjecting the expanded, undifferentiated pluripotent stem cells (e.g., ESCs or iPS cells) to culturing conditions suitable for differentiating the pluripotent stem cells to embryoid bodies. Such differentiation-promoting culturing conditions are substantially devoid of differentiation inhibitory factors which are employed when pluripotent stem cells are to be expanded in an undifferentiated state, such as TGFβ1, $TGF\beta_3$, ascorbic acid, IL-11, CNTF, oncostatin, bFGF and/or the IL6RIL6 chimera.

For EBs formation, the pluripotent stem cells (ESCs or iPS cells) are removed from their feeder-free-culturing systems or suspension cultures and are transferred to a suspension culture in the presence of a culture medium containing serum or serum replacement and being devoid of differentiation-inhibitory factors. For example, a culture medium suitable for EBs formation may include a basic culture medium (e.g., Ko-DMEM or DMEM/F12) supplemented with 20 % FBSd (HyClone, Utah, USA), 1 mM L-glutamine, 0.1 mM β–mercaptoethanol, and 1 % non-essential amino acid stock.

Monitoring the formation of EBs is within the capabilities of those skilled in the art and can be effected by morphological evaluations (e.g., histological staining) and determination of expression of differentiation-specific markers [e.g., using immunological techniques or RNA-based analysis (e.g., RT-PCR, cDNA microarray)].

It will be appreciated that in order to obtain lineage-specific cells from the EBs, cells of the EBs can be further subjected to culturing conditions suitable for lineage-specific cells.

According to some embodiments of the invention, for generating lineage-specific cells from the pluripotent stem cells, the method the method further includes step (c) of subjecting cells of the embryoid bodies to culturing conditions suitable for differentiating and/or expanding lineage specific cells; thereby generating the lineage-specific cells from the embryonic stem cells.

As used herein the phrase "culturing conditions suitable for differentiating and/or expanding lineage specific cells" refers to a combination of culture system, e.g., feeder-free matrix or a suspension culture and a culture medium which are suitable for the differentiation and/or expansion of specific cell lineages derived from cells of the EBs. Non-limiting examples of such culturing conditions are further described hereinunder.

According to some embodiments of the invention, the method of this aspect of the invention further includes isolating lineage specific cells following step (b).

As used herein, the phrase "isolating lineage specific cells" refers to the enrichment of a mixed population of cells in a culture with cells predominantly displaying at least one characteristic associated with a specific lineage phenotype. It will be appreciated that all cell lineages are derived from the three embryonic germ layers. Thus, for example, hepatocytes and pancreatic cells are derived from the embryonic endoderm, osseous, cartilaginous, elastic, fibrous connective tissues, myocytes, myocardial cells, bone marrow cells, vascular cells (namely endothelial and smooth muscle cells), and hematopoietic cells are differentiated from embryonic mesoderm and neural, retina and epidermal cells are derived from the embryonic ectoderm.

According to some preferred embodiments of the invention, isolating lineage specific cells is effected by sorting of cells of the EBs via fluorescence activated cell sorter (FACS).

Methods of isolating EB-derived-differentiated cells via FACS analysis are known in the art. According to one method, EBs are disaggregated using a solution of Trypsin and EDTA (0.025 % and 0.01 %, respectively), washed with 5 % fetal bovine serum (FBS) in phosphate buffered saline (PBS) and incubated for 30 min on ice with fluorescently-labeled antibodies directed against cell surface antigens characteristics to a

specific cell lineage. For example, endothelial cells are isolated by attaching an antibody directed against the platelet endothelial cell adhesion molecule-1 (PECAM1) such as the fluorescently-labeled PECAM1 antibodies (30884X) available from PharMingen (PharMingen, Becton Dickinson Bio Sciences, San Jose, CA, USA) as described in Levenberg, S. et al., (Endothelial cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. USA. 2002. 99: 4391-4396). Hematopoietic cells are isolated using fluorescently-labeled antibodies such as CD34-FITC, CD45-PE, CD31-PE, CD38-PE, CD90-FITC, CD117-PE, CD15-FITC, class I-FITC, all of which IgG1 are available from PharMingen, CD133/1-PE (IgG1) (available from Miltenyi Biotec, Auburn, CA), and glycophorin A-PE (IgG1), available from Immunotech (Miami, FL). Live cells (*i.e.,* without fixation) are analyzed on a FACScan (Becton Dickinson Bio Sciences) by using propidium iodide to exclude dead cells with either the PC-LYSIS or the CELLQUEST software. It will be appreciated that isolated cells can be further enriched using magnetically-labeled second antibodies and magnetic separation columns (MACS, Miltenyi) as described by Kaufman, D.S. et al., (Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. USA. 2001, 98: 10716–10721).

According to some embodiments of the invention, isolating lineage specific cells is effected by a mechanical separation of cells, tissues and/or tissue-like structures contained within the EBs.

For example, beating cardiomyocytes can be isolated from EBs as disclosed in U.S. Pat. Appl. No. 20030022367 to Xu et al. Four-day-old EBs of the present invention are transferred to gelatin-coated plates or chamber slides and are allowed to attach and differentiate. Spontaneously contracting cells, which are observed from day 8 of differentiation, are mechanically separated and collected into a 15-mL tube containing low-calcium medium or PBS. Cells are dissociated using Collagenase B digestion for 60-120 minutes at 37 °C, depending on the Collagenase activity. Dissociated cells are then resuspended in a differentiation KB medium (85 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4$, 1 mM EGTA, 5 mM creatine, 20 mM glucose, 2 mM Na_2ATP, 5 mM pyruvate, and 20 mM taurine, buffered to pH 7.2, Maltsev et al., Circ. Res. 75:233, 1994) and incubated at 37 °C for 15-30 min. Following dissociation cells are seeded into chamber

slides and cultured in the differentiation medium to generate single cardiomyocytes capable of beating.

According to some embodiments of the invention, isolating lineage specific cells is effected by subjecting the EBs to differentiation factors to thereby induce differentiation of the EBs into lineage specific differentiated cells.

Following is a non-limiting description of procedures and approaches for inducing differentiation of EBs to lineage specific cells.

To differentiate the EBs of some embodiments of the invention into neural precursors, four-day-old EBs are cultured for 5-12 days in tissue culture dishes including DMEM/F-12 medium with 5 mg/ml insulin, 50 mg/ml transferrin, 30 nM selenium chloride, and 5 mg/ml fibronectin (ITSFn medium, Okabe, S. et al., 1996, Mech. Dev. 59: 89-102). The resultant neural precursors can be further transplanted to generate neural cells *in vivo* (Brüstle, O. et al., 1997. *In vitro*-generated neural precursors participate in mammalian brain development. Proc. Natl. Acad. Sci. USA. 94: 14809-14814). It will be appreciated that prior to their transplantation, the neural precursors are trypsinized and triturated to single-cell suspensions in the presence of 0.1 % DNase.

EBs of some embodiments of the invention can differentiate to oligodendrocytes and myelinate cells by culturing the cells in modified SATO medium, *i.e.,* DMEM with bovine serum albumin (BSA), pyruvate, progesterone, putrescine, thyroxine, triiodothryonine, insulin, transferrin, sodium selenite, amino acids, neurotrophin 3, ciliary neurotrophic factor and Hepes (Bottenstein, J. E. & Sato, G. H., 1979, Proc. Natl. Acad. Sci. USA 76, 514-517; Raff, M. C., Miller, R. H., & Noble, M., 1983, Nature 303: 390-396]. Briefly, EBs are dissociated using 0.25 % Trypsin/EDTA (5 min at 37 °C) and triturated to single cell suspensions. Suspended cells are plated in flasks containing SATO medium supplemented with 5 % equine serum and 5 % fetal calf serum (FCS). Following 4 days in culture, the flasks are gently shaken to suspend loosely adhering cells (primarily oligodendrocytes), while astrocytes are remained adhering to the flasks and further producing conditioned medium. Primary oligodendrocytes are transferred to new flasks containing SATO medium for additional two days. Following a total of 6 days in culture, oligospheres are either partially dissociated and resuspended in SATO medium for cell transplantation, or completely dissociated and a plated in an oligosphere-conditioned medium which is derived from the previous shaking step [Liu,

S. et al., (2000). Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc. Natl. Acad. Sci. USA. 97: 6126-6131].

For mast cell differentiation, two-week-old EBs of some embodiments of the invention are transferred to tissue culture dishes including DMEM medium supplemented with 10 % FCS, 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, 20 % (v/v) WEHI-3 cell-conditioned medium and 50 ng/ml recombinant rat stem cell factor (rrSCF, Tsai, M. et al., 2000. *In vivo* immunological function of mast cells derived from embryonic stem cells: An approach for the rapid analysis of even embryonic lethal mutations in adult mice *in vivo*. Proc Natl Acad Sci USA. 97: 9186-9190). Cultures are expanded weekly by transferring the cells to new flasks and replacing half of the culture medium.

To generate hemato-lymphoid cells from the EBs of some embodiments of the invention, 2-3 days-old EBs are transferred to gas-permeable culture dishes in the presence of 7.5 % CO_2 and 5 % O_2 using an incubator with adjustable oxygen content. Following 15 days of differentiation, cells are harvested and dissociated by gentle digestion with Collagenase (0.1 unit/mg) and Dispase (0.8 unit/mg), both are available from F.Hoffman-La Roche Ltd, Basel, Switzerland. CD45-positive cells are isolated using anti-CD45 monoclonal antibody (mAb) M1/9.3.4.HL.2 and paramagnetic microbeads (Miltenyi) conjugated to goat anti-rat immunoglobulin as described in Potocnik, A.J. et al., (Immunology Hemato-lymphoid *in vivo* reconstitution potential of subpopulations derived from *in vitro* differentiated embryonic stem cells. Proc. Natl. Acad. Sci. USA. 1997, 94: 10295-10300). The isolated CD45-positive cells can be further enriched using a single passage over a MACS column (Miltenyi).

It will be appreciated that the culturing conditions suitable for the differentiation and expansion of the isolated lineage specific cells include various tissue culture media, growth factors, antibiotic, amino acids and the like and it is within the capability of one skilled in the art to determine which conditions should be applied in order to expand and differentiate particular cell types and/or cell lineages.

As mentioned above, lineage specific cells can be obtained by directly inducing the expanded, undifferentiated pluripotent stem cells such as ESCs or iPS cells to culturing conditions suitable for the differentiation of specific cell lineage.

For example, as described in Examples 10, 11 and 12 of the Examples section which follows, pluripotent stem cells which were expanded and maintained in a suspension culture as single cells devoid of cell clumps are pluripotent as is evidenced *in vitro* by direct differentiation of the pluripotent stem cells to neuronal progenitors of the ectoderm cell lineage (Figures 17A-C, Example 10), mesenchymal stem cells (of the mesoderm lineage (Figures 18A-C, Example 11) and PDX1-expressing cells of the endoderm cell lineage (Figures 20A-B, Example 12).

According to an aspect of some embodiments of the invention there is provided a method of generating lineage-specific cells from pluripotent stem cells. The method is effected by (a) culturing the pluripotent stem cells according to the method of some embodiments of the invention, to thereby obtain expanded, undifferentiated stem cells; and (b) subjecting the expanded, undifferentiated stem cells to culturing conditions suitable for differentiating and/or expanding lineage specific cells, thereby generating the lineage-specific cells from the pluripotent stem cells.

Following are non-limiting examples of culturing conditions which are suitable for differentiating and/or expanding lineage specific cells from pluripotent stem cells (e.g., ESCs and iPS cells).

Mesenchymal stromal cells which are CD73-positive and SSEA-4-negative can be generated from hESCs by mechanically increasing the fraction of fibroblast-like differentiated cells formed in cultures of hESCs, essentially as described in Trivedi P and Hematti P. Exp Hematol. 2008, 36(3):350-9. Briefly, to induce differentiation of hESC the intervals between medium changes are increased to 3-5 days, and the cells at the periphery of the ESC colonies become spindle-shaped fibroblast-looking cells. After 9-10 days under these conditions when about 40-50% of the cells in the culture acquire the fibroblast-looking appearance, the undifferentiated portions of ESC colonies are physically removed and the remaining differentiated cells are passaged to new culture plates under the same conditions.

To induce differentiation of hESCs into dopaminergic (DA) neurons, the cells can be co-cultured with the mouse stromal cell lines PA6 or MS5, or can be cultured with a combination of stromal cell-derived factor 1 (SDF-1/CXCL12), pleiotrophin (PTN), insulin-like growth factor 2 (IGF2) and ephrin B1 (EFNB1) essentially as

described in Vazin T, et al., PLoS One. 2009 Aug 12;4(8):e6606; and in Elkabetz Y., et al., Genes Dev. 2008 January 15; 22: 152–165.

To generate mesencephalic dopamine (mesDA) neurons, hESCs can be genetically modified to express the transcription factor Lmx1a (e.g., using a lentiviral vector with the PGK promoter and Lmx1a) essentially as described in Friling S., et al., Proc Natl Acad Sci U S A. 2009, 106: 7613–7618.

To generate lung epithelium (type II pneumocytes) from hESCs, the ESCs can be cultured in the presence of a commercially available cell culture medium (Small Airway Growth Medium; Cambrex, College Park, MD), or alternatively, in the presence of a conditioned medium collected from a pneumocyte cell line (e.g., the A549 human lung adenocarcinoma cell line) as described in Rippon HJ., et al., Proc Am Thorac Soc. 2008; 5: 717–722.

To induce differentiation of hESCs or human iPS cells into neural cells, the pluripotent stem cells can be cultured for about 5 days in the presence of a serum replacement medium supplemented with TGF-b inhibitor (SB431542, Tocris; e.g., 10 nM) and Noggin (R&D; e.g., 500 ng/ml), following which the cells are cultured with increasing amounts (e.g., 25 %, 50 %, 75 %, changed every two days) of N2 medium (Li XJ., et al., Nat Biotechnol. 2005, 23:215-21) in the presence of 500 ng/mL Noggin, essentially as described in Chambers SM., et al., Nat Biotechnol. 2009, 27: 275–280.

To induce differentiation of hESCs or human iPS cells into neural progenitors, the cells are cultured in suspension, following which the differentiation inhibition factors are removed from the culture medium and 5 X 10^{-5} M Retinoic acid is added for 21 Days. The cells are then transferred to fibronectin coated plates and cultured for additional 5 days before harvesting the cells for analysis. Q-PCR and immunostainings confirm the presence of neuronal progenitor cells (see Example 7 of the Examples section which follows).

To induce differentiation of hESCs or human iPS cells into endoderm cells (including insulin producing cells) the differentiation inhibition factors are removed from the culture medium of the pluripotent stem cells and the cells are exposed to 10 ng/ml Activin for 48 hours, in medium containing cAMP increasers such as forskolin, 8-bromocAMP, GABA, IBMX and DBC. Ten days later the cells are analyzed for endodermal markers. Q-PCR for Sox17 demonstrate significant increase in Sox17

expression in treated cells in compare to none treated controls (see Example 7 of the Examples section which follows).

To induce differentiation of hESCs or human iPS cells into mesenchymal stem cells (MSCs) the pluripotent stem cells are transferred to serum containing medium for 14 days and then plated on either gelatin or Matrigel. 7-14 days later the cells are differentiated into MSCs, which can be either frozen or passaged while using trypsin.

In addition to the lineage-specific primary cultures, EBs of the invention can be used to generate lineage-specific cell lines which are capable of unlimited expansion in culture.

Cell lines of the present invention can be produced by immortalizing the EB-derived cells by methods known in the art, including, for example, expressing a telomerase gene in the cells (Wei, W. et al., 2003. Mol Cell Biol. 23: 2859–2870) or co-culturing the cells with NIH 3T3 hph-HOX11 retroviral producer cells (Hawley, R.G. et al., 1994. Oncogene 9: 1-12).

As described in Example 11 of the Examples section which follows, the pluripotent stem cells which are cultured in suspension as single cells devoid of cell clumps can further differentiate into cells of the mesodermal lineage in a suspension culture (3-D) or in a 2-dimensional culture system.

As described in Example 11 of the Examples section which follows, the present inventors have uncovered a novel method of differentiating pluripotent stem cells to mesenchymal stem cells in suspension.

According to an aspect of some embodiments of the invention there is provided a method of generating a mesenchymal stem cell in a suspension culture. The method is effected by culturing the pluripotent stem cells of some embodiments of the invention (e.g., the PSCs which are cultured in suspension as single cells devoid of clumps, or the PSCs which are cultured in suspension as cell clumps) in a suspension culture under conditions suitable for differentiation of pluripotent stem cells to mesenchymal stem cells, thereby generating the mesenchymal stem cell in the suspension culture.

Any known culture medium suitable for differentiating pluripotent stem cells to MSCs can be used.

The present inventors have uncovered that the following culture media are suitable for differentiation of pluripotent stem cells to mesenchymal stem cells:

(1) Fy enriched medium; consisting of 80% DMEM/F12 (Biological Industries, Beit Haemek, Israel), containing 10% knockout serum replacement, 10% fetal bovine serum (FBS; HyClone or Biological Industries) 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acid stock (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated);

(2) MeSus I medium: consisting of 80% DMEM (Biological Industries, Beit Haemek, Israel), containing 20% FBS (HyClone or Biological Industries) 2 mM L-glutamine, (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated);

(3) MeSus II medium: consisting of 80% αMEM (Biological Industries, Beit Haemek, Israel), containing 20% FBS (HyClone or Biological Industries) 2 mM L-glutamine, (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated);

(4) MeSus III medium: consisting of DMEM/F12 (Biological Industries, Beit Haemek, Israel), 1% ITS (Invitrogen) 2 mM L-glutamine, (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated).

The present inventors have uncovered that the culture conditions should include a gradual transfer of the pluripotent stem cells from the suspension culture with undifferentiating medium to a suspension culture with the MSC differentiating medium. Following are non-limiting methods for transferring the pluripotent stem cells to the differentiating medium:

I. (i) 25% differentiation medium 75% pCM100F for one passage; (ii) 50% differentiation medium 50% pCM100F for one passage; (iii) 75% differentiation medium 25% pCM100F for one passage; (iv) 100% differentiation medium.

II. (i) 50% differentiation medium 50% pCM100F for one passage; (ii) 75% differentiation medium 25% pCM100F for one passage; (iii) 100% differentiation medium

III. (i) 50% differentiation medium 50% pCM100F for one passage; (ii) 100% differentiation medium.

According to an aspect of some embodiments of the invention there is provided an isolated population of mesenchymal stem cells (MSCs) in a suspension culture generated by the method of some embodiments of the invention.

According to some embodiments of the invention, at least about 30% (e.g., 30%), at least about 35% (e.g., 35%), at least about 40% (e.g., 40%), at least about 45% (e.g., 45%), at least about 50% (e.g., 50%), at least about 55% (e.g., 55%), at least about 60% (e.g., 60%), at least about 65% (e.g., 65%), at least about 70% (e.g., 70%), at least about 75% (e.g., 75%), at least about 80% (e.g., 80%), at least about 81% (e.g., 81%), at least about 82% (e.g., 82%), at least about 83% (e.g., 83%), at least about 84% (e.g., 84%), at least about 85% (e.g., 85%), at least about 86% (e.g., 86%), at least about 87% (e.g., 87%), at least about 88% (e.g., 88%), at least about 89% (e.g., 89%), at least about 90% (e.g., 90%), at least about 91% (e.g., 91%), at least about 92% (e.g., 92%), at least about 93% (e.g., 93%), at least about 94% (e.g., 94%), at least about 95% (e.g., 95%), at least about 96% (e.g., 96%), at least about 97% (e.g., 97%), at least about 98% (e.g., 98%), at least about 99% (e.g., 99%), e.g., 100% of the MSCs generated by the method of some embodiments of the invention are characterized by a CD73+/CD31-/CD105+ expression signature.

According to some embodiments of the invention, the MSCs are capable of differentiation in a suspension culture into a cell lineage selected from the group consisting of an adipogenic lineage, an osteoblastic lineage, and a chrondrogenic lineage.

As described in Example 10 of the Examples section which follows, the pluripotent stem cells which are cultured in suspension as single cells devoid of cell clumps can further differentiate into cells of the ectodermal lineage in a suspension culture (3-D) or in a 2-dimensional culture system.

According to an aspect of some embodiments of the invention, there is provided a method of generating a neuronal progenitor cell in a suspension culture, comprising culturing the pluripotent stem cells of some embodiments of the invention (e.g., the pluripotent stem cells which were cultured in a suspension culture as single cells devoid of cell clumps) in a suspension culture under conditions suitable for differentiation of neuronal progenitor cell, thereby generating the neuronal progenitor cell in the suspension culture.

Any known culture medium suitable for differentiating pluripotent stem cells to neuronal progenitor cells can be used. Non-limiting examples include a medium

containing retinoic acid (10^{-3} M) or Noggin (10 ngr/ml), essentially as described under "General Materials and Experimental Methods".

According to an aspect of some embodiments of the invention, there is provided an isolated population of neuronal progenitor cells in a suspension culture generated by the method of some embodiments of the invention.

As described in Example 12 of the Examples section which follows, the pluripotent stem cells which are cultured in suspension as single cells devoid of cell clumps can further differentiate into cells of the endodermal lineage in a suspension culture (3-D) or in a 2-dimensional culture system.

According to an aspect of some embodiments of the invention, there is provided a method of generating an endodermal cell in a suspension culture, comprising culturing the pluripotent stem cells of some embodiments of the invention (e.g., the pluripotent stem cells which were cultured in a suspension culture as single cells devoid of cell clumps) in a suspension culture under conditions suitable for differentiation of the pluripotent stem cells to endodermal cells, thereby generating the endodermal cell in the suspension culture.

Any known culture medium suitable for differentiating pluripotent stem cells to endodermal cells can be used. Non-limiting examples include a medium containing activin A (e.g., at concentration of 10 ng/ml), for 24-48 hours, essentially as described under "General Materials and Experimental Methods".

According to an aspect of some embodiments of the invention, there is provided an isolated population of endodermal cells in a suspension culture generated by the method of some embodiments of the invention.

It will be appreciated that since the lineage-specific cells or cell lines obtained according to the teachings of the invention are developed by differentiation processes similar to those naturally occurring in the human embryo they can be further used for human cell-based therapy and tissue regeneration.

Thus, the invention envisages the use of the expanded and/or differentiated lineage-specific cells or cell lines of some embodiments of the invention for treating a disorder requiring cell replacement therapy (cell based therapy).

For example, oligodendrocyte precursors can be used to treat myelin disorders (Repair of myelin disease: Strategies and progress in animal models. Molecular

Medicine Today. 1997. pp. 554-561), chondrocytes or mesenchymal cells can be used in treatment of bone and cartilage defects (U.S. Pat. No. 4,642,120) and cells of the epithelial lineage can be used in skin regeneration of a wound or burn (U.S. Pat. No. 5,716,411).

For certain disorders, such as genetic disorders in which a specific gene product is missing [e.g., lack of the CFTR gene-product in cystic fibrosis patients (Davies JC, 2002. New therapeutic approaches for cystic fibrosis lung disease. J. R. Soc. Med. 95 Suppl 41:58-67)], ESC-derived cells or iPS cells-derived cells are preferably manipulated to over-express the mutated gene prior to their administration to the individual. It will be appreciated that for other disorders, the ESC-derived cells or iPS-derived cells should be manipulated to exclude certain genes.

Over-expression or exclusion of genes can be effected using knock-in and/or knock-out constructs [see for example, Fukushige, S. and Ikeda, J. E.: Trapping of mammalian promoters by Cre-lox site-specific recombination. DNA Res 3 (1996) 73-50; Bedell, M. A., Jerkins, N. A. and Copeland, N. G.: Mouse models of human disease. Part I: Techniques and resources for genetic analysis in mice. Genes and Development 11 (1997) 1-11; Bermingham, J. J., Scherer, S. S., O'Connell, S., Arroyo, E., Kalla, K. A., Powell, F. L. and Rosenfeld, M. G.: Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. Genes Dev 10 (1996) 1751-62].

The lineage specific cells of some embodiments of the invention can be utilized to produce high amounts (massive production) of proteins such as hormones, cytokines, growth factors and drugs. For example, to produce the proteins the cells should be induced to over-express the protein by transfection for example, and after expansion the protein could be isolated from the culture medium.

The lineage specific cells of some embodiments of the invention can be utilized to prepare a cDNA library. mRNA is prepared by standard techniques from the lineage specific cells and is further reverse transcribed to form cDNA. The cDNA preparation can be subtracted with nucleotides from embryonic fibroblasts and other cells of undesired specificity, to produce a subtracted cDNA library by techniques known in the art.

The lineage specific cells of some embodiments of the invention can be used to screen for factors (such as small molecule drugs, peptides, polynucleotides, and the like) or conditions (such as culture conditions or manipulation) that affect the differentiation of lineage precursor to terminally differentiated cells (e.g., for drug screening). For example, growth affecting substances, toxins or potential differentiation factors can be tested by their addition to the culture medium.

The lineage specific cells of some embodiments of the invention can be used to prepare a vaccine. For example, the pluripotent stem cells, or cells differentiated therefrom, can be inoculated with viral particles and further cultured in a suitable medium until cell lysis occurs and newly produced viral particles are released in the medium. The cells can be used for production of attenuated virus belonging to the family of poxvirus, in particular canarypoxvirus, fowlpoxvirus and vaccinia virus such as native or recombinant vaccinia virus [for example, Modified Vaccinia virus Ankara such as MVA available under ATCC Number VR-1508) or other orthopoxviruses]. For additional description see U.S. Patent Application No. 20040058441 which is fully incorporated herein by reference.

The cell culture of some embodiments of the invention, or the lineage-specific cells generated therefrom can be subject to genetic manipulation by using either infection or transfection of a polynucleotide of interest. The polynucleotide may be included in a nucleic acid construct under the regulation of a promoter.

Methods of introducing the polynucleotide into cells are described in Sambrook et al., [Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992)]; Ausubel et al., [Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Maryland (1989)]; Chang et al., [Somatic Gene Therapy, CRC Press, Ann Arbor, MI (1995)]; Vega et al., [Gene Targeting, CRC Press, Ann Arbor MI (1995)]; Vectors [A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston MA (1988)] and Gilboa et al. [Biotechniques 4 (6): 504-512 (1986)] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors [e.g., using retrovirus, adenovirus (e.g., adenovirus-derived vector Ad-TK, Sandmair et al., 2000. Hum Gene Ther. 11:2197-2205), a chimeric adenovirus/retrovirus vector which combines retroviral and adenoviral components (Pan et al., Cancer Letters 184: 179-188, 2002). See also

United States patent 4,866,042 for vectors involving the central nervous system and also United States patents 5,464,764 and 5,487,992 for positive-negative selection methods for inducing homologous recombination.

According to some embodiments, the numbers described herein are preceded by about.

The tern "ng" refers to nanogram. The term "pg" refers to picogram. The term "ml" refers to milliliter. The term "mM" refers to millimolar. The term "μM" refers to micromolar.

As used herein the term "about" refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges

between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the

literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987: 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

GENERAL MATERIALS AND EXPERIMENTAL METHODS

Induced pluripotent stem (iPS) cell lines - iPS cell lines J1.2-3 (from foreskin fibroblasts; Park et al, Nature 451:P141-147, 2008); C2 and C3 (from foreskin fibroblasts, Germanguz et al, JCMM, 2009); iF4 (from adult skin fibroblast) [Park et al, 2008, Germanguz et al, 2009]; KTN7 and KTN3 (from Kartenocytes, Novac-Petraro Cellular Reprograming, In Press); and KTR13 and KTR13.4 (from Kartenocytes; Novac-Petraro Cellular Reprogramming, In Press) were cultured with inactivated MEF as previously described [Park et al, 2008].

Human embryonic stem cell (hESC) lines – Human ESC lines H9.2, I3, I3.2 and I6.2 (described in Amit et al, J. Anatomy 2002); and human ESC lines H14, H7, H9 (Wisconsin cell lines) were cultured as previously described [Amit et al, 2000].

Human extended blastocyst cell (hEBC) lines - Human extended blastocyst cell lines (described in WO2006/040763) J3 and J6 were cultured as described in Amit et al, Dev Biol, 2000.

Culture media - The following culture medium combinations were tested for their ability to support the growth of iPS, hESC and hEBC lines in attached (2D) cultures or in suspension cultures (three-dimensional, 3D):

yF10 - Basic culture medium consisting of 85% DMEM/F12 (Biological Industries, Beit Haemek, Israel), containing 15% knockout serum replacement (SR), 2 mM L-glutamine, 0.1 mM β–mercaptoethanol, 1% non-essential amino acid stock, and 10 ng/ml basic fibroblast growth factor (bFGF) (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated). This basic culture medium was used as control and for the routine growth of iPS cells and hESCs with inactivated MEF or foreskin fibroblasts as feeder layers in 2D cultures.

yFIL25 - basic medium (yF10) with the addition of 25 ng/ml interleukin 6 (IL6) and IL6 soluble receptor (R&D Biosystems, Minneapolis, MN, USA). It should be mentioned that any gp130 agonist such as Oncostatin, IL11 can be used instead of IL6.

NCM100F - basic medium (yF10) in which instead of knockout serum replacement the serum replacement is the animal free serum replacement (Invitrogen corporation, Knockout SR zeno-free, Catalogue number 12618). In addition the NCM100F medium included 100 ng/ml of the IL6RIL6 [IL6-IL6-receptor chimera (SEQ ID NO:19; which was described in Chebath J, et al., 1997 and WO 99/02552 to Revel

M., et al.]. The 85-Kda IL6RIL6 was produced and purified (Serono International SA, Geneva, Switzerland) and was donated by Merck-Serono group (Nes-Ziona, Israel and Geneva, Switzerland).

NCM100Fp - basic medium (yF10) in which instead of knockout serum replacement the serum replacement is the animal free serum replacement (Invitrogen corporation, Knockout SR xeno-free Catalogue Number 12618). In addition the NCM100Fp medium included 100 pg/ml of the IL6RIL6.

ILCNTF - basic medium (yF10) supplemented with 1 ng/ml interleukin 11 (IL11; R&D Biosystems, Catalogue number 18-IL) and Ciliay Neurotrophic Factor (CNTF; R&D Biosystems, Catalogue number 257-NT).

NILCNTF - basic medium (yF10) in which instead of knockout serum replacement the serum replacement is the animal free serum replacement (Invitrogen corporation, Knockout SR xeno-free Catalogue number 12618), and supplemented with 1 ng/ml IL11 and CNTF (R&D Biosystems).

cmV5b - 10 ng/ml bFGF (Invitrogen corporation), 100 ng/ml IL6IL6-receptor chimera in Nutristem medium (Biological Industries).

cmV5bp - 10 ng/ml bFGF (Invitrogen corporation), 100 pg/ml IL6IL6-receptor chimera in Nutristem (Biological Industries).

cmTeSR – 100 ng/ml IL6IL6-receptor chimera in mTeSR medium (StemCell Technologies).

cmTeSRp – 100 pg/ml IL6IL6-receptor chimera in mTeSR (StemCell Technologies).

cmTeSR2 – 100 ng/ml IL6IL6-receptor chimera in TeSR2 (StemCell Technologies).

cmTeSR2p 100 pg/ml IL6IL6-receptor chimera in TeSR2 (StemCell Technologies).

cmHA13 85% DMEM/F12 (Biological Industries, Beit Haemek, Israel), containing 1% SR3 serum replacement (Sigma), 2 mM L-glutamine, ascorbic acid 50 μg/ml, 1% lipid mixture and 10 ng/ml bFGF and the IL6IL6-receptor chimera at 100 ng/ml. The 85-Kda IL6RIL6 was produced and purified as described and was donated by Merck-Serono group. (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated).

cmHA13p 85% DMEM/F12 (Biological Industries, Beit Haemek, Israel), containing 1% SR3 serum replacement (Sigma), 2 mM L-glutamine, ascorbic acid 50 μg/ml, 1% lipid mixture and 10 ng/ml bFGF and the IL6IL6-receptor chimera at 100 pg/ml. The 85-Kda IL6RIL6 was produced and purified as described and was donated by Merck-Serono group. (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated).

CMrb100F - basic medium (yF10) including; the bFGF concentration was increased to 100ng/ml, 100 ng/ml of the IL6RIL6 (IL6-IL6-receptor chimera; which was described in Chebath J, et al., 1997 and WO 99/02552 to Revel M., et al). The 85-Kda IL6RIL6 was produced and purified (Serono International SA, Geneva, Switzerland) and was donated by Merck-Serono group (Nes-Ziona, Israel and Geneva, Switzerland).

CMrb100Fp - basic medium (yF10) including; the bFGF concentration was increased to 100ng/ml, 100 pg/ml of the IL6RIL6 (IL6-IL6-receptor chimera; which was described in Chebath J, et al., 1997 and WO 99/02552 to Revel M., et al). The 85-Kda IL6RIL6 was produced and purified (Serono International SA, Geneva, Switzerland) and was donated by Merck-Serono group (Nes-Ziona, Israel and Geneva, Switzerland).

NCMrb100F - basic medium (yF10) in which instead of knockout serum replacement the serum replacement is the animal free serum replacement (Invitrogen corporation, Knockout SR xeno-free Catalogue Number 12618) and the bFGF concentration was increased to 100ng/ml. In addition, 100 ng/ml of the IL6RIL6 (IL6-IL6-receptor chimera; which was described in Chebath J, et al., 1997 and WO 99/02552 to Revel M., et al). The 85-Kda IL6RIL6 was produced and purified (Serono International SA, Geneva, Switzerland) and was donated by Merck-Serono group (Nes-Ziona, Israel and Geneva, Switzerland).

NCMrb100Fp - basic medium (yF10) in which instead of knockout serum replacement the serum replacement is the animal free serum replacement (Invitrogen corporation, Knockout SR xeno-free Catalogue Number 12618) and the bFGF concentration was increased to 100ng/ml. In addition the NCM100F medium included 100 pg/ml of the IL6RIL6 (IL6-IL6-receptor chimera; which was described in Chebath J, et al., 1997 and WO 99/02552 to Revel M., et al. The 85-Kda IL6RIL6 was produced and purified (Serono International SA, Geneva, Switzerland) and was donated by Merck-Serono group (Nes-Ziona, Israel and Geneva, Switzerland).

Culture in 2-dimensional culture systems - For feeder layer free culture system the extracellular matrices Matrigel (BD Biosciences) or human fibronectin (Millipore, Billerica, MA) were used.

Initiation of suspension culture - To initiate suspension cultures, the iPS or ES cells were removed from their culture dish using 1.5 mg/ml type IV collagenase (Worthington biochemical corporation, Lakewood, NJ, USA), or using scrapper, further broken into small clumps using 200 – 1000 µl Gilson pipette tips, and cultured in suspension in 58 mm Petri dishes (Greiner, Frickenhausen, Germany) at a cell density of $1 \times 10^6 - 5 \times 10^6$ cells/dish. The Petri dishes were kept static in an incubator at 37 °C in 5 % CO_2. The medium in the suspension culture was changed daily, and the cells were passaged every 5-7 days either by manual cutting of clumps using 27g needles (only at passages 1-3) or by gentle pipetting using 200-1000 µl Gilson pipette tips. Alternatively, the cells were passaged using trypsin EDTA (0.25%, Biological Industries, Beit Haemek, Israel) combined with one hour treatment with 10 M ROCK inhibitor (EMD Biosciences, Inc. La Jolla, CA, USA) before the incubation with trypsin.

Culture in Spinner flasks - Cell clumps cultured in Petri dish for at least one passage were transferred to a 250 ml spinner flask in the tested medium, shaken continuously at 40-110 rounds per minute (rpm) using magnetic plate, and placed in the incubator. Medium was changed every 1-3 days. Every 5-7 days the clumps were split in a ratio of 1:2-1:4.

Culture in a controlled bioreactor - The cells were cultured in a controlled bioreactor Biostat® Cultibag RM (Sartorius North America, Edgewood, New York, USA) (2 litter bag with 1 litter). The reactor parameters included speed of tilting: 16 rounds per minute (rpm); angle 7°; Temperature: 37°C, PH: 7-7.4, O_2 concentration: 50%;

Immunohistochemistry - For fluorescent immunostaining undifferentiated hESCs grown in suspension or re-cultured on MEFs were fixed with 4% paraformaldehyde and exposed to the primary antibodies overnight at 4 °C. Cys 3 conjugated antibodies (Chemicon International, Temecula CA, USA) were used as secondary antibodies (1:200 dilution). The primary antibodies (1:50 dilution) include SSEA 1,3 and 4 (Hybridoma Bank, Iowa, USA), TRA1-60 and TRA1-81 (Chemicon International, Temecula CA, USA), Oct4 (Santa Cruz Biotechnology, Santa Cruz, CA,

USA), Oligodendrocyte marker (O4; from R&D Biosystems), Glial fibrillary acidic protein (GFAP; from Millipore, Billerica, MA, USA), β-tubulin (Covance, Princeton, New Jersey, USA), nestin (Chemicon, Intnl, Inc. Temecula, CA, USA), PDX1 (the primary antibody is Goat anti human PDX1; two secondary antibodies were used: Rabitt anti goat IgG conjugated to FLUOR (green) or Donkey anti goat NL557 (Red), all from R&D Biosystems).

Flow cytometry analysis - Spheres of hPSCs cultured in suspension were dissociated to single cells using trypLE (Invitrogen Corporation products, Grand Island, NY, USA). The single cells were pipetted up and down with 200 µl pipette tip. The cells were stained with anti-h/mSSEA4, anti-h/mSSEA1, h/mTRA-160, h/mTRA1-81 Ab conjugated to Phycoerythrin, Phycoerythrin conjugated Rat IgG2B were used as isotype control (unless otherwise stated, all antibodies were purchased from R&D systems, Minneapolis, MN, USA). The stained cells were then analyzed with FACS calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest software according to the manufacturer's instructions. The anti-CD73 (BD Pharmingen), CD146 (BD Pharmingen), CD105 (BioScince), CD44 (BioScince), CD45 and CD31 (BD Pharmingen) antibodies.

Karyotype analysis - Karyotype analysis (G-banding) was performed on at least 10 cells from each sample, two samples per test, as previously described [Amit et al, 2003]. Karyotypes were analyzed and reported according to the "International System for Human Cytogenetic Nomenclature" (ISCN).

Embryoid Bodies (EBs) formation - For the formation of EBs, hESCs and iPS cells were passaged as described and transferred to 58 mm Petri dishes (Greiner, Frickenhausen, Germany). EBs were grown in medium consisting of 80 % DMEM/F12 (Biological Industries, Beit Haemek, Israel), supplemented with 10 % fetal bovine serum (FBS) (HyClone, Utah, USA), 10 % serum replacement (SR), 2 mM L-glutamine, 0.1 mM µ–mercaptoethanol, and 1% non-essential amino acid stock (Invitrogen Corporation, Grand Island NY, USA). 10-14 day-old EBs were harvested for RNA isolation and histological examination. For histological analysis EBs were fixed in 10 % neutral-buffered formalin, dehydrated in graduated alcohol (70%-100%) and embedded in paraffin. 1-5 µm sections were deparaffinized and stained with hematoxylin/eosin (H&E).

Reverse transcription polymerase chain reaction (RT PCR) - Total RNA was isolated from hESCs and iPS cells grown for at least 5 passages in suspension (three-dimension, 3D) or at 2-dimension (2D) in the tested medium, and from 10-21 day-old EBs (formed from cells grown in suspension or cells cultured in 2D) using Tri-Reagent (Sigma, St. Louis MO, USA), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg total RNA using MMLV reverse transcriptase RNase H minus (Promega, Madison WI, USA). PCR reactions included denaturation for 5 minutes at 94°C followed by repeated cycles (the number of cycles is indicated in Table 1 below) of: danaturation at 94°C for 30 seconds, annealing at a specific annealing temperature (as indicated in Table 1 below) and in the presence of a specific $MgCl_2$ concentration (as indicated in Table 1, below) for 30 seconds; and extension at 72°C for 30 seconds. PCR primers and reaction conditions used are described in Table 1. PCR products were size-fractionated using 2% agarose gel electrophoresis. DNA markers were used to confirm the size of the resultant fragments.

Table 1

RT-PCR primers and conditions

Gene product (GenBank Accession number); SEQ ID NO:	***Forward (F) and reverse (R) primers (5'→3')***	***Reaction Conditions***	***PCR product size [(base pairs (bp)]***
Oct-4 (S81255); SEQ ID NO:1	F: GAGAACAATGAGAACCTTC AGGA (SEQ ID NO:2) R: TTCTGGCGCCGGTTACAGA ACCA (SEQ ID NO:3)	30 cycles; annealing temperature: 60 °C; concentration of $MgCl_2$: 1.5 mM	219

Gene product (GenBank Accession number); SEQ ID NO:	*Forward (F) and reverse (R) primers (5'→3')*	*Reaction Conditions*	*PCR product size [(base pairs (bp)]*
Nanog (NM_024865.2); SEQ ID NO:4	F: ACTAACATGAGTGTGGATCC (SEQ ID NO:5) R: TCATCTTCACACGTCTTCAG (SEQ ID NO:6)	30 cycles; annealing temperature: 61 °C; concentration of $MgCl_2$: 1.5 mM	929
Rex1 (AF450454); SEQ ID NO:7	F: GCGTACGCAAATTAAAGTCCAGA (SEQ ID NO:8) R: CAGCATCCTAAACAGCTCGCAGAAT (SEQ ID NO:9)	30 cycles; annealing temperature: 56 °C; concentration of $MgCl_2$: 1.5 mM	306
FGF4 (NM_002007); SEQ ID NO:10	F: CTACAACGCCTACGAGTCCTACA (SEQ ID NO:11) R: GTTGCACCAGAAAAGTCAGAGTTG (SEQ ID NO:12)	30 cycles; annealing temperature: 52 °C concentration of $MgCl_2$: 1.5 mM	370
Sox2 (Z31560); SEQ ID NO:13	F: CCCCCGGCGGCAATAGCA (SEQ ID NO:14) R: TCGGCGCCGGGGAGATACAT (SEQ ID NO:15)	30 cycles; annealing temperature: 60 °C concentration of $MgCl_2$: 1.5 mM	448

Gene product (GenBank Accession number); SEQ ID NO:	*Forward (F) and reverse (R) primers (5'→3')*	*Reaction Conditions*	*PCR product size [(base pairs (bp)]*
GAPDH (NM_002046); SEQ ID NO:16	F:AATCCCATCACCATCTTC CA (SEQ ID NO:17) R:GCCTGCTTCACCACCTTC T (SEQ ID NO:18)	23 cycles; annealing temperature: 60 °C concentration of $MgCl_2$: 1.5 mM	581
PAX6 (NM_001127612); SEQ ID NO:20	F: AACAGACACAGCCCTCACA AACA (SEQ ID NO:21); R: CGGGAACTTGAACTGGAAC TGAC (SEQ ID NO:22)	35 cycles; annealing temperature: 65 °C concentration of $MgCl_2$: 1.5 mM	274
Nestin (NM_006617.1); SEQ ID NO:23	F: CAGCTGGCGCACCTCAAGA TG (SEQ ID NO:24); R: AGGGAAGTTGGGCTCAGG ACTGC (SEQ ID NO:25)	35 cycles; annealing temperature: 65 °C concentration of $MgCl_2$: 1.5 mM	210
HNF (NM_005382); SEQ ID NO:26	F: GAGCGCAAAGACTACCTG AAGA (SEQ ID NO:28); R: CAGCGATTTCTATATCCAG AGCC (SEQ ID NO:27);	35 cycles; annealing temperature: 65 °C concentration of $MgCl_2$: 1.5 mM	430

Gene product (GenBank Accession number); SEQ ID NO:	*Forward (F) and reverse (R) primers (5'→3')*	*Reaction Conditions*	*PCR product size [(base pairs (bp)]*
Lhx2 (NM_004789.3); SEQ ID NO:29	F: CCAAGGACTTGAAGCAGCTC (SEQ ID NO:30); R:TGCCAGGCACAGAAGTTAAG (SEQ ID NO:31)	35 cycles; annealing temperature: 64 °C concentration of $MgCl_2$: 1.5 mM	285

Table 1. Provided are the genes names (identified by GenBank Accession numbers and sequence identifiers) along with the primers (sequences and sequence identifiers) used to detect the expression level of the genes' transcripts. Also provided are the PCR conditions and the resulting PCR products.

Real time RT-PCR – RNA was extracted using TriReagent (Talron) from cells which were cultured in 2-D, in a suspension culture as cell clumps, or in a suspension culture as single cells. The RNA was then subjected to real time RT-PCR using the RT mix (Applied Biosystems) and the primers provided in Table 2, hereinbelow (Applied Biosystems), according to manufacturer's instructions.

Table 2

Real time RT-PCR primers

Gene symbols	*Gene ID number*	*Gene full name*	*Catalog number*
Oct4	ID: 5460	POU5F1	00999632
NANOG	ID: 79923	Nanog	02387400
Rex1	ID: 132625	ZFP42	00381890
Sox2	ID: 6657	Sox2	01053049
FN1	ID: 2335	Fibronectin 1	01549976
THBS4	ID: 7060	Thopombospondin	00170261
CTNNB1	ID: 1499	Beta catenin	00355049

Gene symbols	*Gene ID number*	*Gene full name*	*Catalog number*
CDH2	ID: 1000	N-cadherin	00983062
CDH1	ID: 999	E-cedherin	00170423
CLDN18	ID: 51208	Claudin18	00212584
CLDN6	ID: 9074	Claudin6	00607528
ITGA2	ID: 3673	Integrin alpha 2	01041011
ITGB5	ID: 3693	Integrin beta 5	00174435
EGFR	ID: 1956	Epidermal growth factor receptor	01076091
FBLN5	ID: 10516	Fibulin 5	00197064
PLXNA2	ID: 5362	Plexin A2	00300697
ITGA7	ID: 3679	Integrin alpha 7	01056475
ITGA6	ID: 3655	Integrin, alpha 6	01041011

Table 2. Provided are the gene symbols, their Gene ID number (Hypertext Transfer Protocol://World Wide Web (dot) ncbi (dot) nlm (dot) nih (dot) gov/gene/), the full gene name, and the Applied Biosystems Catalogue Number of the primers used for real time RT-PCR.

Teratoma formation - Cells from four to six 58 mm dishes, 3-6 wells in 6 wells plate, or 20 ml of a suspension culture were harvested and injected into the hindlimb muscles of four week-old male of severe combined immunodeficiency (SCID)-beige mice. Ten weeks after the injection the resultant teratomas were harvested and prepared for histological analysis using the same method mentioned for EBs.

Testing cloning efficiency of the pluripotent stem cells (PSCs) – PSCs were cultured in 2-D, in a suspension culture as cell clumps or in a suspension culture as single cells were tested for their cloning capacity as follows, wherein for each treatment group, 6 repeats of 96 cells was conducted.

Cloning from 2D cultures with MEFs: H7 cells were trypsinized with 0.05% trypsin 0.53 mM EDTA (Invitrogen) to single cells. Each individual cell was plated in separate well in 96 well plate (Nunc) covered with mitotically inactivated MEFs. 96 cells in each biological repeat were cloned while adding 10 μM/ml Rock inhibitor. 10 days after plating, the number of resulting colonies was calculated. Three colonies were picked up by passage using 1 mg/ml collagenase type IV and 10 mg/ml dispase (both from GibcoBRL). The cloned cultures were grown in pCM100F medium [containing 85% DMEM/F12 (Biological Industries, Beit Haemek, Israel), containing 15%

knockout serum replacement (SR), 2 mM L-glutamine, 0.1 mM b–mercaptoethanol, 1% non-essential amino acid stock, and 4 ng/ml bFGF (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated) supplemented with 100 pg/ml IL6RIL6 chimera] and were routinely passaged every 5-7 days with 1 mg/ml collagenase type IV. After expansion in culture, the resulting 3 clones were examined for ESCs characteristic.

Cloning from 3D cultures as single cells: H7 cells cultured as single cells in suspension were used. Each individual cell was plated in separate low attachment well in 96-well plate (Nunc) or on plates covered with MEFs. 96 cells in each biological repeat were cloned with or without the addition of 10 μM/ml Rock inhibitor as described in Table 4 in Example 9 of the Examples section which follows. 10 days after plating the number of resulting colonies was calculated. Three colonies were picked up by 200 μL tip. Cloned cultures were grown in pCM100F medium containing Serum replacement, IL6IL6receptor chimera and 4 ng/ml bFGF, and were routinely passage every 5-7 days by pipette. After expansion in culture the resulting 3 clones were examined for ESCs characteristic.

Freezing and thawing efficiency - Cells were frozen using one of the following freezing solutions:

1. Serum and animal freezing solution (Biological Industries).
2. DMEM supplemented by 10% DMSO and 20% FBS.
3. DMEM supplemented by 10% DMSO and 30% SR.

After 1-7 days at -80 °C refrigerator (using freezing box) the vials were transferred to liquid nitrogen. Cells were thawed and the viability was tested by tripan blue staining. Three separate experiments were conducted.

Genetic manipulation - Cells were transfected using the following vector: CMV promoter-GFP (based on N1 plasmid). The following methods were used:

1. Electroporation using BTX ECM 2001 electroporator with the following parameters: 40 μgr DNA, 10^7 cells, 3-6 mSc, 220V.
2. Transfection reagent Fugene 6 (Roche) or Lipofectamine (Invitrogen) according to manufacturer instructions (40 μgr DNA for 10^6 cells).

Neural differentiation - To induce neural differentiation single cells cultured in suspension were transferred to a medium without bFGF and the IL6RIL6 chimera.

Either retinoic acid (10^{-3} M) or Noggin (10 ngr/ml) were added for three to seven days. Three weeks after differentiation induction, the cells were plated for staining with fibronectin. The cells were stained for O4 (oligodendrocytes marker), GFAP (Glial fibrillary acidic protein), nestin and β-tubulin.

Media used for differentiation of suspension MSCs to adipogenic, osteogenic, and chondrogenic cell lineages:

Adipogenic medium - DMEM F-12 supplemented with 10% FBS, 1 mM L-glutamine, 0.5 mM IBMX, 10 μg/ml Insulin, 10^{-6} M Dexamethasone, 0.1 mM Indomethacin.

Osteogenic medium - GMEM BHK-21 supplemented with 10% FBS, 1% Sodium pyruvate, 1% Nonessential amino acids, 50 μg/ml L- ascorbic acid, 0.1 mM β-mercaptoethanol, 10 mM β-glycerol-phosphate, and 0.1 μM Dexamethasone.

Chondrogenic medium - DMEM supplemented with 10^{-7} M Dexamethasone, 1% ITS, 50 μg/ml L-ascorbic acid, 1 mM Sodium pyruvate, 4 mM L-proline, and 10 ng/ml TGFβ3.

Differentiation protocols of suspension MSCs to adipogenic, osteogenic, and chondrogenic cell lineages:

Differentiation procedure for adipogenic differentiation and Oil Red O staining - MSC were seeded in density of 20,000 cell/cm^2 in 6 well plates and grown in adipogenic medium for 4 weeks with medium changes twice a week.

Adipogenic differentiation was assessed by observation of the accumulation of lipid– rich vacuoles within the cells after Oil Red O staining.

Oil Red O staining - cells were rinsed once with PBS, fixed with 4% Paraformaldehyde (PFA) for 20 minutes, rinsed again and stained with Oil Red O solution for 10 minutes in room temperature. Staining solution was removed and the cells were washed 5 times with water.

Differentiation procedure for osteogenic differentiation and Alizarin red staining - MSC were seeded in density of 2000-3000 cell/cm^2 in 6 well plate, and grown in osteogenic medium for 4 weeks with medium changes twice a week. Cells cultures were assayed for mineral content by Alizarin red staining.

Alizarin red staining - cells were rinsed once with PBS, fixed with 4% Paraformaldehyde (PFA) for 20 minutes, rinsed again and stained with 2% Alizarin red

solution for 15 minutes in room temperature. Staining solution was removed and the cells were washed a few times with water.

Differentiation procedure for chondrogenic differentiation, hematoxylin and eosin and Alcian blue staining - For chondrogenic differentiation, $2x10^5$ MSC were centrifuged at 300 g for 5 minutes in 15 ml polypropylene falcon tubes to form a cell pellet. The cells were grown in chondrogenic medium for 9 weeks with medium changes twice a week without disturbing the cell mass. Cell sections were made after fixing the cell pellets with 4% PFA and embedding it in low melting agarose (1.5%).

Hematoxylin and eosin (H&E) and Alcian blue stainings - were performed by the pathologic laboratory at Rambam Medical Center.

Differentiation protocols of MSCs in suspension - The same adipogenic, osteogenic, and chondrogenic media (described hereinabove) were used to differentiate the MSCs in suspension, without seeding the MSCs on a 2-D culture system.

EXAMPLE 1

SUSPENSION CULTURE OF PLURIPOTENT STEM CELLS IN THE NOVEL CULTURE MEDIA OF SOME EMBODIMENTS OF THE INVENTION

Culture of pluripotent cells in suspension holds significant advantages over conventional cultures, particularly when aiming to obtain large amounts of cells for cell and tissue transplantation. To initiate suspension cultures from pluripoteny cells grown with MEF or in feeder layer-free conditions [Amit et al, 2004], a number of growth factors and cytokines were employed. Pluripotent cells from different sources were used: iPS cells from newborn (foreskin fibroblasts), iPS cells from adults (fibroblasts) and hESCs.

Experimental results

Suspension cultures -At 24 hours after being placed in a suspension culture in the presence of the following culture media: yFIL25, NCM100F, NCM100Fp, ILCNTF, NILCNTF, cmV5b, cmV5bp, cmTeSR, cmTeSRp, cmTeSR2, cmTeSR2p, cmHA13, CMrb100F, CMrb100Fp, NCMrb100F, or NCMrb100Fp, the pluripotent cells created spheroid clumps or disc-like structures which upon histological examination revealed a homogenous population of small cells with large nuclei. The spheroids grew and were

split mechanically every 5-7 days while maintaining their morphology, allowing expansion of the suspension cultures. All these type of medium were found advantages for culturing ESCs and iPS cells in suspension as single cells or small clumps of less than 100 cells.

Alternatively, by using trypsin –EDTA and ROCK inhibitor treatment, suspended cells could be dissociated into single cells and still formed spheroids of the same morphology and features, thus allowing efficient cell expansion. Cells subjected to the suspension culture with the tested culture media showed similar behavior and spheroid morphology and histology. When returned to 2D culture with MEFs or fibronectin after at least 5 passages in suspension, all of the spheroid clumps adhered to the MEFs or fibronectin matrix, respectively, and after 24-48 hours demonstrated typical pluripotent cells colony morphology, exhibiting high nucleus-to-cytoplasm ratio with a notable presence of one to three nucleoli and with typical spacing between the cells.

Maintenance of undifferentiated stem cell phenotype - Several surface markers typical of primate undifferentiated ESCs and iPS cells were examined using immunofluorescent staining essentially as described in Thomson et al, 1998; Bhattacharya, *et al.* 2004; Kristensen et al, 2005, each of which is fully incorporated herein by reference. Human pluripotent cells cultured in suspension with the tested media for at least 5 passages were found to be still strongly positive for SSEA4, TRA-1-60 and TRA-1-81 and Oct 4. As in other primate ESCs [Thomson et al, 1995 and 1996] and with cells cultured with MEFs, staining for SSEA3 was weak and staining for SSEA1 was negative. Staining for stem cell markers remained high when cells that were cultured in suspension were returned to 2D cultures on MEF feeder cell layers. RT-PCR analyses showed that, similarly to cells cultured with MEFs, pluripotent cells cultured in suspension for at least 5 passages expressed genetic markers of pluripotency [King et al, 2006] including *Oct 4*, *Nanog*, *Sox2*, *Rex1*, and *FGF4*. No significant difference in gene expression was detected between cells cultured in suspension. or with cells re-cultured with MEFs after a continuous culture in suspension.

Maintenance of karyotype - Karyotype analysis by Giemsa banding was carried out on cells after at least 7 passages in suspension, and the cells were found to exhibit normal 46,XY or 46,XX karyotype. Thus, the karyotype of the suspension cell culture remained stable.

Pluripotency - Following prolonged expansion in suspension cultures with the tested medium, pluripotent cells conserved their pluripotent differentiation ability. The developmental potential of the cells was first examined *in vitro* by the formation of EBs. When pluripotent cells cultured in suspension for over 5 passages were transferred to serum-containing medium without the addition of the growth factors, formation of cystic EBs was observed after 7-10 days, similarly to cells cultured with MEFs where cavitated EBs appeared following 10 days in culture [Itskovitz et al, 2000], and cystic EBs after 14-20 days. Within these EBs, there were cell types representative of the three embryonic germ layers typical of pluripotent cells differentiation.

Pluripotency of the suspension pluripotent cells was further demonstrated *in vivo* by teratoma formation. Cells cultured in suspension for about 10 passages were injected into SCID Beige mice, and 10 weeks later tumors were formed. Within these teratomas, tissues representative of the three germ layers were observed.

Shaking suspension cultures - Pluripotent cells were cultured in suspension in spinner flask for at least a month using the tested medium. A examination after one month showed that morphologically the spheroid clumps formed by the cells remained similar to those observed with cells cultured statically using Petri dishes. When re-cultured on MEFs, the cells in the clumps re-attached, forming again typical colonies of pluripotent cells. The karyotype of the cells cultured for one month in the spinner flask was found to be normal.

EXAMPLE 2

TWO-DIMENSIONAL CULTURE OF PLURIPOTENT STEM CELLS IN THE NOVEL CULTURE MEDIA OF SOME EMBODIMENTS OF THE INVENTION

Culturing pluripotent cells in 2D cultures using serum-free, xeno-free and supportive-layers free system - Several possible medium combinations were tested for the ability to support feeder-layer free or animal free (xeno-free, e.g., using foreskin fibroblast as feeders) culture of pluripotent cells. All tested medium (i.e., yFIL25, NCM100F, NCM100Fp, ILCNTF, NILCNTF, cmV5b, cmV5bp, cmTeSR, cmTeSRp, cmTeSR2, cmTeSR2p, cmHA13, CMrb100F, CMrb100Fp, NCMrb100F, or NCMrb100Fp), were found suitable for supporting undifferentiated pluripotent cells cultures. Pluripotent cells were cultured continuously for at least 5 passages while

maintaining their stemness features including undifferentiated proliferation, karyotype stability and pluripotency. No morphological differences could be observed between colonies grown in the tested culture systems and those grown on MEF with the basic medium, correspondingly, morphological features remained unchanged on a single-cell level, rendering cells small and round, exhibiting high nucleus-to-cytoplasm ratio, with a notable presence of one to three nucleoli and typical spacing between the cells. Similar to cells grown on MEFs, cells were passaged routinely every five to seven days, at the same ratio of 1/2 or 1/3, indicating a similar population doubling time. The cells were passage at the same seeding efficiency of about 1 million cells per 10 cm^2, with the same viability rate of over 90%.

Pluripotent stem cells which are cultured on 2-D culture systems in the presence of the novel culture media of some embodiments of the invention maintain expression pattern of undifferentiated cells - Several surface markers typical of primate undifferentiated ESCs and iPS cells were examined using immunofluorescent staining essentially as described in Thomson et al, 1995, 1996, 1998, each of which is fully incorporated herein by reference. Cells cultured with the tested medium for at least 7 passages (e.g., 10, 15 passages) were found to be strongly positive to surface markers SSEA4, TRA-1-60, TRA-1-81 and Oct 4. As in other primate ESCs, staining with SSEA3 was weak and negative for SSEA1.

Pluripotent stem cells which are cultured on 2-D culture systems in the presence of the novel culture media of some embodiments of the invention are capable of differentiation into cell lineages derived from the three embryonic germ layers in vitro and in vivo - The developmental potential of the cells after prolonged culture in the tested conditions was examined *in vitro* by the formation of embryoid bodies (EBs). Pluripotent cells cultured in the tested conditions formed EBs similar to those created by ESCs grown on MEFs. Within these EBs, stem cells differentiated into cell types representative of the three embryonic germ layers (data not shown).

In addition, the pluripotent stem cells were shown capable of differentiation *in vivo*. Thus, following their injection to SCID Beige mice cells cultured under the tested conditions form teratomas containing cell types representative of the three embryonic germ layers *i.e.*, ectoderm, endoderm and mesoderm (data not shown).

EXAMPLE 3

CULTURING OF PLURIPOTENT STEM CELLS AS SINGLE CELLS IN SUSPENSION WITHOUT ENZYMATIC PASSAGING

Experimental Results

Culturing single cells in suspension cultures - Pluripotent cells were cultured in suspension in spinner flask or Petri dishes for at least a month using all of the tested medium (yFIL25, NCM100F, NCM100Fp, ILCNTF, NILCNTF, cmV5b, cmV5bp, cmTeSR, cmTeSRp, cmTeSR2, cmTeSR2p, cmHA13, CMrb100F, CMrb100Fp, NCMrb100F, or NCMrb100Fp), as single cells. An examination after one month showed that the cells exhibit pluripotent cells features including stable karyotype, expression on specific markers and differentiation potential. The cells were passage without the use of ROCK inhibitor and without the use of trypsin and were split mechanically using a pipette. This is the first time human ESCs or iPS were shown capable of culturing in a suspension culture as single cells without the need for enzymatic passaging, since the cell adopted a single cell culturing mode. The system can be used for an industrial processes without passage.

Human ESCs which are cultured in a suspension culture as single cells can be replated on 2-dimensional culture systems, demonstrating typical hESCs morphology - CL1 (13E1) cultured for 17 passages in suspension as single cells were re-plated with inactivated MEFs. During the first passage the colony morphology is not clear. Few weeks after, the cells formed colonies with pluripotent cells morphology of spaces between cells, clear borders and high nucleus to cytoplasm ratio (Figures 11A-B).

EXAMPLE 4

HUMAN ESCS AND IPS CELLS CAN BE SHIPPED WHILE IN A SUSPENSION CULTURE

Shipment of living cells - Cells cultured in suspension as cell clumps using the described method survive shipping at room temperature or at 0-15 Celsius degrees. Using 50 ml tubes with 20-40 ml of culture medium, vented or not vented, 2-10 million cells per tube could be shipped. At least 50% of the cells survived and continued to grow while maintaining all pluripotent features. Other tube size might be use. The medium could be supplemented with anti oxidants and RoCK or other anti apoptotic agents.

EXAMPLE 5

EXPANSION OF PLURIPOTENT STEM CELLS UNDER DYNAMIC CULTURE CONDITIONS IN THE PRESENCE OF THE NOVEL CULTURE MEDIUM OF SOME EMBODIMENTS OF THE INVENTION

The present inventors tested the ability of the novel culture media of some embodiments of the invention to support the growth and expansion of pluripotent stem cells such as iPSCs and ESCs under dynamic culture conditions when cultured as single cells (devoid of cell clumps) or in suspension with cell clumps.

Experimental Methods

Cell lines and seeding concentration: The C2 IPS cell line was used at passage 77, of which 37 passages were in suspension before seeding into the dynamic culture conditions. The IPSCs were seeded (inoculated) at a concentration of 3.7×10^4 cell/ml.

Culture media and conditions: The following culture media were used for the dynamic suspension culture: CM100Fp. The cells were culture in spinner flasks or a controlled bioreactor continuously for 5 days. When cultured in a bioreactor the medium was not changed during the culturing process. When cultured in spinner flasks the medium was changed every day.

Culturing conditions for dynamic growth in suspension:

Culture in a controlled bioreactor - The cells were cultured in a controlled bioreactor Biostat® Cultibag RM (Sartorius North America, Edgewood, New York, USA) (2 litter bag with 1 litter). The reactor parameters included speed of tilting: 16 rounds per minute (rpm); angle 7°; Temperature: 37°C, PH: 7-7.4, O_2 concentration: 50%;

Culture in Spinner flasks - Cell clumps cultured in Petri dish for at least one passage were transferred to a 250 ml spinner flask in the tested medium, shaken continuously at 40-110 rounds per minute (rpm) using magnetic plate, and placed in the incubator. Medium was changed every 1-3 days. Every 5-7 days the clumps were split in a ratio of 1:2-1:4.

Experimental Results

Expansion of pluripotent stem cells in a suspension culture using the culture media according to some embodiments of the invention - The pluripotent stem cells, which were subject to the dynamic culture conditions, were expanded up to about 26-

folds in cell number within 11 days of culture in spinner flasks when grown in a suspension culture with cell clumps, or up to about 50-folds in cell number within 11 days of culture in spinner flasks when grown in a suspension culture as single cells devoid of cell clumps. In addition, the pluripotent stem cells were expanded up to about 64-folds in cell number within 5 days of culture in the controlled bioreactor when grown as single cells (Figures 5A-C and data not shown). These results demonstrate that the novel culture media of some embodiments of the invention is capable of supporting pluripotent cell expansion when cultured in suspension under dynamic conditions.

EXAMPLE 6

PLURIPOTENT STEM CELLS CULTURED IN SUSPENSION RECOVER WELL FROM FREEZE/THAW CYCLES

To test the ability of the pluripotent stem cells cultured in suspension in the presence of the novel culture media of some embodiments of the invention to recover from re-freeze/thaw cycles, the cells were frozen in liquid nitrogen by using the following freezing solutions:

(1) 10% DMSO (Sigma), 10% FBS (HyClone), 10% SR (Invitrogen cooperation), 70% DMEM.

(2) 5% DMSO, 10% FBS, 10% SR, 75% DMEM

(3) 10% DMSO, 90% SR

(4) 5% DMSO, 95% SR

(5) Commercial serum free freezing solution (Biological Industries, Beit HaEmek, Israel)

The frozen cells were initially frozen at -80°C refrigerator, and after 12 hours to three days, were transferred to liquid nitrogen tank for storage.

Experimental Results

The pluripotent stem cells were subject to freezing conditions using the above described freezing solution, and then were thawed, and re-cultured in suspension. Figures 6A-C demonstrate C2 cells cultured for 48 passages in suspension with cmrb100p medium after thawing using three different freezing solutions.

EXAMPLE 7

GENERATION OF LINEAGE SPECIFIC CELLS FROM THE PLURIPOTENT STEM CELLS

Differentiation into neuronal cells - Cells from the four tested cell lines (I3, I4, I6 and H9.2) were cultured in suspension with cell clump for at least 25 passages. Then, the factors were removed from the culture medium and 5 X 10^{-5} M Retinoic acid was added for 21 Days. The cells were then transferred to fibronectin coated plates and cultured for additional 5 days before harvesting the cells for analysis. Quantitative RT-PCR, immunostainings (immuno-fluorescence and FACS) were conducted and the results show expression of genes of the neuronal cell lineage such as PAX6, HNF, nestin, β-tubulin and PSA-NCAM (Figures 7A-C, 8A-B, 9A-G).

Differentiation into endodermal cells - Cells cultured in suspension with cell clumps from C2 cell line (iPS cell line derived from foreskin fibroblast) were cultured in suspension for at least 10 passages. Then the factors were removed from the culture medium and the cells were exposed to 10 ng/ml Activin for 48 hours, in medium containing cAMP increasers such as foreskulin, 8-bromocAMP, GABA, IBMX and DBC. Ten days later the cells were analyzed for endodermal markers. Quantitative RT-PCR for Sox17 demonstrate significant increase in Sox17 expression in treated cells in compare to non-treated controls (Data not shown). As shown in Figures 10A-B the differentiated cells express PDX1, a transcription factor indicating differentiation into endoderm lineage, mainly into β-cells.

Differentiation into mesenchymal stem cells (MSCs) - Cells cultured in suspension with cell clumps in suspension were transferred to serum containing medium for 14 days and then plated on ether gelatin or Matrigel. 7-14 days later the resulted MSCs were either frozen or passage while using trypsin.

EXAMPLE 8

CHARACTERIZATION OF THE EXPRESSION PATTERN OF HUMAN PLURIPOTENT EMBRYONIC STEM CELLS WHICH ARE CULTURED IN A SUSPENSION CULTURE AS SINGLE CELLS

Study design to characterize the novel hESCs which are cultured in a suspension culture as single cells

Three groups of cultured pluripotent stem cells (PSCs) were tested:

1. hESCs cultured with MEFs in two dimensions standard conditions (2D).
2. hESCs cultured as clump (spheroid, more then 200 cells) in suspension (3D)
3. hESCs cultured as single cells (SC, less than 50 cells, most of them as single cells) in suspension (3D).

The cells were tested for expression of pluripotency markers using flow cytometry after culturing of at least 15 passages in the above conditions.

Experimental Results

Human ESCs which are cultured in a suspension culture as single cells exhibit a unique expression pattern similar to that of the "naïve" mouse ESCs – As shown in Figures 12A-J, FACS analyses of pluripotent stem cells cultured in suspension as single cells demonstrate an altered expression pattern as compared to hESCs cultured in 2-D or in a suspension culture as cell clumps. Thus, while the majority of hESCs which are cultured on 2-D or in a suspension culture as cell clumps express the TRA1-60 (Figures 12A, 12C), TRA1-81 (Figures 12B, 12D) and SSEA4 (Figure 12H) markers of pluripotency, the majority of the pluripotent hESCs which are cultured in a suspension culture as single cells do not express the TRA1-60 (Figure 12E), TRA1-81 (Figure 12F) and SSEA4 (Figure 12J) markers. In contrast, while only 11% of the hESCs which are cultured on 2-D or in a suspension culture as cell clumps express SSEA1 (Figure 12G), the majority of the hESCs which are cultured in a suspension culture as single cells express SSEA1 (Figure 12I). Thus, hESCs that were cultured in suspension as single cells exhibit a modified expression pattern as compared to hESCs cultured on 2-D or in a suspension culture as cell clumps. Such an expression pattern resembles that of the more "Naïve" mouse ESCs cells, which do not express TRA1-60, TRA1-81 and SSEA4, but which do express SSEA1.

Table 3, hereinbelow summarizes the results of the FACS analyses.

Table 3

Expression pattern of human pluripotent stem cells under various culturing conditions

	SSEA4	*TRA60*	*TRA81*	*SSEA1*
2D	+	+	+	-
Clumps 3D	+	+	+	-
Single cells 3D	-	-	-	+

Table 3. Provided are the expression signatures of the various pluripotent stem cells.

Cells cultured in suspension as single cells exhibit increased levels of OCT-4 – Real time RT-PCR analysis was performed on hESCs cultured in 2-D, a suspension culture as cell clumps or in a suspension culture as single cells using the primers listed in Table 2 in "General Materials and Experimental Methods" hereinabove. As shown in Figure 13A the expression levels of Nanog is slightly decreased in a single cell suspension culture as compared to hESCs grown in 2-D. On the other hand, OCT4 expression was found to be increased by about 8 folds in hESCs cultured in suspension as SC as compared to hESCs cultured in 2D.

EXAMPLE 9

CHARACTERIZATION OF THE CLONING EFFICIENCY OF HUMAN PLURIPOTENT EMBRYONIC STEM CELLS WHICH ARE CULTURED IN A SUSPENSION CULTURE AS SINGLE CELLS

Experimental Results

Human ESCs which are cultured in suspension as single cells or hESCs which were cultured in 2-D were tested for their cloning efficiency. Cells which were cultured in 2-D were trypsinized and plated as single cells, each in a single well of a 96-well plate covered with MEFs (as described under "General Materials and Experimental Methods" hereinabove), and cells which were grown as single cells in suspension were plated each in a single well of a low-adhesive 96-well plate (as described under General Materials and Experimental Methods" hereinabove).

Human ESCs which are cultured in suspension as single cells exhibit a significantly higher cloning efficiency as compared to hESCs cultured on 2-D - As shown in Table 4, significantly higher cloning efficiency was observed for hESCs cultured in suspension as single cells (95.63%) compared to hESCs cultured on 2-D (4.33%). In addition, while the addition of the ROCK inhibitor increased the cloning efficiency of hESCs cultured on 2-D, the cloning efficiency of hESCs cultured in suspension as single cells was not increased in the presence of the ROCK inhibitor.

Table 4

Cloning efficiency of hESCs under various culture conditions

Culturing conditions	*Cloning efficiency %*
2D + trypsin	4.33
2D + trypsin + RoCK inhibitor	17.7
3D (single cells devoid of cell clumps) without trypsin	95.63
3D (single cells devoid of cell clumps) without trypsin but with RoCK inhibitor	87

Table 4. Provided are the percentage of cell cloning obtained under the various culturing conditions.

Human ESCs which are cultured in suspension as single cells exhibit higher survival to freezing and thawing cycles as compared to hESCs cultured on 2-D - In order to test the ability of the pluripotent stem cells to survive freezing and thawing cycles the hESCs (which were cultured in suspension as single cells) were subjected to a freezing cycle using any of the following freezing solutions:

I. Serum and animal freezing solution (Biological Industries).

II. DMEM supplemented by 10% DMSO and 20% FBS.

III. DMEM supplemented by 10% DMSO and 30% SR (serum replacement).

After freezing for about 1-7 days at -80°C degrees the vials were transferred to liquid nitrogen. The cells were thawed, and the viability was tested by tripan blue staining. The survival of hESCs to the freezing-thawing cycle was about 80% for hESCs cultured in suspension as single cells, which is significantly higher than the

survival of hESCs which are cultured on 2-D to a freezing-thawing cycle under identical assay conditions (up to 50%, data not shown). Figure 15 is a representative image of human ESCs cultured as single cells in a suspension culture after a freezing-thawing cycle.

Human ESCs which are cultured in suspension as single cells exhibit higher survival and efficiency of genetic manipulations as compared to hESCs cultured on 2-D - Cells were transfected using the CMV promoter-GFP nucleic acid construct (based on N1 plasmid) as described under *"GENERAL MATERIALS AND EXPERIMENTAL METHODS"*. Following the genetic manipulation, the survival of the cells was evaluated using phase contrast microscopy. As is shown in Figure 16A, more than 90% of the suspended single cells survived the procedure. In contrast, from the 2D cells cultured with MEFs, only up to 17 cells (of out of 10^7 cells) recovered (data not shown). Moreover, while none of the hESCs that were cultured on 2-D were green (data not shown), a few of the hESCs cultured in 3-D as single cells were green, *i.e.,* expressed the transgene CMV-GFP construct (Figure 16B).

EXAMPLE 10

HUMAN PLURIPOTENT EMBRYONIC STEM CELLS WHICH ARE CULTURED IN A SUSPENSION CULTURE AS SINGLE CELLS ARE CAPABLE OF DIFFERENTIATION INTO NEURAL CELL LINEAGE

Experimental Results

Human ESCs which are cultured in suspension as single cells are capable of differentiating into the neuronal cell linage - To induce neural differentiation, hESCs which are cultured in suspension as single cells were transferred to a neuronal differentiating medium (without bFGF and the IL6RIL6 chimera) which included either retinoic acid (10^{-3} M) or Noggin (10 ngr/ml) as described under "General Materials and Experimental Methods" hereinabove. Differentiation was induced in either 2-D by plating on human plasma fibronectin (HPF)-coated plates (at a concentration of 50 μgr per 10 cm^2 HPF) or in a suspension culture. Three weeks after differentiation induction, the cells were plated for staining with fibronectin, O4, GFAP, nestin and β-tubulin. As show in Figures 17A-C, the cells differentiated into neuronal progenitor cells which

were positively stained with GFAP (Glial fibrillary acidic protein), a marker of astrocytes, O4, a marker of oligodendrocytes, and β-Tubulin and Nestin, markers of neurons. These results conclusively show that hESCs which are cultured in suspension as single cells are capable of differentiating into the ectoderm embryonic germ layer.

EXAMPLE 11

A NOVEL METHOD FOR DIFFERENTIATING MESENCHYMAL STEM CELLS IN SUSPENSION

The present inventors have developed a novel method for differentiating pluripotent stem cells into mesenchymal stem cells in suspension, as follows.

To induce differentiation to MSCs, single cells cultured in suspension were transferred gradually to one of the following media:

(1) Fy enriched; consisting of 80% DMEM/F12 (Biological Industries, Beit Haemek, Israel), containing 10% knockout serum replacement (SR), 10% FBS (HyClone or Biological Industries) 2 mM L-glutamine, 0.1 mM β–mercaptoethanol, 1% non-essential amino acid stock (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated).

(2) MeSus I: consisting of 80% DMEM (Biological Industries, Beit Haemek, Israel), containing 20% FBS (HyClone or Biological Industries) 2 mM L-glutamine, (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated).

(3) MeSus II: consisting of 80% αMEM (Biological Industries, Beit Haemek, Israel), containing 20% FBS (HyClone or Biological Industries) 2 mM L-glutamine, (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated).

(4) MeSus III: consisting of DMEM/F12 (Biological Industries, Beit Haemek, Israel), 1% ITS (Invitrogen) 2 mM L-glutamine, (all from Invitrogen Corporation products, Grand Island NY, USA, unless otherwise indicated).

Human ESCs which were grown in a suspension culture as single cells were transferred to the MSC differentiation medium gradually using any one of the following methods:

I. (i) 25% differentiation medium 75% pCM100F for one passage; (ii) 50% differentiation medium 50% pCM100F for one passage; (iii) 75% differentiation medium 25% pCM100F for one passage; (iv) 100% differentiation medium.

II. (i) 50% differentiation medium 50% pCM100F for one passage; (ii) 75% differentiation medium 25% pCM100F for one passage; (iii) 100% differentiation medium

III. (i) 50% differentiation medium 50% pCM100F for one passage; (ii) 100% differentiation medium.

All of the above described media and transfer methods resulted in efficient differentiation into MSCs.

The cells were then cultured in suspension (Petri dish, Spinner flasks and/or bio-reactors) and passage every 5-10 days by pipette. After the cells were cultured for at least one passage with the differentiation medium, MSCs features were tested. Figures 19A-B depict images of MSCs which were differentiated from PSCs cultured in suspension as single cells for at least 10 passages. When cells were re-plated on Gelatin they demonstrate typical MSCs morphology. Figure 19A shows the CL1 cells that were differentiated in the Fy enriched medium, and Figure 19B shows the CL1 cells that were differentiated in the MeSusII medium.

In order to enrich the MSCs population, magnetic-activated cell sorting (MACS) was employed using an anti- CD73 antibody (Milteniy) according to manufacturer instructions. The CD73-MACS resulted in enrichment of the MSCs from about 40% CD73-positive cells to more than 80% CD73-positive cells.

The MSCs, which were generated by differentiation of hESCs that were cultured in suspension as single cells, exhibit typical MSC expression pattern - As shown in Figures 18A-C, FACS analyses show that when the cells were grown in an animal-free medium, 82.5% of the MSC are CD73-positive and only 4.83% are CD31-positive. In addition, when the MSCs are grown in a serum-containing medium, 99.3% are CD105-positive.

Differentiation of suspension MSCs into an adipogenic cell lineage – The MSCs in suspension were subjected to a differentiation protocol towards the adipogenic lineage on either a 2-D culture system or in a suspension culture, as described in "General Materials and Experimental Methods" hereinabove. Briefly MSC were seeded

in density of 20,000 cell/cm^2 in 6 well plates or in a concentration of 1x10^6-5x10^6 cells/ml in a suspension culture and grown in the presence of the adipogenic medium for 4 weeks with medium changes twice a week. As shown in Figure 19D, MSCs (which were generated by differentiation of hESCs that were cultured in suspension as single cells) were capable of differentiation into the adipogenic cell lineage, exhibiting lipid– rich vacuoles within the cells.

Differentiation of suspension MSCs into an osteogenic cell lineage – The MSCs in suspension were subjected to a differentiation protocol towards the osteogenic lineage on either a 2-D culture system or in a suspension culture, as described in "General Materials and Experimental Methods" hereinabove. Briefly MSC were seeded in density of 2000-3000 cell/cm^2 in 6 well plate, or in a concentration of 1x10^6-5x10^6/ml in a suspension culture and grown in the presence of the osteogenic medium for 4 weeks with medium changes twice a week. As shown in Figure 19C, MSCs (which were generated by differentiation of hESCs that were cultured in suspension as single cells) were capable of differentiation into the osteogenic cell lineage, exhibiting mineralized cells, detected by Alizarin red staining.

Differentiation of suspension MSCs into a chondrogenic cell lineage – The MSCs in suspension were subjected to a differentiation protocol towards the chondrogenic lineage on either a 2-D culture system or in a suspension culture, as described in "General Materials and Experimental Methods" hereinabove. Briefly 2x10^5 MSC were centrifuged at 300 g for 5 minutes in 15 ml polypropylene falcon tubes to form a cell pellet. The cells were grown in chondrogenic medium as a pellet in a tube for 9 weeks with medium changes twice a week without disturbing the cell mass. Cell sections were made after fixing the cell pellets with 4% PFA and embedding it in low melting agarose (1.5%). The cells were stained with Alcian blue, which stains the matrix of chondrocytes of the chondrogenic cell lineage (data not shown), demonstrating the ability of the MSCs to differentiate into the chondrogenic cell lineage.

EXAMPLE 12

HUMAN PLURIPOTENT EMBRYONIC STEM CELLS WHICH ARE CULTURED IN A SUSPENSION CULTURE AS SINGLE CELLS ARE CAPABLE OF DIFFERENTIATION INTO THE ENDODERM CELL LINEAGE

Experimental Results

C2 cells were cultured for more than 10 passages as single cells in suspension in the pCM100F culture medium. For endoderm differentiation, the bFGF and the IL6RIL6 chimera were removed from the culture medium and activin A at concentration of 10 ng/ml was added for 48 hours in a suspension culture. 10 days after exposure to activin A, the cells were plated on Matrigel™ or HFF (human foreskin fibroblast) matrix, or were cultured in a 3-dimensional culture system (in suspension) and were stained to PDX1. When levels of expression of the SOX 17 gene were tested by real time PCR, an increase could be observed during differentiation from day 2 to 10 after exposure to activin A (data not shown). Figures 20A-B show the expression of PDX1 in the cells, demonstrating differentiation into endodermal cells.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

REFERENCES

(ADDITIONAL REFERENCES ARE CITED IN TEXT)

Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, and Thomson JA. "Clonally derived human embryonic stem cells lines maintain pluripotency and proliferative potential for prolonged periods of culture". Dev Biol 227:271-278, 2000.

Amit, M., Shariki, K., Margulets, V., & Itskovitz-Eldor, J. (2004). Feeder and serum-free culture system for human embryonic stem cells. Biol. Reprod. 70, 837-845.

Aoi T, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, Yamanaka S. Generation of Pluripotent Stem Cells from Adult Mouse Liver and Stomach Cells. Science. 2008.

Bhattacharya, B. et al. (2004). Gene expression in human embryonic stem cell lines: unique molecular signature. Blood 103, 2956-2964.

Germanguz I, Sedan O, Zeevi-Levin N, Shtrichman R, Barak E, Ziskind A, Eliyahu S, Meiry G, Amit M, Itskovitz-Eldor J, Binah O. Molecular characterization and functional properties of cardiomyocytes derived from human inducible pluripotent stem cells. J Cell Mol Med. 2009 Dec 11. [Epub ahead of print].

Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science. 2007, 318(5858):1920-1923.

Hanna J, Markoulaki S, Schorderet P, Carey BW, Beard C, Wernig M, Creyghton MP, Steine EJ, Cassady JP, Foreman R, Lengner CJ, Dausman JA, Jaenisch R. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell. 2008,133(2):250-264.

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., Benvenisty, N. (2000). Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Mol. Med. 6, 88-95.

King, T.D., Gandy, J.C. & Bijur, G.N. (2006). The protein phosphatase-1/inhibitor-2 complex differentially regulates GSK3 dephosphorylation and increases

sarcoplasmic/ endoplasmic reticulum calcium ATPase 2 levels. Exp. Cell Res. 312, 3693-3700.

Kristensen, D.M., Kalisz, M., & Nielsen, J.H. (2005). Cytokine signalling in embryonic stem cells. APMIS. 113, 756-772.

Lowry WE, Richter L, Yachechko R, Pyle AD, Tchieu J, Sridharan R, Clark AT, Plath K. Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc Natl Acad Sci U S A, 2008, 105(8):2883-2888.

Meissner A, Wernig M, Jaenisch R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol. 2007, 25(10):1177-1181.

Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol. 2008, 26(1):101-106.

Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors.Nature. 2008, 451(7175):141-146.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006, 126(4):663-676.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007, 131(5):861-872.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S/, Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282, 1145-1147.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., Hearn, J.P. (1995). Isolation of a primate embryonic stem cell line. Proc. Natl. Acad. Sci. USA. 92, 7844-7848.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Hearn, J.P. (1995). Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. Biol. Reprod. 55, 254-259.

Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, Thomson JA. Human induced pluripotent stem cells free of vector and transgene sequences. Science. 2009, 324(5928):797-801.

WHAT IS CLAIMED IS:

1. A method of generating mesenchymal stem cells in a suspension culture, comprising culturing human pluripotent stem cells under conditions suitable for differentiation of said human pluripotent stem cells to the mesenchymal stem cells, wherein said human pluripotent stem cells comprise at least 50% human pluripotent stem cells characterized by an endogenous $OCT4^{+}$/TRA1-60^{-}/TRA1-81^{-}/$SSEA1^{+}$/$SSEA4^{-}$ expression signature and being capable of differentiating into endoderm, ectoderm and mesoderm embryonic germ layers in a suspension culture,

thereby generating the mesenchymal stem cells in the suspension culture.

2. The method of claim 1, wherein said conditions comprise culturing the human pluripotent stem cells in a suspension culture in the presence of a differentiation culture medium suitable for differentiation of said human pluripotent stem cells to the mesenchymal stem cells.

3. The method of claim 2, wherein said culturing comprises a gradual transfer of the human pluripotent stem cells from a culture medium supporting expansion of said human pluripotent stem cells in an undifferentiation state into said differentiation culture medium.

4. The method of claim 3, wherein said gradual transfer comprises:

(i) culturing for one passage in a medium comprising 25% differentiation medium and 75% of said culture medium supporting expansion of said human pluripotent stem cells in said undifferentiation state;

(ii) culturing for one passage in a medium comprising 50% differentiation medium and 50% of said culture medium supporting expansion of said human pluripotent stem cells in said undifferentiation state;

(iii) culturing for one passage in a medium comprising 75% differentiation medium and 25% of said culture medium supporting expansion of said human pluripotent stem cells in said undifferentiation state; and

(iv) culturing the cells in a differentiation medium.

5. The method of claim 4, wherein said culture medium supporting expansion of said human pluripotent stem cells in said undifferentiation state comprises a culture medium which comprises interleukin 11 (IL11) and Ciliay Neurotrophic Factor (CNTF).

6. The method of claim 4, wherein said culture medium supporting expansion of said human pluripotent stem cells in said undifferentiation state comprises a culture medium which comprises basic fibroblast growth factor (bFGF) at a concentration of at least 50 ng/ml and an IL6RIL6 chimera.

7. The method of claim 4, wherein said culture medium supporting expansion of said human pluripotent stem cells in said undifferentiation state comprises a culture medium which comprises an animal contaminant-free serum replacement and an IL6RIL6 chimera.

8. The method of claim 4, wherein said culture medium supporting expansion of said human pluripotent stem cells in said undifferentiation state comprises a serum-free culture medium which comprises a soluble interleukin 6 receptor (sIL6R) and interleukin 6 (IL6), wherein a concentration of said sIL6R is at least 5 ng/ml, and wherein a concentration of said IL6 is at least 3 ng/ml.

9. The method of claim 4, wherein said culture medium supporting expansion of said human pluripotent stem cells in said undifferentiation state comprises a culture medium which comprises interleukin 11 (IL11) and oncostatin.

10. The method of claim 4, wherein said culture medium supporting expansion of said human pluripotent stem cells in said undifferentiation state comprises a culture medium which comprises replacement, basic fibroblast growth factor (bFGF) and 100 pg/ml of an IL6RIL6 chimera.

11. The method of any one of claims 2-10, wherein said differentiation culture medium comprises serum or serum replacement.

12. The method of any one of claims 2-10, wherein said differentiation culture medium comprises serum and serum replacement.

13. The method of claim 11 or 12, wherein said differentiation culture medium further comprises L-glutamine, beta-mercaptoethanol, and non-essential amino acids.

14. The method of any one of claims 1-13, wherein said culturing said cells is performed under conditions devoid of substrate adherence.

15. A method of generating a neuronal progenitor cell in a suspension culture, comprising culturing human pluripotent stem cells under conditions suitable for differentiation of said human pluripotent stem cells to neuronal progenitor cell, wherein said human pluripotent stem cells comprise at least 50% human pluripotent stem cells characterized by an endogenous $OCT4^{+}/TRA1\text{-}60^{-}/TRA1\text{-}81^{-}/SSEA1^{+}/SSEA4^{-}$ expression signature and being capable of differentiating into endoderm, ectoderm and mesoderm embryonic germ layers in a suspension culture,

thereby generating the neuronal progenitor cell in the suspension culture.

16. The method of claim 15, wherein said conditions comprise culturing the human pluripotent stem cells in a suspension culture in the presence of a differentiation culture medium suitable for differentiation of said human pluripotent stem cells to the neuronal progenitor cell.

17. The method of claim 16, wherein said differentiation culture medium suitable for differentiation of said human pluripotent stem cells to the neuronal progenitor cell comprises retinoic acid.

18. The method of claim 16, wherein said differentiation culture medium suitable for differentiation of said human pluripotent stem cells to the neuronal progenitor cell comprises Noggin.

19. The method of any one of claims 16-18, wherein said neuronal progenitor cells expressing Glial fibrillary acidic protein (GFAP), O4, β-Tubulin and/or Nestin.

20. A method of generating an endodermal cell in a suspension culture, comprising culturing human pluripotent stem cells under conditions suitable for differentiation of said human pluripotent stem cells to an endodermal cell lineage, wherein said human pluripotent stem cells comprise at least 50% human pluripotent stem cells characterized by an endogenous $OCT4^{+}/TRA1\text{-}60^{-}/TRA1\text{-}81^{-}/SSEA1^{+}/SSEA4^{-}$ expression signature and being capable of differentiating into endoderm, ectoderm and mesoderm embryonic germ layers in a suspension culture,

thereby generating the endodermal cell in the suspension.

21. The method of claim 20, wherein said conditions comprise culturing the human pluripotent stem cells in a suspension culture in the presence of a differentiation culture medium suitable for differentiation of said human pluripotent stem cells to the endodermal cell.

22. The method of claim 21, wherein said differentiation culture medium suitable for differentiation of said human pluripotent stem cells to the endodermal cell comprises Activin.

23. The method of any of claims 20 to 22, wherein said endodermal cell expresses PDX1.

24. The method of any of claims 20 to 22, wherein said endodermal cell expresses SOX17.

25. A method of generating lineage-specific cells from human pluripotent stem cells, the method comprising:

(a) culturing the human pluripotent stem cells in a suspension culture, wherein said human pluripotent stem cells comprise at least 50% human pluripotent stem cells characterized by an endogenous $OCT4^{+}/TRA1\text{-}60^{-}/TRA1\text{-}81^{-}/SSEA1^{+}/SSEA4^{-}$ expression

signature and being capable of differentiating into endoderm, ectoderm and mesoderm embryonic germ layers in a suspension culture, to thereby obtain expanded, undifferentiated pluripotent stem cells devoid of clumps; and

(b) subjecting said expanded, undifferentiated pluripotent stem cells devoid of clumps to culturing conditions suitable for differentiating and/or expanding lineage specific cells,

thereby generating lineage-specific cells from human pluripotent stem cells.