MEDIA FOR CULTURING STEM CELLS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to culture media, cell cultures and methods of culturing stem cells such as under defined and xeno-free culturing conditions.

Human Embryonic stem cells (hESCs) were traditionally cultured and derived using the conventional methods employed for mouse ESCs, *i.e.*, in the presence of a medium supplemented with fetal bovine serum (FBS) and feeder-layers consisting of inactivated mouse embryonic fibroblasts (MEFs) (Thomson et al, 1998). However, for use in cell-based therapy, hESC cultures should be well-defined and xeno-free (*i.e.*, devoid of any animal contaminant) in terms of culture components. In recent years, extensive investigation into improving the culture systems for hESCs has yielded the following advances: the ability to grow cells in serum-free conditions (Amit et al, 2000); maintenance of the cells in an undifferentiated state on a Matrigel™ matrix with 100 % MEF-conditioned medium (Xu et al, 2001); and the use of either human embryonic fibroblasts, adult Fallopian tube epithelium (Richards et al, 2002) or foreskin fibroblasts (Amit et al, 2003; Hovatta et al, 2003) as feeder layers. However, while the use of MEF-conditioned medium or a Matrigel™ matrix (which contains components from animal cells) may expose the hESCs to animal pathogens, the batch-specific variations may affect the quality of the culture. On the other hand, although human feeder-layer-based culture systems are xeno-free, they require the simultaneous growth of both feeder cell layers and hESCs, which limits the potential of large-scale culturing of hESCs. Moreover, culture systems based on feeder cells or conditioned medium are not well-defined and thus cannot be accurately repeated due to differences between the various lines of feeder-cells.

To overcome such limitations, attempts have been made to culture hESCs in feeder-layer-free culture systems devoid of conditioned medium. Xu C., et al. (Stem Cells, 2005, 23:315-23) developed a culture system based on a Matrigel™ matrix and a medium supplemented with serum replacement™ (SR), basic fibroblast growth factor (bFGF), with or without the addition of the Flt-3 ligand to the culture medium. However, under these conditions, the background differentiation of the ESCs was 20 or 28 %, respectively, which is higher than observed for hESCs when cultured on

MEFs. Another culturing system based a Matrigel™ matrix and a medium supplemented with bFGF and Noggin, an antagonist of bone morphogenetic proteins (BMPs), resulted in a background differentiation of 10 % (Xu RH., et al., 2005, Nat Methods. 2: 185-190). However, since both of these systems rely on Matrigel™ as a culturing matrix, their use for cell-based therapy is limited. To avoid animal contaminants, the present inventors have previously developed a culture system based on a fibronectin matrix and a medium supplemented with 20 % SR, transforming growth factor β1 ($TGF\beta_1$) and bFGF (Amit et al, 2004). Under these conditions, the cells maintained hESC features for more than 32 passages. A further step towards defined culture conditions for hESCs culture was recently achieved by Ludwig and colleague (Ludwig et al, 2006) when using a matrix consisted of the combination of human collagen IV, fibronectin, laminin and vitronectin and a medium supplemented with human serum albumin, bFGF and $TGF\beta_1$. Such conditions enabled the derivation and culturing of hESCs under defined and feeder layer-free culture conditions. However, hESCs cultured in these conditions exhibited chromosomal instability of the cells following extended periods in culture. For example, one of the isolated hESC lines was reported to harbor a karyotype of 47,XXY after 4 months of continuous culturing and a second line, which was initially normal, converted to trisomy 12 between 4 and 7 months of culturing. Thus, improvements of the feeder-free, xeno-free culturing systems of hESCs are highly needed.

Recent studies discussed the possible involvement of several intracellular transduction pathways in hESC renewal and maintenance of "stemness" identity, but the mechanism underlining hESC self-maintenance is still unrevealed. Sato and colleagues (Sato et al, 2004) suggested that the Wnt pathway is involved in hESC self-renewal. A later publication by the same group indicates that the TGFβ pathway plays a crucial role in cell-fate determination and holds interconnections with the Wnt signaling pathway in maintaining hESC features (James, D., et al., 2005). These results are consistent with the feeder layer-free culture method suggested by the present inventors (Amit et al, 2004), which is based on the addition of $TGF\beta_1$, bFGF and/or LIF to a culture medium which includes serum or serum replacement. In addition, the mechanism by which bFGF involves in hESC' self-maintenance has yet to be proven. Another candidate for the role of maintaining hESC properties is

Noggin - an inhibitor of the BMPs signaling pathway (Xu RH., et al, 2005). However, to date, not Noggin itself or any Noggin analog were found in MEF-conditioned media.

Mouse ESCs can be continuously cultured without feeder layers provided that leukemia inhibitory factor (LIF) is added to the culture medium. However, accumulating data regarding hESCs suggest that LIF has no effect on preventing hESC differentiation (Thomson et al, 1998; Reubinof et al, 2000). In addition, activation of key proteins of the LIF cellular pathway, such as signal transducer and activator of transcription 3 (STAT3) was found to be weak or absent in hESCs (Daheron et al, 2004; Humphrey et al, 2004; Sato et al, 2004). The gp130 receptor, which is activated by ligands such as LIF, interleukin 6 (IL-6) and a chimera made of IL-6 and its soluble IL6 receptor (the IL6RIL6 chimera; Chebath et al, 1997), was shown to positively affect the mouse ESCs self-maintenance via STAT3 (Williams et al, 1988; Niwa et al, 1998; Smith et al, 1988). In hematopoietic stem cells, the IL6RIL6 chimera exhibited a much higher affinity for human gp130 and was found to be more potent in increasing proliferation of progenitor cells than the mixture of IL-6 and the soluble IL6 receptor (Kollet et al, 1999). On the other hand, the IL6RIL6 chimera induced differentiation of ESC-derived oligodendrocyte precursors (Zhang PL., et al., 2006, Mol. Cell Neurosci. 31: 387-398). In a recent study, Nichols et al., (1994) demonstrated that the IL6RIL6 chimera is capable of supporting mouse ESC culturing and derivation. On the other hand, Daheron et al. (2004) showed that although the LIFRβ and the signaling subunit gp130 are expressed in hESCs and that human LIF can induce STAT3 phosphorylation and nuclear translocation in hESCs, human LIF is unable to maintain the pluripotent state of hESCs. In addition, Humphrey et al. (2004) found that hESCs rapidly differentiate when cultured in a medium containing members of the IL-6 family of cytokines such as LIF, IL-6 or a complex of the soluble IL-6 receptor and IL-6 (the "hyper-IL-6") and concluded that maintenance of pluropotency in human ESCs is STAT independent. Thus, it is currently accepted that in contrast to mouse ESCs which can be maintained in the undifferentiated state in the presence of activators of the gp130 receptor, culturing of human ESCs in the presence of LIF, IL6 or the hyper-IL-6 results in differentiation of the hESCs.

There is thus a widely recognized need for, and it would be highly advantageous to have, a defined, xeno-free medium suitable for maintaining stable, undifferentiated and pluripotent hESCs devoid of the above limitations.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a culture medium being serum-free, xeno-free, feeder-free and protein carrier-free and capable of maintaining stem cells in an undifferentiated state.

According to another aspect of the present invention there is provided a cell culture comprising a stem cell and a culture medium, said culture medium being serum-free, xeno-free, feeder-free and protein carrier-free and capable of maintaining said stem cells in an undifferentiated state.

According to yet another aspect of the present invention there is provided a method of expanding and maintaining stem cells in an undifferentiated state, the method comprising culturing the stem cells in a culture medium being serum-free, xeno-free, feeder-free and protein carrier-free and capable of maintaining the stem cells in an undifferentiated state, thereby expanding and maintaining the stem cells in the undifferentiated state.

According to still another aspect of the present invention there is provided a method of deriving an embryonic stem cell line, the method comprising: (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 said embryonic stem cell in a culture medium being serum-free, xeno-free, feeder-free and protein carrier-free and capable of maintaining said embryonic stem cell in an undifferentiated state; thereby deriving the embryonic stem cell line.

According to an additional aspect of the present invention there is provided a method of generating lineage-specific cells from embryonic stem cells, the method comprising: (a) culturing the embryonic stem cells in a culture medium being serum-free, xeno-free, feeder-free and protein carrier-free and capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; (b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating and/or

expanding lineage specific cells; thereby generating the lineage-specific cells from the embryonic stem cells.

According to yet an additional aspect of the present invention there is provided a method of generating embryoid bodies from embryonic stem cells, the method comprising: (a) culturing the embryonic stem cells in a culture medium being serum-free, xeno-free, feeder-free and protein carrier-free and capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; and (b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating said embryonic stem cells to embryoid bodies; thereby generating the embryoid bodies from the embryonic stem cells.

According to still an additional aspect of the present invention there is provided a method of generating lineage-specific cells from embryonic stem cells, the method comprising: (a) culturing the embryonic stem cells in a culture medium being serum-free, xeno-free, feeder-free and protein carrier-free and capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; (b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating said expanded, undifferentiated embryonic stem cells to embryoid bodies; and (c) subjecting cells of said 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.

According to a further aspect of the present invention there is provided a culture medium comprising a TGFβ isoform and being devoid of serum, serum replacement and protein carrier, wherein the culture medium is capable of maintaining stem cells in an undifferentiated state.

According to yet a further aspect of the present invention there is provided a cell culture comprising a stem cell and a culture medium, said culture medium comprising a TGFβ isoform and being devoid of serum, serum replacement and protein carrier, wherein said culture medium is capable of maintaining said stem cell in an undifferentiated state.

According to still a further aspect of the present invention there is provided a method of expanding and maintaining stem cells in an undifferentiated state, the method comprising culturing the stem cells in a culture medium which comprises a TGFβ isoform and being devoid of serum, serum replacement and protein carrier, said culture medium is capable of maintaining the stem cells in an undifferentiated state, thereby expanding and maintaining the stem cells in the undifferentiated state.

According to still a further aspect of the present invention there is provided a method of deriving an embryonic stem cell line, the method comprising: (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 said embryonic stem cell in a culture medium which comprises a TGFβ isoform and being devoid of serum, serum replacement and protein carrier, said culture medium is capable of maintaining the embryonic stem cells in an undifferentiated state, thereby deriving the embryonic stem cell line.

According to still a further aspect of the present invention there is provided a method of generating lineage-specific cells from embryonic stem cells, the method comprising: (a) culturing the embryonic stem cells in a culture medium which comprises a TGFβ isoform and being devoid of serum, serum replacement and protein carrier, said culture medium is capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; and (b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating and/or expanding lineage specific cells; thereby generating the lineage-specific cells from the embryonic stem cells.

According to still a further aspect of the present invention there is provided a method of generating embryoid bodies from embryonic stem cells, the method comprising: (a) culturing the embryonic stem cells in a culture medium which comprises a TGFβ isoform and being devoid of serum, serum replacement and protein carrier, said culture medium is capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; and (b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating said embryonic stem cells to embryoid bodies; thereby generating the embryoid bodies from the embryonic stem cells.

According to still a further aspect of the present invention there is provided a method of generating lineage-specific cells from embryonic stem cells, the method comprising: (a) culturing the embryonic stem cells in a culture medium which comprises a TGFβ isoform and being devoid of serum, serum replacement and protein carrier, said culture medium is capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; (b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating said expanded, undifferentiated embryonic stem cells to embryoid bodies; and (c) subjecting cells of said 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.

According to still a further aspect of the present invention there is provided a culture medium comprising an IL6RIL6 chimera, wherein the culture medium is capable of maintaining human embryonic stem cells in an undifferentiated state.

According to still a further aspect of the present invention there is provided a cell culture comprising a human embryonic stem cell and a culture medium, said culture medium comprising an IL6RIL6 chimera, wherein said culture medium is capable of maintaining said human embryonic stem cell in an undifferentiated state.

According to still a further aspect of the present invention there is provided a method of expanding and maintaining human embryonic stem cells in an undifferentiated state, the method comprising culturing the human embryonic stem cells in a culture medium which comprises an IL6RIL6 chimera, said culture medium is capable of maintaining the human embryonic stem cells in an undifferentiated state, thereby expanding and maintaining the embryonic stem cells in the undifferentiated state.

According to still a further aspect of the present invention there is provided a method of deriving a human embryonic stem cell line, the method comprising: (a) obtaining a human embryonic stem cell from a pre-implantation stage blastocyst, post-implantation stage blastocyst and/or a genital tissue of a fetus; and (b) culturing said human embryonic stem cell in a culture medium which comprises an IL6RIL6 chimera, said culture medium is capable of maintaining the human embryonic stem cells in an undifferentiated state, thereby deriving the embryonic stem cell line.

According to still a further aspect of the present invention there is provided a method of generating lineage-specific cells from embryonic stem cells, the method comprising: (a) culturing the embryonic stem cells in a culture medium which comprises an IL6RIL6 chimera, said culture medium is capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; and (b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating and/or expanding lineage specific cells; thereby generating the lineage-specific cells from the embryonic stem cells.

According to still a further aspect of the present invention there is provided a method of generating embryoid bodies from embryonic stem cells, the method comprising: (a) culturing the embryonic stem cells in a culture medium which comprises an IL6RIL6 chimera, said culture medium is capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; and (b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating said embryonic stem cells to embryoid bodies; thereby generating the embryoid bodies from the embryonic stem cells.

According to still a further aspect of the present invention there is provided a method of generating lineage-specific cells from embryonic stem cells, the method comprising: (a) culturing the embryonic stem cells in a culture medium which comprises an IL6RIL6 chimera, said culture medium is capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; (b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating said expanded, undifferentiated embryonic stem cells to embryoid bodies; and (c) subjecting cells of said 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.

According to further features in preferred embodiments of the invention described below, the embryonic stem cells are human embryonic stem cells.

According to still further features in the described preferred embodiments the culture medium comprising a TGFβ isoform.

According to still further features in the described preferred embodiments the culture medium being serum replacement-free.

According to still further features in the described preferred embodiments the culture medium comprising IL6RIL6 chimera.

According to still further features in the described preferred embodiments the culture medium comprising IL6RIL6 chimera and whereas said stem cells are human embryonic stem cells.

According to still further features in the described preferred embodiments the culture medium comprising IL6RIL6 chimera and whereas said embryonic stem cells are human embryonic stem cells.

According to still further features in the described preferred embodiments the stem cells are embryonic stem cells.

According to still further features in the described preferred embodiments the culture medium is capable of expanding said stem cells in an undifferentiated state.

According to still further features in the described preferred embodiments the protein carrier is albumin.

According to still further features in the described preferred embodiments culturing is effected in suspension.

According to still further features in the described preferred embodiments the suspension is devoid of substrate adherence.

According to still further features in the described preferred embodiments culturing is effected on a feeder-layer free matrix.

According to still further features in the described preferred embodiments the feeder-layer free matrix is a fibronectin matrix.

According to still further features in the described preferred embodiments the culturing is effected on feeder cells.

According to still further features in the described preferred embodiments the culture medium is xeno-free.

According to still further features in the described preferred embodiments culturing is effected in xeno-free culturing conditions.

According to still further features in the described preferred embodiments the TGFβ isoform is a TGFβ isoform 1 ($TGF\beta_1$).

According to still further features in the described preferred embodiments the TGFβ isoform is a TGFβ isoform 3 ($TGF\beta_3$).

According to still further features in the described preferred embodiments the $TGF\beta_1$ is provided at a concentration of at least 0.06 ng/ml.

According to still further features in the described preferred embodiments the $TGF\beta_1$ is provided at a concentration of 0.12 ng/ml.

According to still further features in the described preferred embodiments the $TGF\beta_3$ is provided at a concentration of at least 0.5 ng/ml.

According to still further features in the described preferred embodiments the $TGF\beta_3$ is provided at a concentration of 2 ng/ml.

According to still further features in the described preferred embodiments the culture medium comprises basic fibroblast growth factor (bFGF).

According to still further features in the described preferred embodiments the bFGF is provided at a concentration of at least 2 ng/ml.

According to still further features in the described preferred embodiments the bFGF is provided at a concentration of at least 4 ng/ml.

According to still further features in the described preferred embodiments the IL6RIL6 chimera is provided at a concentration of at least 25 ng/ml.

According to still further features in the described preferred embodiments the culture medium comprises serum or serum replacement.

According to still further features in the described preferred embodiments the culture medium is devoid of serum or serum replacement.

According to still further features in the described preferred embodiments the serum or serum replacement is provided at a concentration of at least 10 %.

According to still further features in the described preferred embodiments the method further comprising isolating lineage specific cells following step (b).

According to still further features in the described preferred embodiments isolating lineage specific cells is effected by a mechanical separation of cells, tissues and/or tissue-like structures contained within said embryoid bodies.

According to still further features in the described preferred embodiments isolating lineage specific cells is effected by subjecting said embryoid bodies to differentiation factors to thereby induce differentiation of said embryoid bodies into lineage specific differentiated cells.

According to still further features in the described preferred embodiments the embryonic stem cell is a human embryonic stem cell.

According to still further features in the described preferred embodiments the embryonic stem cell is a primate embryonic stem cell.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a well-defined, xeno-free culture media which comprise a TGFβ isoform or the IL6RIL6 chimera, which are capable of mainataining stem cells in an undifferentiated state.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and 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 not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is 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 the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1a-f are photomicrographs depicting examples of the morphology of undifferentiated ESC colonies and ESC single cells grown in various feeder-free culture systems. Figures 1a-b - Undifferentiated I-3 colony cultured on a fibronectin-based feeder layer free culture system for 12 passages in the presence of a culture medium supplemented with 100 ng/ml of the IL6RIL6 chimera. Note the "auto-feeders" formed at the periphery of the colony (Figure 1a, arrow). Figure 1c - Undifferentiated I-3 colony cultured on a mouse laminin-based feeder layer free culture system for 7 passages in the presence of a culture medium supplemented with 100 ng/ml of the IL6RIL6 chimera. Note the absence of "auto-feeders". Figures 1d-f - undifferentiated I-4 colonies cultured on a fibronectin-based feeder free culture system for 28 passages in the presence of a culture medium supplemented with 100 ng/ml of the IL6RIL6 chimera. Size bar for Figures 1a, c, d, e, f - 20 μM; Size bar for Figure 1b - 50 μM.

FIG. 2 depicts a normal karyotype of an exemplary hESC. The karyotype was examined after 7 passages of culturing in the presence of a culture medium containing 300 ng/ml of the IL6RIL6 chimera on a fibronectin feeder-free culture system. Note the presence of a normal 46,XX karyotype. Normal karyotype was also detected when the hESCs were cultured for 7 passages in the presence of a culture medium containing 100 ng/ml of the IL6RIL6 chimera on a fibronectin feeder-free culture system (not shown). Repeated test at passage 23 was found to be normal (not shown). 40 metaphases were examined from each sample.

FIGs. 3a-c are photomicrographs depicting immunofluorescence staining of undifferentiated colonies stained with surface markers specific to the hESC undifferentiated stage. Undifferentiated I-4 hESCs were cultured on a fibronectin feeder-free culture system for 27 passages in the presence of 100 ng/ml of the IL6RIL6 chimera and were subjected to immunostatining with Tra-1-60 (Figure 3a), Oct 4 (Figure 3b) or SSEA-4 (Figure 3c). Size bar = 20 μM.

FIGs. 4a-l are RT-PCR analyses depicting the expression of representative genes of the undifferentiated stage and of the three embryonic germ layers in hESCs grown on human fibronectin feeder free culture system or in embryoid bodies (EBs) derived therefrom. Lane 1 - Cell line I-3 cultured for 12 passages in CM100 (100

ng/ml of the IL6RIL6 chimera). Lane 2 - Cell line I-3 cultured for 12 passages in 300 ng/ml of the IL6RIL6 chimera. Lane 3 - 10-day-old EBs derived from I-3 cells which were cultured for 10 passages in CM100. Lane 4 - EBs derived from I-3 cells which were cultured for 10 passages in 300 ng/ml of the IL6RIL6 chimera. Figure 4a - Oct4; Figure 4b - Sox2; Figure 4c - Rex1; Figure 4d - Cx43; Figure 4e - FGF4; Figure 4f – Albumin; Figure 4g – Glucagon; Figure 4h - β-Globulin; Figure 4i - Cardiac actin; Figure 4j - Flk1; Figure 4k - AC133; Figure 4l - NFH.

FIGs. 5a-d are photomicrographs depicting the morphology of undifferentiated hES colonies and hES single cells cultured in various culture systems in the presence of the TGFβ-containing culture media. Figure 5a - I4 hESCs cultured for 28 passages on a Matrigel™ matrix with the D1 medium; Figure 5b - I4 hESCs cultured for 9 passages on MEFs with the HA16 medium; Figure 5c - I4 hESCs cultured for 20 passages on foreskins fibroblasts with the D2 medium; Figure 5d - I4 hESCs cultured for 11 passages on a human fibronectin matrix with the D2 medium. Note the undifferentiated morphology after prolonged culturing with the unique TGFβ-containing media types. Magnifications are X 15 for Figures 5a-d.

FIGs. 6a-c are photomicrographs depicting undifferentiated colonies stained with surface markers specific to the hESC undifferentiated stage. I4 hESCs cultured for 36 passages on a Matrigel™ matrix with the medium D1 and stained with TRA-1-60 (Figure 6a), SSEA4 (Figure 6b) and TRA-1-81 (Figure 6c); Magnifications are X 20 for Figures 6a-c.

FIGs. 7a-b are photomicrographs depicting the derivation of a new hESC line under xeno-free conditions on foreskin fibroblasts using the HA16 medium. Figure 7a - the cultured embryo at first passage (p-1), arrow points at the inner cell mass (ICM); Figure 7b - the isolated ICM at passage 2 (p-2). Magnifications are X 20 for Figures 7a-b.

FIGs. 8a-c are photomicrograph depicting immunostaining of hESCs cultured for 3 passages in suspension in the presence of the D2 medium. Shown are immunostaining analyses of Oct4 (Figure 8a), TRA-1-60 (Figure 8b) and TRA-1-81 (Figure 8c); Magnifications are X 63 for Figures 8a-c.

FIGs. 9a-g are photomicrographs depicting histological sections and morphology of suspended hESCs culture. Figure 9a - Histology of a hESC clump (I4

hESC line) cultured for 3 passages in suspension in the presence of the D1 medium and stained with H&E. Note that the hESC clump is homogeneous, containing small cells with large nuclei typical for hESCs morphology. Figures 9b-c - I4 hESCs were cultured for 3 passages in suspension in the presence of the D2 medium and were then re-cultured on MEFs. Shown is the morphology of the colonies after re-culturing on MEFs using an inverted microscope. Note the typical undifferentiated morphology of the hESCs. Figures 9d-e - I4 hESCs were cultured for 16 passages in suspension in the presence of the CM100F medium and were then re-cultured on MEFs. Shown is the morphology of colonies after re-culturing on MEFs. Note the typical undifferentiated morphology of the hESCs. Figures 9f-g – I4 hESCs were cultured for 7 passages in suspension in the presence of the HA19 medium (Figure 9f) or for 10 passages in the presence of the CM100F medium (Figure 9g); Magnifications are X 20 for Figure 9a and X 15 for Figures 9b-g.

FIGs. 10a-d are RT-PCR analyses depicting the expression of representative genes of the undifferentiated state of hESCs cultured in suspension in the presence of the HACM100, CM100F or the HA19 medium. Lane 1 - I-4 hESCs cultured for 1 passage in suspension in the presence of the HACM100 medium (serum or serum replacement-free, IL6RIL6-containing medium). Lane 2 - I-4 hESCs cultured for 1 passage in suspension in the presence of the CM100F medium (IL6RIL6 and serum replacement-containing medium). Lane 3 - I-4 hESCs cultured for 7 passages in suspension in the presence of the HA19 medium (serum or serum replacement-free, protein carrier-free, TGFβ_3-containing medium). Lane 4 - I-4 hESCs cultured for 2 passages in suspension in the presence of the HA19 medium and then re-cultured on MEFs for additional 6 passages. Figure 10a – Oct4; Figure 10b – Rex1; Figure 10c – Sox2; Figure 10d – Nanog; RT mix were tested and found negative for all tested genes. All samples were tested for β–actin and were found evenly positive.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of well-defined, xeno-free culture media which comprise a TGFβ isoform or the IL6RIL6 chimera, which are capable of mainataining stem cells in an undifferentiated state. In addition, the present invention is of cell

cultures comprising the culture media and the stem cells and of methods of expanding and deriving embryonic stem cells in such well-defined, xeno-free culture media. Moreover, the present invention is of methods of differentiating ESCs or EBs formed therefrom for the generation of lineage specific cells. Specifically, the present invention can be used to generate highly reproducible, xeno-free cultures of hESCs which can be used for both cell-based therapy, pharmaceutical screening, identification of drug targets and cell-based compound delivery.

The principles and operation of the culture medium, cell culture and methods according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not 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. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Human embryonic stem cells (hESCs) are proliferative, undifferentiated, stem cells capable of differentiating into cells of all three embryonic germ layers. As such, hESCs are employed as a research model for early human development and hold promise for various applications including cell-based therapy, pharmaceutical screening, identification of drug targets and cell-based compound delivery which require almost indefinite amounts of proliferating, yet pluripotent hESCs. For use in human therapy and for the production of large amounts of hESCs, hESCs need to be cultured in complete animal-free (xeno-free), well-defined culture systems, where exposure to animal (e.g., retroviruses) or human pathogens and batch-dependent variations are avoided.

Attempts to achieve defined and/or xeno-free hESCs culture systems include the development of a culture system which is based on a fibronectin matrix and a medium supplemented with 20 % SR, transforming growth factor β_1 (TGFβ_1) and bFGF [Amit et al, 2004] or the culture system based on a matrix consisted of the combination of human collagen IV, fibronectin, laminin and vitronectin and a medium supplemented with human serum albumin, bFGF and TGFβ_1 (Ludwig et al,

2006). However, a major drawback of the latter culture system is the chromosomal instability of the cells associated with extended periods in culture. Thus, improvements of the feeder-free, xeno-free culturing systems of hESCs are highly desirable.

One attractive approach of achieving a defined, xeno-free culture system is using a culture medium which is based on isolated, recombinant factors. For example, mouse ESCs can be continuously cultured without feeder layers provided that leukemia inhibitory factor (LIF) is added to the culture medium. However, accumulating data regarding hESCs suggest that LIF has no effect on preventing hESC differentiation [Thomson et al, 1998; Reubinof et al, 2000]. A recombinant polypeptide, which includes the IL-6 and the soluble IL-6 receptor (the IL6RIL6 chimera, Chebath J, et al., 1997), was shown to support mouse ESC culturing and derivation (Nichols et al., 1994). However, a complex of the soluble hIL-6R and hIL- ("hyper IL6") failed to maintain human ESCs in the undifferentiated state when provided with a DMEM/serum replacement (DSR)/FGF2 medium on a laminin matrix (Humphrey et al, 2004). Thus, the common knowledge is that in contrast to mouse ESCs which can be maintained in the undifferentiated state in the presence of activators of the gp130 receptor, culturing of human ESCs in the presence of LIF, IL6 or the "hyper IL6" results in differentiation of the hESCs.

While reducing the present invention to practice, the present inventors have uncovered through laborious experimentations, a well-defined, xeno-free, serum or serum replacement-free and protein carrier-free culture medium which comprises a TGFβ isoform and which can be used for maintaining hESCs in a pluripotent and undifferentiated state. As is shown in Figures 5a-d and 6a-c and is described in Example 2 of the Examples section which follows, hESCs cultured on a human fibronectin matrix in the presence of a well-defined TGFβ-containing culture medium which is devoid of serum, serum replacement and a protein carrier (e.g., albumin) exhibited the morphology of undifferentiated ESC colonies and ESC single cells (Figures 5a-d) and expressed typical hESC surface markers specific to the undifferentiated state (Figures 6a-c). In addition, the present inventors have uncovered that the new TGFβ-containing culture medium is suitable for the

successful derivation of new hESC lines (Figures 7a-b, Example 3 of the Examples section which follows) on a complete xeno-free culture system.

Thus, according to one aspect of the present invention, there is provided a culture medium. The culture medium comprises a TGFβ isoform and being devoid of serum, serum replacement and protein carrier, wherein the culture medium is capable of maintaining stem cells in an undifferentiated state.

As used herein the phrase "culture medium" refers to a solid or a liquid substance used to support the growth of stem cells and maintain them in an undifferentiated state. Preferably, the phrase "culture medium" as used herein refers to a liquid substance capable of maintaining the stem cells in an undifferentiated state. The culture medium used by the present invention 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 stem cells in an undifferentiated state. For example, a culture medium according to this aspect of the present invention can be a synthetic tissue culture medium such as Ko-DMEM (Gibco-Invitrogen Corporation products, Grand Island, NY, USA), DMEM/F12 (Biological Industries, Biet Haemek, Israel), Mab ADCB medium (HyClone, Utah, USA) or DMEM/F12 (Biological Industries, Biet Haemek, Israel) supplemented with the necessary additives as is further described hereinunder. Preferably, all ingredients included in the culture medium of the present invention are substantially pure, with a tissue culture grade.

As used herein the phrase "stem cells" refers to cells which are capable of differentiating into other cell types or remaining in an undifferentiated state. Preferably, the phrase "stem cells" encompasses embryonic stem cells (ESCs), adult stem cells and hematopoietic stem cells.

The phrase "embryonic stem cells" refers to embryonic cells which are capable of differentiating into cells of all three embryonic germ layers *(i.e.*, endoderm, ectoderm and mesoderm), or remaining in an undifferentiated state. The phrase "embryonic stem cells" may comprise 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 to the present inventors] and embryonic germ (EG) cells which are obtained from the genital tissue of a fetus any time during gestation, preferably before 10 weeks of gestation. Adult stem cells (also called "tissue stem cells") include stem cells derived from any adult or fetal tissue such as adipose tissue, skin, kidney, liver, prostate, pancreas, intestine and bone marrow. Hematopoietic stem cells, which may also referred to as adult tissue stem cells, include stem cells obtained from blood or bone marrow tissue of an individual at any age or from cord blood of a newborn individual. Preferred stem cells according to this aspect of the present invention are embryonic stem cells, preferably of a human or primate (e.g., monkey) origin.

The embryonic stem cells of the present 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 (http://escr.nih.gov). Non-limiting

examples of commercially available embryonic stem cell lines are BG01, BG02, BG03, BG04, CY12, CY30, CY92, CY10, TE03 and TE32.

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.

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.

Adult tissue stem cells can be isolated using various methods known in the art such as those disclosed by Alison, M.R. [J Pathol. 2003 200(5): 547-50], Cai, J. et al., [Blood Cells Mol Dis. 2003 31(1): 18-27], Collins, A.T. et al., [J Cell Sci. 2001; 114(Pt 21): 3865-72], Potten, C. S. and Morris, R. J. [Epithelial stem cells *in vivo*. 1988. J. Cell Sci. Suppl. 10, 45-62], Dominici, M et al., [J. Biol. Regul. Homeost. Agents. 2001, 15: 28-37], Caplan and Haynesworth [U.S. Pat. No. 5,486,359] Jones E.A. et al., [Arthritis Rheum. 2002, 46(12): 3349-60]. Fetal stem cells can be isolated using various methods known in the art such as those disclosed by Eventov-Friedman S, et al., PLoS Med. 2006, 3: e215; Eventov-Friedman S, et al., Proc Natl Acad Sci U S A. 2005, 102: 2928-33; Dekel B, et al., 2003, Nat Med. 9: 53-60; and Dekel B, et al., 2002, J. Am. Soc. Nephrol. 13: 977-90. Hematopoietic stem cells can be isolated using various methods known in the arts such as those disclosed by "Handbook of Stem Cells" edit by Robert Lanze, Elsevier Academic Press, 2004, Chapter 54,

pp609-614, isolation and characterization of hematopoietic stem cells, by Gerald J Spangrude and William B Stayton.

It will be appreciated that stem cells in an undifferentiated state are of a distinct morphology, which is clearly distinguishable by the skilled in the art from that of differentiated cells of embryo or adult origin. Typically, undifferentiated stem cells have high nuclear/cytoplasmic ratios, prominent nucleoli and compact colony formation with poorly discernable cell junctions. Additional features of the undifferentiated state of the stem cells are further described hereinunder.

The culture medium according to this aspect of the present invention comprises a TGFβ isoform and being devoid of serum, serum replacement and protein carrier.

As used herein the phrase "TGFβ isoform" refers to any isoform of the transforming growth factor beta (β) including $TGF\beta_1$ (e.g., homo sapiens $TGF\beta_1$, GenBank Accession No. NP_000651), $TGF\beta_2$ (e.g., homo sapiens $TGF\beta_2$, GenBank Accession No. NP_003229) and $TGF\beta_3$ (e.g., homo sapiens $TGF\beta_3$, GenBank Accession No. NP_003230) which function 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 preferred embodiments of the present invention the TGFβ isoform which is included in the culture medium of the present invention is $TGF\beta_1$ or $TGF\beta_3$. Such TGFβ isoforms can be obtained from various commercial sources such as R&D Systems Minneapolis MN, USA.

As is shown in Example 2 of the Examples section which follows, the present inventors have used various culture media which contain $TGF\beta_1$ (e.g., the D1 medium which contains 0.12 ng/ml $TGF\beta_1$) or $TGF\beta_3$ (e.g., the D2 medium, the HA16 medium or the HA19 medium which contain 2 ng/ml $TGF\beta_3$) to successfully culture hESCs and maintain them in the undifferentiated state.

Preferably, $TGF\beta_1$ which is included in the culture medium of this aspect of the present invention is provided at a concentration of at least 0.06 ng/ml, more preferably, at least 0.07 ng/ml, more preferably, at least 0.08 ng/ml, more preferably, at least 0.09 ng/ml, more preferably, at least 0.1 ng/ml, more preferably, at least 0.11 ng/ml, even more preferably, at least 0.12 ng/ml.

Preferably, $TGF\beta_3$ which is included in the culture medium of this aspect of the present invention is provided at a concentration of at least 0.5 ng/ml, more preferably, at least 0.6 ng/ml, more preferably, at least 0.8 ng/ml, more preferably, at least 0.9 ng/ml, more preferably, at least 1 ng/ml, more preferably, at least 1.2 ng/ml, more preferably, at least 1.4 ng/ml, more preferably, at least 1.6 ng/ml, more preferably, at least 1.8 ng/ml, even more preferably, at least 2 ng/ml.

Preferably, the TGFβ-containing culture medium of this aspect of the present invention further includes other growth factors such as basic fibroblast growth factor (bFGF). bFGF can be obtained from any commercial supplier of tissue culture ingredients such as Invitrogen Corporation products, Grand Island NY, USA.

As is shown in Example 2 of the Examples section which follows, a DMEM/F12 – based culture medium (e.g., HA16 or HA19 medium which includes $TGF\beta_3$ and 4 ng/ml bFGF) or Mab ADCB medium – based culture medium (e.g., D1 or D2 medium which includes $TGF\beta_1$ or $TGF\beta_3$, respectively, and 10 ng/ml bFGF) were capable of maintaining hESCs in the undifferentiated state in culture. It should be mentioned that a Mab ADCB medium – based culture medium which includes a $TGF\beta_1$ or $TGF\beta_3$ isoform and bFGF at a concentration of 8 ng/ml was also capable of maintaining hESCs in the undifferentiated state for at least 5 passages (data not shown).

Preferably, the bFGF which is included in TGFβ-containing culture medium of this aspect of the present invention is provided at a concentration of at least 2 ng/ml, at least 3 ng, at least 4 ng/ml, at least 5 ng/ml, at least 6 ng/ml, at least 7 ng, at least 8 ng/ml, at least 9 ng/ml, at least 10 ng/ml.

As mentioned, the culture medium of this aspect of the present invention is devoid of a protein carrier (*i.e.*, protein carrier-free). A 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 cultures is limited by batch-specific variations and/or exposure to pathogens. On the other hand, the recombinant human albumin, which is substantially pure and devoid of animal contaminants is highly expensive,

thus not commonly used in hESCs cultures. Thus, a culture medium which is devoid of a protein carrier is highly advantageous since it enables a truly defined medium that can be manufacture from recombinant or synthetic materials.

In addition, as mentioned hereinabove, the culture medium of this aspect of the present invention is also devoid of serum (*i.e.*, serum-free) or serum replacement (*i.e.*, serum replacement-free). It should be noted that serum or serum replacement are added to most culture media which are designed for culturing stem cells, and particularly, embryonic stem cells, in order to provide the cells with the optimal 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 or an adult tissue of a postnatal individual). However, while 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 individuals and the risk of having xeno contaminants (in case of an animal serum is used), the use of the more defined composition such as the currently available serum replacement™ (Gibco-Invitrogen Corporation, Grand Island, NY USA) may be limited by the presence of Albumax (Bovine serum albumin enriched with lipids) which is from an animal source within the composition (International Patent Publication No. WO 98/30679 to Price, P.J. et al).

Thus, a culture medium which comprises a TGFβ isoform as described hereinabove and is devoid of serum, serum replacement and a protein carrier is highly desirable for both cell-based therapy and pharmaceutical industry, e.g., for pharmaceutical screening, identification of drug targets and cell-based compound delivery.

Preferably, the culture medium of this aspect of the present invention is capable of expanding the stem cells while maintaining them in the undifferentiated state. As used herein the term "expanding" refers to obtaining a plurality of cells from a single or a population of stem cells. Preferably, expanding embryonic stem cells refers also to increasing the number of embryonic stem cells over the culturing period. It will be appreciated that the number of stem cells which can be obtained from a single stem cell depends on the proliferation capacity of the stem cell. The proliferation capacity of a 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 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).

For example, as described in Example 2 of the Examples section which follows, hESCs could be maintained in the undifferentiated state in the presence of the D1 TGFβ-containing culture medium for at least 53 passages. Given that each passage occurs every 4-7 days, the hESCs were maintained for 265 days (*i.e.*, 6360 hours). Given that the hESCs doubling time was 36 hours, a single hESC cultured under these conditions could be expanded to give rise to 2^{176} (*i.e.*, 9.57×10^{52}) hESCs.

Preferably the stem cells which are maintained and expanded in the culture medium of the present invention exhibit stable karyotype (chromosomal stability) while in culture. For example, hESCs cultured in the presence of an IL6RIL6-containing medium (e.g., CM100) or a TGFβ-containing medium (e.g., D1, D2 or HA16) exhibited normal karyotype following at least 23 or 15 passages, respectively.

While further reducing the present invention to practice, the present inventors have uncovered that a culture medium which includes the IL6RIL6 chimera is also capable of maintaining human ESCs in the undifferentiated state. This is in sharp contrast to the teachings of Humphrey R., et al., (2004) which failed to maintain hESCs in the undifferentiated state when using the "hyper IL6" complex and thus concluded that maintenance of pluropotency in human ESCs is STAT independent. Thus, Humphrey R., et al., (2004) teaches away the present invention.

As is shown in Figures 1a-f, 2, 3a-c and 4a-l and described in Example 1 of the Examples section which follows, a culture system based on a fibronectin or laminin feeder layer-free matrix and a culture medium which includes the IL6RIL6 chimera, serum replacement and bFGF was capable of maintaining hESCs in the undifferentiated state for at least 43 (on a fibronectin matrix) or 7 (on a laminin matrix) passages while preserving all hESCs characteristics and pluripotency. On the other hand, as is further shown in Example 1 of the Examples section which follows, a medium containing serum replacement, bFGF and the unconjugated chimera components, *i.e.*, IL-6 (GenBank Accession No. CAG29292) and soluble IL-6 receptor (GenBank Accession No. AAH89410), failed to support hESC prolonged culture and resulted in differentiation of hESCs within 3-5 passages. In addition,

hESCs cultured in a medium containing the IL6RIL6 chimera and serum replacement, in the absence of bFGF, exhibited low proliferation capacity and could not be maintained in culture beyond 1-2 passages. Thus, these results demonstrate, for the first time, that hESCs can be cultured and maintained in the undifferentiated state in a feeder-layer free culture system in the presence of a culture medium which comprises the IL6RIL6 chimera.

Thus, according to another aspect of the present invention there is provided a culture medium which comprises an IL6RIL6 chimera, wherein the culture medium is capable of maintaining human embryonic stem cells in an undifferentiated state.

As used herein the term "IL6RIL6" 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) (e.g., a portion of the soluble IL6 receptors as set forth by amino acids 112-355 of GenBank Accession No. AAH89410) and the interleukin-6 (IL6) (e.g., human IL-6 as set forth by GenBank Accession No. CAG29292) or a biologically active fraction thereof (e.g., a receptor binding domain). Preferably, the IL6RIL6 chimera used by the method according to this aspect of the present invention is capable of supporting the undifferentiated growth of human embryonic stem cells, while maintaining their pluripotent capacity. It will be appreciated 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). Preferably, 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:31 and in Figure 11 of WO 99/02552 to Revel M., et al., which is fully incorporated herein by reference.

It will be appreciated that any of the proteinaceous factors used in the culture medium of the present invention (e.g., the IL6RIL6 chimera, bFGF, $TGF\beta_1$, $TGF\beta_3$) 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.

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 (e.g., the IL6RIL6 chimera) 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.

For example, to generate the IL6RIL6 chimera, a polynucleotide sequence encoding the IL6RIL6 chimera (e.g., the polypeptide set forth by SEQ ID NO:31) is preferably ligated into a nucleic acid construct suitable for expression in a host cell [*i.e.*, a cell in which the polynucleotide encoding the polypeptide-of-choice (e.g., the IL6RIL6 chimera) is expressed]. Preferably, to generate an IL6RIL6 chimera with the amount and pattern of glycosylation as of the naturally occurring IL6 and IL6-R, the host cell employed is a eukaryotic host cell, more preferably a mammalian host cell such as human cell or CHO cell).

For expression in mammalian cells [e.g., CHO cells, human HEK 293 cells (ATCC CRL 1573)] a number of mammalian expression vectors can be used. Examples include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

Expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses can be also used. SV40 vectors include pSVT7 and pMT2. Vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors

derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A$^+$, pMTO10/A$^+$, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Various methods can be used to introduce the expression vector of the present invention into host cells. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4-(6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Transformed cells are cultured under effective conditions, which allow for the expression of high amounts of the recombinant polypeptide (e.g., the IL6RIL6 chimera). Following a predetermined time in culture, recovery of the recombinant polypeptide is effected. The phrase "recovery of the recombinant polypeptide" used herein refers to collecting the whole fermentation medium containing the polypeptide and need not imply additional steps of separation or purification.

Thus, polypeptides of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

The polypeptide of the present invention (*e.g.*, the IL6RIL6 chimera) is preferably retrieved in "substantially pure" form. As used herein, the phrase "substantially pure" refers to a purity that allows for the effective use of the

polypeptide of the present invention (e.g., the IL6RIL6 chimera) in maintaining the human embryonic stem cells in an undifferentiated state while in culture.

As shown in Figures 1-4 and described in Example 1 of the Examples section which follows, a medium supplemented with the 100 or 300 ng/ml of the IL6RIL6 chimera enabled the prolonged culturing of hESCs in the undifferentiated state for at least 43 passages, while preserving normal karyotype and pluripotent capacity [as evidenced by the formation of embryoid bodies (EBs) which express markers of all three embryonic germ cells].

Preferably, the IL6RIL6 chimera which is included in the culture medium of this aspect of the present invention is provided at a concentration of at least 25 ng/ml, at least 50 ng/ml, at least 100 ng/ml, preferably, at least 200 ng/ml, preferably, at least 300 ng/ml. It should be noted that the concentration of the IL6RIL6 chimera can vary depending on the purity of the chimeric polypeptide following its synthesis or recombinant expression and those of skills in the art are capable of adjusting the optimal concentration depending on such purity.

Preferably, the IL6RIL6-containing culture medium of this aspect of the present invention includes at least 2 ng/ml bFGF, at least 3 ng/ml, at least 4 ng/ml, at least 5 ng/ml, at least 6 ng/ml, at least 7 ng, at least 8 ng/ml, at least 9 ng/ml, at least 10 ng/ml bFGF. For example, as shown in Example 1 of the Examples section which follows, a culture medium which includes 4 ng/ml bFGF along with 100 ng/ml of the IL6RIL6 chimera was capable of maintaining hESCs in an undifferentiated state for at least 43 passages.

As mentioned, the IL6RIL6-containing culture medium described in Example 1 of the Examples section which follows included serum replacement. It should be noted that such a culture medium can also include serum (e.g., such as human serum) instead of serum replacement and yet maintain hESCs in culture in the undifferentiated state. Thus, serum or serum replacement which are included in the IL6RIL-containing culture medium of this aspect of the present invention can be provided at various concentrations, such as a concentration of at least 10 %, e.g., a concentration of at least 15 %, at least 20 %, at least 25 % or at least 30 %.

Alternatively and currently more preferred, in order to achieve more defined culture conditions of the hESCs, the IL6RIL6-containing culture medium of this aspect of the present invention is preferably devoid of serum or serum replacement.

A non-limiting example of such a culture medium can be the HACM100 culture medium described in Examples 2 and 4 of the Examples section which follows.

Thus, any of the culture media described hereinabove, which are based on the TGFβ isoform or the IL6RIL6 chimera present, for the first time, a well-defined, xeno-free culture medium which is highly suitable for culturing hESCs for applications such as cell-based therapy and for use in the pharmaceutical industry.

Thus, according to yet another aspect of the present invention there is provided a culture medium which is devoid of serum (*i.e.*, serum-free), xeno contaminant (*i.e.*, xeno-free), feeder layers (*i.e.*, feeder layer-free) and protein carrier (*i.e.*, protein carrier-free) and yet capable of maintaining stem cells in an undifferentiated state.

As mentioned hereinabove and described in Examples 1 and 2 of the Examples section which follows, the present inventors have uncovered that stem cells such as human embryonic stem cells can be expanded using any of the culturing media described hereinabove and be maintained in the undifferentiated state while in culture.

Thus, according to an additional aspect of the present invention there is provided a cell culture which comprises the stem cell and any of the culture media described hereinabove, while the culture medium is capable of maintaining said stem cells in an undifferentiated state.

According to still an additional aspect of the present invention there is provided a method of expanding and maintaining stem cells in an undifferentiated state. The method is effected by culturing the stem cells in any of the culture media described hereinabove, thereby expanding and maintaining the stem cells in the undifferentiated state.

Culturing according to this aspect of the present invention is effected by plating the stem cells onto a matrix 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 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 Examples 1 and

2 of 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.

As mentioned before, stem cells can be cultured on feeder cells or on feeder-layer free culturing systems using a matrix instead of a feeder cell layer. As used herein, the term "matrix" refers to any substance to which the 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 stem cells can attach and thus it provides a suitable culture substrate.

Particularly suitable for use with the present invention are extracellular matrix components derived from basement membrane or extracellular matrix components that form part of adhesion molecule receptor-ligand couplings. Non-limiting examples of suitable matrices which can be used by the method of this aspect of the present invention include Matrigel® (Becton Dickinson, USA), laminin, fibronectin, proteoglycan, entactin, heparan sulfate, and the like, alone or in various combinations. 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 matrices include, for example, human-derived fibronectin, recombinant fibronectin, human-derived laminin, foreskin fibroblast matrix or a synthetic fibronectin matrix which can be obtained from Sigma, St. Louis, MO, USA or can be produced using known recombinant DNA technology. Preferred matrices of the present invention are fibronectin derived matrices.

Thus, stem cells cultured using the teachings of the present invention can be expanded while maintaining in the undifferentiated state. For example, as described in Example 2 of the Examples section which follows, culturing of hESCs on a Matrigel™ in the presence of the TGFβ-containing culture medium (the D1 medium) for at least 53 passages (8-9 months) resulted in an expansion factor of 2^{180} (*i.e.*, 1.5 x 10^{54}) given that the hESC doubling time is 36 hours and that passaging occurs every 4-6 days.

Alternatively, as is shown in Figures 8a-c, 9a-g and 10a-d and is described in Example 4 of the Examples section which follows and in U.S. provisional application

No. 60/834,795 to the present inventors (filed Aug. 2nd, 2006), the present inventors have uncovered that human ESCs can be expanded in a suspension culture devoid of substrate adherence and as such can be maintained in the undifferentiated, pluripotent state.

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

Thus, the culture of the present invention is "devoid of substrate adherence" in which the stem cells (e.g., ESCs) are capable of expanding without adherence to an external substrate such as components of extracellular matrix, a glass microcarrier or beads.

Thus, hESCs cultured in a suspension culture devoid of substrate adherence can be expanded for at least 17 passages in the CM100F medium and maintain their undifferentiated, pluripotent state (Example 4 and data not shown).

When cultured according to the teachings of the present invention, stem cell growth is monitored to determine their differentiation state. The differentiation state can be determined using various approaches including, for example, morphological evaluation (e.g., as shown in Figures 1a-f, 5a-d and 9a-g) 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 cultured according to the method of this aspect of the present invention revealed the expression of Oct4, stage-specific embryonic antigen (SSEA) 4, the tumour-rejecting antigen (TRA)-1-60 and TRA-1-81 (Figures 3a-c, 6a-c and 8a-c). 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 (as shown in Figures 4a-l and 10a-d) 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).

As is further shown in Figures 7a-b and described in Example 3 of the Examples section which follows, the present inventors were capable of deriving a new line of human ESCs in complete xeno-free culturing conditions using the TGFβ-based culture medium.

Thus, according to yet an additional aspect of the present invention there is provided a method of deriving an embryonic stem cell line. The method 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 any of the culture media described hereinabove, thereby deriving the embryonic stem cell line.

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

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, as described hereinabove and in the "General Materials and Experimental Methods" of the Examples section which follows. Briefly, the zona pellucida is removed from a 5-7 day-old blastocyst using Tyrode's acidic solution (Sigma, St Louis MO, USA), the trophoblast layer is specifically removed either by immunosurgery or mechanically using 27g needles and the exposed ICM is either directly cultured in a suitable culture system (e.g., feeder layers, feeder-free matrix or a suspension culture) in the presence of any of the culture media described hereinabove (e.g., the CM100F, HA16 or D2 medium) for 4-10 days (in case a preimplantation blastocyst is used) or subject to *in vitro* implantation by culturing the ICM for 6-8 days (to obtain cells of a 13 day-old blastocyst in case a post-implantation/pre-gastrulation blastocyst is used) on feeder layers or a feeder-free

culturing system which allow implantation of the blastocyst to the surface, following which the implanted cells are isolated and can be further cultured on feeder layers, feeder-free matrix or a suspension culture in the presence of any of the culture media described hereinabove (e.g., the CM100F, HA16 or D2 medium) as described hereinunder. When using the genital tissue of a fetus, the genital ridges are dissociated and cut into small chunks which are thereafter disaggregated into cells by mechanical dissociation. The single cell EG cells are then cultured in any of the culture media described hereinabove for 4-10 days).

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

Preferably, the cell culture of the present invention is characterized by at least 40 %, at least 50 %, at least 60 %, more preferably at least 70 %, more preferably at least 80 %, most preferably at least 85 % of undifferentiated stem cells.

It will be appreciated that an established embryonic 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 the Examples section which follows, using 15 % SR and 10 % DMSO, hESCs were successfully frozen and thawed.

As is shown in Figures 4a-l and is described in Examples 1, 2 and 4 of the Examples section which follows, hESCs 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). Thus, hESCs 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 ESCs 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 yet an additional aspect of the present invention there is provided a method of generating embryoid bodies from embryonic stem cells. The method is effected by (a) culturing the embryonic stem cells in any of the culture

media described hereinabove which is capable of maintaining the embryonic stem cells in an undifferentiated state, to thereby obtain expanded, undifferentiated embryonic stem cells; and (b) subjecting the expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating the embryonic stem cells to embryoid bodies; thereby generating the embryoid bodies from the embryonic stem cells.

As used herein the phrase "embryoid bodies" refers to morphological structures comprised of a population of ESCs, extended blastocyst cells (EBCs) and/or embryonic germ cells (EGCs) which have undergone differentiation. EBs formation initiates following the removal of differentiation blocking factors from ES cell cultures. In the first step of EBs formation, ESCs proliferate into small masses of cells which then proceed with differentiation. In the first phase of differentiation, following 1-4 days in culture for human ESCs, 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 of this aspect of the present invention involves the culturing of ESCs in any of the culture media described hereinabove in order to obtain expanded, undifferentiated embryonic stem cells and then subjecting the expanded, undifferentiated ESCs to culturing conditions suitable for differentiating the ESCs to embryoid bodies. Such culturing conditions are substantially devoid of differentiation inhibitory factors which were employed during step (a), e.g., a TGFβ isoform or the IL6RIL6 chimera.

For EBs formation, the ESCs are removed from their feeder cell layers, feeder-free-culturing systems (e.g., the fibronectin or laminin matrix) 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, essentially as described in Examples 1, 2 and 4 of the Examples section which follows. 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 can be effected by morphological evaluations (e.g., histological staining), determination of expression of differentiation-specific markers [e.g., using immunological techniques or RNA-based analysis (e.g., RT-PCR, cDNA microarray) as shown in Figures 4a-l], essentially as described in the Examples section which follows.

Thus, as is shown in Figures 4f-l, cells harvested from EBs according to the method of this aspect of the present invention exhibited markers of all three embryonic germ layers, such as albumin and glucagon (typical of the embryonic endoderm), α-cardiac actin, β-globulin and Flk1 (typical of the embryonic mesoderm), and AC133 and neurofilament (NFH) (typical of the embryonic ectoderm).

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.

Preferably, the method of this aspect of the present invention 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 cell layers, 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.

Preferably, the method of this aspect of the present 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 one preferred embodiment of the present invention, isolating 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 yet an additional preferred embodiment of the present invention, isolating 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 still an additional preferred embodiment of the present invention, isolating 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 a number of procedures and approaches for inducing differentiation of EBs to lineage specific cells.

To differentiate the EBs of the present 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 the present 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 the present 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 the present 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.

Additionally or alternatively, lineage specific cells can be obtained by directly inducing the expanded, undifferentiated ESCs to culturing conditions suitable for the differentiation of specific cell lineage.

In addition to the lineage-specific primary cultures, EBs of the present 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).

It will be appreciated that since the lineage-specific cells or cell lines obtained according to the teachings of the present 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 present invention envisages the use of the expanded and/or differentiated lineage-specific cells or cell lines of the present invention for treating a disorder requiring cell replacement 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 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 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].

In addition to cell replacement therapy, the lineage specific cells of the present invention can also 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 the present 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. For example, growth affecting substances, toxins or potential differentiation factors can be tested by their addition to the culture medium.

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

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate 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); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "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.

EXAMPLE 1

CULTURING HUMAN EMBRYONIC STEM CELL WITH A MEDIUM CONTAINING THE IL-6 - IL-6 RECEPTOR (IL6RIL6) CHIMERA

To optimize culturing conditions of hESCs on feeder-layer free culturing systems, the present inventors have tested various combinations of growth factors, as follows.

Materials and Experimental Methods

hESC culture – Human embryonic stem cell (hESC) lines I-6 and I-3 [Amit & Itskovitz-Eldor, 2002] were cultured for 46 and 39 passages respectively, with inactivated mouse embryonic fibroblasts (MEFs) in an "hESC basic culture medium" consisting of 85 % Ko-DMEM, supplemented with 15 % serum replacement (SR), 2 mM L-glutamine, 0.1 mM β–mercaptoethanol, 1 % non-essential amino acid stock, and 4 ng/ml bFGF (all Gibco Invitrogen Corporation products, Grand Island NY, USA).

To test various media combinations on feeder-layer free culturing systems, hESCs were transferred to 50 μg per 10 cm^2 fibronectin-covered plates (human plasma fibronectin, Chemicon International, Temecula CA, USA) in the presence of the "hESC basic culture medium" with the following combinations of cytokines:

(i) "CM6 medium" - 0.3 ng/ml Interleukin-6 (IL6) and 0.5 ng/ml IL6 soluble receptor (both from R&D Systems Minneapolis MN, USA);

(ii) "IL-6-IL-6 receptor (IL6RIL6) chimera" - 50 ng/ml, 100 ng/ml, 200 ng/ml or 300 ng/ml of IL6RIL6 chimera (Chebath J, et al., 1997 and WO 99/02552 to Revel M., et al. SEQ ID NO:31). When used with 100 ng/ml of the IL6RIL6 chimera, this medium is also called CM100.

(iii) Control medium – 4 ng/ml bFGF (Gibco Invitrogen Corporation, Grand Island NY, USA).

Cells were passaged every four to six days using 1 mg/ml type IV collagenase (Gibco Invitrogen Corporation, Grand Island NY, USA). Cells were frozen in liquid nitrogen using a freezing solution consisting of 10 % DMSO (Sigma, St Louis MO, USA), 15 % SR (Chemicon International, Temecula CA, USA) and 75 % Ko-DMEM (Gibco-Invitrogen Corporation, Grand Island NY, USA) [Amit et al, 2004].

Immunohistochemistry - Undifferentiated hESCs grown in the feeder-free culture system and differentiated cells dissociated using trypsin-EDTA from 14-day-old EBs were fixed with 4 % paraformaldehyde and incubated overnight at 4 °C with 1:50 dilutions of the following primary antibodies: stage-specific embryonic antigen (SSEA) 1 (SSEA-1), 3 (SSEA-3) and 4 (SSEA-4) (Hybridoma Bank, Iowa, USA), tumor recognition antigen (TRA) 1-60 and TRA1-81 (Chemicon International, Temecula CA, USA) and Oct4 (Santa Cruse). Cys 3 conjugated antibodies (Chemicon International, Temecula CA, USA) were used as secondary antibodies at a 1:100 dilution.

Karyotype analysis - Karyotype analysis (G-banding) was performed on at least 20 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).

EB formation - For the formation of EBs, one to four confluent wells were used in a six-well plate (40 cm^2). ESCs were removed from their culture dish using 1 mg/ml type IV collagenase, further broken into small clumps using 1000 µl Gilson pipette tips, and cultured in suspension in 58 mm petri dishes (Greiner, Frickenhausen, Germany). EBs were grown in medium consisting of 80 % Ko-DMEM, supplemented with 20 % FBSd (HyClone, Utah, USA), 1 mM L-glutamine, 0.1 mM β–mercaptoethanol, and 1 % non-essential amino acid stock (all but FBSd

from Gibco Invitrogen Corporation, Grand Island NY, USA). 10 day-old EBs were harvested for RNA isolation and histological examination.

RT PCR analysis - Total RNA was isolated from hESCs grown for 10-15 passages in feeder-free conditions, or from 10 day-old EBs (formed from cells grown in the feeder-free system) using Tri-Reagent (Sigma, St. Louis MO, USA), according to the manufacturer's instructions. 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 of 94 °C for 30 seconds, annealing for 30 seconds at a temperature specified in Table 1, hereinbelow, and extension at 72 °C for 30 seconds. PCR primers and reaction conditions used are described in Table 1, hereinbelow. 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 conditions

Gene product (Accession number)	*Forward (F) and reverse (R) primers (SEQ ID NO:)*	*Reaction Condition*	*Size (bp)*
Oct-4 (S81255)	F: 5'-GAGAACAATGAGAACCTTCAGGA (SEQ ID NO:1) R: 5'-TTCTGGCGCCGGTTACAGAACCA (SEQ ID NO:2)	30 cycles at 60 °C in 1.5 mM $MgCl_2$	219
Albumin (AF542069)	F: 5'-TGCTTGAATGTGCTGATGACAGGG (SEQ ID NO:3) R: 5'-AAGGCAAGTCAGCAGCCATCTCAT (SEQ ID NO:4)	35 cycles at 60 °C in 1.5 mM $MgCl_2$	302
α-fetoprotein (BC027881)	F: 5'-GCTGGATTGTCTGCAGGATGGGGAA (SEQ ID NO:5) R: 5'-TCCCCTGAAGAAAATTGGTTAAAAT (SEQ ID NO:6)	30 cycles at 60 °C in 1.5 mM $MgCl_2$	216
NF-68KD (NFH (AY156690; X15307; X15309)	F: 5'-GAGTGAAATGGCACGATACCTA (SEQ ID NO:7) R: 5'-TTTCCTCTCCTTCTTCACCTTC (SEQ ID NO:8)	30 cycles at 60 °C in 2 mM $MgCl_2$	473
α-cardiac actin (NM_005159)	F: 5'-GGAGTTATGGTGGGTATGGGTC (SEQ ID NO:9) R: 5'-AGTGGTGACAAAGGAGTAGCCA (SEQ ID NO:10)	35 cycles at 65 °C in 2 mM $MgCl_2$	486
β - Actin (NM_001101)	F: 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG (SEQ ID NO:11) R: 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC (SEQ ID NO:12)	35 cycles at 62 °C in 1.5 mM	838

		$MgCl_2$	
Sox2 (Z31560)	5' CCCCCGGCGGCAATAGCA (SEQ ID NO:13) 3' TCGGCGCCGGGGAGATACAT (SEQ ID NO:14)	35 cycles at 60 °C in 1.5 mM $MgCl_2$	448
Rex1 (AF450454)	5' GCGTACGCAAATTAAAGTCCAGA (SEQ ID NO:15) 3' CAGCATCCTAAACAGCTCGCAGAAT (SEQ ID NO:16)	35 cycles at 56 °C in 1.5 mM $MgCl_2$	306
CX43 (NM_000165)	5' TACCATGCGACCAGTGGTGCGCT (SEQ ID NO:17) 3'GAATTCTGGTTATCATCGGGGAA (SEQ ID NO:18)	35 cycles at 61 °C in 1.5 mM $MgCl_2$	295
FGF4 (NM_002007)	5' CTACAACGCCTACGAGTCCTACA (SEQ ID NO:19) 3' GTTGCACCAGAAAAGTCAGAGTTG (SEQ ID NO:20)	35 cycles at 52 °C in 1.5 mM $MgCl_2$	370
Glucagon (X03991)	5' CTCAGTGATCCTGATCAGATGAACG (SEQ ID NO:21) 3' AGTCCCTGGCGGCAAGATTATCAAG (SEQ ID NO:22)	35 cycles at 65 °C in 1.5 mM $MgCl_2$	370
β-globulin (V00499)	5' ACCTGACTCCTGAGGAGAAGTCTGC (SEQ ID NO:23) 3' TAGCCACACCAGCCACCACTTTCTG (SEQ ID NO:24)	35 cycles at 65 °C in 1.5 mM $MgCl_2$	410
Flk1 (NM_002253)	5' ATGCACGGCATCTGGGAATC (SEQ ID NO:25) 3' GCTACTGTCCTGCAAGTTGCTGTC (SEQ ID NO:26)	35 cycles at 65 °C in 1.5 mM $MgCl_2$	537
AC133 (NM_006017)	5' CAGTCTGACCAGCGTGAAAA (SEQ ID NO:27) 3' GGCCATCCAAATCTGTCCTA (SEQ ID NO:28)	35 cycles at 65 °C in 1.5 mM $MgCl_2$	200
Nanog (NG_004095)	5' ACTAACATGAGTGTGGATCC (SEQ ID NO:29) 3' TCATCTTCACACGTCTTCAG (SEQ ID NO:30)	35 cycles at 61 °C in 1.5 mM $MgCl_2$	800

Table 1: RT-PCR primers and PCR conditions are provided along with the GenBank Accession numbers of the amplified transcripts.

Experimental Results

Medium supplemented with a combination of 100 ng/ml IL6RIL6 chimera and 4 ng/ml bFGF is most suitable to support undifferentiated proliferation of hESCs on a feeder-free culture system - Several concentrations of the IL6RIL6 chimera were tested for the ability to support the feeder-layer free culture of hESCs. Initially, two measures were used to estimate the ability of the hESCs to grow in the feeder-free culture system, namely percentage of differentiation and rate of growth. The medium supplemented with the combination of 100 ng/ml IL6RIL6 chimera and 4 ng/ml bFGF was found to be the most suitable to support undifferentiated feeder-layer free hESC proliferation. Culturing hESCs with a culture medium consisting of 4 ng/ml bFGF and the chimera components IL-6 and IL-6 soluble receptor failed to support hESC prolonged culture and resulted in differentiation of hESCs within 3-5 passages (data not shown). Although 70 % of the hESCs remained in the undifferentiated stage in these conditions for a few passages, the proliferation rate was low and with each passage the number of surviving hESCs decreased. In addition, culturing of hESCs in a medium containing the IL6RIL6 chimera and serum replacement, in the absence of bFGF, resulted in low proliferation of cells and failure to maintain the hESCs beyond 1-2 passages (data not shown). However, in medium supplemented with either 100 ng/ml or 300 ng/ml IL6RIL6 chimera and 4 ng/ml bFGF, the hESCs could be cultured continuously in feeder-layer free conditions for at least 43 passages. hESCs cultured in these conditions (in the presence of the IL6RIL6 chimera medium) maintained their ESC features, including undifferentiated proliferation, karyotype stability and pluripotency as was tested following 28 passages. Based on morphology, the background spontaneous differentiation rates were about 15 %, similar to those occurring in other feeder layer-free culture methods [Xu et al, 2001, 2005; Amit et al, 2004]. Examples of undifferentiated colonies cultured in feeder-layer free conditions are illustrated in Figures 1a-f. Interestingly, when cultured in a medium supplemented with the IL6RIL6 chimera and bFGF using fibronectin as substrate for the feeder-free culture system, the cells differentiated at the periphery of the colonies and formed an outgrowth of feeder-like cells (Figure 1a). In similar cultures, laminin did not induce such differentiation (Figure 1c) but, human and mouse laminin matrices were found to be inconsistent in their ability to support hESC undifferentiated growth and, therefore, fibronectin appears preferable despite

the formation of feeder-like cells. Former studies on feeder-layer free cultures of hESCs also reported the formation of feeder-like cells [Xu et al, 2001].

Feeder-free culture system in the presence of a culture medium containing the IL6RIL6 chimera supports hESCs with typical morphology - When compared to hESCs cultures on exogenous feeder layers, no morphological differences could be observed between colonies grown in the feeder layer-free culture system (a fibronectin matrix and a medium supplemented with IL6RIL6 chimera and bFGF) and those grown on MEFs (Figures 1a-f and data not shown). Correspondingly, the morphological features at the single-cell level remained unchanged, the cells remained small and round, and exhibited high nucleus-to-cytoplasm ratio, with a notable presence of one to three nucleoli and with typical spacing between the cells (Figures 1a-f). The hESC population doubling time was similar to that observed when grown on MEFs (about 36 hours), and in the present feeder-free conditions the cells could be passaged routinely every four to six days, at a ratio of 1/2 or 1/3, similar to the splitting ratio employed when the hESCs are cultured on MEFs. The cells were passaged at the same seeding efficiency as with MEF of about 1 million cells per 10 cm^2, with the same viability rate of over 90 %. Using 15 % SR and 10 % DMSO, cells were successfully frozen and thawed.

The fibronectin feeder-layer free culture system supplemented with a medium containing the IL6RIL6 chimera supports hESCs with normal karyotype for at least 23 passages - Karyotype analysis by Giemsa banding was carried out on two separate cultures, representing the two medium conditions: medium supplemented with 4 ng/ml bFGF and either 100 ng/ml or 300 ng/ml IL6RIL6 chimera 23 passages after transferring the cells into the feeder-layer environment which is based on fibronectin. At least 20 cells were tested from each sample, 40 cells from each medium combination. All examined cells were found to sustain normal karyotype of 46, XX and 46,XY for cell lines I3 and I6, respectively. Examples of chromosomes from examined cells are illustrated in Figure 2. Overall, these results demonstrate that the cells' karyotype remains stable in the fibronectin based feeder free culture system supplemented with the IL6RIL6 chimera medium, similarly to ESCs grown with MEFs.

hESCs cultured on fibronectin feeder free culture systems supplemented with the IL6RIL6 chimera medium express typical hESC markers of

undifferentiated cells - Several surface markers typical of primate undifferentiated ES cells were examined using immunofluorescent staining as described elsewhere [Thomson et al, 1995, 1996, 1998]. hESCs cultured on the fibronectin feeder-free culture system for 27 passages with medium supplemented with 100 ng/ml or 300 ng/ml of the IL6RIL6 chimera were found to be strongly positive to surface markers TRA-1-60 (Figure 3a) Oct4 (Figure 3b), SSEA4 (Figure 3c), and TRA-1-81 (data not shown). As in other primate ES cells, staining with SSEA3 was weak and negative for SSEA1 (data not shown).

The IL6RIL6 chimera medium is capable of supporting pluripotent hESCs as evident by the in vitro differentiation into embryoid bodies (EBs) - The developmental potential of the cells after prolonged culture in feeder layer-free conditions was examined *in vitro* by the formation of EBs. When cultured in suspension, after 7 passages in medium supplemented with either 100 ng/ml or 300 ng/ml IL6RIL6 chimera, hESCs formed EBs similar to those created by ES cells grown on MEFs. Within these EBs, stem cells differentiated into cell types representative of the three embryonic germ layers.

EBs formed from the hESCs cultured on the IL6RIL6 chimera expressed typical differentiation markers of all embryonic cell lineages – To further define the expression pattern of the undifferentiated hESCs grown on the fibronectin-based feeder free culture system in the presence of a medium containing the IL6RIL6 chimera, RT-PCR analysis was performed using primers specific to both differentiation and undifferentiated cells (primers employed are listed in Table 1, hereinabove). As is shown in Figures 4a-l, while undifferentiated cells cultured on the fibronectin feeder free culture system using medium supplemented with the IL6RIL6 chimera expressed undifferentiated genetic markers such as Oct 4 (Figure 4a), Nanog (data not shown), Sox2 (Figure 4b), Rex1 (Figure 4c), Cx43 (Figure 4d) and FGF4 (Figure 4e) [Bhattacharya et al, 2004], cells harvested from 10 day-old EBs expressed genes such as albumin (Figure 4f) and glucagon (Figure 4g) (typical of the embryonic endoderm), α-cardiac actin (Figure 4i), β-globulin (Figure 4h) and Flk1 (Figure 4j) (typical of the embryonic mesoderm), and AC133 (Figure 4k) and neurofilament (NFH; Figure 4l) (typical of the embryonic ectoderm).

Altogether, these results demonstrate the identification of a defined medium based on the IL6RIL6 chimera which is suitable for maintaining hESCs in a pluripotent and undifferentiated state on a feeder-free culture system such as the fibronectin based feeder free system.

Analysis and Discussion

Human ESCs, like mouse ES cells, are traditionally cultured with MEFs, which may expose them to animal pathogens. In this study, the present inventors have demonstrated a defined medium and feeder layer-free culture system based on the use of serum replacement™, bFGF, IL6RIL6 chimera and human fibronectin as a matrix substitute.

Several concentrations of the IL6RIL6 chimera were tested for their ability to maintain hESCs in an undifferentiated state. The most suitable medium was that supplemented with 100 ng/ml of the IL6RIL6 chimera together with 4 ng/ml bFGF, in which the three transferred hESC lines continued to proliferate while retaining normal hESC features throughout the prolonged culture.

Cells cultured in these conditions maintained all characteristics of ES cells. After prolonged culture of up to 28 passages, the cells remained undifferentiated, as demonstrated by the colony and single cell morphology, and by the expression of surface markers typical of undifferentiated primate ESCs [Thomson et al, 1995, 1996, 1998; Reubinoff et al, 2000]. In addition, while cultured in these conditions, hESCs expressed specific markers for the undifferentiated stage such as Oct 4, Sox 2, Rex1 and FGF4, as demonstrated by immunofluorescence (for Oct4) and RT-PCR analyses (for Oct4, Sox 2, Rex1 and FGF4).

Karyotype analysis carried out on representative cell samples demonstrated that the hESCs' karyotype remained stable in the proposed conditions (after 23 passages at the tested conditions). None of the examined 40 cells exhibited karyotype abnormalities.

The cells' pluripotency was examined *in vitro*. Cells cultured in the feeder layer-free culture system for more than 10 passages, formed EBs similar to those created when grown on MEFs [Itskovitz-Eldor et al, 2000]. RT-PCR analysis demonstrated that cells within these EBs differentiated into different cell types representative of the three embryonic germ layers.

These results demonstrate that hESCs can be maintained as undifferentiated cells in defined feeder- and serum-free conditions which include the IL6RIL6 chimera while exhibiting hESC features.

The mechanism by which hESCs self-maintain is still not clear. In mouse ESCs the role of LIF and other members of the IL-6 family, acting through gp130 and the JAK/STAT3 pathway, in maintaining ESCs undifferentiated prolonged culture is well known [Smith et al, 1988; Williams et al, 1988; Rose et al, 1994; Conover et al, 1993; Niwa et al, 1998]. Furthermore, to date the only method for deriving new mouse ESC lines in feeder layer-free conditions is based on the addition of factors from the IL-6 family [Nichols et al, 1994]. The IL6RIL6 chimera was demonstrated as the most potent factor in supporting the feeder layer-free isolation of mouse ESC lines (Nichols et al, 1994). Previous studies did not demonstrate a significant effect of the IL-6 family, including a fusion protein of portions of IL-6 and the IL-6 receptor, on the self-maintenance of undifferentiated hESCs [Daheron et al, 2004; Humphrey et al, 2004; Sato et al, 2004]. The specific conditions for hESC cultures described here may explain why the IL6RIL6 chimera was effective in supporting the proliferation of hESC with minimal differentiation. One difference being the use of fibronectin substrate as a matrix (and not mouse laminin) and another being the precise schedule of hESC passaging. Further research is required to elucidate the underlying mechanisms of action of the IL6RIL6 chimera at the level of signal transduction, its time course and intensity at which different pathways (JAK/STAT, PI3 kinase, MAPK, see Hirano et al, 1997) are activated.

Future clinical uses of hESCs will require a reproducible, well-defined and xeno-free culture system. Although the serum replacement (SR) used in the present study is considered such, it contains "Albumax" which is a lipid-enriched bovine serum albumin and, therefore, is not animal-free. The well-defined conditioned media demonstrated in the present study are suitable for culturing hESCs and may be advantageous for undertaking research on the mechanisms of ESC self-maintenance, especially of the possible roles of the LIF/STAT3 pathway and various integrins as fibronectin receptors. Other studies using hESCs, such as the research on differentiation pathways and mechanisms, will benefit from the availability of a well-defined and reproducible culture system.

This culture system is a further step forward towards fully defined culture conditions for hESCs, and promotes the development of a xeno-free culture system for hESCs.

EXAMPLE 2

CULTURING HUMAN EMBRYONIC STEM CELL WITH A MEDIUM CONTAINING TGF-BETA ISOFORMS DEVOID OF SERUM, SERUM REPLACEMENT AND ALBUMIN

Materials and Experimental Methods

ESC culture – Human embryonic stem cell (hESC) lines I-6, I4 and I-3 [Amit & Itskovitz-Eldor, 2002] were cultured with inactivated mouse embryonic fibroblasts (MEFs) for 40-60 passages in a "basic hESC culture medium" consisting of 85 % DMEM/F12 (Biological Industries, Biet Haemek, Israel) supplemented with 15 % serum replacement (SR), 2 mM L-glutamine, 0.1 mM β–mercaptoethanol, 1 % non-essential amino acid stock, and 4 ng/ml basic fibroblast growth factor (bFGF) (all Gibco Invitrogen Corporation products, Grand Island NY, USA).

To test the ability of various culture media to support the growth of hESC in an undifferentiated yet pluripotent state the hESCs were transferred to several culture systems:

(i) ***Fibronectin feeder-free culture system*** - 50 μg per 10 cm^2 fibronectin-covered plates (human plasma fibronectin, Chemicon International, Temecula CA, USA);

(ii) ***Matrigel™ feeder-free culture system*** - Matrigel™ (BD Biosciences, Bedfrod, MA, USA);

(iii) ***MEFs*** – mouse embryonic fibroblast feeder layer system;

(iv) ***Foreskins fibroblasts*** – foreskin fibroblasts feeder layer system.

Tested media - The tested medium were based on:

(i) ***D1 medium*** - Mab ADCB medium (HyClone, Utah, USA) supplemented with 2 mM L-glutamine (Invitrogen Corporation products, Grand Island NY, USA), 0.12 ng/ml $TGF\beta_1$ (from R&D Systems Minneapolis MN, USA), and 10 ng/ml bFGF (Invitrogen Corporation products, Grand Island NY, USA).

(ii) ***D2 medium*** - Mab ADCB medium (HyClone, Utah, USA) supplemented with 2 mM L-glutamine (Invitrogen Corporation products, Grand Island NY, USA), 2 ng/ml TGFβ_3 and 10 ng/ml bFGF (Invitrogen Corporation products, Grand Island NY, USA).

(iii) ***HA16 medium*** - 96 % DMEM/F12 (Biological Industries, Biet Haemek, Israel) supplemented with 1:1000 dilution of the ITS Premix [the ITS premix is a X1000 stock solution obtained from BD Biosciences, Bedford, MA, USA and consists of 12.5 mg Insulin, 12.5 mg Transferrin and 12.5 mg Selenius acid], 2 mM L-glutamine, 2 ng/ml TGFβ_3 (from R&D Systems Minneapolis MN, USA), 4 ng/ml bFGF, 500 ng/ml ascorbic acid (Sigma, Steinheim, Germany), and a 1:1000 dilution of a lipid mixture (Sigma Cat. No. L5146, Steinheim, Germany) (all but those otherwise specified were obtained from Gibco Invitrogen Corporation products, Grand Island NY, USA).

(iv) ***HA19 medium*** - 96 % DMEM/F12 (Biological Industries, Beth Haemek, Israel) supplemented with 1:1000 dilution of the ITS premix (BD Biosciences, Bedford, MA, USA), 2 mM L-glutamine, 2 ng/ml TGFβ_3 (from R&D Systems Minneapolis MN, USA), 4 ng/ml bFGF, 500 ng/ml ascorbic acid (Sigma, Steinheim, Germany), a 1:1000 dilution of a lipid mixture (Sigma Cat. No. L5146, Steinheim, Germany) and a 1:100 dilution of Simfronic 68 (Pluronic F-68 solution, P5556 from Sigma, Steinheim, Germany, the stock is 10 %, the F-68 in culture is provided at a concentration of 0.1 %) (Sigma, Steinheim, Germany) (all but those otherwise specified were obtained from Gibco Invitrogen Corporation products, Grand Island NY, USA).

(v) ***CM100F medium*** - 85 % DMEM/F12 (Biological Industries, Biet Haemek, Israel) supplemented with 15 % serum replacement (SR), 2 mM L-glutamine, 0.1 mM β–mercaptoethanol, 1 % non-essential amino acid stock, 4 ng/ml basic fibroblast growth factor (bFGF) and 100 ng/ml IL6RIL6 chimera (SEQ ID NO:31, a kind gift from Prof. Revel M, the Weizmann Inst. Rehovot, Israel) (all but those otherwise specified were obtained from Gibco Invitrogen Corporation products, Grand Island NY, USA). As a control, the same culture media was used with the removal of the growth factors (except for bFGF which remained in the control culture medium) and the IL6RIL6 chimera.

(vi) ***"IL6-IL-6 receptor (IL6RIL6) chimera"*** - 85 % Ko-DMEM, supplemented with 15 % serum replacement (SR), 2 mM L-glutamine, 0.1 mM β–mercaptoethanol, 1 % non-essential amino acid stock, 4 ng/ml bFGF and 50 ng/ml, 100 ng/ml, 200 ng/ml or 300 ng/ml of IL6RIL6 chimera (Chebath J, et al., 1997 and WO 99/02552 to Revel M., et al. SEQ ID NO:31) (all Gibco Invitrogen Corporation products, Grand Island NY, USA). When used with 100 ng/ml of the IL6RIL6 chimera, this medium is also called CM100.

(vii) ***HACM100 medium*** - 96 % DMEM/F12 (Biological Industries, Biet Haemek, Israel) supplemented with a 1:1000 dilution of the ITS premix (BD Biosciences, Bedford, MA, USA), 2 mM L-glutamine, 4 ng/ml bFGF, 500 ng/ml ascorbic acid (Sigma, Steinheim, Germany), a 1:1000 dilution of a lipid mixture (Sigma Cat. No. L5146, Steinheim, Germany) and 100 ng/ml of IL6RIL6 chimera.

Cells were passaged every four to six days using 1.5 mg/ml type IV collagenase (Worthington biochemical corporation, Lakewood, NJ, USA). Cells were frozen in liquid nitrogen using a freezing solution consisting of 10 % DMSO (Sigma, St Louis MO, USA), 40 % human serum (HyClone, Utah, USA) and 50 % DMEM/F12 (Biological Industries, Beit Haemek, Israel).

Derivation of new hESC lines

Blastocyst cultivation - Zygotes were donated by couples undergoing pre-implantation genetic diagnosis (PGD) or *in vitro* fertilization (IVF) at Cornell Medical College, NY, who signed informed consent forms. The couples underwent the traditional IVF procedure after ovarian stimulation with gonadotropins and oocyte retrieval. Zygotes were cultured to the blastocyst stage according to IVF laboratory standard protocol: under oil using specialized C1/C2 media for insemination, growth and blastocyst development (Cornell).

Derivation of hESC lines - After digestion of the zona pellucida by Tyrode's acidic solution (Sigma, St Louis MO, USA) or its mechanical removal, the exposed blastocysts were placed in whole on a mitotically inactivated foreskin fibroblasts feeder layer (line F21 which was cultured in an animal free medium since its derivation until used). For the derivation and initial passages, cells were grown in the D2 or HA16 culture medium. The cells were initially passaged mechanically every four to ten days.

Immunohistochemistry - Undifferentiated hESCs grown in the tested culture system were fixed with 4 % paraformaldehyde and exposed to the primary antibodies (1:50) overnight at 4 °C. Stage-specific embryonic antigen (SSEA) 1, 3 and 4 (Hybridoma Bank, Iowa, USA), tumor recognition antigen (TRA) 1-60 and TRA1-81 (Chemicon International, Temecula CA, USA) and Oct 4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary antibodies. Cys 3 conjugated antibodies (Chemicon International, Temecula CA, USA) were used as secondary antibodies (1:200);

Karyotype analysis - Karyotype analysis (G-banding) was performed on at least 20 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).

EB formation - For the formation of EBs, one to three confluent wells were used in a six-well plate (30 cm^2). ESCs were removed from their culture dish using 1 mg/ml type IV collagenase, further broken into small clumps using 1000 μl Gilson pipette tips, and cultured in suspension in 58 mm petri dishes (Greiner, Frickenhausen, Germany). EBs were grown in differentiation medium consisting of 80 % DMEM/F12 (Biological Industries, Beit Haemek, Israel), supplemented with 20 % FBSd (HyClone, Utah, USA), and 1 mM L-glutamine (Invitrogen Corporation, Grand Island NY, USA).

RT PCR - Total RNA was isolated from hESCs grown for over 10 passages in feeder-free conditions, or from 10 day-old EBs (created from cells grown in the tested culture system for more then 10 passages) using Tri-Reagent (Sigma, St. Louis MO, USA), according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using MMLV reverse transcriptase RNase H minus (Promega, Madison WI, USA). PCR reaction included denaturation for 5 minutes at 94 °C followed by repeated cycles of 94 °C for 30 seconds, annealing for 30 seconds at a temperature as specified in Table 1 and extension at 72 °C for 30 seconds. PCR primers and reaction conditions used are described in Table 1 (see Example 1, hereinabove). PCR products were size-fractionated using 2 % agarose gel electrophoresis. DNA markers were used to confirm the size of the resultant fragments.

Teratoma formation - For teratoma formation, cells cultured in the offered culture methods for more than 15 passages, were injected into the rear leg muscle of 4-week-old male SCID-beige mice (two mice for each tested culture system). Cell numbers ranged from 5 x 10^6 cells to 10^7 cells per injection. Three to eight to 12 weeks after injection the mice were sacrificed and the resulting teratomas examined histologically.

Experimental Results

In this study the ability of few medium combinations, HA16, HA19, D1, D2, and CM100 to support undifferentiated and prolonged culture of hESCs in different culture conditions was examined. The basic medium, D1 or D2, is a commercial medium design for industrial and clinical proposes for the culture of hybridomas in suspension. The medium is free from animal, serum products and proteins. HA16 and HA19 are based on defined materials only.

The effect of two isoforms of TGFβ, TGFβ$_1$ and TGFβ$_3$, in supporting hESCs undifferentiated culture, was examined. Initially, two measures were used to estimate the ability of hESCs to grow in several culture systems, namely percentage of differentiation and rate of growth. The culture system used were: (1) feeder layer-free culture system based on fibronectin or Matrigel™ which are the most used matrices; (2) MEF, and (3) foreskin fibroblast. Based on these two parameters, the media supplemented with TGFβ$_3$, D2, HA16 and HA19, were found to be the most suitable to support undifferentiated hESC proliferation in all tested culture methods. Culture medium supplemented with 10 ng/ml bFGF only, failed to support hESC prolonged culture, in all the tested culture conditions. Although 60 % of the hESCs remained at the undifferentiated stage in these conditions for a few passages, the proliferation rate was low and with each passage the number of surviving hESCs decreased and the percentage of background differentiation was increased.

D1 medium on a feeder layer-free system is capable of maintaining all hESCs features along with high proliferation rate - When cultured in the feeder layer-free systems in the presence of the D1 medium, which is supplemented with TGFβ$_1$, the hESCs maintained all hESCs features including high proliferation rates. When cultured on the tested feeder layers in the presence of the D1 medium, the hESCs demonstrated a relatively high background differentiation rate of 20 % and

low proliferation abilities as compared to hESCs cultured at the same feeder layers systems with the D2 HA19 or HA16 medium.

D1, D2 and HA16 media in feeder layer-free are capable of maintaining hESCs in a proliferative, undifferentiated state, with chromosomal stability and pluripotency - Human ESCs grown in the presence of the D1, D2 or HA16 medium in feeder-layer free conditions were cultured continuously for up to 53, 24 or 10 passages, respectively, while maintaining their ESC features, including undifferentiated proliferation, chromosomal stability (as tested by karyotype analysis, not shown) and pluripotency. The background differentiation rates were found to be less than 10 %, which is similar to the differentiation rates occurring when hESCs are cultured in the traditional culture system based on MEFs as the feeder layer and medium supplemented with serum replacement and 4 ng/ml bFGF [Amit et al, 2000]. Examples of undifferentiated colonies cultured with D1, D2 or HA16 medium in feeder-layer free conditions and with the D2 or HA16 medium with the tested feeder layers are illustrated in Figures 5a-d.

hESCs cultured on feeder layer-free systems in the presence of the D1 or the D2 medium are devoid of autofeeder - Interestingly, when the hESCs were cultured in either the D1 or D2 medium on the feeder layer-free system the cells did not differentiate at the periphery of the colonies and did not form an outgrowth of feeder-like cells (also called "autofeeder") (Figure 5d), as described in other reports on feeder layer-free culture methods for hESCs (Xu et al, 2001). No morphological differences could be observed between colonies grown in the feeder layer-free culture system and those grown with feeder layers (Figures 5a-d). Correspondingly, morphological features remained unchanged on a single-cell level, rendering cells small and round, and exhibiting high nucleus-to-cytoplasm ratio, with a notable presence of one to three nucleoli and typical spacing between the cells (Figures 5a-d).

The D1, D2 or HA16 media are capable of maintaining hESCs with normal population doubling - Similar to cells grown on MEFs, cells cultured with D2 or HA16 medium in all tested culture methods, and the D1 medium in the feeder layer-free systems, were passaged routinely every four to six days, at the same ratio of 1/2 or 1/3, indicating a similar population doubling time as of hESCs grown on MEFs. 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 95 %. Using 40 % human serum and 10 % DMSO, cells were successfully frozen and thawed.

Karyotype analysis revealed normal karyotype of hESCs grown with the D1, D2, CM100 or HA16 media - 15 passages and more after transferring the cells into the tested environments, karyotype analysis was performed by Giemsa banding on two separate cultures, representing the four medium conditions, D1, D2, CM100 and HA16 at the different culture methods. At least 20 cells were tested from each sample, 40 cells from each medium combination. All examined cells were found to sustain normal karyotype of 46,XX for cell lines I3 and I4 and 46,XY for cell line I6 (data not shown). Overall, these results suggest that the cells' karyotype remains stable in the tested conditions, similarly to ESCs grown with MEFs using traditional methods (Amit et al, 2000).

hESCs cultured with the D1, D2 or HA16 express typical cell surface markers - Several surface markers typical of primate undifferentiated ES cells were examined using immunofluorescent staining (Thomson et al, 1995, 1996, 1998). hESCs cultured with the D1, D2 or HA16 medium for more than 20 passages, while using the tested culture conditions, were found to be strongly positive to surface markers TRA-1-60 (Figure 6a), SSEA4 (Figure 6b), TRA-1-81 (Figure 6c) and Oct 4 (data not shown). As in other primate ES cells, staining with SSEA3 was weak and negative for SSEA1 (data not shown).

hESCs cultured with the D1, D2 or HA16 medium are pluripotent as tested by EBs formation in vitro - The developmental potential of the cells after prolonged culture in the tested culture methods was examined *in vitro* by the formation of embryoid bodies (EBs). After more than 15, 20 and 30 passages in medium D1, D2 and HA16, respectively hESCs formed EBs similar to those created by hESCs grown on MEFs (not shown). Within these EBs, stem cells differentiated into cell types representative of the three embryonic germ layers as described for EBs formed from hESCs cultured on other culture systems (Itskovitz-Eldor et al, 2000).

EBs formed from the hESCs cultured on the D1, D2 or HA16 medium are capable of differentiating into the ectoderm, endoderm and mesoderm cell lineages - While undifferentiated cells cultured in the tested medium, feeder layers and matrices, expressed undifferentiated genetic markers such as Oct 4, Nanog, Sox2, Rex1, Cx43 and FGF4 (not shown) [Bhattacharya et al, 2004], cells harvested from 10

day-old EBs expressed genes such as albumin and glucagon (endoderm), α-cardiac actin, β-globulin and *Flk1* (mesoderm), and AC133 and neurofilament (ectoderm) as demonstrated by RT-PCR analysis (data not shown).

hESCs cultured with the D1, D2 or HA16 medium are pluripotent as tested by teratomas formation in vivo - The cells pluripotency was also tested *in vivo* by teratomas formation. hESCs cultured for over 12 passages in the HA16, D1 or D2 medium, in the tested culture systems formed teratomas following their injection into SCID-Beige mice. Within these teratomas, hESCs differentiated to representative tissues of the three embryonic germ layers including; cartilage, muscle, bone and fat (mesoderm), stratified epithelium, melanin containing epithelium (ectoderm), and kidney like structure (endoderm and mesoderm), and epithelium of endoderm origin (data not shown). Teratomas formation rates of 90 %, and the number of injected cells were identical to those demonstrated by cells cultured using traditional methods (Amit et al, 2000).

Altogether, these results demonstrate that hESCs cells subjected to prolonged culture in the tested culture systems demonstrated all hESCs features including; pluripotency, chromosomal stability, expression of specific genes and surface markers and indefinite proliferation as undifferentiated cells.

EXAMPLE 3

EVALUATION OF THE CAPACITY OF THE TGFβ-CONTAINING MEDIA DEVOID OF SERUM, SERUM REPLACEMENT AND ALBUMIN TO SUPPORT DERIVATION OF hESC LINE ON FORESKIN FIBROBLASTS IN A COMPLETE XENO-FREE SYSTEM

Materials and Experimental Methods – as in Example 2, hereinabove.

Experimental Results

The HA16 and D2 media are suitable for derivation of hESC line on foreskin fibroblast feeder layers in a complete xeno-free system - The medium combinations of the present invention were also tested for the ability to support hESC line derivation. While using the HA16 or D2 medium on foreskin fibroblasts as a supportive layer, new hESC lines were successfully derived and maintained for at

least 2 passages (in the presence of the D2 medium) or at least 18 passages (in the presence of the HA16 medium). The hESC line derived on foreskin in the presence of the HA16 culture medium demonstrated stem cells morphology at passage 18 (and the culture is still ongoing), normal XY karyotype and pluripotency as evidenced by the formation of EBs (Figures 3a-b and data not shown). The growth and success rates were similar to those obtained while using traditional culture methods. Since the used foreskin fibroblasts line, F21, were derived without any animal products, this new hESC lines were derived under complete xeno-free conditions. Although the new hESC lines still need to be tested for additional hESCs features, their morphology and proliferation rates indicate a typical hESCs culture.

EXAMPLE 4

TGFβ-CONTAINING MEDIUM DEVOID OF SERUM, SERUM REPLACEMENT AND ALBUMIN IS SUITABLE FOR EXPANDING AND MAINTAINING hESCs IN SUSPENSION

To examine the possibility of using the TGFβ-containing media devoid of serum, serum replacement and protein carrier (albumin) for expanding and maintaining hESCs in an undifferentiated state, hESCs were cultured in suspension, as follows.

Materials and Experimental Methods

ESCs and culture media - ESC cultures and the tested media: D1 medium, D2 medium, HA16 medium, HA19 medium and HACM100 medium, which do not contain serum or serum replacement; and the CM100F medium which contains serum replacement, were as described in Example 2, hereinabove.

Culture in suspension - To examine the possibility of using the TGFβ-containing medium which is devoid of serum, serum replacement and albumin for scalable culture of hESCs in suspension, hESCs were cultured in suspension in 58 mm petri dishes (Greiner, Frickenhausen, Germany) in a cell density of 1.5×10^6 to 6×10^6. The HA16 medium was supplemented with 0.1 % F68 (Sigma, St. Louis, MO, USA) for the suspended culture. The cells were passage every 5-7 days using either 30-60 minute incubation with 1.5 mg/ml type IV Collagenase (Worthington biochemical corporation, Lakewood, NJ, USA) or 25 minutes incubation with 1.5

mg/ml type IV Collagenase followed by five minutes incubation with 1 mg/ml Dispase (Invitrogen Corporation products, Grand Island NY, USA), and further broken into small clumps using 200 µl Gilson pipette tips. Alternatively, the cells were passaged mechanically using 27g needles. The medium was changed on a daily basis. Following continuous culturing under these conditions the cells were tested for hESC characteristics. The basic media used for culturing hESCs in suspension (which can be further supplemented with the additive and growth factors as described hereinabove) were DMEM, ko-DMEM, DMEM/F12, MabADCB or NCTC medium.

Derivation of new hESC lines in a suspension culture with the TGFβ-containing medium devoid of serum, serum replacement and albumin

Blastocyst cultivation - Zygotes were donated by couples undergoing PGD or *in vitro* fertilization (IVF) at Cornell Medical College, NY, who signed informed consent forms. The couples underwent the traditional IVF procedure after ovarian stimulation with gonadotropins and oocyte retrieval. Zygotes were cultured to the blastocyst stage according to IVF laboratory standard protocol: under oil using specialized C1/C2 media for insemination, growth and blastocyst development (Cornell).

Derivation of hESC lines in a suspension culture - Following the removal of the zona pellucida using Tyrode's acidic solution (Sigma, St Louis MO, USA), the trophoblast layer is specifically removed either by immunosurgery or mechanically using 27g needles. The exposed ICM is further cultured in suspension culture with a suitable culture medium (e.g., the CM100F, HA16 or D2) for 4-10 days. Initially, the cells are mechanically split using 27g needles.

RT PCR analysis - Total RNA was isolated from hESCs grown for 10-15 passages in the suspension culture using Tri-Reagent (Sigma, St. Louis MO, USA), according to the manufacturer's instructions. 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 of 94 °C for 30 seconds, annealing for 30 seconds at an annealing temperature as specified in Table 1, hereinabove and extension at 72 °C for 30 seconds. PCR primers and reaction conditions used are described in Table 1, hereinbelow. PCR products were size-fractionated using 2 % agarose gel

electrophoresis. DNA markers were used to confirm the size of the resultant fragments.

EB formation from hESCs cultured in suspension - For the formation of EBs, one to three 58 mm petri dishes (Greiner, Frickenhausen, Germany) containing ESCs in suspension cultures were transferred to new 58 mm petri dishes containing EBs-differentiation medium consisting of 80 % DMEM/F12 (Biological Industries, Beit Haemek, Israel), supplemented with 20 % FBSd (HyClone, Utah, USA), and 1 mM L-glutamine (Invitrogen Corporation, Grand Island NY, USA). Alternatively, prior to their transfer to the EB-differentiation medium, the ESCs were subject to treatment with 1 mg/ml type IV collagenase and further broken into small clumps using 1000 µl Gilson pipette tips. 10 day-old EBs were harvested for RNA isolation and histological examination.

Immunohistochemistry, karyotype analysis and Teratoma formation – were performed as described in Example 2, hereinabove.

Experimental Results

The CM100F, HA16, D1, D2 and HA19 media are suitable for culturing hESCs in suspension - hESCs were cultured in suspension using the newly developed TGFβ-containing medium types which are devoid of serum, serum replacement and albumin. To date, the highest passage of hESCs grown in suspension in the tested medium types were 3 passages in the D1 medium, 7 passages in the D2 medium, 10 passages in the HA19 medium and 17 passages in the CM100F medium. All hESCs exhibited undifferentiated morphology at these passages and can be further cultured in these media and maintain hESCs features. Histological sections of the hESCs clumps formed in the suspension cultures illustrated homogeneous cell population, of round cells with large nucleus (Figures 9a-g). In addition, when the cells were plated back on MEFs, they created colonies with typical hESCs morphology (Figures 9b-e), and if returned to suspension cultures, they continued proliferation as undifferentiated cells (data not shown). When hESCs were cultured in a suspension culture in the presence of the serum or serum replacement-free, IL6RIL6-containing HACM100 medium, the cells were expanded and maintained in the undifferentiated state for at least 1-2 passages (data not shown).

hESCs cultured in suspension in the presence of the D1, D2, HA19 or CM100F media express markers of undifferentiated hESCs - Cells cultured in suspension in the presence of the D2 medium for 3 passages as small clumps of 200-1500 cells expressed stem cells markers such as Oct 4 (Figure 8a), TRA-1-60 (Figure 8b), TRA-1-81 (Figure 8c) and SSEA4 (data not shown). Similar results were obtained with the CM100F, D1 or D2 medium at passage 5 (p-5) (data not shown). When cultured in suspension culture in the presence of the CM100F or the HA19 medium the cells expressed high levels of typical stem cells markers such as Oct 4 (Figure 10a), Rex1 (Figure 10b), Sox2 (Figure 10c), Nanog (Figure 10d) and FGF4 (data not shown) as demonstrated by RT-PCR analysis.

ESCs cultured in suspension are capable of forming EBs - When removed from the D1, D2 or HA16 medium and transferred to EBs medium (80 % DMEM/F12 supplemented with 20 % FBSd and 1 mM L-glutamine), the cells formed EBs containing representative tissues of three embryonic germ layers.

Rhesus ESCs can be also cultured in the suspension cultures of the present invention - Similar results with Rhesus ESCs (monkey embryonic stem cells, line R366.4, University of Wisconsin, primate center, Thomson lab, Madison, Wisconsin), which are regarded as good candidate for transgenic model to human diseases, were obtained when the Rhesus ESCs were cultured in suspension in the HA16, D1 and D2 TGFβ-containing culture media (data not shown).

Thus the new TGFβ-containing medium, which is devoid of serum, serum replacement and albumin, or the IL6RIL6-containing medium are capable of supporting the undifferentiated culture of hESCs, while maintaining hESCs characteristics, and provide methods for massive culture of these cells for industrial and clinical purposes.

Analysis and Discussion

hESCs, like mouse ES cells, are traditionally cultured with MEFs, which may expose them to animal pathogens. In this study, the present inventors have demonstrated, for the first time, a defined animal, serum and feeder layer-free culture system for hESCs, based on the use of commercial medium supplemented with either $TGF\beta_3$ or $TGF\beta_1$ and bFGF, and human fibronectin matrix as substitute. This medium

is designed for massive cultivation of cells in GMP for industrial or clinical purposes. All medium types of the present invention (with $TGF\beta_3$ or $TGF\beta_1$), support hESCs culture. The culture medium with the TGFβ isoform 3 was superior of the culture medium with the TGFβ1 isoform in terms of less background differentiation. All medium types of the present invention support the culture with feeders as good as with the regular serum containing media. Cells retained the same proliferation rates and the same background differentiation percentages as hESCs cultured with MEFs using traditional culture methods. Furthermore, the medium can also be used for massive suspended culture of undifferentiated hESCs.

Two isoforms of TGFβ, $TGF\beta_3$ and $TGF\beta_1$, were tested for their ability to maintain hESCs in an undifferentiated state using various culture conditions. $TGF\beta_3$ (D2 and HA16 media) was found to be the most suitable medium supplement, supporting undifferentiated culture of hESCs while using all the tested culture possibilities. All hESCs, from three different cell lines, continued to proliferate while retaining normal hESC features throughout the prolonged culture. Medium supplemented with $TGF\beta_1$ (D1 medium) on the contrary, was demonstrated to support undifferentiated hESC culture only while using feeder layer free culture systems.

Cells cultured while using these media (D1, D2, and HA16) maintained all the characteristics of ESCs. After prolonged culture of more than 20 passages, the cells remained undifferentiated, as demonstrated by the colony and single cell morphology, and by the expression of markers typical of undifferentiated primate ESCs [Thomson et al, 1995, 1996, 1998; Reubinoff et al, 2000]. In addition, while cultured in these conditions, hESCs expressed specific markers for the undifferentiated stage such as Oct 4, Sox 2, Rex1 and Nanog, as demonstrated by RT-PCR.

Karyotype analysis carried out on representative cell samples demonstrated that the hESCs' karyotype remained stable in the proposed conditions. None of the examined cells exhibited any karyotype abnormalities.

The cells' pluripotency was examined *in vitro*. Cells cultured in the tested culture systems for more than 10 passages, formed EBs similar to those created when grown on MEFs [Itskovitz-Eldor et al, 2000]. RT-PCR analysis demonstrated that cells within these EBs differentiated into different cell types representative of the three germ layers. Furthermore, following their injection to SCID-Beige mice, hESCs

formed teratomas containing a multitude of tissues types (D1 and D2, HA16 in process). The teratoma formation rates were identical to those of cells cultured with MEFs. Thus, the pluripotency of the cells culture continuously in the tested culture methods remained intact.

Additionally, and most importantly, the same measurements were used to characterize cells cultured with the D1, D2 and HA16 medium in suspension. ESCs cultured under these conditions for more than 7 passages exhibited undifferentiated markers and when transferred to differentiation promoting conditions, demonstrated pluripotency. Thus, these media can enable massive culture of undifferentiated hESCs, and facilitate the development of control bioprocesses in industrial bioreactors.

These results demonstrate that hESCs can be maintained as undifferentiated cells in the proposed defined animal- and serum-free medium combination, without any feeder cells (D1, D2 and HA16) or alternatively, with commonly used acceptable feeder layers (D2 and HA16). Thus, these media can facilitate hESCs culture for research, industrial and clinical purposes. Moreover, these novel culture media were found to support suspended culture of undifferentiated hESCs, the first and primary step in developing a massive culture system for their growth and scale-up, a crucial step for any industrial and clinical uses.

The mechanism by which hESCs self-maintain is still unclear. Accumulating data suggest the involvement of TGFβ family members in hESCs renewal [Amit et al, 2004; Ludwig et al, 2006; James et al, 2005; Chen et al, 2006, Valdimarsdottir & Mummery, 2006]. Further complementary research is required to explain the underlying mechanisms of action of TGFβ at the level of signal transduction, and the fact that $TGF\beta_3$ is more potent than $TGF\beta_1$.

Future clinical uses of hESCs will require a reproducible, well-defined and xeno-free culture system. The culture method described in this study of fibronectin and D1, D2 or HA16 medium and foreskins fibroblast meet these needs. The well-defined media demonstrated in the present study are suitable for culturing hESCs and may be advantageous for undertaking research on the mechanisms of ESC self-maintenance, especially of the possible roles of the TGFβ pathway. Other studies

using hESCs, such as the research on differentiation pathways and mechanisms, will benefit from the availability of a well-defined and reproducible culture system.

Thus, the present invention discloses for the first time:

1. A defined, xeno-free, serum, serum replacement or albumin-free system suitable for both culture and derivation of hESCs.

2. Defined medium combinations, highly effective in supporting hESCs culture in variety of culture conditions. Priority of $TGF\beta_3$ over $TGF\beta_1$. $TGF\beta_3$ was never demonstrated to promote self-renewal of stem cells.

3. A culture system that allows hESC culturing in suspension as undifferentiated without a carrier (without substrate adherence).

4. A scalable culture system, suitable for developing control bioprocesses in industrial bioreactors.

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.

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.

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WHAT IS CLAIMED IS:

1. A culture medium comprising a transforming growth factor beta (TGFβ) isoform and being serum-free, animal contaminant-free, feeder-free and protein carrier-free and capable of maintaining stem cells in an undifferentiated state under animal contaminant-free conditions.

2. A cell culture comprising a stem cell and a culture medium, said culture medium comprises a transforming growth factor beta (TGFβ) isoform and being serum-free, animal contaminant-free, feeder-free and protein carrier-free and capable of maintaining said stem cells in an undifferentiated state under animal contaminant-free conditions.

3. A method of expanding and maintaining stem cells in an undifferentiated state, the method comprising culturing the stem cells in a culture medium comprising a transforming growth factor beta (TGFβ) isoform and being serum-free, animal contaminant-free, feeder-free and protein carrier-free and capable of maintaining the stem cells in an undifferentiated state under animal contaminant-free conditions, thereby expanding and maintaining the stem cells in the undifferentiated state.

4. A method of deriving an embryonic stem cell line, the method comprising:

culturing an embryonic stem cell, which has been obtained from a pre-implantation stage blastocyst, post-implantation stage blastocyst and/or a genital tissue of a fetus in a culture medium comprising a transforming growth factor beta (TGFβ) isoform and being serum-free, animal contaminant-free, feeder-free and protein carrier-free and capable of maintaining said embryonic stem cell in an undifferentiated state under animal contaminant-free conditions;

thereby deriving the embryonic stem cell line.

5. A method of generating lineage-specific cells from embryonic stem cells, the method comprising:

(a) culturing the embryonic stem cells in a culture medium comprising a transforming growth factor beta (TGFβ) isoform and being serum-free, animal contaminant-free, feeder-free and protein carrier-free and capable of maintaining the embryonic stem cells in an undifferentiated state under animal contaminant-free conditions, to thereby obtain expanded, undifferentiated embryonic stem cells; and

(b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating and/or expanding lineage specific cells;

thereby generating the lineage-specific cells from the embryonic stem cells.

6. A method of generating embryoid bodies from embryonic stem cells, the method comprising:

(a) culturing the embryonic stem cells in a culture medium comprising a transforming growth factor beta (TGFβ) isoform and being serum-free, animal contaminant-free, feeder-free and protein carrier-free and capable of maintaining the embryonic stem cells in an undifferentiated state under animal contaminant-free conditions, to thereby obtain expanded, undifferentiated embryonic stem cells; and

(b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating said embryonic stem cells to embryoid bodies;

thereby generating the embryoid bodies from the embryonic stem cells.

7. A method of generating lineage-specific cells from embryonic stem cells, the method comprising:

(a) culturing the embryonic stem cells in a culture medium comprising a transforming growth factor beta (TGFβ) isoform and being serum-free, animal contaminant-free, feeder-free and protein carrier-free and capable of maintaining the embryonic stem cells in an undifferentiated state under animal contaminant-free conditions, to thereby obtain expanded, undifferentiated embryonic stem cells;

(b) subjecting said expanded, undifferentiated embryonic stem cells to culturing conditions suitable for differentiating said expanded, undifferentiated embryonic stem cells to embryoid bodies; and

(c) subjecting cells of said 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.

8. The culture medium of claim 1, being devoid of serum replacement.

9. A cell culture comprising a stem cell and the culture medium of claim 8.

10. The method of claim 3, 4, 5, 6 or 7, wherein said culture medium is devoid of serum replacement.

11. The cell culture of claim 2 or 9, wherein said stem cells are embryonic stem cells.

12. The culture medium of claims 1, or 8, wherein said protein carrier is albumin.

13. The method of any of claims 3, 4, 5, 6, 7, or 10, wherein said culturing is effected in suspension.

14. The method of any of claims 3, 4, 5, 6, 7, or 10, wherein said culturing is effected on a feeder- free matrix.

15. The method of claim 14, wherein said feeder- free matrix is a fibronectin matrix.

16. The method of any of claims 3, 4, 5, 6, 7, or 10, wherein said culturing is effected on feeder cells.

17. The culture medium of claim 1 or 8, wherein said TGFβ isoform is a TGFβ isoform 1 ($TGF\beta_1$).

18. The culture medium of claim 1 or 8, wherein said TGFβ isoform is a TGFβ isoform 3 ($TGF\beta_3$).

19. The culture medium of claim 17, wherein said $TGF\beta_1$ is provided at a concentration of at least 0.06 ng/ml.

20. The culture medium of claim 17, wherein said $TGF\beta_1$ is provided at a concentration of 0.12 ng/ml.

21. The culture medium of claim 18, wherein said $TGF\beta_3$ is provided at a concentration of at least 0.5 ng/ml.

22. The culture medium of claim 18, wherein said $TGF\beta_3$ is provided at a concentration of 2 ng/ml.

23. The culture medium of any of claims 1, 8, 12, and 17-22, wherein said culture medium comprises basic fibroblast growth factor (bFGF).

24. The culture medium of claim 23, wherein said bFGF is provided at a concentration of at least 2 ng/ml.

25. The culture medium of claim 23, wherein said bFGF is provided at a concentration of at least 4 ng/ml.

26. The method of any of claims 7, 14 and 13-16, further comprising isolating lineage specific cells following step (b).

27. The method of claim 26, wherein said isolating lineage specific cells is effected by a mechanical separation of cells, tissues and/or tissue-like structures contained within said embryoid bodies.

28. The method of claim 26, wherein said isolating lineage specific cells is effected by subjecting said embryoid bodies to differentiation factors to thereby induce differentiation of said embryoid bodies into lineage specific differentiated cells.

29. The method of any of claims 4, 5, 6, 7, and 13-16, and 26-28, wherein the embryonic stem cell is a human embryonic stem cell.

30. The method of any of claims 4, 5, 6, 7, 13-16 and 26-28, wherein the embryonic stem cell is a primate embryonic stem cell.

31. The cell culture of any of claims 2, 9 and 11, wherein said stem cells are human embryonic stem cells.

32. The culture medium of any of claims 1, 17, 19, 20, and 23-25, wherein said culture medium comprises 0.12 ng/ml TGFβ1, 10 ng/ml bFGF and glutamine.

33. The culture medium of any of claims 1, 18, and 21-25, wherein said culture medium comprises 2 ng/ml TGFβ3, 10 ng/ml bFGF and glutamine.

34. The culture medium of any of claims 1, 18, and 21-25, wherein said culture medium comprises 2 ng/ml TGFβ3, 4 ng/ml bFGF, insulin, transferrin, selenius acid, glutamine, ascorbic acid and a lipid mixture.

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35 40 45

Gly Val Glu Pro Glu Asp Asn Ala Thr Val His Trp Val Leu Arg Lys
50 55 60

Pro Ala Ala Gly Ser His Pro Ser Arg Trp Ala Gly Met Gly Arg Arg
65 70 75 80

Leu Leu Leu Arg Ser Val Gln Leu His Asp Ser Gly Asn Tyr Ser Cys
85 90 95

Tyr Arg Ala Gly Arg Pro Ala Gly Thr Val His Leu Leu Val Asp Val
100 105 110

Pro Pro Glu Glu Pro Gln Leu Ser Cys Phe Arg Lys Ser Pro Leu Ser
115 120 125

Asn Val Val Cys Glu Trp Gly Pro Arg Ser Thr Pro Ser Leu Thr Thr
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Lys Ala Val Leu Leu Val Arg Lys Phe Gln Asn Ser Pro Ala Glu Asp
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Phe Gln Glu Pro Cys Gln Tyr Ser Gln Glu Ser Gln Lys Phe Ser Cys
165 170 175

Gln Leu Ala Val Pro Glu Gly Asp Ser Ser Phe Tyr Ile Val Ser Met
180 185 190

Cys Val Ala Ser Ser Val Gly Ser Lys Phe Ser Lys Thr Gln Thr Phe
195 200 205

Gln Gly Cys Gly Ile Leu Gln Pro Asp Pro Pro Ala Asn Ile Thr Val
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Thr Ala Val Ala Arg Asn Pro Arg Trp Leu Ser Val Thr Trp Gln Asp
225 230 235 240

Pro His Ser Trp Asn Ser Ser Phe Tyr Arg Leu Arg Phe Glu Leu Arg

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Tyr Arg Ala Glu Arg Ser Lys Thr Phe Thr Thr Trp Met Val Lys Asp
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Val Val Gln Leu Arg Ala Gln Glu Glu Phe Gly Gln Gly Glu Trp Ser
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Glu Trp Ser Pro Glu Ala Met Gly Thr Pro Trp Thr Glu Ser Arg Ser
305                 310                 315                 320

Pro Pro Ala Glu Asn Glu Val Ser Thr Pro Met Gln Ala Leu Thr Thr
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Asn Lys Asp Asp Asp Asn Ile Leu Phe Arg Asp Ser Ala Asn Ala Thr
            340                 345                 350

Ser Leu Pro Val Glu Phe Met Pro Val Pro Pro Gly Glu Asp Ser Lys
        355                 360                 365

Asp Val Ala Ala Pro His Arg Gln Pro Leu Thr Ser Ser Glu Arg Ile
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Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile Ser Ala Leu Arg Lys
385                 390                 395                 400

Glu Thr Cys Asn Lys Ser Asn Met Cys Glu Ser Ser Lys Glu Ala Leu
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Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala Glu Lys Asp Gly Cys
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Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu Val Lys Ile Ile Thr
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Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr Leu Gln Asn Arg Phe
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Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln Met Ser Thr Lys Val
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Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn Leu Asp Ala Ile Thr
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Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu Thr Lys Leu Gln Ala
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Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His Leu Ile Leu Arg Ser
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Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala Leu Arg Gln Met
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