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(54) Title: CULTURE MEDIUM

(57) Abstract: The invention relates to a culture medium that is suitable for use in the propagation of mammalian pluripotent stem cells in a mesoderm-biased phenotype, and to methods for propagating mammalian pluripotent stem cells in a mesoderm-biased phenotype. The invention further relates to a population of mammalian pluripotent stem cells wherein said population of cells displays a mesoderm-biased phenotype.

# Culture Medium

## Field of the Invention

The invention relates to a culture medium for the propagation of mammalian pluripotent stem cells in a mesoderm-biased phenotype; to a method of propagating mammalian stem cells in a mesoderm-biased phenotype; and to a population of mammalian pluripotent stem cells wherein said population of cells display a mesoderm-biased phenotype.

## Background of the Invention

Pluripotent stem cells (which include embryonic stem cells, induced pluripotent stem cells, naïve pluripotent stem cells, epiblast stem cells and primed pluripotent stem cells) are defined as cells that (i) possess the capacity to differentiate into any of the three germ layers (i.e. endoderm, which develops into e.g. the interior stomach lining, gastrointestinal tract, and the lungs; mesoderm, which develops into e.g. muscle, bone, blood, and urogenital organs; or ectoderm, which develops into, e.g. epidermal tissues and the nervous system); and (ii) are self-renewing, i.e. they possess the ability to go through numerous cycles of cell division whilst maintaining the differentiative capacity of their ancestral cells.

Techniques to culture and maintain the undifferentiated state of pluripotent stem cells such as human embryonic stem cells have been established and developed. Such stem cell culture techniques originally relied upon the exposure of the stem cell line, either directly or indirectly, to mouse embryonic fibroblast (MEF) feeder cells as a feeder layer on which human embryonic stem cells could be cultured. However, feeder-free embryonic stem cell culture media have now been developed. European Patent Application No. 1 749 091 discloses such a feeder-free stem cell culture medium for maintaining the cells in an undifferentiated state, the medium comprising salts, vitamins, amino acids, glucose, a fibroblast growth factor and a bone morphogenetic protein.

The components required for human embryonic stem cell and induced pluripotent stem cell propagation have been extensively examined in order to develop chemically defined cell culture systems. A completely defined, 8 component (including

DMEM/F12) mixture was disclosed for this purpose in Chen, *et al*. 2011. This medium, referred to in the art as E8, has become a standard within industry of stem cell propagation, and can be obtained commercially under the trademark Essential 8™ and TeSR™-E8™ or it can be prepared according to the formulation published in Chen, *et al*. 2011.

In addition to stem cell propagation methods, techniques for differentiating stem cells into specific germ layers and further into cell lineages have been developed. For example, US Pat. No. 9,708,582, to StemCell Technologies Inc., discloses the use of specific media formulations that allow one to obtain the desired differentiated cell type from a population of mammalian pluripotent cells in a selective and standardized manner. StemCell Technologies Inc. also commercially produce STEMDiff™ mesoderm induction medium (https://www.stemcell.com/stemdiff-mesoderm-induction-medium.html), which is designed to encourage human / induced pluripotent stem cell differentiation towards a mesodermal lineage. However, it does not maintain the stem cells themselves in a biased, i.e. pre-complete differentiation, state and, consequently, expansion of lineage-biased cells is not possible using this technology.

However, there is no known medium that enables the passaging of mammalian pluripotent stem cells in a reversible state of bias towards a particularly differentiated state and so it has not been possible to maintain such cells in such a biased state for an extended period of time.

It would be desirable in various areas of stem cell research to maintain mammalian stem cell populations in a state of bias towards a particularly differentiated state, particularly whilst cell numbers are passaged upwards towards experimentally useful numbers. Therefore, there is clear need in the industry for a cell culture medium that enables the propagation of mammalian pluripotent stem cells in a mesodermal biased phenotype.

## Statements of Invention

According to a first aspect of the invention there is provided a culture medium for the propagation of mammalian pluripotent stem cells in a mesoderm-biased phenotype, said medium comprising:

a. one or more inhibitor of the Hippo signalling pathway;
b. one or more canonical WNT signalling agonist; and
c. one or more inhibitor of endogenous WNT signalling.

By including each of the above components in a mammalian stem cell culture medium, it has been shown that mammalian pluripotent stem cells can be propagated for multiple passages, i.e. undergo numerous cycles of cell division, whilst exhibiting a mesoderm-biased phenotype.

As used herein, the term "mesoderm-biased" refers to a pluripotent stem cell that retains the ability to form all germ layers but when induced to differentiate, particularly under "neutral" conditions tends to favour the formation of mesodermal derivatives. The state is characterised by the expression pluripotency-associated genes and co-expression or upregulation of mesendodermal-associated genes. Pluripotency-associated genes may include but are not limited to *POU5F1, NANOG, SOX2* and/or *DNMT3B*. Mesendodermal-associated genes may include but are not limited to *MIXL1, T, TBX3, EOMES, CDX2, NODAL, SOX17, GATA4, GATA6, LEFTY1* and/or *LEFTY2.* The state also expresses a panel of pluripotency-associated surface markers that may include but are not limited to Alkaline Phosphatase, BF4, CD9, SSEA-3, SSEA-4, THY1, TRA-1-60 and/or TRA-1-81. The state has the ability to make self-renewing clonal lines which retain pluripotent potential. Further, cells in this state can be transitioned into standard stem cell culture conditions with transcriptional changes reverting back to levels seen in traditional stem cell cultures.

Inhibitors of the Hippo signalling pathway are any molecules that have an inhibitory effect of the Hippo pathway. Such inhibitors include, but are not limited to, G protein-coupled receptor signalling molecules, and are preferably selected from Lysophosphatidic Acid (LPA), Sphingosine-1-phosphate (S1P), or a combination of LPA and S1P. In some embodiments, inhibitors of the Hippo signalling pathway is an agonist of a G protein-coupled receptor. Such agonists include, but are not limited to, agonists of LPA G protein-coupled receptor(s) and/or S1P G protein-coupled receptor(s). Preferably said agonist is a selective agonist of LPA G protein-coupled receptors and, more preferably, is a selective agonist of $LPA_2$. An exemplary example of a selective agonist of $LPA_2$ is GRI977143 ('GRI', also known as 2-[[3-(1,3-Dioxo-

1H-benz[de]isoquinolin-2(3H)-yl)propyl]thio}-benzoic acid), which is particularly advantageous for use in stem cell media because this non-lipid chemical would be less prone to breakdown by the cell culture in comparison to, e.g. the G protein-coupled receptor signalling molecules LPA or S1P.

Inhibitors of the Hippo signalling pathway may also be selected from small molecule inhibitors that target one or more components of the protein cascade of the Hippo signalling pathway. Such small molecules inhibitors are preferably selected from XMU-MP1 and 9E1.

Canonical WNT signalling agonists are any molecules that have an activating effect on canonical WNT signalling pathway. The well-known canonical WNT signalling pathway causes an accumulation of β-catenin in the cytoplasm followed by its translocation into the nucleus. Glycogen Synthase Kinase 3 (GSK3) inhibitors, in particular GSK3-β inhibitors, are known to be highly potent activators of the canonical WNT signalling pathway. Therefore, in preferred embodiments, the one or more canonical WNT signalling agonist is or includes a GSK3 inhibitor, more preferably a GSK3-β inhibitor. Exemplary GSK3 inhibitors are selected from CHIR 98014, CHIR 99021, AR-A0144-18, TDZD-8, SB216763, and combinations thereof.

Recombinant WNT ligands are also known to be potent activators of canonical WNT signalling. Therefore, as an alternative to GSK3 inhibition, one or more recombinant WNT ligands may be utilised as the canonical WNT signalling agonist(s).

Inhibitors of endogenous WNT signalling are any molecules that have an inhibitory effect on the activation of canonical WNT signalling by endogenous WNT ligands produced by cells in culture. As the release / secretion of WNT ligands is governed by the Porcupine (PORCN) protein, in preferred embodiments the inhibitor(s) of endogenous WNT signalling is/are a PORCN protein inhibitor, which can be used to block the secretion of endogenous WNT ligands and thus prevent the activation of WNT signalling. Exemplary PORCN protein inhibitors are selected from IWP-1, IWP-2, IWP-3, IWP-4, IWP-5, IWP-6, IWP-7, IWP-8, IWP-9, IWP-10, and combinations thereof.

As an alternative to a PORCN protein inhibitor, the inhibitor of endogenous WNT signalling may be a recombinant ligand that inhibits the interaction between endogenous WNT ligands and their associated receptors. A preferred example of such a recombinant ligand is recombinant Dickkopf-related protein 1 (DKK1).

The cell culture medium typically comprises, in addition to the essential components identified above, one or more basal medium components that are typically found in mammalian stem cell culture medium. As would be readily understood by those of ordinary skill in the art, such additional components typically include: one or more sources of L-glutamine, preferably a stabilized form of L-glutamine such as L-alanyl-L-glutamine, which is commercially available as GlutaMAX™; insulin; transferrin, basic fibroblast growth factor (FGF2); and/or more member of the transforming growth factor (TGF)-β superfamily, preferably TGF-β1.

The basal medium components typically also comprise a nutrient mixture that is appropriate for use in the propagation of mammalian stem cell cultures. Such nutrient mixtures are well known to those skilled in the art and are not particularly limited. However, the commercially available nutrient mixture DMEM/F12 is particularly suitable. As used herein, “DMEM” refers to Dulbecco's modified eagle medium which was developed in 1969 and has since become a standard medium for cell culture that is commercially available from various sources. As used herein, “F12” refers to Ham's nutrient mixture F12 medium. This medium is also a standard cell culture medium that is commercially available from various sources (e.g. Sigma Aldrich (catalogue number D6421 or D6434), and was initially designed to cultivate a wide variety of mammalian and hybridoma cells. As used herein, “DMEM/F12” refers to a 1:1 mixture of DMEM with F12, which has become a widely used basal medium for supporting the growth of many different mammalian cells and is commercially available from various suppliers. An alternative, and non-limiting, example of a suitable nutrient mixture for use in the medium of the invention is KnockOut™ DMEM, which is commercially available from ThermoFischer Scientific (catalog number 10829018).

A particularly preferred basal medium, to which the essential components discussed above can be added to prepare the culture medium of the present invention, is the defined stem cell culture medium E8 which is commercially available from ThermoFisher Scientific under the name Essential 8™ (catalog number A1517001)

and Stem Cell Technologies under the name TeSR™-E8™ (catalog number #05990) in prepared form or can be made in house according to the E8 formulation published in Chen, *et al*. 2011.

The culture medium of the present invention preferably further comprises one or more albumin, which may or may not be a component of the basal medium. Preferably, the albumin is a bovine serum albumin, and is more preferably a fatty acid free bovine serum albumin.

The culture medium of the present invention is preferably feeder cell free. As is readily understood in the field of stem cell cultivation, attachment factors, i.e. additional elements that allow attachment of stem cells to the culture vessel, are preferably employed for the propagation of pluripotent cells in such a feeder cell-free medium. Said attachment factors, which are conventionally considered as separate elements from the culture medium itself, are commonly used to pre-treat the culture vessel prior to the addition and subsequent incubation of a population of mammalian pluripotent stem cells in the culture medium. Said attachment factors may also be added to the culture medium, either in lieu of or in addition to pre-treatment of the culture vessel. Particularly suitable attachment factors include but are not limited to vitronectin, Matrigel (Corning), Geltrex (Thermofisher) and laminin 521 (Biolamin).

In a particularly preferred embodiment, the culture medium of the present invention comprises DMEM/F12, to which the components shown in the table below are added. This culture medium is hereinafter referred to for ease of reference as “2xPrimo” medium. This 2xPrimo medium may also be used in diluted form by the incorporation of additional DMEM/F12 such that the concentration of LPA is reduced from 0.96 μM to 0.48 μM, with the resultant medium being referred to for ease of reference as “1xPrimo” medium. As would be apparent to one of ordinary skill in the art, other dilutions of the 2xPrimo medium may be made by modification of the DMEM/F12 content.

| Component | Concentration |
|---|---|
| L-ascorbic acid | 64 mg/L |
| Selenium | 14 µg/L |
| Transferrin | 10.7 mg/L |
| $NaHCO_3$ | 543 mg/L |
| Insulin | 19.4 mg/L |
| L-Glutamine (Glutamax) | 10 ml/L |
| FGF2 | 100 µg/L |
| TGF-β1 | 2 µg/L |
| Albumin (fatty acid free bovine serum albumin (BSA)) | 0.2% |
| Cholesterol | 4 µM |
| LPA | 0.96 µM |
| CHIR99021 | 3 µM |
| IWP-2 | 1 µM |

Further exemplary culture media comprise DMEM/F12, to which the following components are added:

(i) 64 mg/L L-ascorbic acid; 14 µg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 µg/L FGF2; 2 µg/L TGF-β1; 0.1% Albumin (fatty acid free bovine serum albumin (BSA)); 2 µM Cholesterol; 0.48 µM LPA; 3 µM CHIR99021; and 1 µM IWP-2; or

(ii) 64 mg/L L-ascorbic acid; 14 µg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 µg/L FGF2; 2 µg/L TGF-β1; 0.2% Albumin (fatty acid free bovine serum albumin (BSA)); 4 µM Cholesterol; 0.96 µM LPA; 10 µM SB216763; and 1 µM IWP-2; or

(iii) 64 mg/L L-ascorbic acid; 14 µg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 µg/L FGF2; 2 µg/L TGF-β1; 0.2% Albumin (fatty acid free bovine serum

albumin (BSA)); 4 μM Cholesterol; 0.96 μM LPA; 3 μM CHIR99021; and 100 ng/mL DKK1; or

(iv) 64 mg/L L-ascorbic acid; 14 μg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 μg/L FGF2; 2 μg/L TGF-β1; 0.4% Albumin (fatty acid free bovine serum albumin (BSA)); 8 μM Cholesterol; 1.92 μM S1P; 3 μM CHIR99021; and 1 μM IWP-2; or

(v) 64 mg/L L-ascorbic acid; 14 μg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 μg/L FGF2; 2 μg/L TGF-β1; 0.4% Albumin (fatty acid free bovine serum albumin (BSA)); 8 μM Cholesterol; 4 μM GRI; 3 μM CHIR99021; and 1 μM IWP-2.

As indicated above, the culture medium of the present invention can be used to propagate a population of mammalian pluripotent stem cells for multiple passages whilst said cells exhibit a mesoderm-biased phenotype. Preferably the culture medium is a human stem cell culture medium. Preferably the culture medium is an embryonic stem cell, induced pluripotent stem cell, naïve pluripotent stem cell, epliblast stem cell or primed pluripotent stem cell culture medium.

As would be apparent to one of ordinary skill in the art, and without wishing to be bound by theory, the specific identity and/or absolute concentration of each of the key components of the culture medium of the invention, i.e. the inhibitor of the Hippo signalling pathway, the canonical WNT signalling agonist and the inhibitor of endogenous WNT signalling, is not critical to the ability of said culture to propagate mammalian pluripotent stem cells in a mesoderm-biased state. Instead, it is important that a balance between the concentration of the Hippo signalling pathway inhibitor (which tends to drive the cultured cells towards an unbiased pluripotent state) and the canonical WNT signalling agonist (which tends to drive the culture cells towards a differentiated state) is achieved. For example, a higher concentration of CHIR99021 can be balanced with a higher concentration of LPA to provide an alternative media for propagation of mammalian pluripotent stem cells in a mesoderm-biased state. Establishing the minimum concentration and/or balancing the relative concentrations of the inhibitor of the Hippo signalling pathway, the canonical WNT signalling agonist

and the inhibitor of endogenous WNT signalling is well within the repertoire of the skilled reader in light of the teachings of the present application.

According to a second aspect of the invention, there is provided a method of propagating mammalian pluripotent stem cells in a mesoderm-biased phenotype, the method comprising: incubating a population of mammalian pluripotent stem cells in a culture medium according to the first aspect of the invention and passaging the stem cell population multiple times.

According to a third aspect, the invention provides the use of one or more inhibitor of the Hippo signalling pathway, one or more canonical WNT signalling agonist and one or more inhibitor of endogenous WNT signalling for the propagation of a population of mammalian pluripotent stem cells in a mesoderm-biased phenotype.

According to a fourth aspect, the invention provides the use of one or more inhibitor of the Hippo signalling pathway, one or more canonical WNT signalling agonist and one or more inhibitor of endogenous WNT signalling for the manufacture of a medium for the propagation of a population of mammalian pluripotent stem cells in a mesoderm-biased phenotype.

According to a fifth aspect of the invention, there is provided a population of mammalian pluripotent stem cells, wherein said population of cells display a mesoderm-biased phenotype.

In each of the second, third, fourth and fifth aspects of the invention, said mammalian pluripotent stem cells are preferably human pluripotent stem cells.

In each of the second, third, fourth and fifth aspects of the invention, said mammalian pluripotent stem cells are preferably selected from embryonic stem cells, induced pluripotent stem cells, naïve pluripotent stem cells, epiblast stem cells and primed pluripotent stem cells. In particularly preferred embodiments said mammalian pluripotent stem cells are human embryonic stem cells.

Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of the words, for example “comprising” and “comprises”,

mean "including but not limited to" and do not exclude other moieties, additives, components, integers or steps. Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

All references, including any patent or patent application, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. Further, no admission is made that any of the prior art constitutes part of the common general knowledge in the art.

Preferred features of each aspect of the invention may be as described in connection with any of the other aspects.

Other features of the present invention will become apparent from the following examples. Generally speaking, the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein, unless incompatible therewith. Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

The Invention will now be described by way of example only with reference to the Examples below and to the following Figures wherein:

**Figure 1: Functional assessment of cells grown in Primo medium**

**A.** Schematic diagram of the experimental process. Human Embryonic stem cells were grown on recombinant vitronectin coated flasks in 2xPrimo medium for 3 days then cells were taken, unsorted or sorted for MIXL1(+)/SSEA-3(+) and MIXL1(-)/SSEA-3(+), and put through a "neutral" EB assay, analysed after 7 days. As is understood within in the field of stem cell technology, the "neutral"

EB assay involves aggregating cells in a basal medium containing no cytokines or signalling factors to direct differentiation. Separately, single MIXL1(+)/SSEA-3(+) cells were sorted from cells grown in 2xPrimo conditions and clonal lines were established, these lines were then assessed by the same "neutral" EB assay.

**B.** Flow Cytometry density plots of SSEA-3 versus *MIXL1*-GFP from HES3 *MIXL1* cells grown in 2xPrimo conditions. Red box and green boxes indicates the sorting gates.

**C.** Representative colony growth post first passage, immunofluorescence images of live TRA-1-81 staining (RED) and *MIXL1*-GFP expression in (Green).

**D.** Flow cytometry density plots of clone 10-A4 grown in E8 conditions for surface markers, BF4, CD9, SSEA-3, SSEA-4, THY1 and TRA-1-81 CH8 versus *MIXL1*-GFP.

**E.** Bar chart displaying the algorithm score for each sample, for self renewal and three lineages, ectoderm, mesoderm and endoderm. The algorithm score is calculated based on the qPCR values for genes of a given lineage, the 0 baseline is based on the average value of undifferentiated samples. EB samples have been normalised to their undifferentiated counterparts (bars are mean ±SD, significance assessed by two-way ANOVA analysis).

**F.** Flow cytometry density plot of SSEA-3 versus *T*-Venus expression of H9 *T*-Venus reporter line grown in E8 medium and 2xPrimo after 3 passages. SSEA-3 expression remains high in both conditions, but T-Venus expression abundantly detected in 2xPrimo only.

**G.** Algorithm score for EBs generated from H9 T-Venus and Miff1 (iPS line) grown in Primo medium for 3 passages.

**Figure 2. Assessment of multiple passages in Primo medium**

**A.** A schematic diagram outlines how cells could be maintained over multiple passages in Primo medium with the ability to revert to a MIXL1(-) population via bulk and single cell transition.

**B.** Phase contrast and immunofluorescence analysis of a representative colony from single cell cloning. Shown is a 4x brightfield image, 4x Hoechst image, then 10x Hoechst, and NANOG expression. The merged image displays Hoechst in blue and NANOG in red.

C. Flow cytometry density plots of *MIXL1*-GFP versus a given pluripotency associated surface marker for HES3 *MIXL1*-GFP at passage 7 in 2xPrimo conditions. Markers analysed are BF4, CD9, SSEA-3, SSEA-4, THY1 and TRA-1-81. Cells show high expression of all markers analysed and a *MIXL1* positive population present.

D. Immunofluorescence analysis of Hoechst, *MIXL1*-GFP, and NANOG expression of HES3 *MIXL1*-GFP cells in 2xPrimo and E8 alone for 3 days post to 9 passages in 2xPrimo. A merged image of all four channels is present below Hoechst (Blue), *MIXL1*-GFP (Green), and NANOG (red).

E. Stacked percentage bar charts displaying cell profiler analysis of 3 wells for each condition (Bars are mean ± SD) for *MIXL1*-GFP, and NANOG expression grown in 2xPrimo, 2xBCL, E8 alone and E8 with 1μM IWP-2 added, for 3 days post to 9 passages in 2xPrimo.

F. Representative image of G-banded metaphase spreads for HES3 *MIXL1*-GFP at 10 passages in 2xPrimo.

**Figure 3 Human PSC Scorecard Heatmap Analysis**

Heatmap of samples assessed by the human PSC scorecard. Ct values for each gene are normalised to *ACTB.* Heatmap colouring is done after mean centring the genes across the samples. Hierarchical clustering was performed on the samples. Samples are colour coded. Self Renewal (SR) refers to samples grown in E8V conditions. Genes are ordered according to the grouping indicated in the gene group colour key. Three main clusters were identified by hierarchical clusters. Cluster one consisted of samples from self-renewal and Primo conditions. Cluster two contains EBs generated from self-renewal conditions (E8). Cluster 3 contains EBs generated from Primo conditions.

**Figure 4: Gene expression changes in Primo Cultures**

Cells growing in Primo for 10 passages display maintenance of pluripotency-associated genes and upregulation of mesendodermal-associated genes. A,B) 1 divided by delta (normalised to *ACTB*) cycle threshold (1/dCt) values for given genes from HES3 MIXL1 grown in standard hESC culture (E8 conditions) (black bars) and Primo for ten passages (purple bars). A) A collection of pluripotency-associated

genes. B) A collection of mesendodermal-associated genes. X axis starts at 0.05 as this indicates the value for undetected expression

**Figure 5: Substitution of LPA, CHIR99021 and/or IWP 2 components of Primo other components targeting the same pathways**

A-C. Flow cytometry density plots of SSEA-3 vs T-Venus expression of HG9 T-Venus reporter line grown in SB, DKK, S1P or GRI media.

**A.** IWP2 was replaced for DKK1 at 100ng/mL, density plot reveals high double expression 4 days after the first passage.

**B.** CHIR99021 was replaced with SB216763 at 10μM, density plot reveals high double expression after 3 days of induction.

**C.** LPA was replaced with S1P, density plots demonstrate the ability of S1P to block differentiation, 1.92μM S1P maintained a high proportion of double positive cells after 3 days of induction (optimal S1P concentration boxed).

**D.** LPA was replaced with GRI977143, density plots demonstrate the ability of GRI to block differentiation, 4μM GRI maintained a high proportion of double positive cells after 3 days of induction (optimal GRI concentration boxed).

**ABBREVIATIONS**

In the examples which follow, the following abbreviations are used.

| | |
|---|---|
| EB | Embryoid body |
| MEF / KOSR | Mouse Embryonic Fibroblasts / KnockOut™ Serum Replacement |
| E8 | E8 medium described in Chen et al, 2011 |
| E8V | E8 medium on recombinant Vitronectin xeno-free matrix |
| BCL | E8 medium containing Bovine Serum Albumin (BSA), Cholesterol and Lysophosphatidic Acid. |
| hPSC | Human pluripotent stem cell |
| hESC | Human embryonic stem cell |
| LPA | Lysophosphatidic Acid |
| iPS | Induced pluripotent stem cell |
| S1P | Sphingosine-1-phosphate |
| GRI | GRI977143 |

## MATERIALS AND METHODS

### Cell Lines

**HES3**, human embryonic stem cell line, a gift from Adam Hirst. First described in Cooper et al, 2002 derived using the methods described in Reubinoff et al., 2000.

**HES3 *MIXL1*-GFP**, a gift from Andrew Elefanty, Monash University, Australia, reporter human ESC line developed in Davis et al, 2008.

**H9 T-Venus**, a gift from Roger Pedersen and Daniel Ortmann, published in Mendjan et al 2014. This reporter was generated in the human embryonic stem cell line, H9 (WA09) line, published in Thomson et al, 1998.

**MIFF1**, induced pluripotent stem cell line generated by mRNA transfection of Yamanaka factors (OCT4, KLF4, SOX2 and MYC) into human fibroblast cell line. Published in Desmarais et al, 2016.

### Human Pluripotent Stem Cell Culture

Flask/plates of human ESC were grown in humidified incubators at 37°C and 5% $CO_2$. For feeder free systems we used two matrices Geltrex (ThermoFisher) and Vitronectin (Stem cell Technologies). This was combined with E8 medium (made in house, adapted from Chen et al, 2011).

### Coating growth vessels

#### Vitronectin coating:

Vitronectin XF (Stem Cell Technologies) was resuspended in PBS (w/o Ca+, Mg++), at a 1:50 dilution. Diluted vitronectin solution was added to flasks/plates and incubated for 30-60 minutes at room temperature. Vessels were either used directly or stored at 4°C for no longer than one week.

#### Geltrex coating:

Concentrated Geltrex vials were defrosted on ice. 500µL aliquots were placed in cryovials and frozen at -80°C. When Geltrex/Matirgel was need an aliquot was thawed by adding 1ml of cold DMEM into the cryovial. The solution was diluted further to 1:60 by adding DMEM up to 30ml. Vessels were coated with diluted Geltrex/Matrigel, 1ml per 6 well and left to set at room temperature for 2 hours. Vessels were either used directly or stored at 4°C for no longer than one week.

**Preparation of E8 Medium.**

E8 media was made in house with a recipe adapted from Chen et al, 2011. Specifically, the standard glutamine component was replaced with GlutaMax (Thermofisher). Glutamax is a thermostable form of glutamine, which is bound to alanine to increase stability. Large batches of 50X E8 supplements were prepared and frozen as 10ml aliquots at -20°C. For 50X supplements components were added to DMEM/F12 without glutamine and phenol red (Sigma, D6434). Defrosted 10ml aliquots were added to 490ml of DMEM/F12 without glutamine and filtered using a stericup (Millipore) 0.22μM filter. The table below details the concentration of components in the 50x supplements and subsequent 1x final media.

**Composition of E8 Media**

| | Component | 50X concentrate | Final concentrations per 1 Litre of E8 | Company | Catalogue Number |
|---|---|---|---|---|---|
| E8 | DMEM/F12 | - | - | Sigma | D6421 |
| | L-ascorbic acid | 3200mg/L | 64mg/L | Sigma | A8960 |
| | Sodium selenium | 700ug/L | 14ug/L | Sigma | S5261 |
| | Insulin | 970mg/L | 19.4mg/L | ThermoFisher | A11382IJ |
| | $NaHCO_3$ | 27.15g/L | 543mg/L | Sigma | S5761 |
| | Transferrin | 535mg/L | 10.7mg/L | Sigma | T0665 |
| | Glutamax | 50X | 10ml/L | ThermoFisher | 35050038 |
| | FGF2 | 5mg/L | 100ug/L | Peprotech | 100-18B |
| | TGFB1 | 100ug/L | 2ug/L | Peprotech | 100-21 |

**LPA containing media preparation**

The LPA containing media was first made as a 10X stock, 10X BCL (BSA, Cholesterol, LPA). This was then diluted in E8 medium as needed. For production of Primo media CHIRON, a GSK3β inhibitor, was added at 3μM and IWP-2, a porcupine inhibitor, was added at 1μM. Two formulations of Primo media were commonly used 2xPrimo (0.96μM LPA) and 1xPrimo (0.48μM LPA). CHIR99021

(Tocris, #4423) and IWP-2 (Tocris, #3533) were resuspended in DMSO (Sigma, #D2650) at 10mM and 5mM, respectively. Cholesterol (Synthechol, Sigma, #C1231) was resuspended at 20mM in 100% ethanol. Oleoyl-L-α-lysophosphatidic acid sodium salt (LPA) (Sigma, #L7260) was resuspended in PBS (w/o Ca+, Mg++), with 0.1% Fatty Acid free BSA (Probumin, Millipore, #810664) at 122 μM. Components were added to E8 medium at the concentrations indicated in the table below for 10X supplements. 10X supplements were then diluted in E8 medium to desired concentrations.

**Composition of Primo Medium at various concentrations**

| | | | Concentration | | |
|---|---|---|---|---|---|
| Media | Component | Company | **10X BCL Stock (4.8μM LPA)** | **2X Primo (0.96μM LPA)** | **1X Primo (0.48μM LPA)** |
| BCL | E8 | Homemade | - | - | - |
| | Fatty Acid free BSA | Millipore | 1% | 0.2% | 0.1% |
| | Cholesterol | Sigma-Aldrich | 20μM | 4 μM | 2μM |
| | LPA | Sigma-Aldrich | 4.8μM | 0.96μM | 0.48μM |
| Primo | CHIR99021 | Tocris | - | 3μM | 3μM |
| | IWP-2 | Tocris | - | 1μM | 1μM |

As alternatives to Primo medium, two additional formulations ('SB Media' and 'DKK Media') were prepared based on 2xPrimo, wherein CHIR99021 was replaced with SB216763 (an alternative GSK3β inhibitor), or wherein the porcupine inhibitor IWP-2 was replaced with DKK1 (a recombinant ligand that inhibits the interaction between endogenous WNT ligands and their associated receptors), respectively.

**Composition of SB Media and DKK Media**

| | | Concentration | |
|---|---|---|---|
| Component | Company | **SB Media** | **DKK Media** |
| E8 | Homemade | - | - |
| Fatty Acid free BSA | Millipore | 0.2% | 0.2% |
| Cholesterol | Sigma-Aldrich | 4 μM | 4 μM |
| LPA | Sigma-Aldrich | 0.96 μM | 0.96 μM |
| CHIR99021 | Tocris | 0 | 3 μM |
| SB216763 | Sigma-Aldrich | 10 μM | 0 |
| IWP-2 | Tocris | 1μM | 0 |
| DKK1 | Peprotech | 0 | 100 ng/mL |

**Preparation of S1P containing media ('S1P Media') and GRI977143 containing media ('GRI Media')**

As further alternatives to the LPA-based Primo medium, additional formulations ('S1P Media' and 'GRI Media') were prepared based on 2xPrimo, wherein the LPA component was replaced with S1P (an alternative G protein-coupled receptor signalling molecule), or GRI977143 (an LPA receptor 2 chemical agonist). Three S1P Media were prepared and tested, in which the S1P concentration varied from 0.48 – 4.8 μM. Similarly, three GRI Media were prepared and tested, in which the GRI concentration varied from 1 – 10 μM. Much like LPA containing Primo, a 10x BCL stock was prepared which included 1% BSA and 20 μM Cholesterol and the highest concentration of S1P or GRI, this is then diluted to desired concentration with E8 medium. CHIRON, a GSK3β inhibitor, was added at 3μM and IWP-2, a porcupine inhibitor, was added at 1μM after dilution. The use of BSA is particularly crucial to act as a binding agent for S1P, which prevents it from precipitating out of the medium.

**Composition of S1P Media**

| | | Concentration | | |
|---|---|---|---|---|
| Component | Company | S1P Media A | S1P Media B | S1P Media C |
| E8 | Homemade | - | - | - |
| Fatty Acid free BSA | Millipore | 0.1% | 0.4% | 1% |
| Cholesterol | Sigma-Aldrich | 2 µM | 8 µM | 20 µM |
| CHIR99021 | Tocris | 3µM | 3µM | 3µM |
| IWP-2 | Tocris | 1µM | 1µM | 1µM |
| S1P | Sigma-Aldrich | 0.48 µM | 1.92 µM | 4.8 µM |

**Composition of GRI Media**

| | | Concentration | | |
|---|---|---|---|---|
| Component | Company | GRI Media A | GRI Media B | GRI Media C |
| E8 | Homemade | - | - | - |
| Fatty Acid free BSA | Millipore | 0.1% | 0.4% | 1% |
| Cholesterol | Sigma-Aldrich | 2 µM | 8 µM | 20 µM |
| CHIR99021 | Tocris | 3µM | 3µM | 3µM |
| IWP-2 | Tocris | 1µM | 1µM | 1µM |
| GRI | Sigma-Aldrich | 1 µM | 4 µM | 10 µM |

**Routine Passaging:**

Cells grown in E8, Primo, SB, DKK, S1P or GRI media on vitronectin were passaged with a non-enzymatic disassociation solution ReLeSR (Stem Cell Technologies). Media was aspirated from flasks/plates and cells were washed once with PBS before the addition of ReLeSR (Stem Cell Technologies). ReleSR was left on the cells for ~30 seconds and then aspirated. Flasks were left to incubate at room temperature for 4-6 minutes before the addition of fresh media. Flasks were gently tapped to dislodge colonies and the media was gently pipetted up and down to break colonies

into smaller aggregates. Dissected colonies were resuspended in fresh media and divided amongst new flask at a ratio on average between 1:3-1:6.

**Primary Antibodies**

| Antibody | Antigen | Iso-type | Summary | Host Species | Dilution | Reference | Source |
|---|---|---|---|---|---|---|---|
| **BF4** | BF4 | IgM | sialated, non-ceramide | Mouse Monoclonal | 1:5 | Wright et al 2011 | In-House Hybridoma |
| **CH8** | CD9 | IgG | non-sialated, non-ceramide | Mouse Monoclonal | 1:20 | Wright et al 2011 | In-House Hybridoma |
| **MC631-2C2** | SSEA-3 | IgM | globoseries glycoplipid | Rat Monoclonal | 1:10 | Shevinsky et al 1982 | In-House Hybridoma |
| **813-70** | SSEA-4 | IgG3 | globoseries glycoplipid | Mouse Monoclonal | 1:100 | Kannagi et al 1983 | In-House Hybridoma |
| **Anti-THY1** | THY1 | IgG | CD90 | Mouse Monoclonal | 1:10 | McKenzie and Fabre 1981 | In-House Hybridoma |
| **TRA-1-81** | TRA-1-81 | IgM | non-sialated, non-ceramide | Mouse Monoclonal | 1:10 | (Andrews et al., 1984)(Wright et al., 2011) | In-House Hybridoma |
| **Anti-NANOG** | NANOG | IgG | (D73G4) XP® #4903 | Rabbit Monoclonal | 1:400 | - | Cell Signalling Technology |

**Secondary Antibodies**

| Antibody | Fluorophore | Summary | Dilution | Source |
|---|---|---|---|---|
| **Goat anti Mouse Affinipure IgG+IgM (H+L)** | AlexaFluor 647 | 115-605-044-JIR | 1:200 | Stratech (Jackson ImmunoResearch) |
| **Goat anti Rabbit Affinipure IgG (H+L)** | AlexaFluor 594 | 111-585-003-JIR | 1:200 | Stratech (Jackson ImmunoResearch) |

**i. Intracellular staining**

When plates were to be immunostained they were first fixed with 4% paraformaldehyde (PFA) at room temperature for 15 minutes. Cells were treated with permeablisation buffer consisting of 10% FCS, 0.1% BSA and 0.5% Triton X-100 (Sigma-Aldrich) in PBS (w/o Ca+, Mg++). After Fixation and permeabilization, cells were incubated in blocking buffer consisting of 10% FCS and 0.1% BSA in PBS (w/o Ca+, Mg++) for 1 hour at room temperature. Primary and secondary antibodies were diluted separately at the relevant concentration depicted in in blocking buffer, Hoescht 33342 (ThermoFisher, #H3570) was added at 1:1000 to the diluted secondary antibody solution. Diluted primary antibody was added to wells and plates were incubated overnight at 4°C on an orbital shaker. After primary antibody incubation wells were washed once with blocking buffer before diluted secondary antibody with Hoescht was added. Plates were incubated for 2 hours at 4°C on an orbital shaker. After secondary antibody incubation cells were washed twice with PBS and wells were filled with PBS. Plates were either imaged immediately or stored in sealed bags at 4°C until they were imaged. Plates were imaged using the INCell analyser 2200 (GE Healthcare). Quantifying expression of images was performed by pipelines designed in CellProfiler (Carpenter et al., 2006).

**1.1.1. Antibody staining for Fluorescent flow cytometry analysis.**

Cells were harvested as single cells by treating with Accutase (Thermofisher) for 10 minutes and passed through a 70μM filter (Millipore) to remove larger cell aggregates. Cells were resuspended in DMEM (without phenol red) and 10% FCS

at a density of $1x10^7$ per mL. 100µL of the samples was dispensed into 5ml tubes and antibodies were added at the appropriate dilution. After addition of the primary antibody cells were incubated at 4°Cfor 30 minutes. Cells were then washed with DMEM/FCS and centrifuged at 1000 rpm for 3 minutes. After pelleting, the cells are resuspended in 200µL of DMEM/FCS. Secondary antibody was added at the appropriate dilution and incubated at 4°C for 30 minutes. Cells were then washed with DMEM/FCS, centrifuged for 3 minutes at 1000rpm and resuspended in fresh DMEM/FCS for analysis by flow cytometry.

To set baselines for *MIXL1*-GFP and negative secondary 647, unlabelled HES3 line was harvested and stained for P3X. P3X is an IgG1 antibody which is secreted from the parent myeloma which all in house antigens were derived. P3X shows minimal reactivity to human cells (Kohler and Milstein, 1975). Positive gates were set according to HES3 P3X negative controls. Samples were also stained for P3X to assess non-specific binding. All flow cytometry analysis contained P3X samples for baseline setting.

**Fluorescence activated cell sorting (FACS)**

**Bulk Cell sorts**

The staining method for sorting was the same as described above. Following laser alignment, Accudrop beads were run through the machine to set the drop delay for sorting. Cells were analysed on the machine and sorting gates were set within the population of interest. The gates were positioned to allow a suitable margin between to populations to ensure accurate separation. Cells were sorted into the appropriate vessels and post sort the samples were reanalysed on the flow cytometer. Only samples that had high efficiency percentages were used in further experiments.

**Single Cell Cloning by FACS into 96 well plates.**

Sorts were performed on the BD FACS Jazz. 96 well plates were coated with gelatin and a layer of mouse embryonic feeders. Cells were harvested as single cells by treating with accutase (Thermofisher) for 10 minutes and passed through a 70µM filter (Millipore) to remove larger cell aggregates. After staining for SSEA-3, DAPI (ThermoFisher, #62248) was added at 1:10,000 and used for live/dead discrimination. After gating on the BD sortware program the desired population was sorted as single cells directly into 96 well plates. The sort was indexed to retain information regarding

the *MIXL1*-GFP and SSEA-3 expression levels. For single cell cloning the medium was a culture medium containing Knock-Out Serum Replacement (KOSR). During initial plating 10μM Rock Inhibitor, Y-27632, was added to the medium. Immediately after sorting into the wells the plates were centrifuged at 1000 rpm for 1 minute to aid attachment of the cells. After two days the medium was replaced with fresh medium to remove the ROCK inhibitor. Colonies were left to develop over 9-12 days before passaging the wells which looked to contain typical human PSC colonies. Colonies were passaged from 96 well plate into a 48 well plate by manual scrapping with a p200 tip, then aspirating and dispensing the dissected colony into one well of a 48 well plate.

**Embryoid Bodies**

**i. Formation**

To assess the trilineage potential of either established clonal lines or particular *MIXL1* positive substates we used an approach that entailed the formation of Embryoid Bodies (EB) under "neutral" conditions, in this context, neutral simply indicates that no exogenous cytokines or chemicals were added to guide differentiation. Cells were either used directly from flasks or after they had been FACS sorted for a particular population. In either situation cells were centrifuged at 1000rpm for 3 minutes before being resuspended in APEL 2 medium (Stem Cell Technologies) containing 10μM ROCKi. Cells were resuspended at 3,000 cells per 50μL. 50μL of cells were added to the inner 60 wells of a non-adherent Grenier U bottom 96 well plate. The outer 36 wells were filled with PBS as to prevent the inner wells from drying out. After adding the cells plates were centrifuged at 1000rpm for 3 minutes, to pellet cells. Plates were incubated at 37°C, %CO2 for 7 days.

**ii. Harvesting**

After 7 days, the medium and EBs were collected from all 60 wells and transferred into a 15ml falcon tube. The wells are then washed with 5 mL of PBS and transferred to the 15mL tube to collect any EBs that had been left behind. The sample is then left for 15 minutes at room temperature to allow EBs to sediment by gravity. The supernatant is then removed and 1mL of Trizol is added with a 1000μL pipette tip. The solution is pipetted up and down vigorously to break up EBs. The solution is then

incubated for 3 minutes before another session of vigorous pipetting. Samples are then transferred to -80°C until RNA extraction.

### iii. RNA Extraction

RNA was extracted from Trizol treated samples. Trizol samples were defrosted on ice before 200µl of chloroform was added per ml of trizol sample. Samples were then vortexed vigorously until solution appeared milky pink in colour. Samples were incubated at room temperature for 10 minutes before centrifugation at 14,000g for 30 minutes at 4°C, to facilitate phase separation. After this the clear aqueous phase was transferred in to an RNA binding column from a Norgen Total RNA Purification Kit (Norgen, 37500) and processed as per manufacturer's instructions. RNA was eluted into 50µl of elution buffer. RNA samples were then analysed for RNA concentration using a Nanodrop lite (Thermofisher).

### iv. RNA to cDNA Conversion

Prior to cDNA conversion, samples were first treated with DNA-free DNA Removal kit (Thermofisher, AM1906). This removes contaminating genomic DNA to ensure better qPCR results. Samples were diluted to 200µg per mL and processed as per manufacturer's instructions. After DNA removal remaining samples were diluted to 1µg in 225µL and 25µL was dispensed into all wells of an 8 well 0.2mL PCR tube strip. 25µL of reaction master mix was added from the High Capacity cDNA Reverse Transcription Kit (Thermofisher, 4368814) to each well. Samples were then loaded into a PCR machine (QuantStudio 12k Flex, Applied Biosystems) and run on the following cycle: 10 Minutes 25°C, 120 Minutes 37°C, 5 minutes 85°C and hold at 4°C.

### v. Human PSC Scorecards

Human PSC scorecards were purchased from ThermoFisher(A15870). These scorecards are designed to assess the trilineage potential of human PSC lines, based on the work from Alex Meissner (Bock et al., 2011). The scorecard analyses each sample for 96 genes, related to pluripotency, endoderm, mesoderm, ectoderm and some housekeeping genes as well. Purified cDNA samples are diluted as per manufacturer's instructions and 10µL is added to each well, which contains desiccated primer and probe for a given gene. The scorecards are then loaded and run on the QuantStudio Flex 12K Thermocycler. The cycle conditions are shown below in the table below. After the run had finished, data was uploaded to

Thermofisher for analysis using their software (https://www.thermofisher.com/uk/en/home/life-science/stem-cell-research/taqman-hpsc-scorecard-panel/scorecard-software.html).

**Human PSC scorecard qPCR Cycle Conditions**

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| **Hold** | 50°C | 2 minutes | - |
| **Hold** | 95°C | 10 minutes | - |
| **Melt** | 95°C | 15 seconds | 40 |
| **Aneal/Extend** | 60°C | 1 minute | |

**EXAMPLES**

***Functional assessment of cells growing in 2xPrimo medium***

2xPrimo medium was produced as a result of optimisation studies to identify a culture medium that allows for the maintenance of a sizeable proportion of *MIXL1*(+) cells in culture (data not shown). After these optimisation trials had been completed, a functional assessment of these 2xPrimo medium cultured cells with respect to their self-renewal and differential potential was completed. Figure 1A is a schematic representation of the analysis process: HES3 MIXL1 cells were grown in 2xPrimo medium for 3 days, single MIXL1(+)/SSEA-3(+) cells were taken for cloning experiments and bulk samples were taken for "neutral" embryoid body differentiation. The clonal lines established were also assessed by EB differentiation. The EBs were harvested for qPCR analysis using the hPSC scorecard from Thermofisher.

For clonal line generation 384 MIXL1(+)/SSEA-3(+) single cells into 96 well plates and, from these, obtained stem cell like colonies in 38 wells. All 38 colonies were passaged further, of which 24 of those survived and were positive for TRA-1-81 staining, a representative colony is depicted in Figure 1C. Further to this, six clonal lines were established. After expansion in MEF/KOSR conditions and transition into E8V (E8 medium on Vitronectin xeno-free matrix) conditions, lines were assessed for their pluripotency associated surface marker expression and *MIXL1*-GFP expression. All lines displayed high expression levels of the pluripotency associated markers, BF4, CD9, SSEA-3, SSEA-4, THY-1, TRA-1-81 and relatively no MIXL1-GFP expression

(Figure 1D). Under "Neutral" EB conditions clonal lines generated EBs containing signatures of all three germ layers (Figure 1E).

This demonstrated that cells cultured in 2xPrimo can revert back to a more pristine pluripotent state upon transition into E8V conditions, i.e. standard feeder free stem cell culture conditions. As a further assessment, it was evaluated whether cultured cells taken directly from 2xPrimo conditions exhibited trilineage protentional or a particular lineage bias. The same "neutral" EB approach to assess the cells, assessing unsorted cultures as well as the *MIXL1* positive and negative fractions separately. HES3 *MIXL1*-GFP were cultured in 2xPrimo conditions for 3 days and then unsorted and sorted *MIXL1*(-)/SSEA-3(+) and *MIXL1*(+)/SSEA-3(+) cells (FIGURE 1A, sorting gates depicted in FIGURE 1B) were taken from these cultures for EB assessment. EB scorecard analysis indicated significantly enhanced mesoderm signatures from all samples grown in Primo conditions. This mesoderm signature was not limited to the *MIXL1*(+)/SSEA-3(+) fraction either but also present in the *MIXL1*(-)/SSEA-3(+) and unsorted fractions (FIGURE 1E).

The MIXL1(-)/SSEA-3(+) cells from 2xPrimo conditions still generated EBs with enhanced mesoderm signatures, differing with the same fractions taken from E8 conditions. To assess this disparity another hPSC line H9 was cultured, carrying a reporter for the mesoderm associated gene *T* (Mendjan et al., 2014). As with the HES3 *MIXL1*-GFP line we were able to maintain good cell growth and colony morphology for H9 T-Venus grown in 2xPrimo medium. After three passages in the Primo medium we assessed the T-Venus and SSEA-3 expression of the cells. Almost 90% of the cells in this condition were double positive for T-Venus and SSEA-3 compared to E8 conditions which had less than 1% double positive cells (FIGURE 1F). So, although not all the cells are MIXL1, the data obtained with this reporter indicates a large proportion might be T positive. "Neutral" EBs generated from the H9 T-Venus cells and from another iPS line, Miff1, after 3 passages in 2xPrimo, exhibited enhanced mesoderm signatures (FIGURE 1G).

***Multiple passages in Primo medium***

Following optimisation of the medium and the demonstration that cultured cells retain the ability to revert to a *MIXL1*(-)/SSEA-3(+) state, it was assessed if cells could be maintained in 2xPrimo medium for multiple passages (FIGURE 2A). A relatively low

passage number HES3-*MIXL1* line with a normal karyotype was chosen for propagation. The 2xPrimo (0.96μM LPA) formulation was used for passaging, as improved maintenance of SSEA-3 post passage was observed using this formulation. The formulation for the first 3 passages also contained 2-mercaptoethanol but much like Chen et al, 2011, it was found to be detrimental to growth so it was removed after this point (data not shown). Single cells were sorted from the *MIXL1*(+)/SSEA-3(+) fraction at passage 3 and 11% of these cells formed colonies. Intracellular immunostaining demonstrated that we could still obtain NANOG positive colonies, from cells at passage 3 (FIGURE 2B).

The cells were continually passaged in 2xPrimo and at passage 7, the expression of a panel of pluripotency associated surface markers was assessed. Cells were stained for BF4, CD9, SSEA-3, SSEA-4, THY1 and TRA-1-81. All antigens showed high levels of expression, over 95%. The culture also still exhibits a *MIXL1* positive population, approximately 20% (FIGURE 2C).

After the tenth passage in 2xPrimo medium, the cells were assessed within 2xPrimo conditions and the effect on the cells when split into different culture media was also observed. Cells were split into 2xPrimo to assess the maintenance of the lineage biased population, *MIXL1*(+). Cells were also split into 2xBCL (E8 with LPA), E8 with and without 1μM IWP-2 to assess the cells ability to revert to a *MIXL1*(-) state. Immunofluorescent analysis of NANOG expression revealed high expression in all conditions, while *MIXL1*-GFP expression was mainly confined to 2xPrimo conditions only (FIGURE 2D). When assessing the coexpression of NANOG with *MIXL1*-GFP there were colonies within the 2xPrimo conditions which showed their coexpression, with ~45% of the cells in culture being identified as double positive. The other conditions, including E8 alone, exhibited a significant reduction in *MIXL1*-GFP expression, but a maintenance of high percentage of NANOG expression (FIGURE 2E).

Genetic variants can arise in standard hPSC culturing, common such changes involve the gaining of parts or whole chromosomes 1, 12, 17 and 20. These changes can have an effect on functional aspects of the cells including proliferation, cloning efficiency, and their ability to differentiate. A genetic variant which resisted differentiation might be selected for in a lineage priming condition. Therefore, with

this in mind, cells from passage ten of 2xPrimo were analysed by metaphase spreads, for karyotypical changes. However, after ten passages in the Primo medium the cells had maintained a normal diploid karyotype, 46 XX, when 30 metaphases were analysed (FIGURE 2F). This maintenance of a normal diploid karyotype was also seen using the H9 *T*-Venus reporter line grown for 3 passages in Primo conditions (data not shown).

***Gene Expression of Passage 10 Samples***

After 9 passages in 2xPrimo some cells were also passaged into 1xPrimo conditions for assessment along with the conditions described previously. Cells grown in 1xPrimo (10P 1xP, denoting 10 passages in Primo medium with the last passage in 1xPrimo), 2xPrimo (10P 2xP, denoting 10 passages in 2xPrimo), 2xBCL (9P BCL SR, denoting 9 passages in Primo medium and the last passage into (SR) self renewal conditions, BCL) and E8 + IWP-2 (9P IWP2 SR, denoting 9 passages in Primo medium and the last passage into (SR) self renewal conditions, E8 with IWP-2) were harvested for gene expression analysis by human PSC scorecards (Figure 3). This gene expression was compared to data obtained from pluripotent and differentiated cells. For comparisons to the pluripotent state, gene expression data for cells grown in self-renewal conditions (E8 and Vitronectin) were used, including the parental HES3 *MIXL1*-GFP line and three Primo clones, 10-A8, 11-E4 and 12-F11. For comparisons to differentiated states, gene expression data from Embryoid Bodies generated from cells grown in self-renewal (Standard EB) and Primo (Primo EB) conditions were used. Hierarchical clustering segregated the cells into 3 clear clusters (Figure 3).

The first cluster contained samples grown under standard self-renewal conditions, this included three clonal lines derived from Primo *MIXL1*(+)/SSEA-3(+) fraction. The cells at passage ten from all conditions showed high expression of pluripotency-associated genes and clustered with parental HES3 *MIXL1* line grown under self-renewal conditions, E8V. The samples which had been growing in Primo for 9 passages and then reverted to *MIXL1*-GFP negative, in the presence of IWP-2 or BCL (LPA) formed a sub-cluster with the standard self-renewal cells. Both 10 passage cells grown in 1xPrimo and 2x Primo were within the main cluster but were separated into their own sub-cluster. Cells from both 1x and 2x Primo had elevated expression of differentiation

associated markers such as *EOMES*, *FOXA2* and *T* whilst maintaining similar levels of *NANOG, POU5F1,* and *SOX2* to the other samples in this first cluster.

The second cluster contained EBs made from cells grown under standard self-renewal conditions (E8 and Vitronectin). EBs from clonal lines derived from Primo *MIXL1*(+)/SSEA-3(+) fraction and grown in E8 and vitronectin clustered with the EBs derived from parental HES3 and HES3 *MIXL1*-GFP line in self-renewal conditions prior to EB formation. EBs generated from cells growing in Primo for 9 passages and then reverted to *MIXL1*-GFP negative, in the presence of LPA (BCL) also resided in this cluster albeit as an outgroup of this cluster.

The third cluster encompassed all the samples which came directly from 2xPrimo conditions, including both unsorted and sorted samples *MIXL1*(-)/SSEA-3(+) and *MIXL1*(+)/SSEA-3(+). This cluster has some upregulation of ectoderm and endoderm associated genes but has a greater upregulation of genes associated with mesoderm. Downstream mesoderm markers such as *HAND1* and *HAND2* are strongly upregulated in these samples.

Analysis using the human PSC scorecards demonstrates a stark difference between the EBs generated from self-renewal conditions and that of EBs generated from Primo conditions, in respect to expression of mesoderm associated genes, whilst also demonstrating that both clonal lines and reverted samples exhibit similar EB gene expression patterns as EBs generated from self-renewal conditions.

When cells from 10 passages in Primo conditions were compared to cells grown in standard hESC conditions (E8 conditions) expression was maintained for the pluripotency-associated genes and there was an upregulation of mesendodermal-associated genes (Figure 4). Some pluripotency associated factors exhibited lower expression levels such as DNMT3B and SOX2 whereas some exhibited increased expression including NANOG and TRIM22 (Figure 4A). Genes associated with mesendodermal differentiation exhibited high upregulation including *CDX2, EOMES, GATA6, NODAL* and *T*, with all genes exhibiting increased expression compared to hESCs grown in standard culture (Figure 4B).

***Functional assessment of cells growing in SB, DKK, S1P and GRI media***

In order to assess whether the LPA, CHIR99021 and/or IWP-2 components of the optimised recipe of 2xPrimo could be substituted with alternative reagents targeting the same key pathways whilst maintaining the ability of the media to generate a mesoderm biased state, the H9 T-Venus reporter cell line was cultured in variety of media in which either (i) the IWP-2 component was substituted with DKK1 ('DKK Media'); (ii) the CHIR99021 component was substituted with SB216763 ('SB Media'); (iii) the LPA component was substituted with S1P ('S1P Media'); or (iv) the LPA component was substituted with GRI977143 ('GRI Media'), with T-Venus and SSEA-3 expression being assessed, wherein double positive T-Venus and SSEA-3 cells are indicative of the mesoderm biased state.

Notably after a single passage in DKK Media, in which the PORCN protein inhibitor component of 2xPrimo (IWP-2) was substituted with 100 ng/mL DKK1 (a recombinant ligand that inhibits the interaction between endogenous WNT ligands and their associated receptors), cells expressed over 99% SSEA-3 and 70% of these SSEA-3 positive cells also expressed T-Venus (Figure 5 A).

Further, upon culturing in SB media, in which the GSK3-β inhibitor component of 2xPrimo (CHIR 99021) was substituted with an alternative GSK3-β inhibitor (10μM SB216763), 96% T-Venus/SSEA-3 double positive cells was observed after 3 days of induction (Figure 5B).

The H9 T-Venus reporter cell line was also cultured in various LPA free media, wherein the G protein-coupled receptor signalling molecule component of 2xPrimo (LPA) was substituted with an alternative G protein-coupled receptor signalling molecule (S1P) or with an LPA receptor 2 chemical agonist (GRI) as alternative inhibitors of the Hippo signalling pathway.

T-Venus/SSEA-3 expression was assessed using a range of S1P Media, in which the concentration of S1P varied from 0.48 μM to 4.8 μM. It was observed that lower levels of S1P (0.48 μM) did result in expression of T-Venus but with a loss of SSEA3 expression, and that higher levels of S1P (4.8 μM) maintained SSEA3 expression but blocked the expression of T-Venus. Notably, inclusion of S1P at 1.92 μM was able to maintain high levels (83%) of T-Venus/SSEA-3 double positive cells.

Similarly, T-Venus/SSEA-3 expression was assessed using a range of GRI Media, in which the concentration of GRI varied from 1 μM to 10 μM. Analogous to the S1P results, it was observed that lower levels of GRI (1 μM) did result in expression of T-Venus but with a loss of SSEA3 expression, and that higher levels of GRI (10 μM) maintained SSEA3 expression but blocked the expression of T-Venus. Notably, inclusion of GRI at 4 μM was able to maintain high levels (72%) of T-Venus/SSEA-3 double positive cells.

The cell expression patterns observed using S1P Media and GRI Media were similar to that observed using LPA-based media such as 2xPrimo, albeit at slightly different concentrations.

## SUMMARY

We report the discovery that pluripotent mammalian cells can be maintained in a mesoderm biased, self-renewing state in a culture medium comprising the combination of one or more inhibitor of the Hippo signaling pathway, one more canonical WNT signaling agonist, and one or more inhibitor of endogenous WNT signaling. Further, we report that cells in this mesoderm-biased state, following transition into standard stem cell culture conditions, recover their trilineage potential (i.e. the ability to differentiate into mesoderm, ectoderm or endoderm germ layers), with transcriptional changes reverting to levels seen in traditional stem cell cultures.

## REFERENCES

Andrews, P.W., Banting, G., Damjanov, I., Arnaud, D., and Avner, P. (1984). Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. Hybridoma *3*, 347-361.

Andrews, P. W., Goodfellow, P. N. and Bronson, D. L. (1983). Cell surface characteristics and other markers of differentiation of human teratocarcinomas in culture. In Teratocarcinoma Stem Cells, M.G. Silver LM, Strickland S, ed. (Cold Spring Harbor Conferences on Cell Proliferation), pp. 579-590.

Bock, C., Kiskinis, E., Verstappen, G., Gu, H., Boulting, G., Smith, Z.D., Ziller, M., Croft, G.F., Amoroso, M.W., Oakley, D.H., Gnirke, A., Eggan, K., and Meissner, A. (2011). Reference Maps of Human ES and iPS Cell Variation Enable High-Throughput Characterization of Pluripotent Cell Lines. Cell *144*, 439-452.

Carpenter, A.E., Jones, T.R., Lamprecht, M.R., Clarke, C., Kang, I.H., Friman, O., Guertin, D.A., Chang, J.H., Lindquist, R.A., Moffat, J., Golland, P., and Sabatini D. M. (2006). CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biology *7*, R100.

Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Diol, N.R., Propson, N.E., Wagner, R., Lee, G.O., Antosiewicz-Bourget, J., Teng, J. M., and Thomson, J. A. (2011). Chemically defined conditions for human iPSC derivation and culture. Nature methods *8*, 424-429.

Cooper, S., Bennett, W., Andrade, J., Reubinoff, B.E., Thomson, J., and Pera, M.F. (2002). Biochemical properties of a keratan sulphate/chondroitin sulphate proteoglycan expressed in primate pluripotent stem cells. Journal of anatomy *200*, 259-265.

Davis, R.P., Ng, E.S., Costa, M., Mossman, A.K., Sourris, K., Elefanty, A.G., and Stanley, E.G. (2008). Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors. Blood *111*, 1876-1884.

Desmarais, J.A., Unger, C., Damjanov, I., Meuth, M., and Andrews, P. (2016). Apoptosis and failure of checkpoint kinase 1 activation in human induced pluripotent stem cells under replication stress. Stem Cell Research & Therapy *7*, 17-23.

Henderson, J.K., Draper, J.S., Baillie, H.S., Fishel, S., Thomson, J.A., Moore, H., and Andrews, P.W. (2002). Preimplantation Human Embryos and Embryonic Stem Cells

Show Comparable Expression of Stage-Specific Embryonic Antigens. Stem cells (Dayton, Ohio) *20*, 329-337.

Kannagi, R., Cochran, N.A., Ishigami, F., Hakomori, S., Andrews, P.W., Knowles, B.B., and Solter, D. (1983). Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. The EMBO journal *2*, 2355-2361.

Kohler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. 1975. Journal of immunology (Baltimore, Md : 1950) *174*, 2453-2455.

McKenzie, J.,L. and Fabre, J., L. (1981) Human Thy1: Unusual localisation and possible functional significance in lymphoid tissues. J. Immunol. *126*, 843–850.

Mendjan, S., Mascetti, Victoria L., Ortmann, D., Ortiz, M., Karjosukarso, Dyah W., Ng, Y., Moreau, T., and Pedersen, Roger A. (2014). NANOG and CDX2 Pattern Distinct Subtypes of Human Mesoderm during Exit from Pluripotency. Cell stem cell *15*, 310-325.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nature biotechnology *18*, 399-404.

Shevinsky, L.H., Knowles, B.B., Damjanov, I., and Solter, D. (1982). Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. Cell *30*, 697-705.

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

Wright, A., Andrews, N., Bardsley, K., Nielsen, J.E., Avery, K., Pewsey, E., Jones, M., Harley, D., Nielsen, A.R., Moore, H., *et al.* (2011). Mapping the stem cell state: eight novel human embryonic stem and embryonal carcinoma cell antibodies. International journal of andrology *34*, e175-187; discussion e187-178.

## CLAIMS

1. A culture medium for the propagation of mammalian pluripotent stem cells in a mesoderm-biased phenotype, said medium comprising:
    a. one or more inhibitor of the Hippo signalling pathway;
    b. one or more canonical WNT signalling agonist; and
    c. one or more inhibitor of endogenous WNT signalling.

2. The culture medium according to claim 1, wherein said one or more inhibitor of the Hippo signalling pathway is or includes a G protein-coupled receptor signalling molecule.

3. The culture medium according to claim 2, wherein said G protein-coupled receptor signalling molecule is selected from Lysophosphatidic Acid (LPA), Sphingosine-1-phosphate (S1P), or a combination thereof.

4. The culture medium according to any of the preceding clams, wherein said one or more inhibitor of the Hippo signalling pathway is or includes an agonist of a G protein-coupled receptor.

5. The culture medium according to claim 4, wherein said one or more inhibitor of the Hippo signalling pathway is or includes an agonist of LPA G protein-coupled receptor(s) and/or S1P G protein-coupled receptor(s).

6. The culture medium according to claim 5, wherein said agonist is a selective agonist of $LPA_2$ such as GRI977143.

7. The culture medium according to any of the preceding claims, wherein said one or more inhibitor of the Hippo signalling pathway is or includes an inhibitor that targets one or more components of the protein kinase cascade of said Hippo signalling pathway.

8. The culture according to claim 7, wherein said inhibitor is selected from XMU-MP1 and 9E1.

9. The culture medium according to any of the preceding claims, wherein said one or more canonical WNT signalling agonist is or includes a Glycogen Synthase Kinase 3 (GSK3) inhibitor, preferably a GSK3-β inhibitor.

10. The culture medium according to claim 9, wherein said GSK3 inhibitor is selected from CHIR 98014, CHIR 99021, AR-A0144-18, TDZD-8, SB216763, or a combination thereof.

11. The culture medium according to any of the preceding claims, wherein said one or more canonical WNT signalling agonist is or includes a recombinant WNT ligand.

12. The culture medium according to any of the preceding claims, wherein said one or more inhibitor of endogenous WNT signalling is a Porcupine (PORCN) protein inhibitor.

13. The culture medium according to claim 12, wherein said PORCN protein inhibitor is selected from IWP-1, IWP-2, IWP-3, IWP-4, IWP-5, IWP-6, IWP-7, IWP-8, IWP-9, IWP-10 or a combination thereof.

14. The culture medium according to any of claims 1 to 11, wherein said one or more inhibitor of endogenous WNT signalling is a recombinant ligand that inhibits the interaction between endogenous WNT ligands and their associated receptors.

15. The culture medium according to claim 14, wherein said recombinant ligand is recombinant Dickkopf-related protein 1 (DKK1).

16. The culture medium according to any of the preceding claims, further comprising: one or more sources of L-glutamine; insulin; transferrin, basic fibroblast growth factor (FGF2); and/or more member of the transforming growth factor (TGF)-β superfamily, preferably TGF-β1.

17. The culture medium according to any of the preceding claims, further comprising one or more albumin.

18. The culture medium according to any of the preceding claims, further comprising DMEM/F12 - a mixture of Dulbecco's Modified Eagle Medium and Ham's nutrient mixture F-12.

19. The culture medium according to any of the preceding claims, wherein said medium is feeder cell free.

20. The culture medium according to claim 19, comprising DMEM/F12 - a mixture of Dulbecco's Modified Eagle Medium and Ham's nutrient mixture F-12, to which the components shown below are added:

| Component | Concentration |
|---|---|
| L-ascorbic acid | 64 mg/L |
| Selenium | 14 µg/L |
| Transferrin | 10.7 mg/L |
| $NaHCO_3$ | 543 mg/L |
| Insulin | 19.4 mg/L |
| L-Glutamine (Glutamax) | 10 ml/L |
| FGF2 | 100 µg/L |
| TGF-β1 | 2 µg/L |
| Albumin (fatty acid free bovine serum albumin (BSA)) | 0.2% |
| Cholesterol | 4 µM |
| LPA | 0.96 µM |
| CHIR99021 | 3 µM |
| IWP-2 | 1 µM |

21. The culture medium according to claim 19, comprising DMEM/F12, to which the following components are added:

(i) 64 mg/L L-ascorbic acid; 14 µg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 µg/L FGF2; 2 µg/L TGF-β1; 0.1% Albumin (fatty acid free bovine serum

albumin (BSA)); 2 μM Cholesterol; 0.48 μM LPA; 3 μM CHIR99021; and 1 μM IWP-2; or

(ii) 64 mg/L L-ascorbic acid; 14 μg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 μg/L FGF2; 2 μg/L TGF-β1; 0.2% Albumin (fatty acid free bovine serum albumin (BSA)); 4 μM Cholesterol; 0.96 μM LPA; 10 μM SB216763; and 1 μM IWP-2; or

(iii) 64 mg/L L-ascorbic acid; 14 μg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 μg/L FGF2; 2 μg/L TGF-β1; 0.2% Albumin (fatty acid free bovine serum albumin (BSA)); 4 μM Cholesterol; 0.96 μM LPA; 3 μM CHIR99021; and 100 ng/mL DKK1; or

(iv) 64 mg/L L-ascorbic acid; 14 μg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 μg/L FGF2; 2 μg/L TGF-β1; 0.4% Albumin (fatty acid free bovine serum albumin (BSA)); 8 μM Cholesterol; 1.92 μM S1P; 3 μM CHIR99021; and 1 μM IWP-2; or

(v) 64 mg/L L-ascorbic acid; 14 μg/L Selenium; 10.7 mg/L Transferrin; 543 mg/L $NaHCO_3$; 19.4 mg/L Insulin; 10 ml/L L-Glutamine (Glutamax); 100 μg/L FGF2; 2 μg/L TGF-β1; 0.4% Albumin (fatty acid free bovine serum albumin (BSA)); 8 μM Cholesterol; 4 μM GRI; 3 μM CHIR99021; and 1 μM IWP-2.

22. A human stem cell culture medium according to any of the preceding claims.

23. An embryonic stem cell, induced pluripotent stem cell, naïve pluripotent stem cell, epiblast stem cell or primed pluripotent stem cell culture medium according to any of the preceding claims.

24. A method of propagating mammalian pluripotent stem cells in a mesoderm-biased phenotype, the method comprising: incubating a population of mammalian pluripotent stem cells in a culture medium according to any of the preceding claims; and passaging the stem cell population multiple times.

25. Use of one or more inhibitor of the Hippo signalling pathway, one or more canonical WNT signalling agonist and one or more inhibitor of endogenous WNT signalling for the propagation of a population of mammalian pluripotent stem cells in a mesoderm-biased phenotype.

26. Use of one or more inhibitor of the Hippo signalling pathway, one or more canonical WNT signalling agonist and one or more inhibitor of endogenous WNT signalling for the manufacture of a medium for the propagation of a mesoderm biased mammalian pluripotent stem cell population.

27. A population of mammalian pluripotent stem cells, wherein said population of cells display a mesoderm-biased phenotype.

28. The method of claim 24, the use according to claim 25 or claim 26, or the population of mammalian pluripotent stem cells according to claim 27, wherein said mammalian pluripotent stem cells are selected from embryonic stem cells, induced pluripotent stem cells, naïve pluripotent stem cells, epiblast stem cells and primed pluripotent stem cells.

29. The method of claim 24 or claim 28, the use according to any of claims 25, 26 or 28, or the population of mammalian pluripotent stem cells according to claim 27 or 28, wherein said mammalian pluripotent stem cells are human pluripotent stem cells.

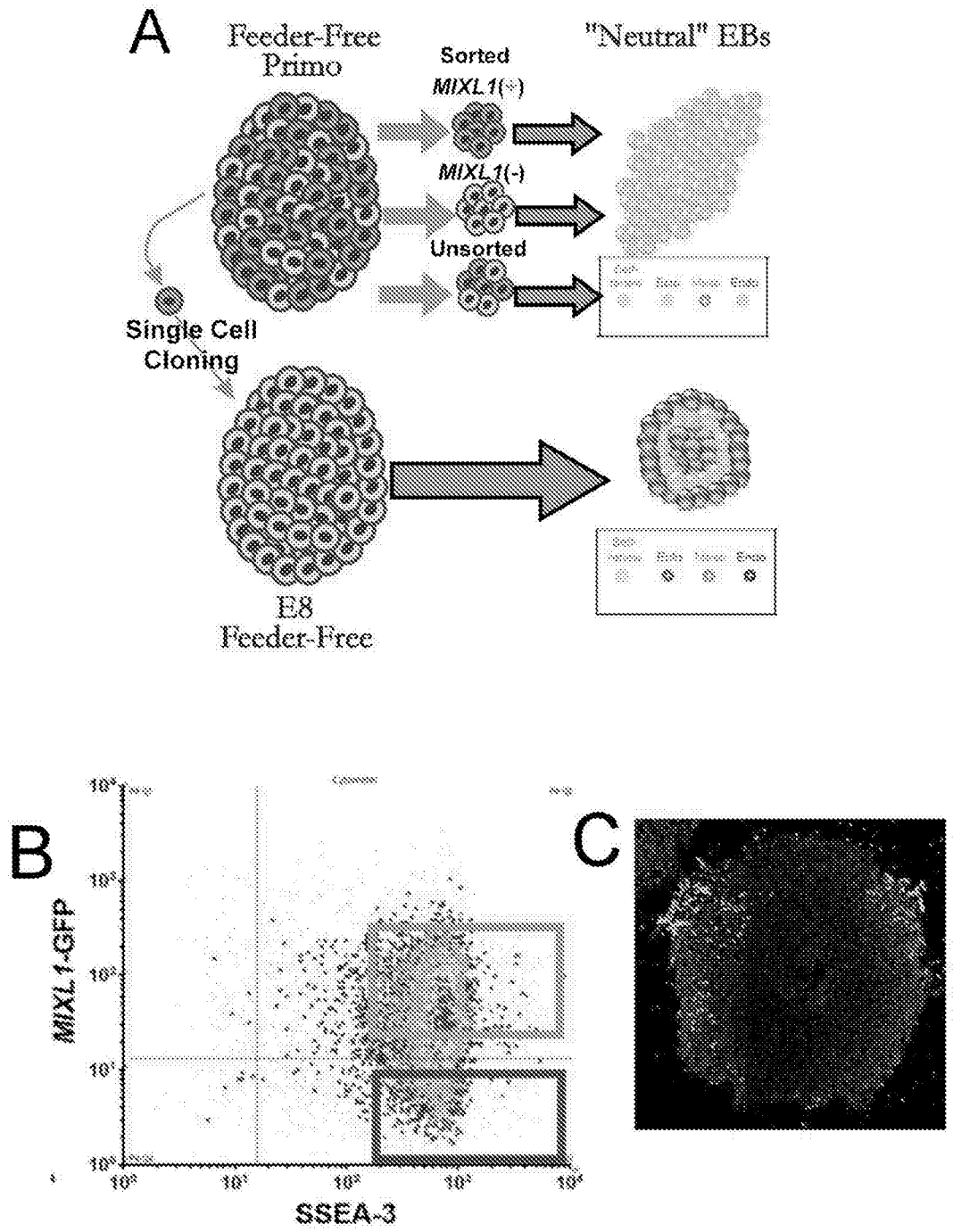
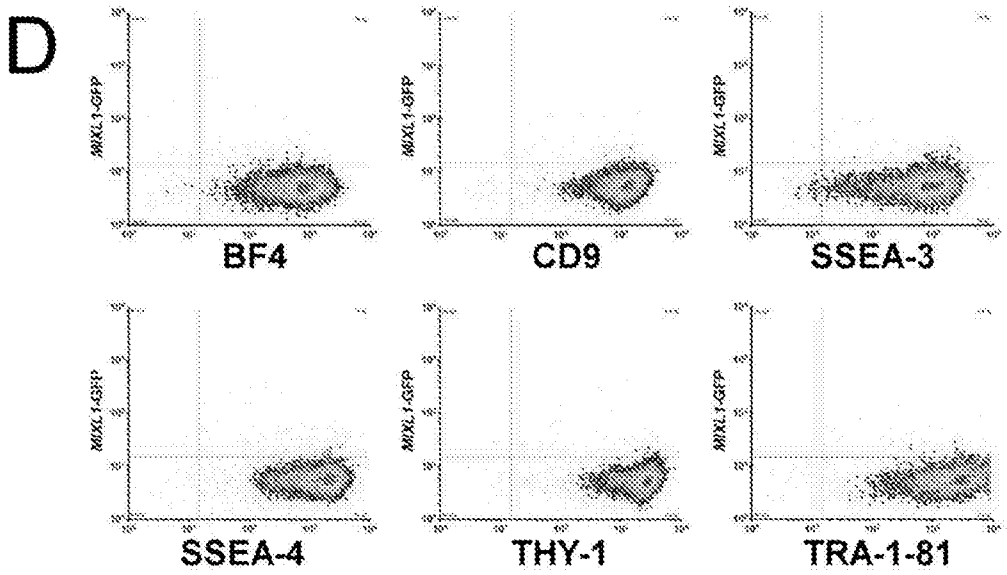

A
Feeder-Free
Primo
Sorted
MIXL1(+)
MIXL1(-)
Unsorted
"Neutral" EBs
Single Cell
Cloning
E8
Feeder-Free
B
MIXL1-GFP
SSEA-3
C

Fig. 1


D
MIXL1-GFP
BF4
CD9
SSEA-3
SSEA-4
THY-1
TRA-1-81

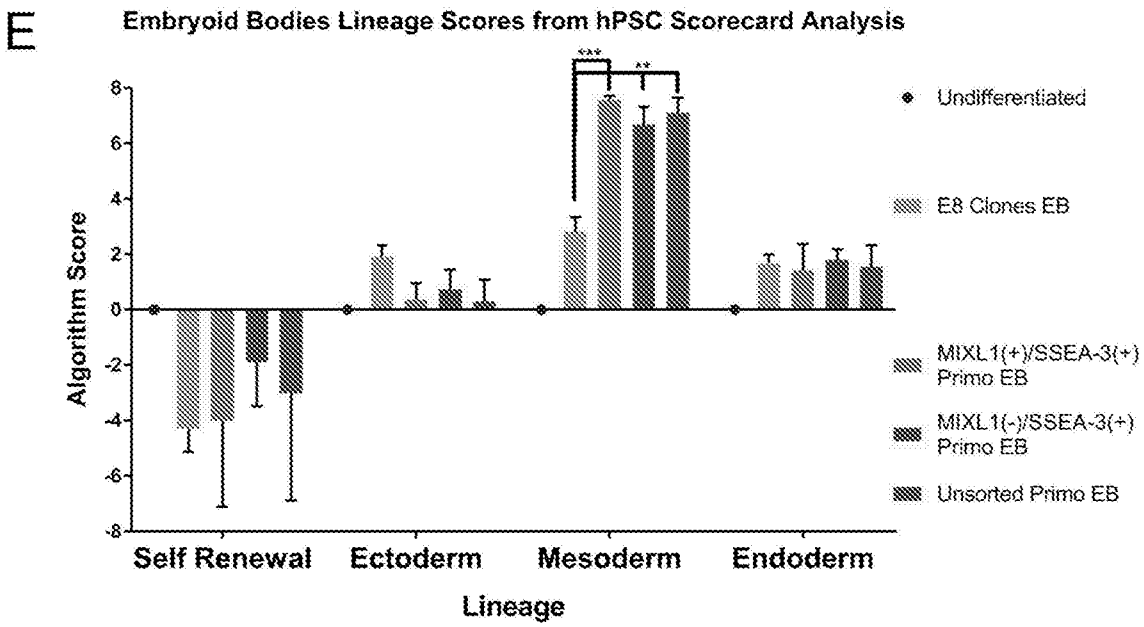

E
Embryoid Bodies Lineage Scores from hPSC Scorecard Analysis
***
**
Algorithm Score
8
6
4
2
0
-2
-4
-6
-8
Undifferentiated
E8 Clones EB
MIXL1(+)/SSEA-3(+) Primo EB
MIXL1(-)/SSEA-3(+) Primo EB
Unsorted Primo EB
Self Renewal
Ectoderm
Mesoderm
Endoderm
Lineage

Fig. 1 (cont)

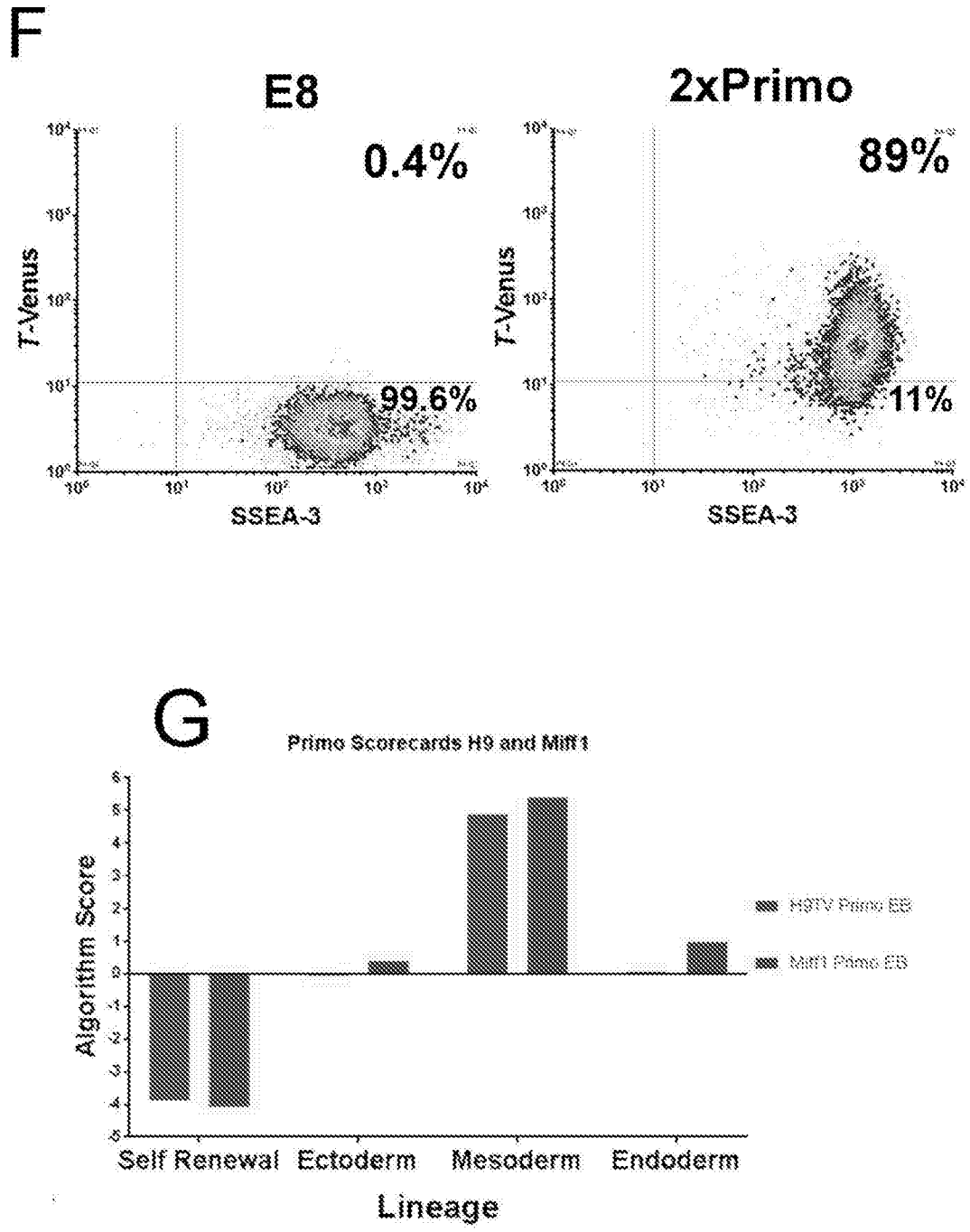
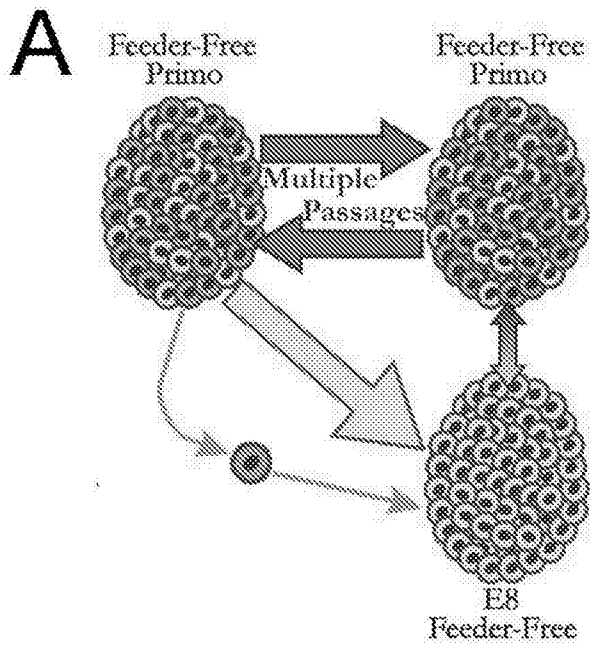

F
E8
0.4%
99.6%
T-Venus
SSEA-3
2xPrimo
89%
11%
T-Venus
SSEA-3
G
Primo Scorecards H9 and Miff1
Algorithm Score
Self Renewal
Ectoderm
Mesoderm
Endoderm
Lineage
H9TV Primo EB
Miff1 Primo EB

Fig. 1 (cont)


A
Feeder-Free
Primo
Feeder-Free
Primo
Multiple
Passages
E8
Feeder-Free

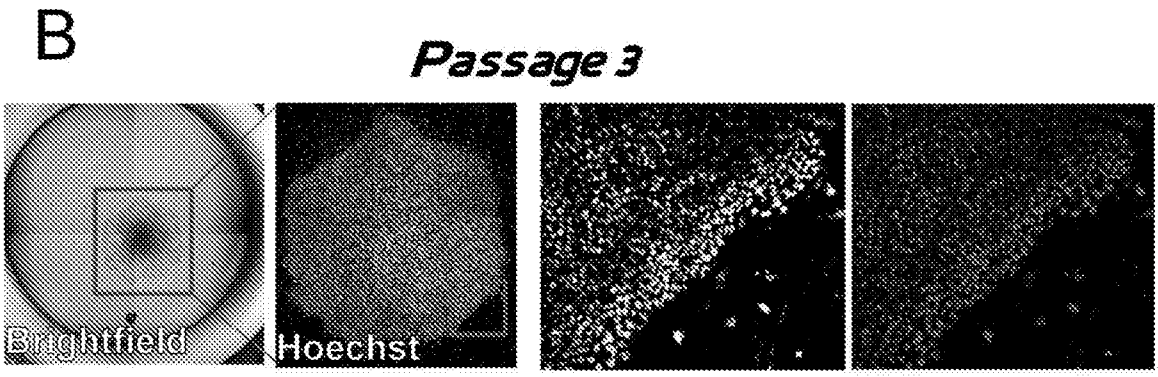
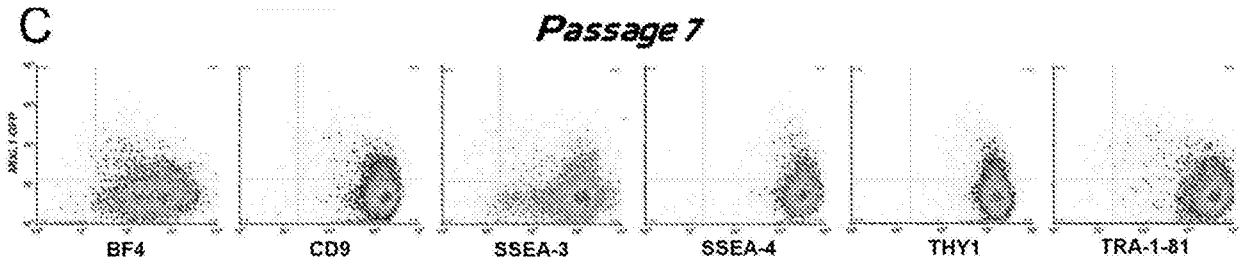

B
Passage 3
Brightfield
Hoechst

Fig. 2


C
Passage 7
BF4
CD9
SSEA-3
SSEA-4
THY1
TRA-1-81

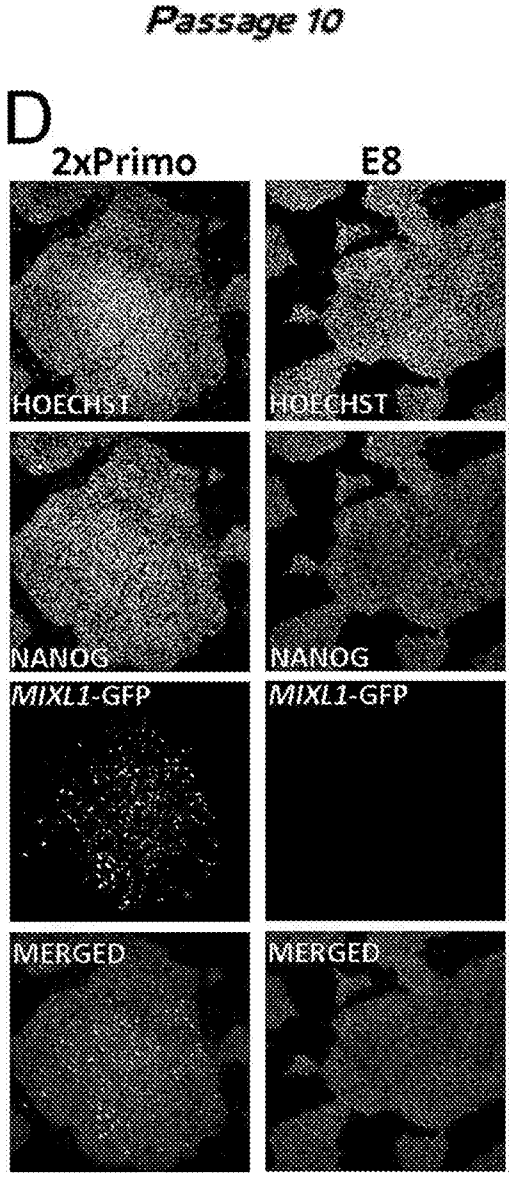
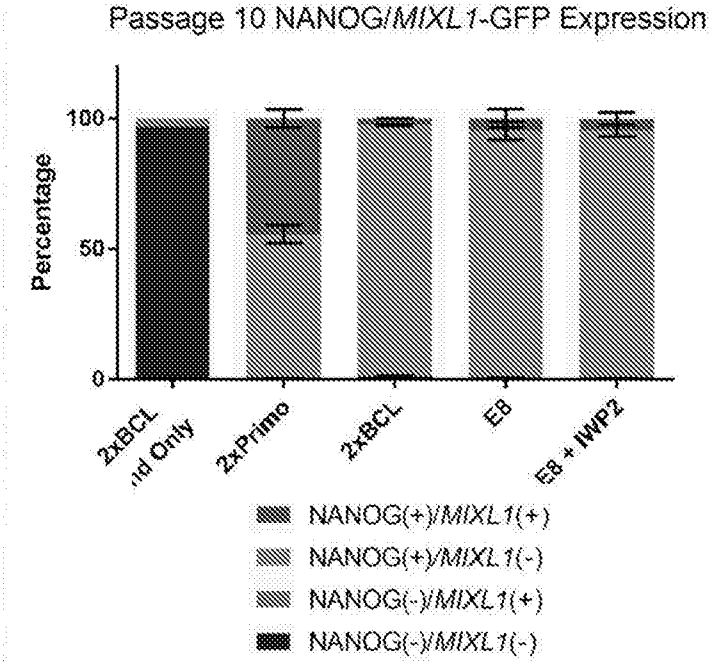

Passage 10
D
2xPrimo
E8
HOECHST
HOECHST
NANOG
NANOG
MIXL1-GFP
MIXL1-GFP
MERGED
MERGED

Fig. 2 (cont)

E


Passage 10 NANOG/MIXL1-GFP Expression
Percentage
100
50
0
2xBCL
nd Only
2xPrimo
2xBCL
E8
E8 + IWP2
NANOG(+)/MIXL1(+)
NANOG(+)/MIXL1(-)
NANOG(-)/MIXL1(+)
NANOG(-)/MIXL1(-)

F

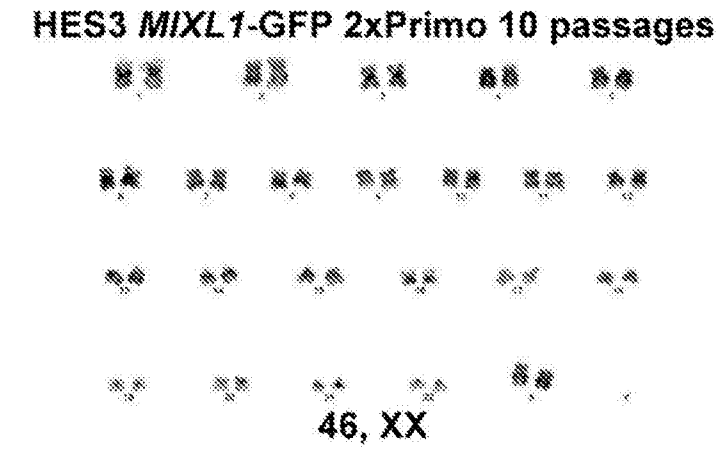

HES3 MIXL1-GFP 2xPrimo 10 passages
46, XX

Fig. 2 (cont)

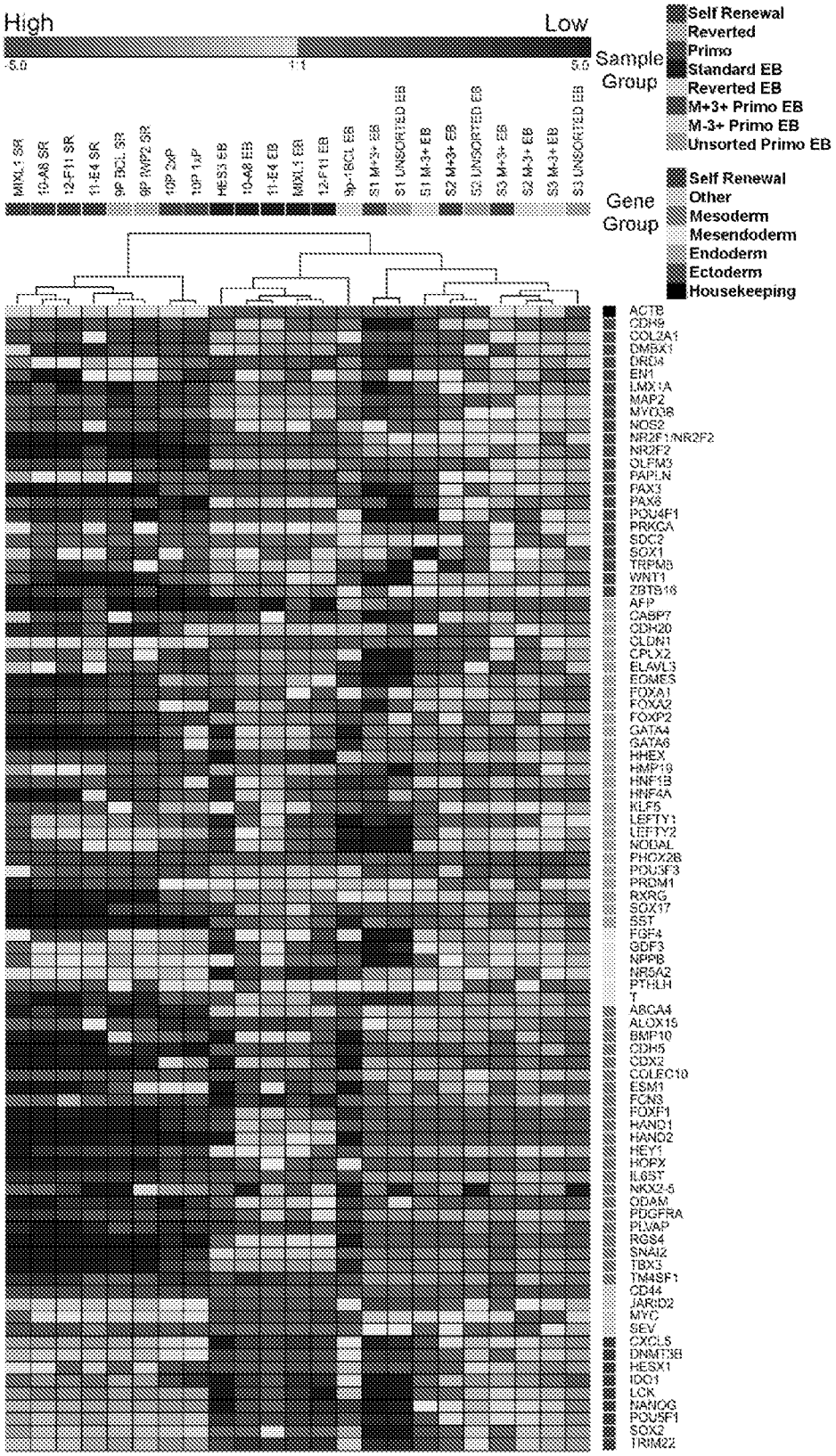

High
Low
-5.0
1.1
5.0
Sample Group
Self Renewal
Reverted
Primo
Standard EB
Reverted EB
M+3+ Primo EB
M-3+ Primo EB
Unsorted Primo EB
MIXL1 SR
10-A8 SR
12-F11 SR
11-E4 SR
9P BCL SR
9P IWP2 SR
10P 2xP
10P 1xP
HES3 EB
10-A8 EB
11-E4 EB
MIXL1 EB
12-F11 EB
9p-1BCL EB
S1 M+3+ EB
S1 UNSORTED EB
S1 M-3+ EB
S2 M+3+ EB
S2 UNSORTED EB
S3 M+3+ EB
S2 M-3+ EB
S3 M-3+ EB
S3 UNSORTED EB
Gene Group
Self Renewal
Other
Mesoderm
Mesendoderm
Endoderm
Ectoderm
Housekeeping
ACTB
CDH9
COL2A1
DMBX1
DRD4
EN1
LMX1A
MAP2
MYO3B
NOS2
NR2F1/NR2F2
NR2F2
OLFM3
PAPLN
PAX3
PAX6
POU4F1
PRKCA
SDC2
SOX1
TRPM8
WNT1
ZBTB16
AFP
CASP7
CDH20
CLDN1
CPLX2
ELAVL3
EOMES
FOXA1
FOXA2
FOXP2
GATA4
GATA6
HHEX
HMP19
HNF1B
HNF4A
KLF5
LEFTY1
LEFTY2
NODAL
PHOX2B
POU3F3
PRDM1
RXRG
SOX17
SST
FGF4
GDF3
NPPB
NR5A2
PTHLH
T
ABCA4
ALOX15
BMP10
CDH5
CDX2
COL9A3
ESM1
FCN3
FOXF1
HAND1
HAND2
HEY1
HOPX
IL6ST
NKX2-5
ODAM
PDGFRA
PLVAP
RGS4
SNAI2
TBX3
TM4SF1
CD44
JARID2
MYC
SEV
CXCL5
DNMT3B
HESX1
IDO1
LCK
NANOG
POU5F1
SOX2
TRIM22

Fig. 3

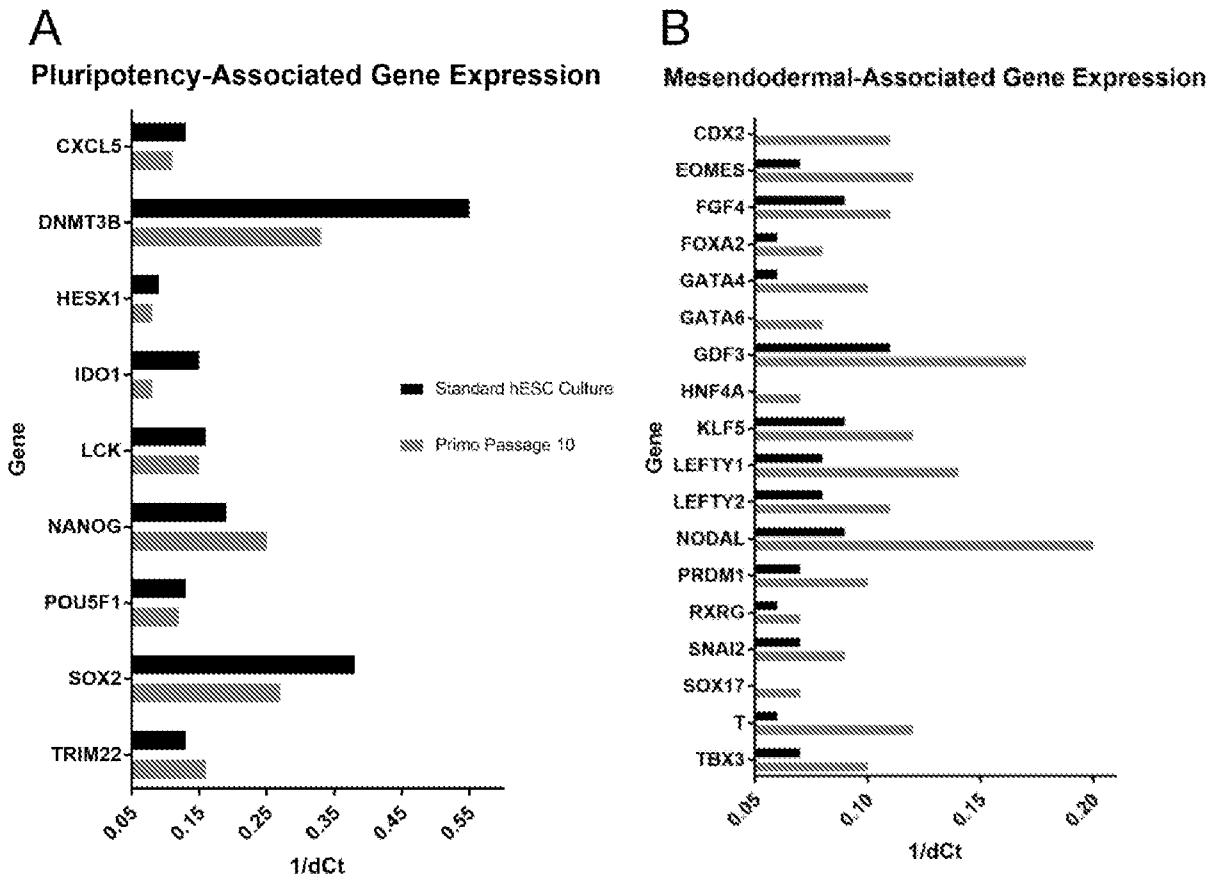
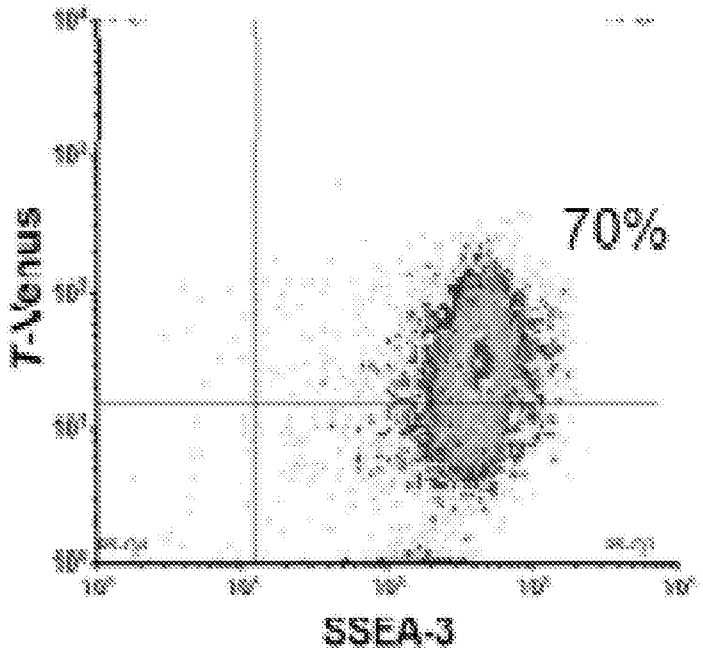

A
Pluripotency-Associated Gene Expression
CXCL5
DNMT3B
HESX1
IDO1
LCK
NANOG
POU5F1
SOX2
TRIM22
Gene
Standard hESC Culture
Primo Passage 10
0.05
0.15
0.25
0.35
0.45
0.55
1/dCt
B
Mesendodermal-Associated Gene Expression
CDX2
EOMES
FGF4
FOXA2
GATA4
GATA6
GDF3
HNF4A
KLF5
LEFTY1
LEFTY2
NODAL
PRDM1
RXRG
SNAI2
SOX17
T
TBX3
Gene
0.05
0.10
0.15
0.20
1/dCt

Fig. 4

A




B

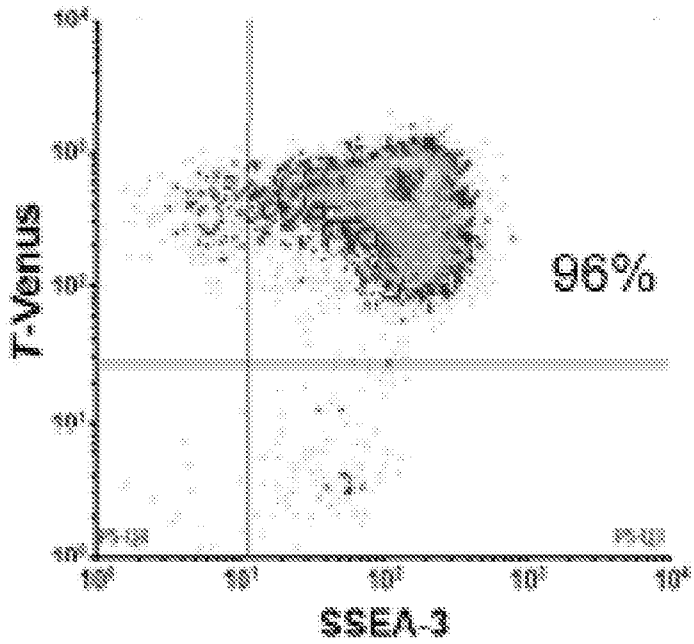



Fig. 5

C

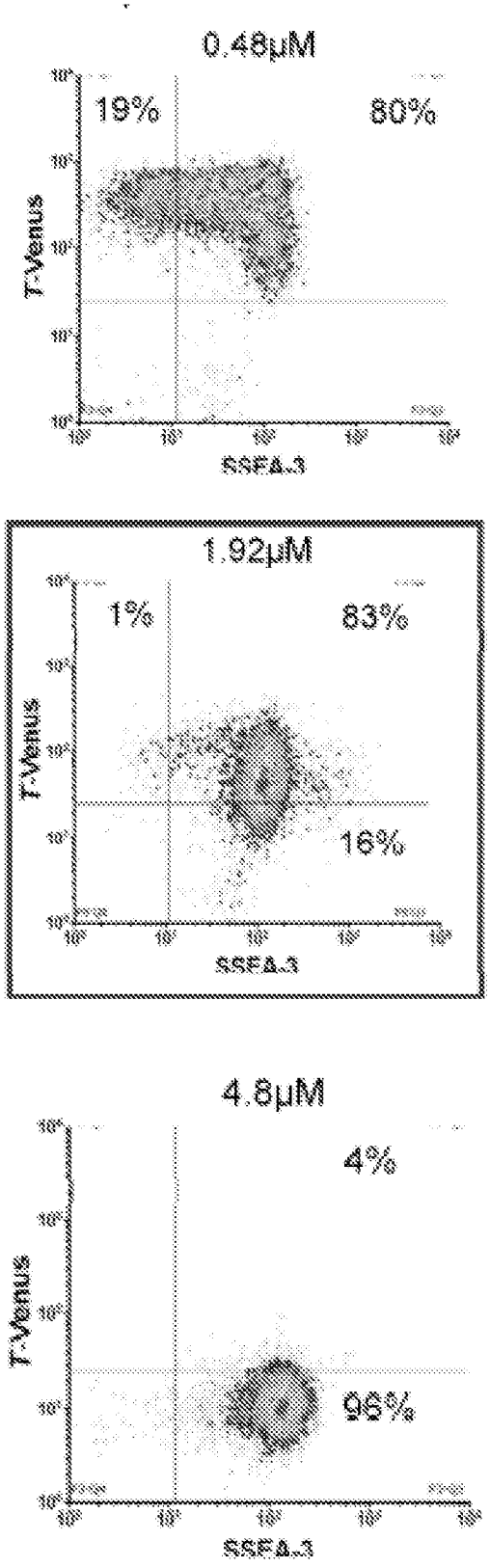

0.48µM
19%
80%
T-Venus
SSEA-3
1.92µM
1%
83%
16%
T-Venus
SSEA-3
4.8µM
4%
96%
T-Venus
SSEA-3

Fig. 5 (cont)

D

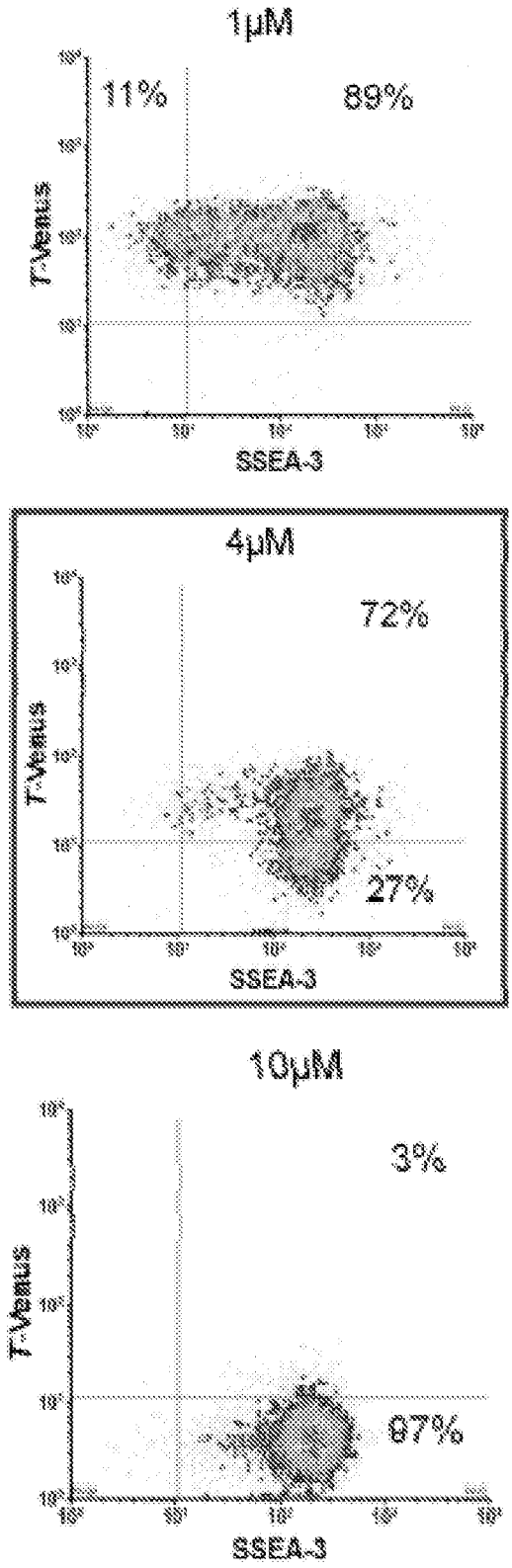

1μM
11%
89%
T-Venus
SSEA-3
4μM
72%
27%
T-Venus
SSEA-3
10μM
3%
97%
T-Venus
SSEA-3

Fig. 5 (cont)

# INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2019/052413

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/00 C12N5/0735
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | TIMOTHY A. BLAUWKAMP ET AL: "Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors", NATURE COMMUNICATIONS, vol. 3, 18 September 2012 (2012-09-18), page 1070, XP055267159, DOI: 10.1038/ncomms2064 page 2, left-hand column, paragraph 2 - right-hand column, paragraph 2 page 6, left-hand column, paragraph 1 - page 7, left-hand column, paragraph 1; figures 3 a, b, i | 1-29 |
| A | US 2018/201904 A1 (LIU PENTAO [GB] ET AL) 19 July 2018 (2018-07-19) paragraphs [0188], [0190], [0200], [0204] | 1-29 |

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 November 2019 | 04/12/2019 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Petri, Bernhard |

1

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# INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2019/052413

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | US 2017/275593 A1 (HANNA YAQUB [IL] ET AL) 28 September 2017 (2017-09-28) paragraphs [0330] - [0331] paragraphs [0313], [0318] | 1-29 |
| A | US 2013/224857 A1 (BLAK ALEXANDRA A [DE] ET AL) 29 August 2013 (2013-08-29) cited in the application the whole document | 1-29 |
| T | JAMES HACKLAND: "Top-Down Inhibition (TDi) and Baseline Activation (BLa): Controlling Signal Transduction When Endogenous Cytokines are Ruining Your Differentiation", CURRENT PROTOCOLS IN STEM CELL BIOLOGY, vol. 51, no. 1, 5 September 2019 (2019-09-05), XP055640712, US ISSN: 1941-7322, DOI: 10.1002/cpsc.98 page 3, right-hand column, paragraph 2; figure 1B | |
| T | Dylan Stavish ET AL: "ABSTRACT", bioRxiv, 24 October 2019 (2019-10-24), XP55640641, DOI: 10.1101/813071 Retrieved from the Internet: URL:https://www.biorxiv.org/content/biorxiv/early/2019/10/24/813071.full-text.pdf page 6, paragraph 137-175 | |

1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2019/052413

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US 2018201904 A1 | 19-07-2018 | EP 3221446 A1 | 27-09-2017 |
| | | US 2018201904 A1 | 19-07-2018 |
| | | WO 2016079146 A1 | 26-05-2016 |
| US 2017275593 A1 | 28-09-2017 | CN 108064274 A | 22-05-2018 |
| | | EP 3194572 A1 | 26-07-2017 |
| | | JP 2017525351 A | 07-09-2017 |
| | | US 2017275593 A1 | 28-09-2017 |
| | | WO 2016016894 A1 | 04-02-2016 |
| US 2013224857 A1 | 29-08-2013 | NONE | |