(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 September 2013 (12.09.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/134378 A1

(51) International Patent Classification:
C12N 5/0735 (2010.01) *C12N 5/02* (2006.01)

(21) International Application Number:
PCT/US2013/029360

(22) International Filing Date:
6 March 2013 (06.03.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/607,706 7 March 2012 (07.03.2012) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

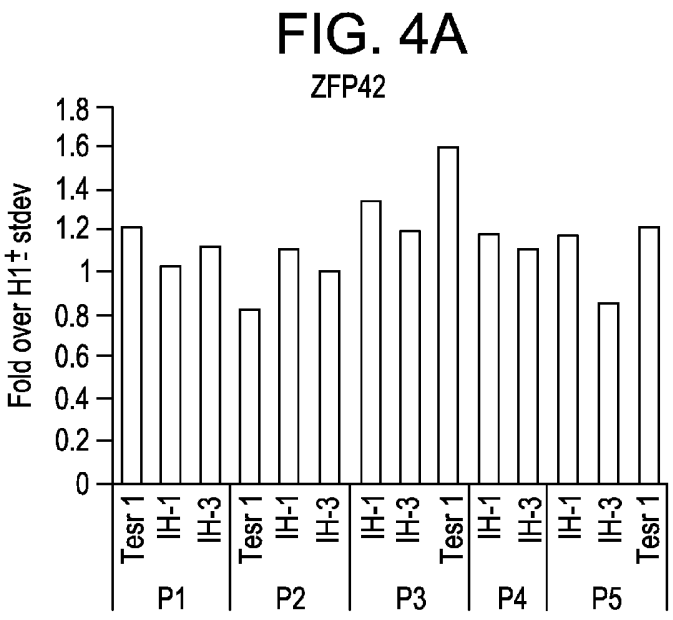
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*

[Continued on next page]

(54) Title: DEFINED MEDIA FOR EXPANSION AND MAINTENANCE OF PLURIPOTENT STEM CELLS



(57) Abstract: The present invention provides methods to promote the proliferation of undifferentiated pluripotent stem cells in defined media. Specifically, the invention provides a defined cell culture formulation for the culture, maintenance, and expansion of pluripotent stem cells, wherein culturing stem cells in the defined cell culture formulation maintains the pluripotency and karyotypic stability of the cells for at least 10 passages. Further disclosed is a cell population grown under defined media conditions that express OCT4, SOX2, NANOG, and FOXA2.

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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

DEFINED MEDIA FOR EXPANSION AND MAINTENANCE OF PLURIPOTENT STEM CELLS

CROSS REFERENCE TO RELATED APPLICATION

[001] The present application claims the benefit of U.S. Provisional Patent Application Serial No. 61/607,706, filed March 7, 2012, which is incorporated herein by reference in its entirety for all purpose.

FIELD OF THE INVENTION

[002] The present invention is in the field of proliferation and maintenance of pluripotent stem cells under defined media conditions.

BACKGROUND

[003] Expansion of undifferentiated pluripotent stem cells has been traditionally employed "feeder" cells which provide sufficient factors to support attachment, proliferation and maintenance of pluripotency markers. Early methods for the generation and culture of human embryonic stem cells required the use of mouse embryonic fibroblast (MEF) feeder cells. Subsequent techniques included use of "conditioned media" and an extracellular matrix coating to replace feeder cells. Conditioned media is media that has been modified by feeder cells, such as MEFs. However, both methods suffer from inconsistencies in batches of conditioned media or feeder cells to continually support expansion of pluripotent stem cells. Furthermore, both systems provide undefined factors that may work differently on different pluripotent stem cells. Accordingly, establishing a defined, cheap, reproducible culture media that supports continual expansion of pluripotent stem cells is of great interest in the regenerative medicine field.

[004] A defining feature of human embryonic stem cells (hES cells) is that the cells have a tendency to differentiate into various lineages. This unwanted differentiation can hamper uniform and directed differentiation required to subsequently generate desired specific cell types. In fact, both feeder cells and conditioned media culture conditions typically result in some level of unwanted

differentiation, particularly around the edges of the growing ES cell colony or in the center of the colony.

[005] Recent efforts have resulted in replacement of feeder cells or conditioned media with a host of replacement culture conditions, such as: knock-out serum replacer (KSR) in the media (2005, Nature Methods, 2:185-189). KSR contains a crude undefined fraction of bovine serum albumin (BSA). Others have shown long-term maintenance of pluripotency in a chemically defined media with FGF2, activin A, and insulin (Vallier et al., 2005, J Cell Sci, 118:4495-4509) Commercially available media formulations including mTeSR®1 media (StemCell Technologies, Vancouver, Canada) and StemPro™ (Invitrogen, CA) have also been previously used to maintain and proliferate human pluripotent stem cells. Additional prior art focusing on development of defined media include US7449334, US7442548, US7005252, US2008/0268534, US7410798, US7297539, and US6800480. Furthermore, a recent publication further refined the mTeSR®1 media to eight components (Nature Methods, 2011, 8:424-424) highlighting that even in defined media there exists unnecessary agent(s) that may actually slow the proliferation of ES cells or reduce their pluripotency state. The refined mTeSR®1 media consists of DMEM/F12 basal media supplemented with insulin, selenium, transferrin, ascorbic acid, FGF2 (bFGF), and TGFβ or nodal, having the pH adjusted with $NaHCO^3$.

[006] It is therefore clear that there is still a need for fully defined media conditions that provide consistency regarding expansion of pluripotent cells while having minimal number of added components.

SUMMARY

[007] The present invention provides a defined cell culture formulation for the culture, maintenance, and expansion of pluripotent stem cells, wherein the defined cell culture formulation comprises basal medium, insulin, transferrin, selenium, fatty-acid free albumin, a TGF-β ligand, bFGF, and ascorbic acid; and wherein culturing stem cells in the defined cell culture formulation maintains the pluripotency and karyotypic stability of the stem cells for at least 10 passages. In some embodiments of the invention, the cell culture formulation further comprises insulin growth factor 1

(IGF-1). In some embodiments of the invention, the cell culture formulation comprises DMEM-F12.

[008] The invention provides a defined cell culture formulation for the culture, maintenance, and expansion of pluripotent stem cells, wherein the defined cell culture formulation comprises basal medium, insulin, transferrin, selenium, fatty-acid free albumin, a TGF-β ligand, bFGF, ascorbic acid, Trace Elements C, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, lithium chloride, glucose, Defined Lipids, and L-alanyl-L-glutamine dipeptide; and wherein culturing stem cells in the defined cell culture formulation maintains the pluripotency and karyotypic stability of the stem cells for at least 10 passages. In some embodiments of the invention, the cell culture formulation comprises MCDB-131.

[009] In some embodiments of the invention ITS-X provides the insulin, transferrin, and selenium for the defined cell culture formulation of the invention. In some embodiments of the invention, the ITS-X is present from about 0.5% to about 2%. In some embodiments of the invention, the ITS-X is present at about 1%. In some embodiments of the invention, the fatty acid free albumin is reagent grade. In some embodiments of the invention, the reagent grade fatty acid-free BSA is present from about 0.2% to about 2.5%. In some embodiments of the invention, the reagent grade fatty acid-free BSA is present at about 2%.

[0010] In some embodiments, the TGF-β ligand in the defined cell culture formulation of the invention is TGF-β1. In some embodiments of the invention, the TGF-β1 is present from about 0.5 ng/ml to about 10 ng/ml. In some embodiments of the invention, the TGF-B1 is present at about 1 ng/ml.

[0011] In some embodiments of the invention, the bFGF is present in the cell culture formulation from about 50 ng/ml to about 100 ng/ml. In some embodiments of the invention, the bFGF is present in the defined cell culture formulation at about 50 ng/ml. In some embodiments, the bFGF is present in the defined cell culture formulation at about 100 ng/ml.

[0012] In some embodiments of the invention, the insulin growth factor 1 (IGF-1) is present from about 10 ng/ml to about 50 ng/ml. In some embodiments of

the invention, the IGF-1 is present in the defined cell culture formulation at about 20 ng/ml.

[0013] In some aspects of the invention, ascorbic acid is present in the defined cell culture formulation from about 0.2 mM to about 0.3 mM. In some aspects of the invention, ascorbic acid is present in the defined cell culture formulation at about 0.25 mM.

[0014] In an embodiment, the invention concerns a defined cell culture formulation consisting essentially of DMEM-F12 basal medium, ITS-X (to provide insulin, transferrin, and selenium), fatty-acid free albumin, a TGF-β ligand, bFGF, insulin growth factor 1 (IGF-1), and ascorbic acid.

[0015] In an embodiment, the invention relates to a defined cell culture formulation consisting essentially of MCDB-131, ITS-X (as a source of insulin, transferrin, and selenium), fatty-acid free albumin, a TGF-β ligand, bFGF, ascorbic acid, Trace Elements C, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, lithium chloride, glucose, Defined Lipids, and L-alanyl-L-glutamine dipeptide.

[0016] In an embodiment, the invention concerns a method for the expansion of human pluripotent stem cells, where the method comprises culturing the human pluripotent stem cells on a feeder-free matrix in a defined cell culture formulation; where the defined cell culture formulation comprises basal medium, insulin, transferrin, selenium, fatty-acid free albumin, a TGF-β ligand, bFGF, and ascorbic acid; and where culturing the stem cells in the defined cell culture formulation maintains the pluripotency and karyotypic stability of the cells for at least 10 passages. In some embodiments, the defined cell culture formulation further comprises insulin growth factor 1 (IGF-1). In some embodiments, the cell culture formulation comprises DMEM-F12.

[0017] In an embodiment, the invention relates to a method for the expansion of human pluripotent stem cells, where the method comprises culturing the human pluripotent stem cells on a feeder-free matrix in a defined cell culture formulation; where the defined cell culture formulation comprises basal medium, insulin, transferrin, selenium, fatty-acid free albumin, a TGF-β ligand, bFGF, ascorbic acid, IGF-1, Trace Elements C, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid,

lithium chloride, glucose, Defined Lipids, and L-alanyl-L-glutamine dipeptide. In some embodiments, the cell culture formulation used in the method for the expansion of human pluripotent stem cells, comprises MCDB-131.

[0018] An embodiment of the present invention is an in vitro cell population wherein greater than 50% of the cell population is positive for protein expression of OCT4, SOX2, NANOG, FOXA2 with negative or low protein expression of SSEA-4 and ZFP42. The population is obtained by culturing pluripotent stem cells in a defined cell culture formulation comprising basal media supplemented with IGF-1, insulin, bFGF, TGF-B ligand, and fatty-acid free albumin; and where the defined cell culture formulation does not comprise ascorbic acid.

[0019] In some embodiments of the invention, the defined cell culture formulation comprises DMEM/F12 basal media. In some embodiments of the invention the cell culture formulation comprises insulin as ITS-X. In some embodiments of the invention, the ITS-X is present from about 0.5% to about 2%. In some aspects of the invention, the ITS-X is present at about 1%. In some embodiments of the invention, the fatty acid free albumin is reagent grade. In some aspects of the invention, the reagent grade fatty acid-free albumin is present from about 0.2% to about 2.5%. In some embodiments of the invention, the reagent grade fatty acid-free albumin is present at about 2%. In some aspects of the invention, the TGF-B ligand is TGF-B1. In some embodiments of the invention, the TGF-B1 is present from about 0.5 ng/ml to about 10 ng/ml. In some aspects of the invention, the TGF-B1 is present at about 1 ng/ml.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1A to Figure 1D show phase-contrast images of H1 cells cultured for 3 passages in IH-3 (FIG 1A), IH-1 (FIG 1B), IH-6 (FIG 1C), and mTeSR®1 (FIG 1D).

[0021] Figure 2A to Figure 2C show phase-contrast images of H1 cells cultured for 10 passages in IH-3 (FIG 2A), IH-1 (FIG 2B), and mTeSR®1 (FIG 2C) media.

[0022] Figure 3A to Figure 3C show phase-contrast images of H1 cells cultured for 18 passages in IH-3 (FIG 3A), IH-1 (FIG 3B), and mTeSR®1 (FIG 3C) media.

[0023] Figure 4A to Figure 4F show data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 cultured in media described in Example 1 and harvested at passages 1 to 5 (P1-P5); ZFP42 (FIG 4A), SOX2 (FIG 4B), POU5F1 (OCT4) (FIG 4C), Nanog (FIG 4D), FOXA2 (FIG 4E), and AFP (FIG 4F).

[0024] Figure 5A to Figure 5B show data from real-time PCR analyses of the expression of Nanog, POU5F1 (OCT4), SOX2, and ZFP42 (FIG 5A), and of AFP and FOXA2 (FIG 5B) in cells of the human embryonic stem cell line H1 cultured in media described in Example 1 and harvested at Passage 10.

[0025] Figure 6A and Figure 6B show data from real-time PCR analyses of the expression of ZFP42, SOX2, POU5F1 (OCT4), and Nanog (FIG 6A), and of AFP and FOXA2 (FIG 6B) in cells of the human embryonic stem cell line H1 cultured in media described in Example 1 and harvested at Passage 18.

[0026] Figure 7A to Figure 7F show FACS histogram expression profiles of the following markers in cells cultured for 18 passages in IH-3 media described in Example 1: Isotype control (FIG 7A); KI-67 (FIG 7B); OCT4 (FIG 7C); SOX17 (FIG 7D); FOXA2 (FIG 7E); and SOX2 (FIG 7F). Percentage expression for each marker is shown on each histogram.

[0027] Figure 8A to Figure 8F show images of cells cultured for 18 passages in IH-3 media described in Example 1 and immunostained for OCT-4, FOXA2, SOX2, and fluorescent labeling of DNA using DAPI. Images obtained for OCT4 (Fig 8A), FOXA2 (FIG 8B), and DAPI-stained DNA (FIG 8C) were obtained from the same optical field but with different filters. Similarly, images for SOX2 (FIG 8D), FOXA2 (FIG 8E), and DAPI stained DNA (FIG 8F) were obtained from the same optical field but with different filters

[0028] Figure 9A to Figure 9F depict phase-contrast images of H1 cells cultured for five passages in mTeSR®1 media (FIG 9A) and in IH-3 (FIG 9B), IH-3-1

(FIG 9C), IH-3-2 (FIG 9D), IH-3-3 (FIG 9E), and IH-3-4 (FIG 9F) formulations described in Example 2.

[0029] Figure 10A to Figure 10E show data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 cultured in media described in Example 2 and harvested at Passage 5: ZFP42 (FIG 10A), SOX2 (FIG 10B), FOXA2 (FIG 10C), Nanog (FIG 10 D), and POU5F1 (OCT4) (FIG 10E).

[0030] Figure 11A to Figure 11D depict phase-contrast images of H1 cells cultured for 20 passages in mTeSR®1 media (Fig 1A), IH-3 (FIG 11B), IH-1 (FIG 11C), and IH-3RT (FIG 11D) media formulations described in Example 3.

[0031] Figure 12A to Figure 12F show data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 cultured for 15 passages in media described in Example 3: AFP (FIG 12A), FOXA2 (FIG 12B), SOX2 (FIG 12C), Nanog (FIG 12D), POU5F1 (OCT4) (FIG 12E), and ZFP42 (FIG 12F).

[0032] Figure 13A to Figure 13F show data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 cultured for 20 passages in mTeSR®1 media, and IH-1 and IH-3 media described in Example 3: AFP FIG 13A), FOXA2 (FIG 13B), NANOG (FIG 13C), POU5F1 (OCT4) (FIG 13D), SOX2 (FIG 13E), and ZFP42 (FIG 13F).

[0033] Figure 14A and Figure 14B depict phase-contrast images of H1 cells cultured for 4 days in media formulations described in Example 5 containing Sigma BSA (FIG 14A) or containing fatty acid free BSA (FIG 14B).

[0034] Figure 15A and Figure 15B depict phase-contrast images of H1 cells cultured for three passages in media formulations described in Example 5 containing Sigma BSA (FIG 15A) or containing fatty acid free BSA (FIG 15B).

[0035] Figure 16A to Figure 16C show data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 cultured for three passages in media formulations described in Example 5

containing Sigma BSA or fatty acid free BSA: AFP (FIG 16A), MIXL1 (FIG 16B), and T (BRY) (FIG 16C).

[0036] Figure 17A to Figure 17D show data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 cultured for ten passages in media formulations described in Example 6: SOX2 (FIG 17A), POU5F1 (FIG 17B), NANOG (FIG 17C), and FOXA2 (FIG 17C).

[0037] Figure 18A to Figure 18E depict phase-contrast images of H1 cells cultured for 10 passages in IH-3 (FIG 18A), IH-3P-2 (FIG 18B), IH-3P-3 (FIG 18C), IH-3P-4 (FIG 18D), and IH-3P-5 (FIG 18E) media formulations described in Example 6.

DETAILED DESCRIPTION

[0038] For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections that describe or illustrate certain features, embodiments or applications of the present invention.

Definitions

[0039] Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny cells, including self-renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate *in vitro* into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts.

[0040] Stem cells are classified by their developmental potential as: (1) totipotent, meaning able to give rise to all embryonic and extra-embryonic cell types; (2) pluripotent, meaning able to give rise to all embryonic cell types; (3) multipotent, meaning able to give rise to a subset of cell lineages but all within a particular tissue, organ, or physiological system (for example, hematopoietic stem cells (HSC) can produce progeny that include HSC (self- renewal), blood cell restricted oligopotent

progenitors, and all cell types and elements (e.g., platelets) that are normal components of the blood); (4) oligopotent, meaning able to give rise to a more restricted subset of cell lineages than multipotent stem cells; and (5) unipotent, meaning able to give rise to a single cell lineage (e.g., spermatogenic stem cells).

[0041] Differentiation is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, for example, a nerve cell or a muscle cell. A differentiated or differentiation-induced cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term “committed”, when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. De-differentiation refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, i.e., which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest.

[0042] "Markers", as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

[0043] “Basal Medium” refers to a solution of salts, nutrients, and vitamins that can support the growth of pluripotent stem cells in culture. Basal media may be selected among others from Dulbecco’s modified Eagle's media (DMEM), MCDB media, RPMI. DMEM may also be DMEM/F12 (also referred to as DM-F12), or DMEM-high glucose (also referred to as DMEM-hg). MCDB media may be selected

from any of the MCDB media available, and specifically MCDB-131. Alternatively, basal media may be selected by mixing the basl media formulations listed above in the appropriate ratio to allow for proliferation and maintenance of pluripotency of embryonic stem cells.` In some embodiments, the basal media in the defined cell culture formulation of the invention is DMEM-F12. In some embodiments, the basal media in the cell culture formulation of the invention is MCDB-131.

[0044] "Feeder Cells" refers to non-pluripotent stem cells on which pluripotent stem cells are plated. The feeder cells provide sufficient soluble and insoluble factors to support for attachment, proliferation, and maintenance of pluripotency markers by pluripotent stem cells.

[0045] "Conditioned Medium" refers to a medium that is further supplemented with soluble factors derived from feeder cells.

[0046] "Extracellular Matrix" or "Defined Matrix" or "Synthetic Matrix" refers to one or more substances that can provide for attachment, proliferation, and maintenance of pluripotency markers by pluripotent stem cells. Used interchangeably herein are "IGF" and "IGF-1" which stand for Insulin-like growth factor 1. In humans this protein is made by the liver and is responsible for much of what is attributed to the human growth hormone.

[0047] As used herein, "FGF2" and "bFGF" are used interchangeably to identify the human basic fibroblast growth factor.

[0048] Used interchangeably herein are "TGF beta", "TGF-B", and "TGF-β". A TGF-β ligand may be selected from bone morphogenetic proteins (BMPs), growth and differentiation factor (GDFs), activins (Activin A, Activin AB, Activin B, Activin C), nodal and TGF-βs. A TGF-β may be selected from TGF-β1, TGF-β2, activin A, and TGF-β3.

Isolation, Expansion and Culture of Pluripotent Stem Cells

Characterization of Pluripotent Stem Cells

[0049] Pluripotent stem cells may express one or more of the stage-specific embryonic antigens (SSEA) 3 and 4, and markers detectable using antibodies

designated Tra-1-60 and Tra-1-81 (Thomson *et al.*, Science 282:1145, 1998). Differentiation of pluripotent stem cells *in vitro* results in the loss of SSEA-4, Tra 1-60, and Tra1-81 expression (if present) and increased expression of SSEA-1. Undifferentiated pluripotent stem cells typically have alkaline phosphatase activity, which can be detected by fixing the cells with 4% paraformaldehyde, followed by developing with Vector Red as a substrate, as described by the manufacturer (Vector Laboratories, Burlingame CA). Undifferentiated pluripotent stem cells also typically express OCT4 and TERT, as detected by RT-PCR.

[0050] Another desirable phenotype of propagated pluripotent stem cells is a potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Pluripotency of stem cells can be confirmed, for example, by injecting cells into severe combined immunodeficient (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining them histologically for evidence of cell types from the three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers.

[0051] Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published karyotypes of the corresponding primate species. It is desirable to obtain cells that have a "normal karyotype," which means that the cells are euploid, wherein all human chromosomes are present and not noticeably altered. Pluripotent cells may be readily expanded in culture using various feeder layers or by using matrix protein coated vessels. Alternatively, chemically defined surfaces in combination with defined media such as mTeSR®1 media (StemCell Technologies, Vancouver, Canada) may be used for routine expansion of the cells. Pluripotent cells may be readily removed from culture plates using enzymatic, mechanical or use of various calcium chelators such as EDTA (Ethylenediaminetetraacetic acid). Alternatively, pluripotent cells may be expanded in suspension in the absence of any matrix proteins or a feeder layer.

Sources of Pluripotent Stem Cells

[0052] The types of pluripotent stem cells that may be used include established lines of pluripotent cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10 to 12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell Research Institute, Madison, WI). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are inducible pluripotent cells (IPS) or reprogrammed pluripotent cells that can be derived from adult somatic cells using forced expression of a number of pluripotent related transcription factors, such as OCT4, Nanog, Sox2, KLF4,and ZFP42 (Annu Rev Genomics Hum Genet, 2011, 12:165-185).

[0053] Human embryonic stem cells may be prepared as described by Thomson *et al.* (U.S. Patent No. 5,843,780; Science, 1998; 282:1145-1147; Curr Top Dev Biol, 1998; 38:133-165; 1995, Proc Natl Acad Sci USA 92:7844-7848).

[0054] Characteristics of pluripotent stem cells are well known to those skilled in the art, and additional characteristics of pluripotent stem cells continue to be identified. Pluripotent stem cell markers include, for example, the expression of one or more of the following: ABCG2, cripto, FOXD3, CONNEXIN43, CONNEXIN45, OCT4, SOX2, NANOG, hTERT, UTF1, ZFP42, SSEA-3, SSEA-4, Tra 1-60, Tra 1-81.

[0055] Differentiation markers typically present in cultures of embryonic stem cells include for example, AFP, FOXA2, SOX17, T(BRY), and MIXL1.

[0056] In an embodiment of the present invention, human pluripotent stem cells are cultured in a defined media comprising ascorbic acid, IGF, insulin, bFGF, TGF-B ligand, and fatty-acid free albumin to sustain proliferation of the pluripotent

stem cells while maintaining pluripotency and karyotypic stability of the expanded cells for at least 10 passages.

[0057] An embodiment of the present invention is an in vitro cell population wherein greater than 50% of the cell population is positive for protein expression of OCT4, SOX2, NANOG, and FOXA2 positive but low protein expression of SSEA-4 and ZFP42.

[0058] Another aspect of the present invention describes an in vitro defined cell culture formulation comprising IGF, insulin, bFGF, TGF-B, fatty-acid free albumin, and no ascorbic acid that results in a cell population wherein greater than 50% of the cell population is positive by protein staining for OCT4, SOX2, NANOG, FOXA2 and low protein expression of SSEA-4 and ZFP42.

[0059] The present invention is further illustrated, but not limited, by the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Publications cited throughout this document are hereby incorporated by reference in their entirety.

Example 1

Testing of various culture conditions to identify optimal media components for proliferation of undifferentiated embryonic stem cells

[0060] Cells of the human embryonic stem cell line H1 (at passage 35 to passage 40), cultured on MATRIGEL™ (1:30 dilution; BD Biosciences, Franklin Lakes, NJ) coated dishes in mTeSR®1 media (StemCell Technologies, Vancouver, Canada) and passaged using EDTA, were used as the starting population to test

various media compositions. Cells were passaged as small colonies using 5-10 min EDTA treatment at room temperature. Cultures were routinely split in a ratio of 1:6 to 1:10 at each passage. Table I lists the initial media formulations tested for their ability to proliferate H1 cells while maintaining their undifferentiated morphology and pluripotency markers.

Table I

Media Formulations Evaluated

Media Number	Basal Media	Added Components*
IH-1	MCDB-131	1 X Trace Elements C**, 0.25 mM ascorbic acid, 10 mM HEPES, 1 mM lithium chloride, 10 mM Glucose, 1:500 X Defined Lipids***, 1 X ITS-X, 2% reagent grade fatty acid free BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 1X GlutaMAX™
IH-2	MCDB-131	1X Trace Elements C**, 0.25 mM ascorbic acid, 10 mM HEPES, 1 mM lithium chloride, 10 mM Glucose, 1:500 X Defined Lipids***, 1 X ITS-X, 2% lipid rich BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 1X GlutaMAX™
IH-3	DM-F12	1 X ITS-X, 2% reagent-grade fatty acid free BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 20 ng/ml IGF-1
IH-4	DM-F12	1 X Trace Elements C**, 0.25 mM ascorbic acid, 10 mM HEPES, 1 mM lithium chloride, 10 mM Glucose, 1:500 X Defined Lipids***, 1 X ITS-X, 2% BSA (New Zealand origin), 1 ng/ml TGF-B1, 100 ng/ml bFGF, 1X GlutaMAX™

Media Number	Basal Media	Added Components*
IH-5	DM-F12	1 X Trace Elements C**, 0.25 mM ascorbic acid, 10 mM HEPES, 1 mM Lithium chloride, 10 mM Glucose, 1:500 X Defined Lipids***, 1 X ITS-X, 2% standard grade BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 1X GlutaMAX™
IH-6	DM-F12	1 X Non-essential amino acids, 1 X ITS-X, 20 ng/ml bFGF, 0.1 mM β-mercaptoethanol, 0.95 μM CHIR99021, 0.4 μM PD0325901, and 10 μM Y-27632

[0061] *:Trace Elements C** (Mediatech, Manassas, VA), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Invitrogen, Carlsbad, CA), LiCl (Sigma, Saint Louis, MO), glucose (Sigma), Defined Lipids*** (Invitrogen), reagent-grade fatty acid free BSA (Proliant, Ankeny, IA), TGF-β1 (R & D Systems, Minneapolis, MN), bFGF (R & D Systems), IGF-1 (R & D Systems), GlutaMAX™ (200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl; Invitrogen), Lipid rich BSA- Albumax (Invitrogen), ITS-X (Insulin, transferrin, selenium-X-supplement; Invitrogen), standard grade New Zealand BSA (Lampire Biological Laboratories, Coopersburg, PA), standard grade BSA (Lampire), NEAA (Invitrogen), mercaptoethanol (Invitrogen), CHIR99021 (Stemgent, Cambridge, MA) ,PD0325901 (Sigma), Y2763 (Sigma).

[0062] ** Mediatech Trace Elements C Catalog No. 99-176 1000x liquid contains: 1.20 mg/L $AlCl_3 \cdot 6H^2O$, 0.17 mg/L $AgNO_3$, 2.55 mg/L $Ba(C_2H_3O_2)^2$, 0.12 mg/L KBr, 2.28 mg/L $CdCl_2$, 2.38 mg/L $CoCl_2 \cdot 6H_2O$, 0.32 mg/L $CrCl_3$ (anhydrous), 4.20 mg/L NaF, 0.53 mg/L GeO_2, 0.17 mg/L KI, 1.21 mg/L RbCl, and 3.22 mg/L $ZrOCl_2 \cdot 8H_2O$.

[0063] *** Invitrogen Chemically Defined Lipid Concentrate Catalog No. 11905031 contains 100.0 ml/L ethyl alcohol (200 proof) and 2 mg/L Arachidonic Acid, 220 mg/L Cholesterol, 70 mg/L DL-alpha-Tocopherol Acetate, 0 mg/L Ethyl Alcohol 100%, 10 mg/L Linoleic Acid, 10 mg/L Linolenic Acid, 10 mg/L Myristic Acid, 10 mg/L Oleic Acid, 10 mg/L Palmitic Acid, 10 mg/L Palmitoleic Acid, 90000 mg/L Pluronic F-68, 10 mg/L Stearic Acid, and 2200 mg/L Tween 80® (ICI Americas, Inc. Bridgewater, NJ).

[0064] Use of IH-4 and IH-5 were discontinued for further evaluation because cells cultured using IH-4 and IH-5 failed to grow past passage 2. At passage 2, cells grown in IH-2 showed significant change in morphology consistent with differentiated cells and loss of packed colonies. Media IH-1, IH-3, and IH-6 were selected for further evaluation. At passage 3-5, cells cultured in IH-6 showed morphological evidence of differentiated cells at the periphery of the ES colonies (compare FIG 1C with FIG 1A, FIG 1B, and FIG 1D).

[0065] After passage 5, only IH-1 and IH-3 were further compared to the cells cultured in mTeSR®1 media. At passages 5 to 18 samples were collected from IH-1, IH-3, and mTeSR®1 cultures and evaluated by FACS, PCR, karyotype analysis (G-banding or FISH), and immune fluorescence staining. The results from FISH analysis are shown in Table II. These results show that H1 cells cultured in IH-1 media or IH-3 media showed normal karyotype, whereas cells cultured in mTeSR®1 media displayed abnormal trisomy 12 at passage 10 and 18.

Table II

Results of FISH Analysis of Chromosome 12 and Chromosome 17by CellLineGenetics (Madison, WI)

Media	P5	P10	P18
IH-1	Normal	Normal	Normal
IH-3	Normal	Normal	Normal
mTeSR®1	Normal	14% Trisomy 12, normal 17	14% Trisomy 12, normal 17

[0066] Furthermore, similar to cells grown in mTeSR®1 media, cells passaged continuously in IH-1 media maintained characteristic ES colony morphology with very few differentiated cells surrounding the colonies. However, cells grown in IH-3 media started to lose the characteristic ES colony morphology beyond passage 10 (See FIG 1A, FIG 2A, and FIG 3A).

[0067] Evaluation of surface and internal markers attributed to pluripotency was used to assess the impact of the tested formulations on maintenance of pluripotency. As shown in Table III, at passage 5, cells cultured in IH-1 and IH-3 showed similar profile of surface markers as cultures expanded in mTeSR®1 media. However, by passage 10, H1 cells cultured in IH-3 media showed a significant drop in expression of SSEA-4 and a modest drop in expression of TRA1-60 and 1-81. H1 cells cultured in IH-1 media for 10 passages maintained similar expression pattern to those cultured in mTeSR®1 media.

Table III

FACS Results at Passage 5 and Passage 10 for Surface Markers Related to the Pluripotency State of the Cells

	P5			
	%CD9	**%SSEA-4**	**%TRA 1-60**	**%TRA 1-81**
IH-1	80	98	50	54
IH-3	83	87	39	50
mTeSR®1	60	99	56	63
	P10			
IH-1	83	95	55	44
IH-3	93	15.7	42	31
mTeSR®1	58	97	55	62

[0068] Surprisingly, similar to H1 cells cultured in mTeSR®1 and IH-1 media, H1 cells cultured in IH-3 media maintained strong expression of OCT4 and SOX2 markers at passage 11 (Table IV). This was despite a very low expression level of SSEA-4 for H1 cells cultured in IH-3 media.

Table IV

Internal and surface markers of cells cultured for 11 passages in IH-1, IH-3 and mTeSR®1 media

	%Sox2	%SSEA-4	%Oct3/4
IH-1	97	97	92
IH-3	98	4.2	96
mTeSR®1	98	98	92

[0069] As shown in Figure 4, mRNA expression of core pluripotency markers, such as Nanog (FIG 4D), OCT4 (FIG 4C), SOX2 (FIG 4B), and ZPF42 (FIG 4A) were maintained through passage 5 for H1 cells cultured in IH-1, and IH-3 media to the same level as H1 cells cultured in mTeSR®1. However, by passages 10 to 18 there was a significant decrease in expression of ZFP42 while expression of OCT4, Nanog, and SOX2 were not significantly changed for cells grown in IH-3 media as compared to H1 cells cultured in IH-1 or mTeSR®1 media (See FIG 5A and FIG 6A). Furthermore, FACS analysis of H1 cells cultured in IH-3 media for 18 passages showed >97% of cells were OCT4+ (FIG 7C), SOX2+ (FIG 7F), and KI-67+ (FIG 7B).Approximately 1% of the cells were SOX17+ (FIG 7D) and ~85% of the cells were FOXA2+ (FIG 7E). Figure 8A to Figure 8F show images of immunofluorescence staining of H1 cells cultured in IH-3 media for 18 passages. These images illustrate that a significant number of OCT4 and SOX2 positive cells were also FOXA2+. H1 cells cultured in IH3 media had acquired a phenotype where at least 70% of the cells were Oct4+ NANOG+ SOX2+ KI-67+ ZFP42- and FOXA2+. This represents a population of cells not yet described in the art.

Example 2

Culturing of H1 Cells in IH-3 Media Spiked with Ascorbic Acid Restores Major Features of Undifferentiated Embryonic Stem Cells

[0070] In order to identify the cause for the drop in SSEA-4 and ZPF42 for H1 cells cultured in IH-3 vs those cultured in IH-1 and mTeSR®1 media, a gap analysis

was conducted to identify the major reagents present in mTeSR®1 and IH-1 but absent in IH-3 media. IH-3 media was supplemented with Trace Elements C, ascorbic acid, lithium chloride, or Defined lipids as indicated in Table V.

TABLE V

Modifications to IH-3 Media

Media	Additions to IH-3 Media
IH-3-1	1x Trace Elements C
IH-3-2	0.25 mM ascorbic acid
IH-3-3	1 mM lithium chloride
IH-3-4	1:500 X Defined Lipids

[0071] H1 cells cultured for 14 passages in IH-3 were subsequently cultured in the above media formulations and compared to cells cultured in IH-3 media. At various passages, H1 cells cultured using various media formulations were assayed for pluripotency markers. As shown Table VI, following five additional passages, H1 cells cultured in IH-3-2 (IH-3 supplemented with ascorbic acid) media recovered a small percentage of their SSEA-4 expression as compared to cells cultured in the other tested media.

Table VI

FACS Results at Five Passages Beyond Passage 15 for Surface Markers Related to the Pluripotency State of the H1 Cells.

	CD9	SSEA-4
mTeSR®1	26	96.9
IH-1	82.9	96.9
IH-3	89.7	0.8
IH-3-1	90.4	0.9
IH-3-2	91.6	4.2
IH-3-3	87.6	0.7
IH-3-4	88.8	0.6

[0072] As shown in FIG 9D, H1 cells cultured in IH-3-2 media retained typical embryonic stem cell morphology similar to cells cultured in mTeSR®1 (FIG

9A) media. However, H1 cells cultured in IH-3, IH-3-1, IH-3-3, and IH-3-4 showed loose colony morphology (See FIG 9B, FIG 9C, and FIG 9F). PCR analysis of cells cultured in the above media formulations further confirmed that H1 cells cultured in IH-3-2 media regained some of the expression of ZFP42 and down regulated expression of FOXA2 (see FIG 10A to FIG 10E). The above data shows that presence of ascorbic acid is required to maintain pluripotency of ES cells along with their characteristic colony/cell morphology and low expression of differentiation markers. Based on this data, subsequent cultures of H1 cells in IH-3 media were further supplemented with 0.25 mM ascorbic acid.

[0073] Cells cultured in IH-3-2 recovered some of the characteristic colony morphology of ES cells whereas cells cultured in other IH media formulations displayed a looser morphology.

Example 3

Long-Term Cultures of H1 Cells in IH-3 and IH-1 Media Maintain Pluripotency and Stable Karyotype

[0074] Cells of the human embryonic stem cells line H1 (passage 35 to passage 40), cultured on MATRIGEL™ (1:30 dilution) coated dishes in mTeSR®1 media and passaged using EDTA, as described in Example 1, were used as the starting population to evaluate long-term cultures using IH-1, IH-3-2 and mTeSR®1 media. Cells were passaged as small colonies using 5-10 minute EDTA treatment at room temperature. The components of the tested media are listed in Table VII.

Table VII

Ingredients used in IH-1, IH-3-2, and IH-3RT media formulations.

Media number	Basal Media	Added components*
IH-1	MCDB-131	1X Trace Elements C, 0.25 mM ascorbic acid, 10 mM HEPES, 1 mM lithium chloride, 10 mM Glucose, 1:500 X Defined Lipids, 1 X ITS-X, 2% reagent grade fatty acid free BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 1X GlutaMAX™
IH-3-2	DM-F12	1 X ITS-X, 2% reagent-grade fatty acid free BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 20 ng/ml IGF-1, 0.25 mM ascorbic acid
IH-3RT	DM-F12	2% reagent-grade fatty acid free BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 20 ng/ml IGF-1, 0.25 mM ascorbic acid, 5.5 μg/ml Recombinant Human Transferrin (Millipore), 10 μg/ml insulin (Invitrogen), 0.0067 μg/ml sodium selenite (Invitrogen)

[0075] As seen in FIG 11A to FIG 11D, H1 cells cultured for 20 passages in IH-1, IH-3-2, and IH-3RT retained typical ES morphology. The results of PCR analysis of H1 cells cultured for 15 passages in IH-1, IH-3-2, and IH-3RT are shown in FIG 12A to FIG 12F. The results of PCR analysis of H1 cells cultured for 20 passages in IH-1, IH-3-2, and IH-3RT are shown in FIG 13A to FIG13F. These analyses confirmed that, similar to H1 cells cultured in mTeSR®1 media, cells cultured for 15 or 20 passages in IH-1, IH-3-2, and IH-3RT (recombinant human

transferrin) media retained all core pluripotency markers while showing very low expression of FOXA2 and AFP. FACS analysis at Passage 15 and Passage 20 also confirmed expression of surface markers related to pluripotent cells to the same levels as H1 cells cultured in mTeSR®1 media (See Table VIII).

Table VIII

FACS Results for Cells Tested at Passage 15 and Passage20 for Surface Markers Related to the Pluripotency State of the Cells

	P15			
	%CD9	**%SSEA-4**	**%TRA 1-60**	**%TRA 1-81**
IH-1	93	99	59	59
IH-3-2	72	99	55	52
IH-3RT	65	99	50	48
mTeSR®1	63	99	49	49
	P20			
IH-1	91	96	52	54
IH-3-2	91	99	49	53
mTeSR®1	66	97	57	63

[0076] H1 cells cultured continuously in IH-1, IH-3-2, and IH-3RT showed normal karyotype as measured by G-banding and FISH analysis. However, H1 cells cultured for 10 to 20 passages in mTeSR®1 showed abnormal chromosomal counts (See Table IX).

Table IX

FISH and G-banding Analysis of H1 Cells Cultured in IH-1, IH-3, IH-3RT, and mTeSR®1.

Media	P10 (G-banding and FISH)	P15 (FISH)	P20 (FISH)
IH-1	46 XY, Normal 12 and 17 chromosomes	Normal	Normal
IH-3-2	46 XY, Normal 12 and 17 chromosomes	Normal	Normal
IH-3RT	46 XY, Normal 12 and 17 chromosomes	Normal	ND
mTeSR®1	48, XY, +12, +14[2], /46, XY[18]- 20% trisomy 12 by FISH	11% Trisomy 12, normal 17	20% Trisomy 12, normal 17

Example 4

Equivalent Proliferation Rate for H1 Cells Cultured in IH-1, IH-3, and mTeSR®1 Media

[0077] In order to compare the proliferation rate of cells cultured in previously tested media, H1 cells cultured in IH-1, IH-3-2 and mTeSR®1 media were released by using TrypLE (Invitrogen) and seeded at a density of 5 X 10^5 cells per 10 cm MATRIGEL™ -coated dishes. In order to reduce apoptosis of single cells and enhance attachment, released cells were pretreated with 10 μM Rock inhibitor (Sigma). Media was changed daily until three days post-seeding. On day 3, cells were released as single cells and counted using a hemocytometer. As shown in Table X, cells cultured in all three media formulations showed equivalent doubling times

Table X

Doubling Times of H1 Cells Cultured in mTeSR®1, IH-1, and IH-3-2 Media Formulations.

	mTeSR®1	IH-1	IH-3-2
0 h	0.5 x 10^6 cells	0.5 x 10^6 cells	0.5 x 10^6 cells
72 h	6.7 x 10^6 cells	4.2 x 10^6 cells	6.8 x 10^6 cells
Cell Doubling Time	19.23h	23.45h	19.12h

Example 5

High Quality Fatty-Acid Free BSA Allows for Expansion of Pluripotent Cells

[0078] Cells of the human embryonic stem cells line H1 (passage 35 to passage 40), cultured on MATRIGEL™ (1:30 dilution) coated dishes in mTeSR®1 media and passaged using EDTA, were used as the starting population to evaluate short-term cultures using IH-3-2 media supplemented with either 2% Sigma BSA (catalog No. A2153; Lot: 061M1804V) or fatty-acid free BSA (Proliant, Catalog No. 7500804; Lot: 11G54001). Cells were passaged as small colonies using 5-10 minute EDTA treatment at room temperature. Figure 14A and Figure 14B depict phase-contrast images of H1 cells cultured for 4 days in media formulations containing Sigma BSA (FIG 14A) or fatty acid free BSA (FIG 14B). Figure 15A and Figure 15B depict phase-contrast images of H1 cells cultured for three passages in media formulations containing Sigma BSA (FIG 15A) or fatty acid free BSA (FIG 15B). As seen in FIG 14A, as early as day 4 following seeding, there was morphological evidence of differentiated cells in cultures using Sigma BSA. However, there was no gross differentiated cell morphology evident in cultures treated with fatty acid-free BSA (see FIG 14B)). The same trend was noted at passage 3, there was morphological evidence of differentiated cells in cultures using Sigma BSA (see FIG 15A), while there was no gross differentiated cell morphology evident in cells cultured in media comprising fatty acid-free BSA (see FIG 15B). Furthermore, there was a significant drop in confluency of cells cultured in media comprising Sigma

BSA as compared to cells cultured in media comprising reagent grade fatty-acid BSA (compare FIG 15A and FIG 15B).

[0079] Data from real-time PCR analyses of the expression of AFP (FIG 16A), MIXL1 (FIG 16B), and T (BRY) (FIG 16C) in cells of the human embryonic stem cell line H1 cultured for three passages in media formulations containing Sigma BSA or fatty acid free BSA are shown in FIG 16A, 16B, and 16C. PCR data at passage 3 clearly showed significant upregulation of markers associated with a differentiated cell for cells cultured in media comprising Sigma BSA. This data clearly demonstrates that use of fatty-acid-free BSA is critical in the maintenance of pluripotency, colony morphology, and proliferation of cells.

Example 6

Pluripotent Stem Cells can be Propagated and Maintain Pluripotency in IH-3 Media Using a Wide Range of Fatty Acid Free BSA and bFGF Concentrations

[0080] Cells of the human embryonic stem cells line H1 (passage 35 to passage 40), cultured on MATRIGEL™ (1:30 dilution) coated dishes in mTesr®1 media and passaged using EDTA, were used as the starting population to evaluate short and long-term cultures using IH-3 media supplemented as indicated in Table XI.

Table XI

Ingredients used in IH-3 media supplemented with varying doses of BSA and bFGF

Media number	Basal Media	Added components*
IH-3-2	DM-F12	1X ITS-X, 2% reagent-grade fatty acid free BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 20 ng/ml IGF-1, 0.25 mM ascorbic acid

Media number	Basal Media	Added components*
IH-3P-2	DM-F12	1X ITS-X, 2% reagent-grade fatty acid free BSA, 1 ng/ml TGF-B1, 50 ng/ml bFGF, 20 ng/ml IGF-1, 0.25 mM ascorbic acid
IH-3P-3	DM-F12	1X ITS-X, 1% reagent-grade fatty acid free BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 20 ng/ml IGF-1, 0.25 mM ascorbic acid
IH-3P-4	DM-F12	1X ITS-X, 0.5% reagent-grade fatty acid free BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 20 ng/ml IGF-1, 0.25 mM ascorbic acid
IH-3P-5	DM-F12	1X ITS-X, 0% reagent-grade fatty acid free BSA, 1 ng/ml TGF-B1, 100 ng/ml bFGF, 20 ng/ml IGF-1, 0.25 mM ascorbic acid

[0081] At passage 10, cells were evaluated morphologically by PCR for pluripotency and differentiation-associated genes. Furthermore, cells were evaluated for karyotypic stability using FISH analysis for chromosomes 12 and 17. Figure 17A to Figure 17D show data from real-time PCR analyses of the expression of SOX2 (FIG 17A), POU5F1 (FIG 17B), NANOG (FIG 17C), and FOXA2 (FIG 17C) in cells of the human embryonic stem cell line H1 cultured for ten passages in media formulations listed in Table XI. As shown in these figures, all of the above formulations retained strong expression of pluripotency markers relative to cells grown in mTeSR®1 media. However, cells grown in 0-0.5% BSA showed higher expression of FOXA2 indicating a higher level of spontaneous differentiation in these cultures as compared to the other tested formulations. Figure 18A to Figure 18E depict phase-contrast images of H1 cells cultured for 10 passages in IH-3-2 (FIG 18A), IH-3P-2 (FIG 18B), IH-3P-3 (FIG 18C), IH-3P-4 (FIG 18D), and IH-3P-5 (FIG

18E) media formulations listed in Table XI. As indicated in these figures, all formulations tested in this example allowed for formation of ES colonies with minimal evidence of gross differentiated morphology.

Table XII

FISH analysis of chromosome 12 and 17 analyzed by CellLineGenetics

Media	P10
IH-3-2	Normal
IH-3P-2	Normal
IH-3P-3	Normal
IH-3P-4	Normal
IH-3P-5	Normal

[0082] As seen in Table XII, H1 cells cultured for ten passages in media formulations listed in Table XI retained normal counts for chromosome 12 and 17 as measured by FISH analysis. The above data indicates that defined media consisting of DMEM/F12 basal media supplemented with ITS-X, reagent-grade fatty acid-free BSA, TGF-B1, IGF-1, and ascorbic acid allows for expansion of pluripotent cells while maintaining pluripotency of the cells when using a wide range of concentrations of fatty acid –free BSA and bFGF.

CLAIMS

What is claimed is:

1. A defined cell culture formulation for the culture, maintenance, and expansion of pluripotent stem cells, wherein the defined cell culture formulation comprises basal medium, insulin, transferrin, selenium, fatty-acid free albumin, a TGF-β ligand, bFGF, and ascorbic acid; and wherein culturing stem cells in the defined cell culture formulation maintains the pluripotency and karyotypic stability of the cells for at least 10 passages.

2. The defined cell culture formulation of claim 1, wherein the cell culture formulation further comprises insulin growth factor 1 (IGF-1).

3. The defined cell culture formulation of claim 1 or 2, wherein the cell culture formulation comprises DMEM-F12.

4. The defined cell culture formulation of claim 1, wherein the cell culture formulation further comprises Trace Elements C, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, lithium chloride, glucose, Defined Lipids, and L-alanyl-L-glutamine dipeptide.

5. The defined cell culture formulation of claim 4, wherein the cell culture formulation comprises MCDB-131.

6. The defined cell culture formulation of any one of claims 1 to 5, wherein ITS-X provides the insulin, transferrin, and selenium.

7. The defined cell culture formulation of any one of claims 1 to 6, wherein the fatty acid free albumin is reagent grade.

8. The defined cell culture formulation of any one of claims 1 to 7, wherein the TGF-β ligand is TGF-β1.

9. A defined cell culture formulation consisting essentially of DMEM-F12 basal medium, insulin, transferrin, selenium, fatty-acid free albumin, a TGF-β ligand, bFGF, and IGF-1.

10. A defined cell culture formulation consisting essentially of DMEM-F12 basal medium, insulin, transferrin, selenium, fatty-acid free albumin, a TGF-β ligand, bFGF, IGF-1, and ascorbic acid.

11. A defined cell culture formulation consisting essentially of MCDB-131, Trace Elements C, ascorbic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, lithium chloride, glucose, defined lipids, insulin, transferrin, selenium, fatty acid free albumin, a TGF-β ligand, bFGF, and L-alanyl-L-glutamine dipeptide.

12. A method for the expansion of human pluripotent stem cells, wherein the method comprises culturing the human pluripotent stem cells on a feeder-free matrix in a defined cell culture formulation; wherein the defined cell culture formulation comprises basal medium, insulin, transferrin, selenium, fatty-acid free albumin, a TGF-β ligand, bFGF, and ascorbic acid; and wherein culturing the stem cells in the defined cell culture formulation maintains the pluripotency and karyotypic stability of the cells for at least 10 passages.

13. The method of claim 12, wherein the defined cell culture formulation further comprises insulin growth factor 1 (IGF-1).

14. The method of claim 12 or 13, wherein the cell culture formulation comprises DMEM-F12.

15. The method of claim 12, wherein the defined cell culture formulation further comprises Trace Elements C, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, lithium chloride, glucose, Defined Lipids, and L-alanyl-L-glutamine dipeptide.

16. The method of claim 15, wherein the defined cell culture formulation comprises MCDB-131.

17. An in vitro population of pluripotent cells cultured in DMEM/F12 medium comprising ITS-X, fatty acid-free albumin, TGF-B1, bFGF, and IGF-1, wherein at least 70% of the cells in the population are Oct4+, NANOG+, SOX2+, KI67+, FOXA2+, and ZFP42-.

FIG. 1A

IH-3

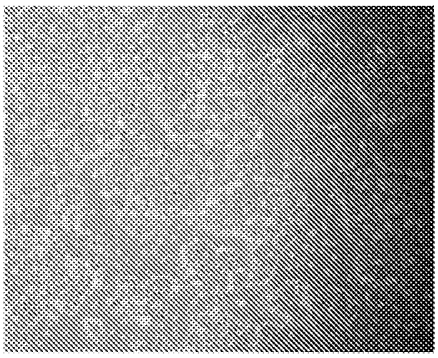


FIG. 1B

IH-1

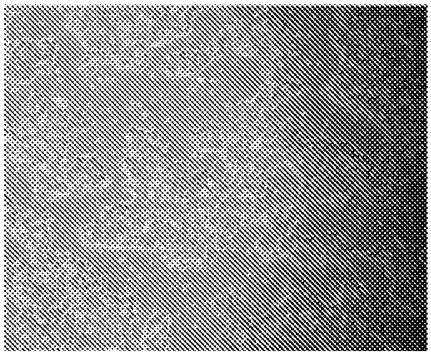


FIG. 1C

IH-6

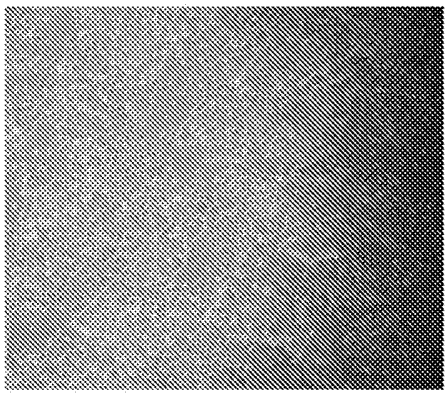


FIG. 1D

TeSR®1

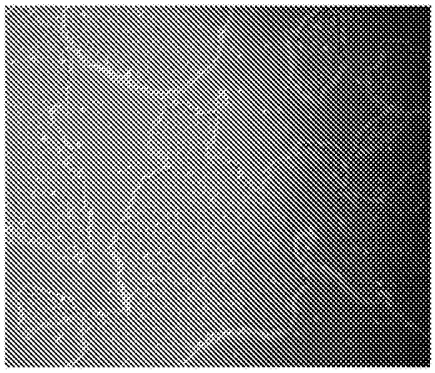


FIG. 2A

IH-3

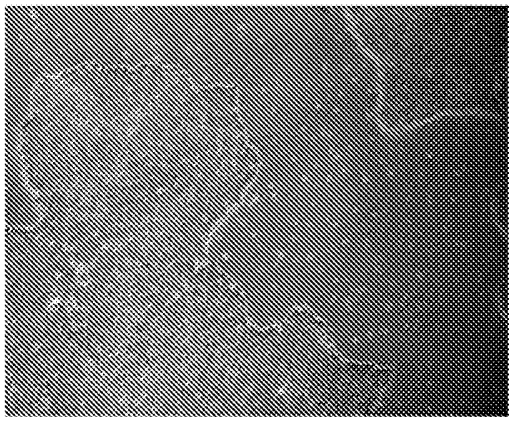


FIG. 2B

IH-3

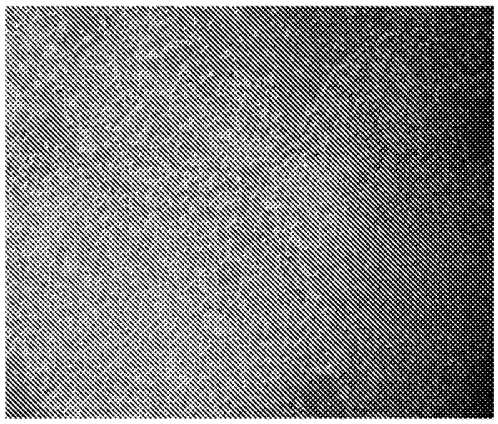


FIG. 2C

TeSR®1

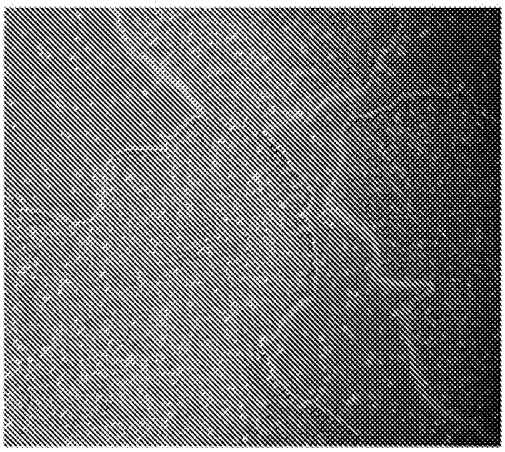


FIG. 3A

IH-3

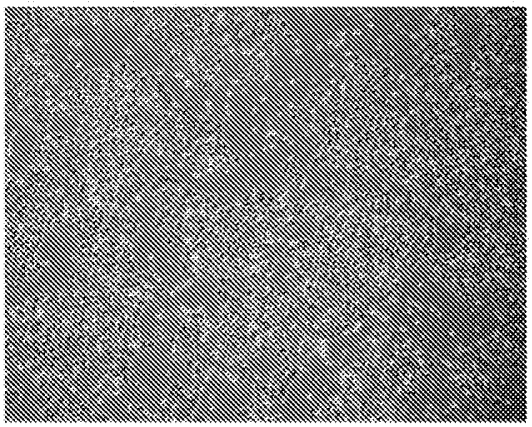


FIG. 3B

IH-1

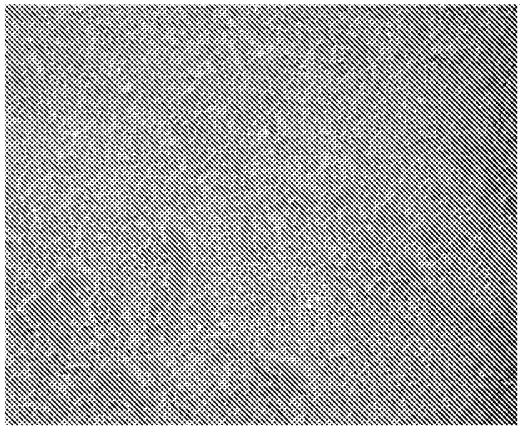


FIG. 3C

TeSR®1

FIG. 4A

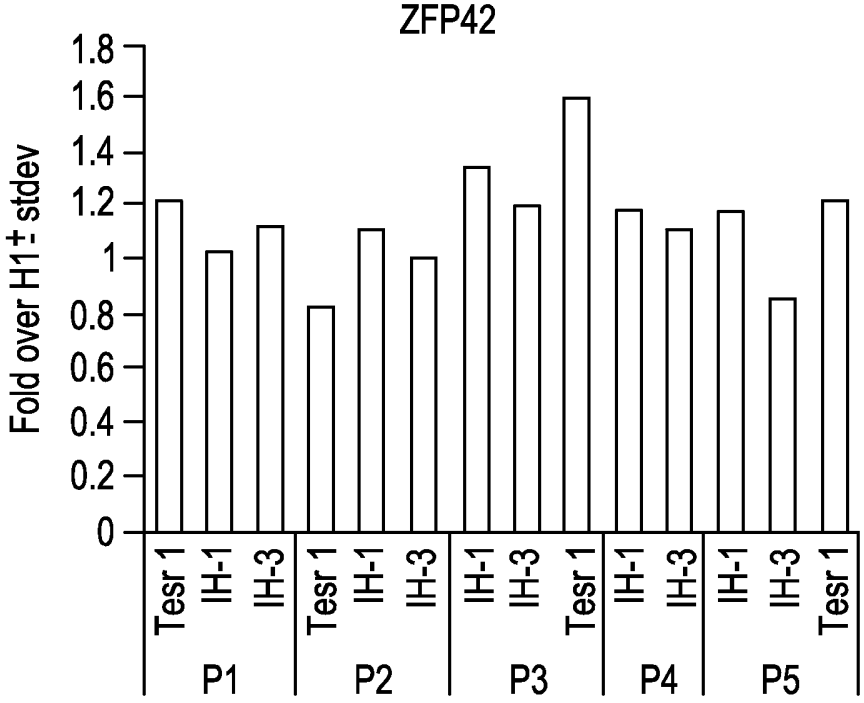

ZFP42
Fold over H1 ± stdev
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Tesr 1
IH-1
IH-3
P1
P2
P3
P4
P5

FIG. 4B

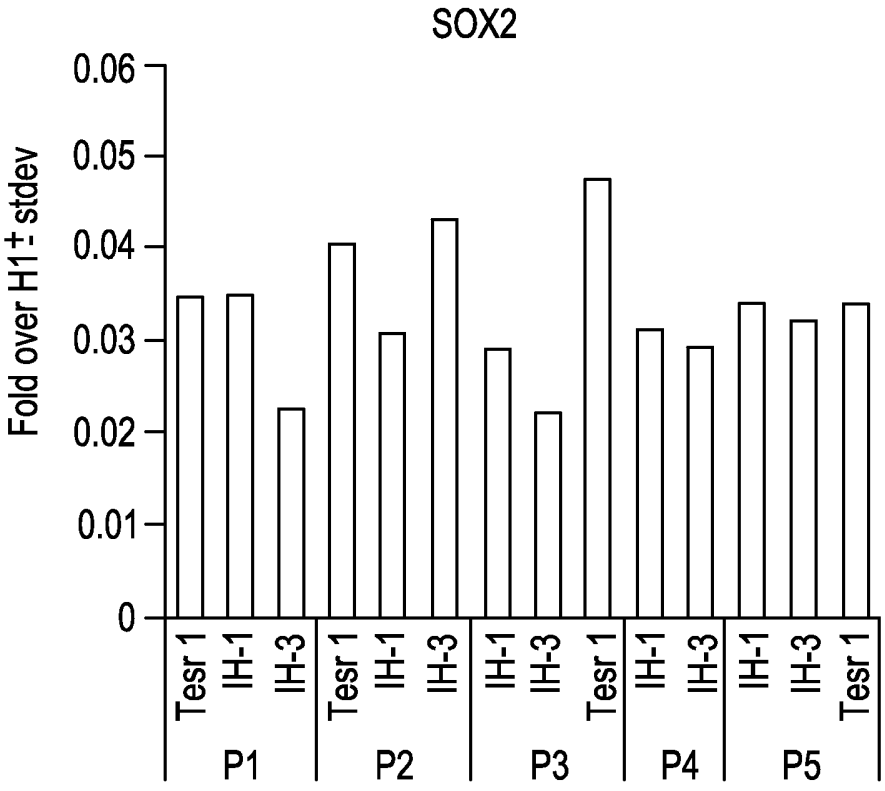

SOX2
Fold over H1 ± stdev
0.06
0.05
0.04
0.03
0.02
0.01
0
Tesr 1
IH-1
IH-3
P1
P2
P3
P4
P5

FIG. 4C

POU5F1

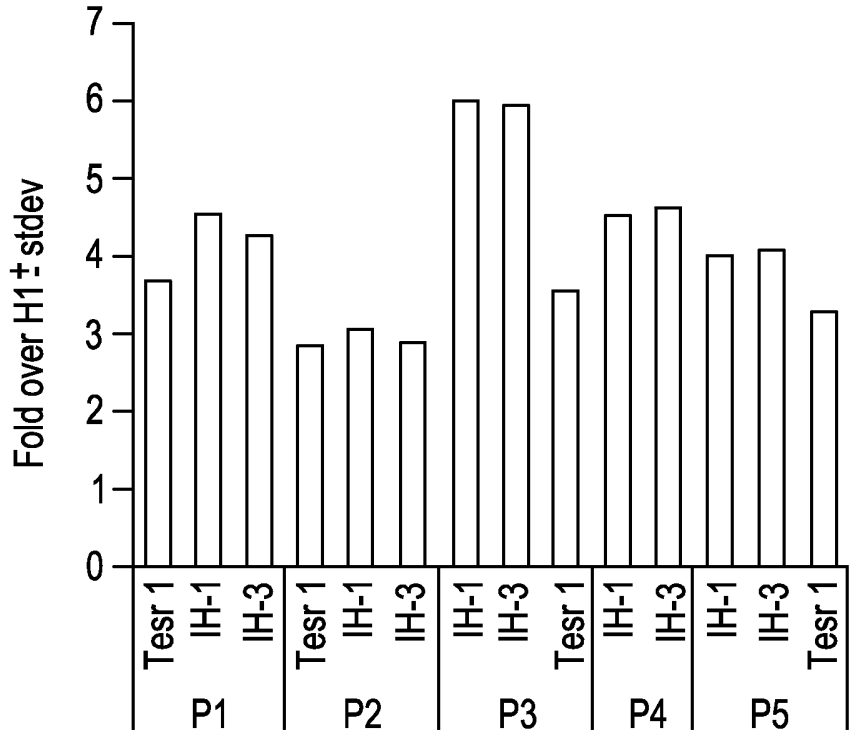


FIG. 4D

NANOG

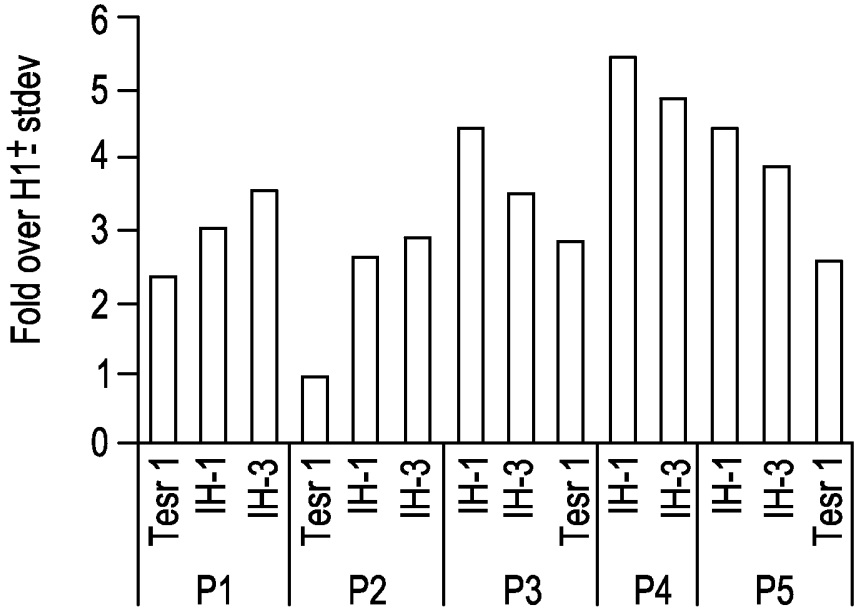


FIG. 4E

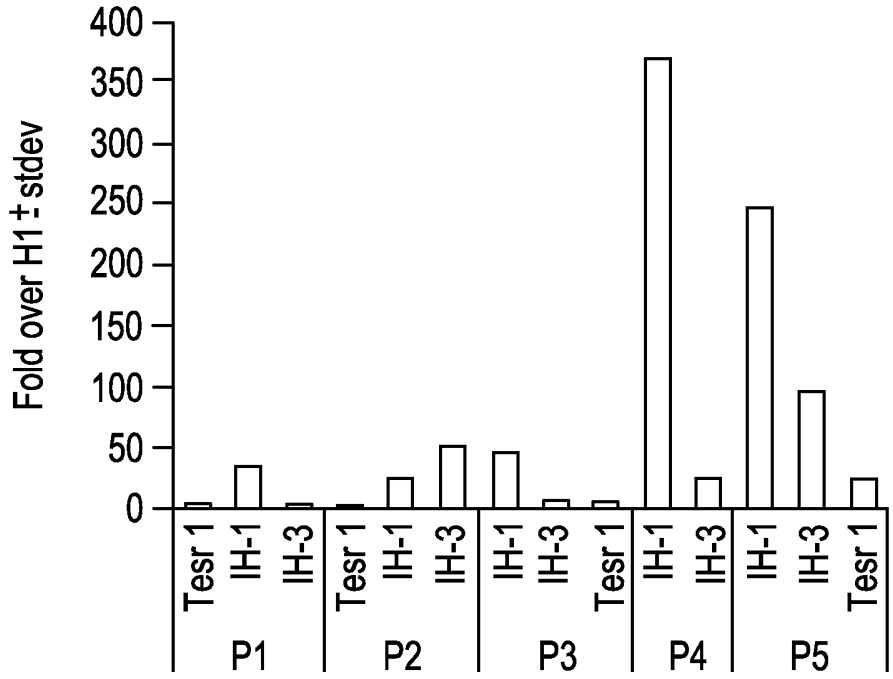

FOXA2
Fold over H1 ± stdev
400
350
300
250
200
150
100
50
0
Tesr 1
IH-1
IH-3
P1
P2
P3
P4
P5

FIG. 4F

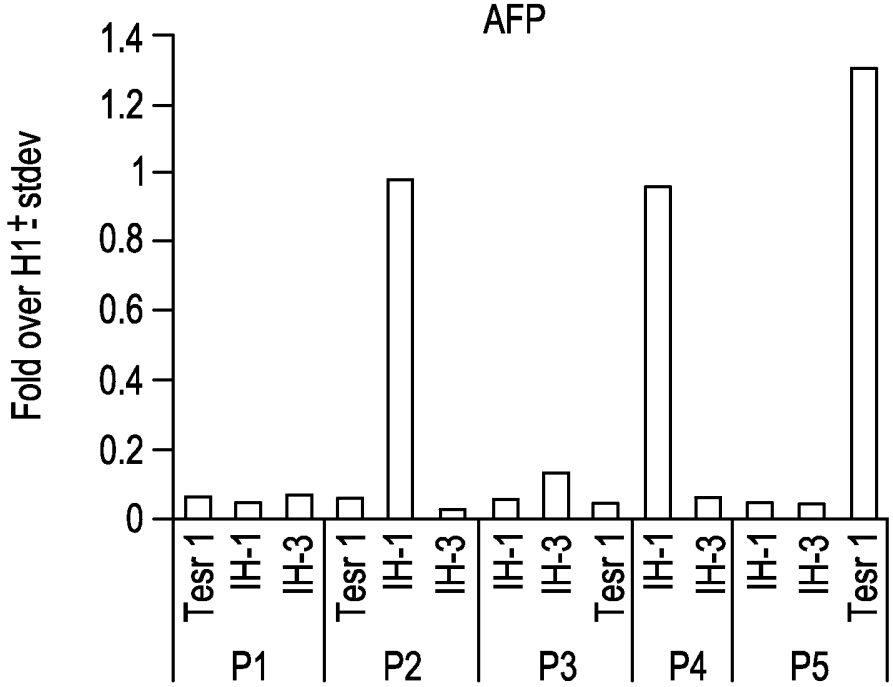

AFP
Fold over H1 ± stdev
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Tesr 1
IH-1
IH-3
P1
P2
P3
P4
P5

FIG. 5A

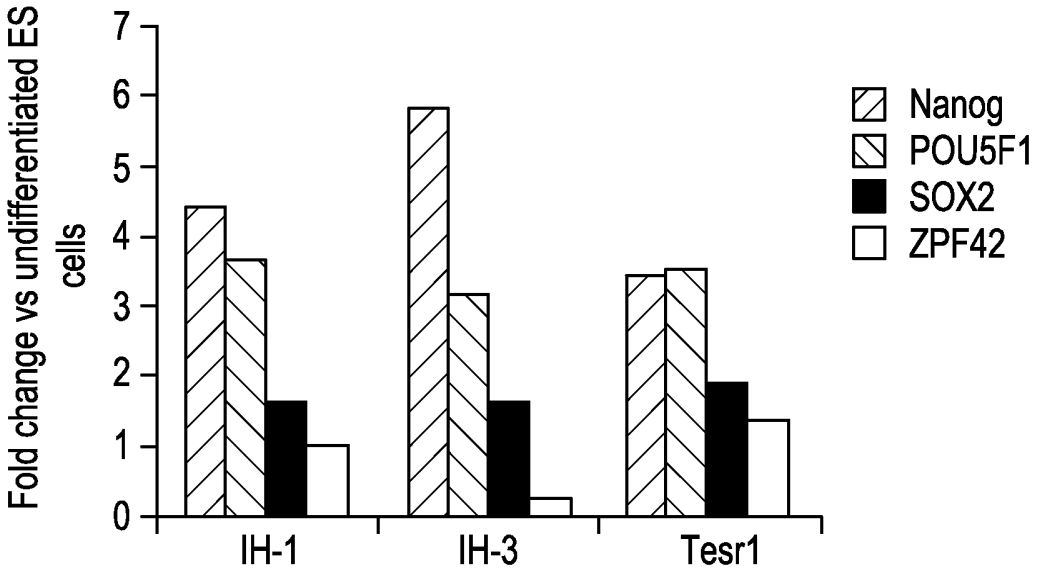

Fold change vs undifferentiated ES cells
0
1
2
3
4
5
6
7
IH-1
IH-3
Tesr1
Nanog
POU5F1
SOX2
ZPF42

FIG. 5B

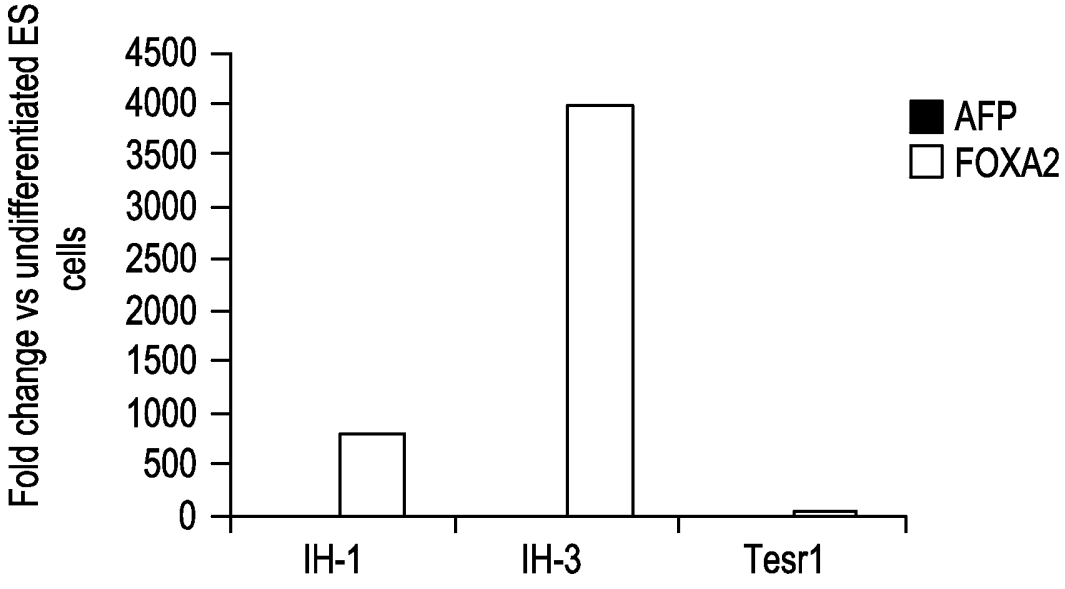

Fold change vs undifferentiated ES cells
0
500
1000
1500
2000
2500
3000
3500
4000
4500
IH-1
IH-3
Tesr1
AFP
FOXA2

FIG. 6A

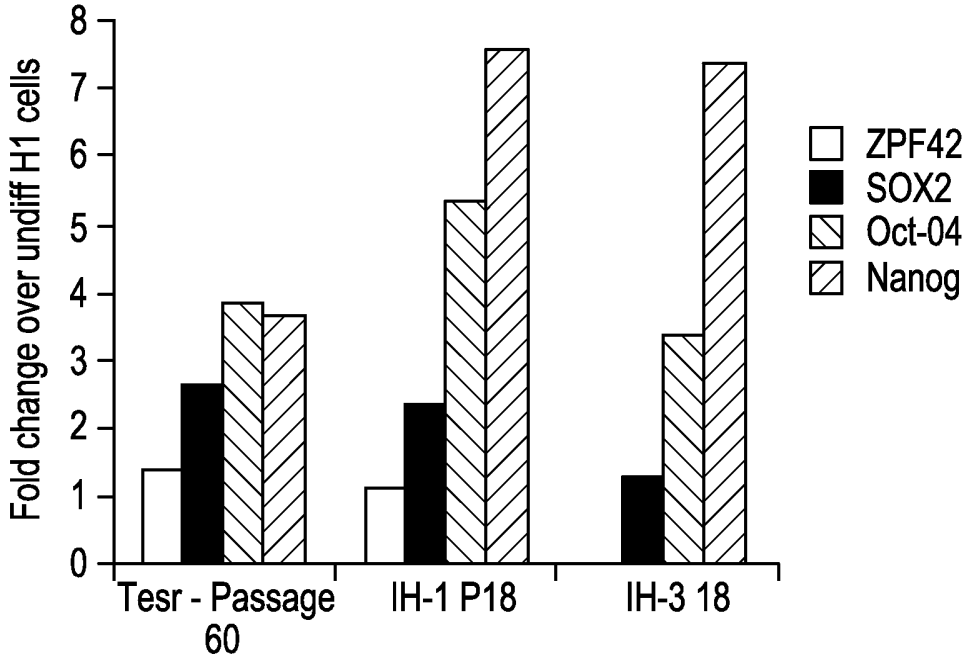

Fold change over undiff H1 cells
0
1
2
3
4
5
6
7
8
Tesr - Passage 60
IH-1 P18
IH-3 18
ZPF42
SOX2
Oct-04
Nanog

FIG. 6B

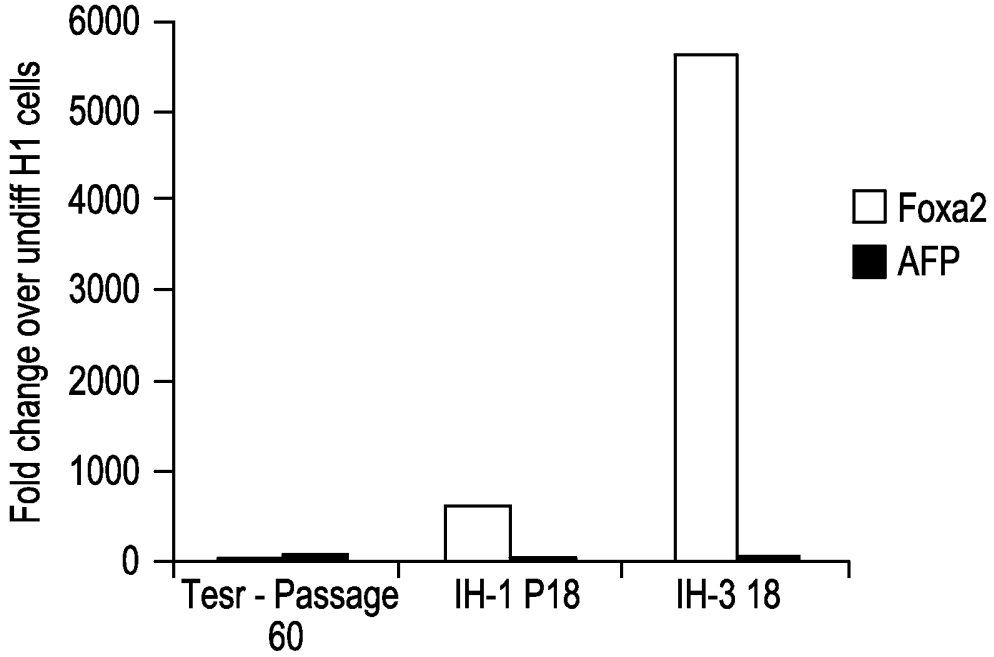

Fold change over undiff H1 cells
0
1000
2000
3000
4000
5000
6000
Tesr - Passage 60
IH-1 P18
IH-3 18
Foxa2
AFP

FIG. 7A

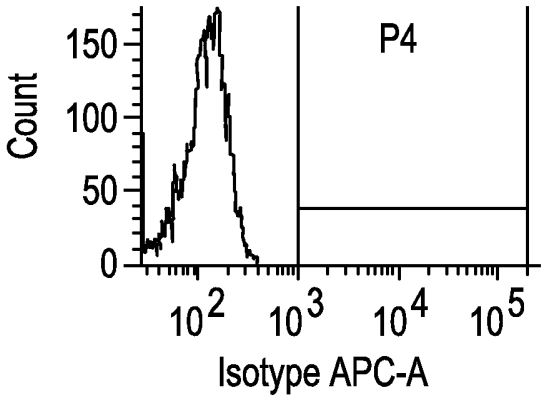


FIG. 7B

KI67

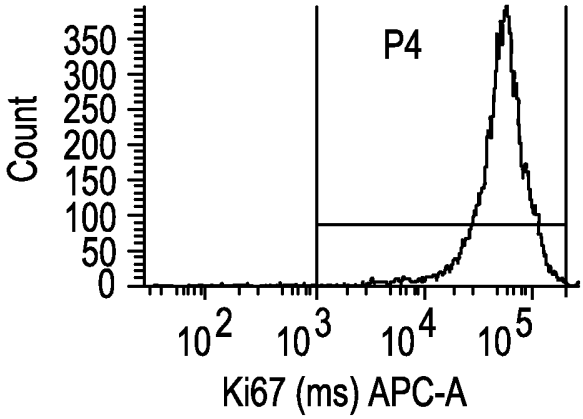


FIG. 7C

OCT4

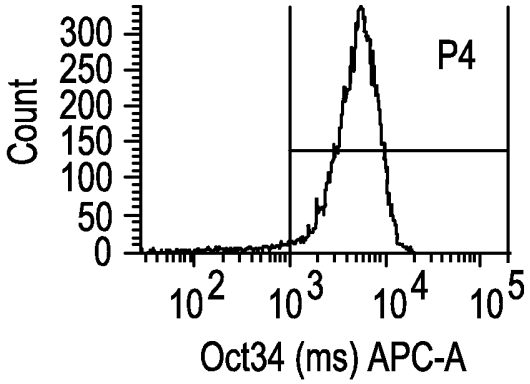


FIG. 7D

SOX17

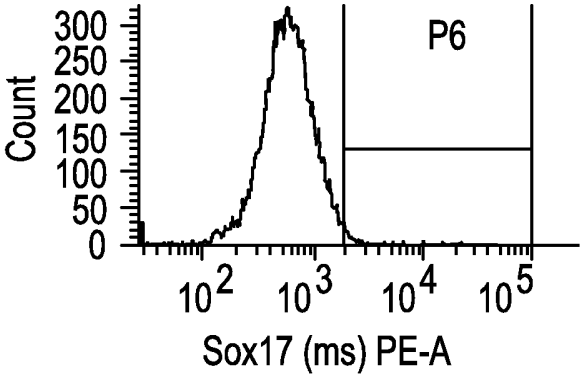


FIG. 7E

FOXA2

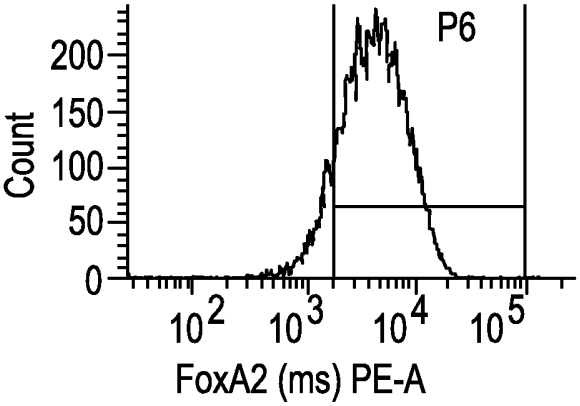


FIG. 7F

SOX2

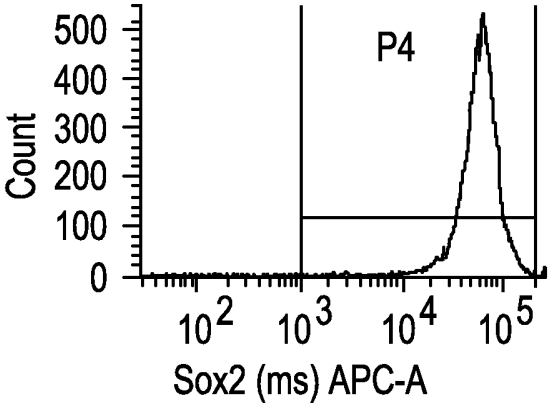


FIG. 8A

OCT4

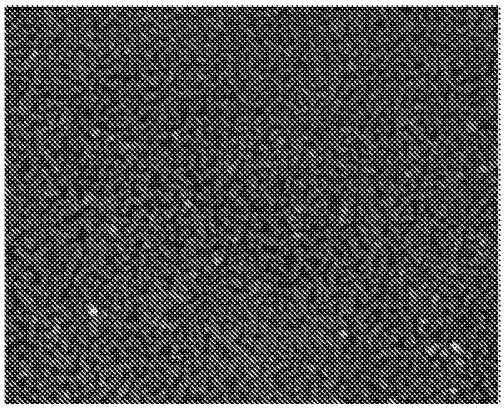


FIG. 8B

FOXA2

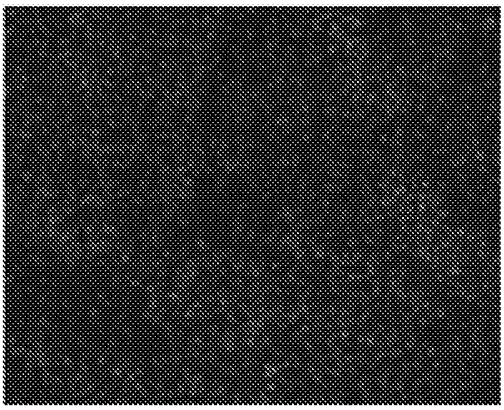


FIG. 8C

DAPI

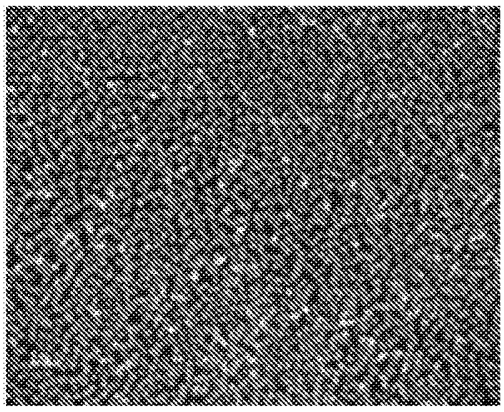


FIG.8D

SOX2

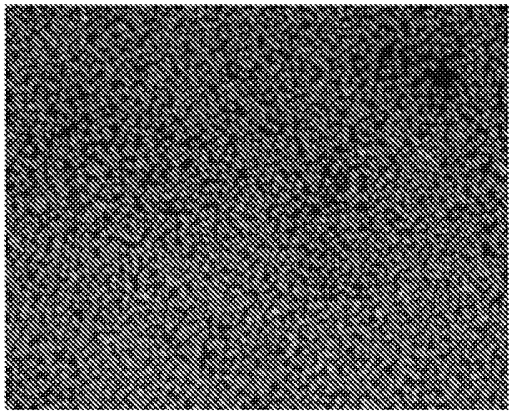


FIG. 8E

FOXA2

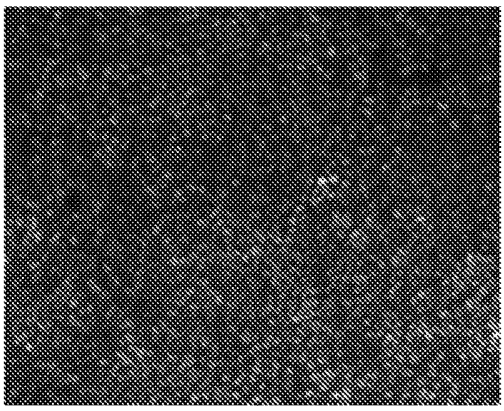


FIG. 8F

DAPI

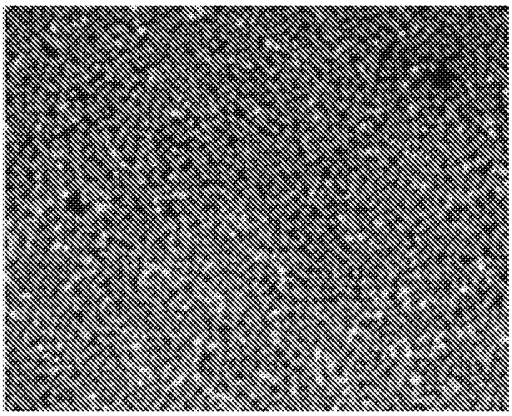


FIG. 9A

Tesr1

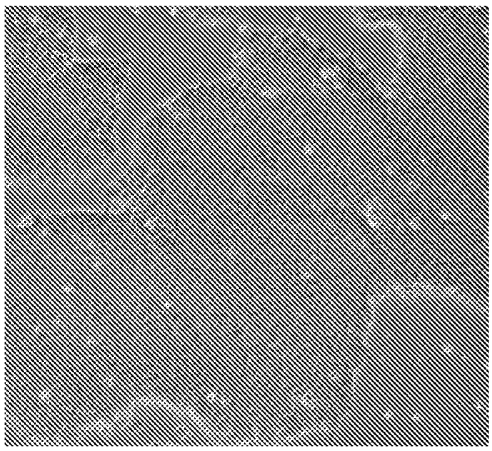


FIG. 9B

IH-3

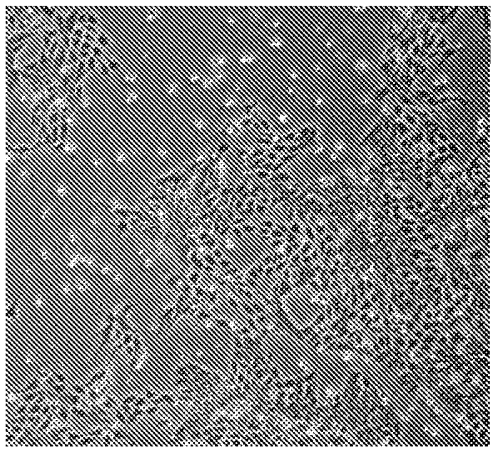


FIG. 9C

IH-3-1

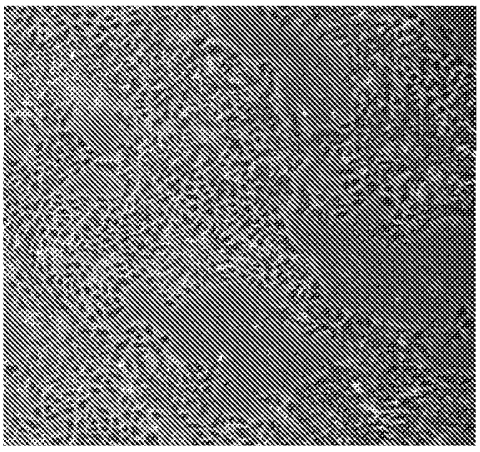


FIG. 9D

IH-3-2

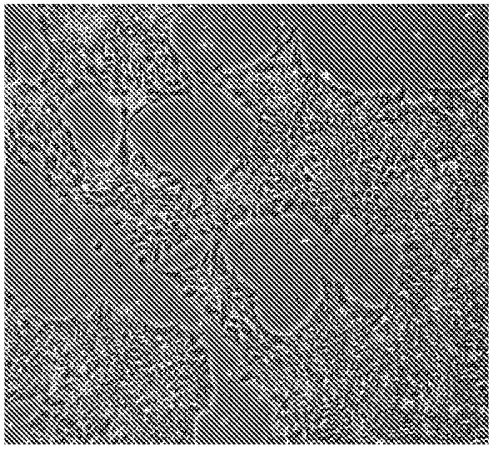


FIG. 9E

IH-3-3

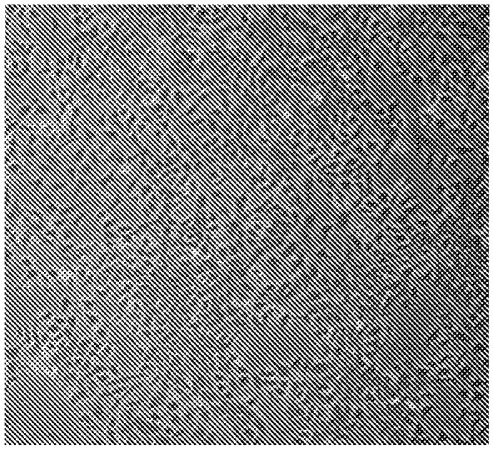


FIG. 9F

IH-3-4

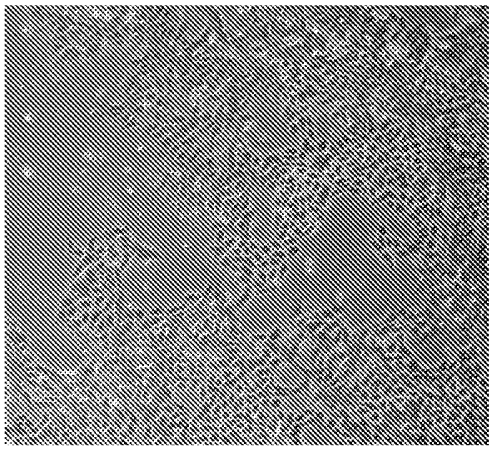


FIG.10A

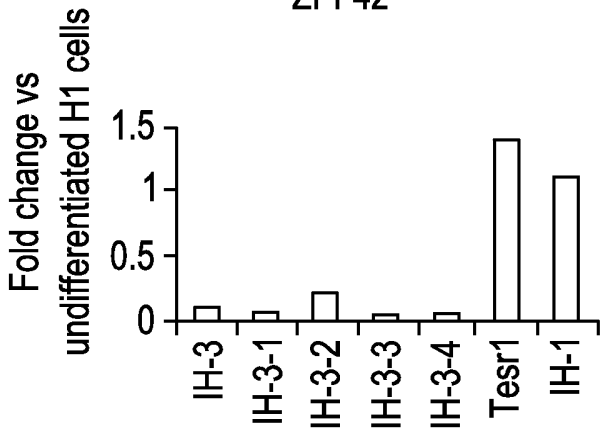


FIG.10B

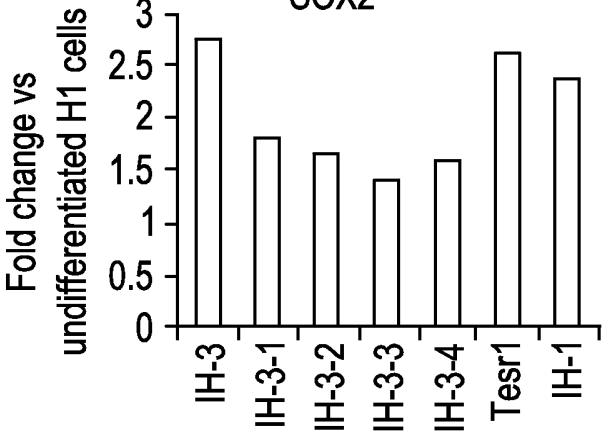


FIG.10C

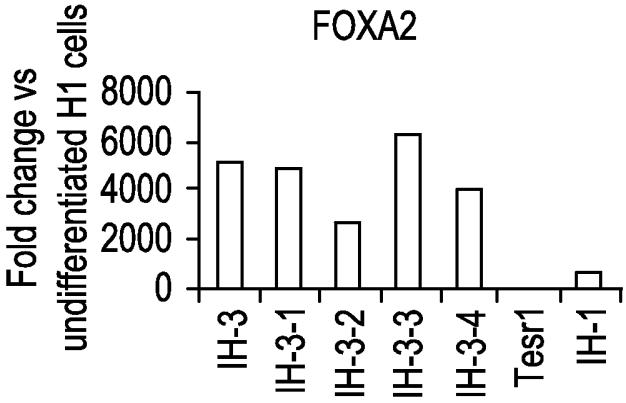


FIG.10D

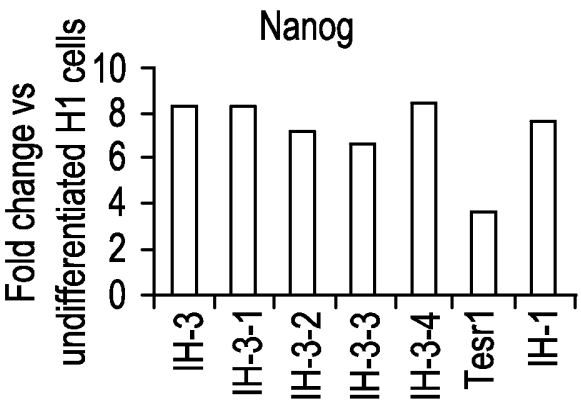


FIG.10E

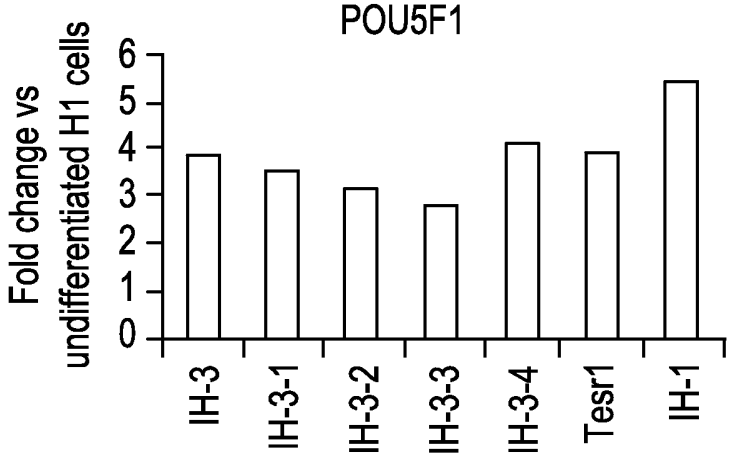


FIG. 11A

Tesr1

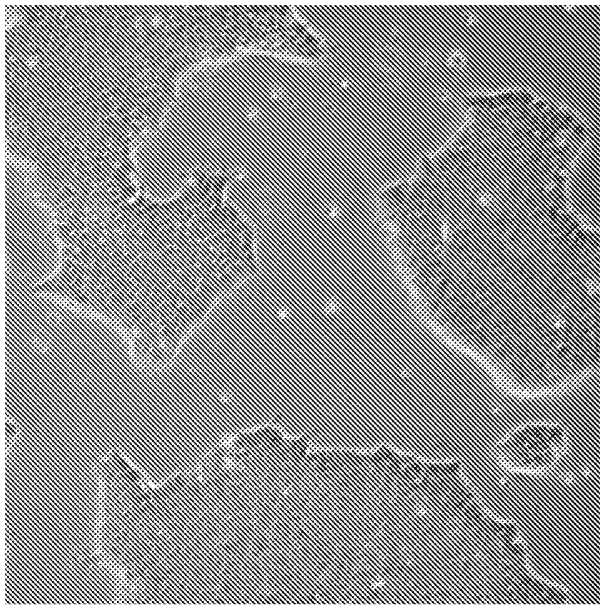


FIG. 11B

IH-3

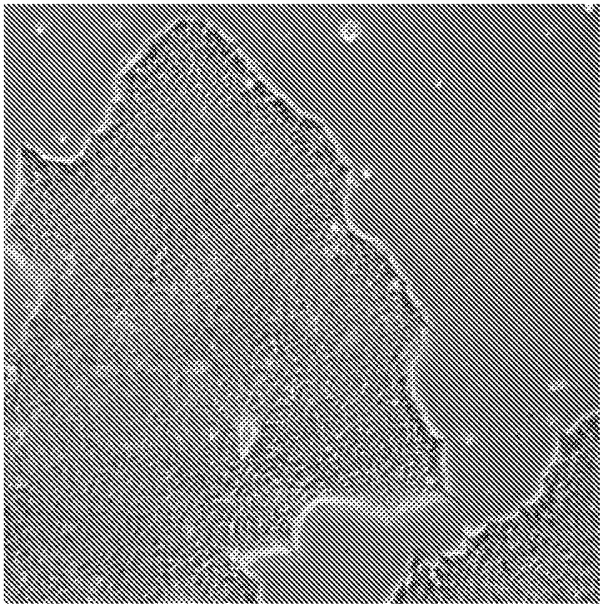


FIG. 11C

IH-1

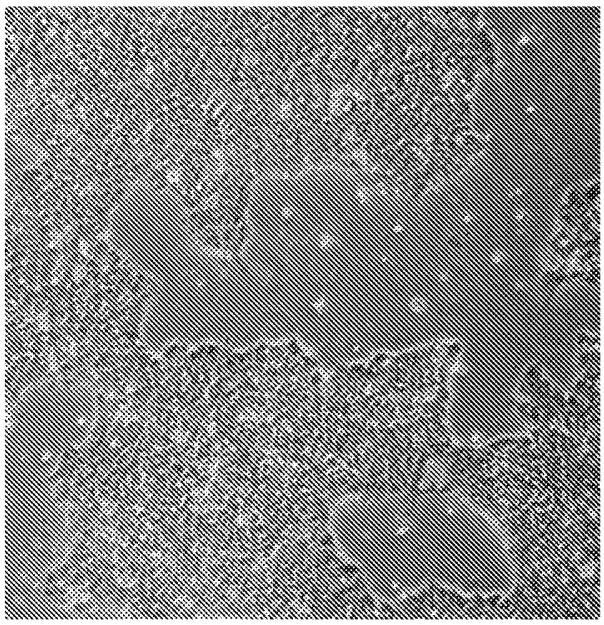


FIG. 11D

IH-3RT

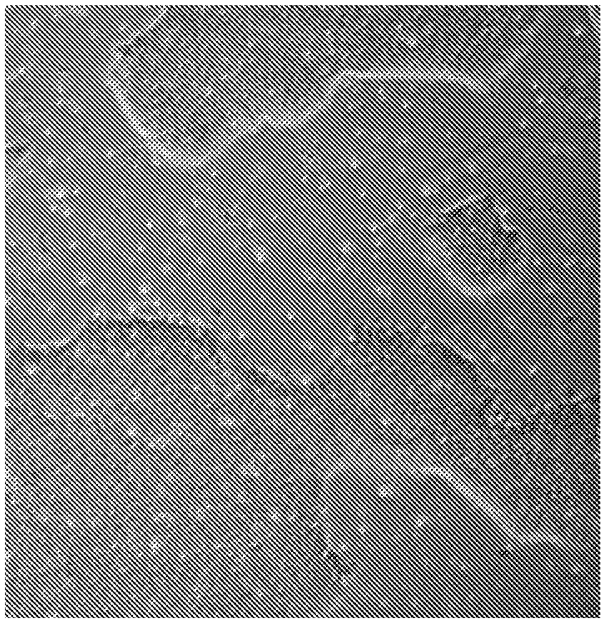


FIG. 12A

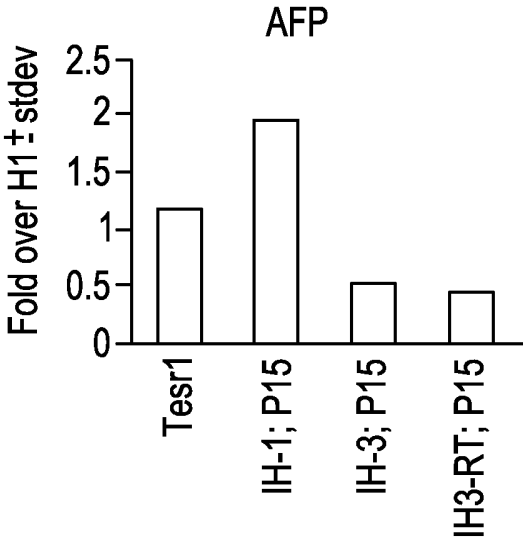


FIG. 12B

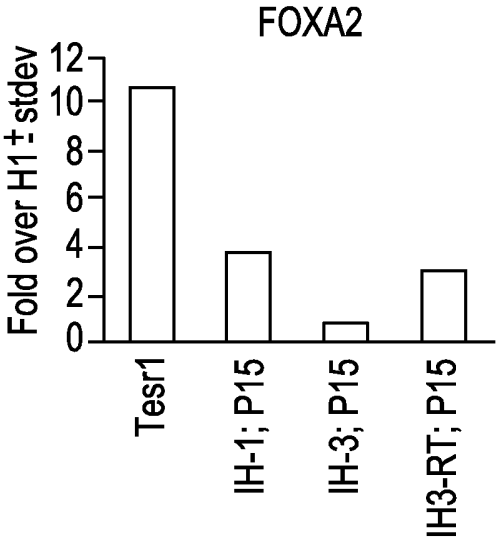


FIG. 12C

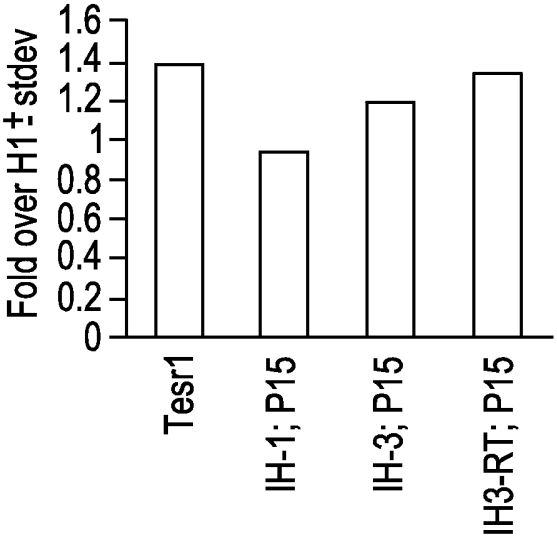


FIG. 12D

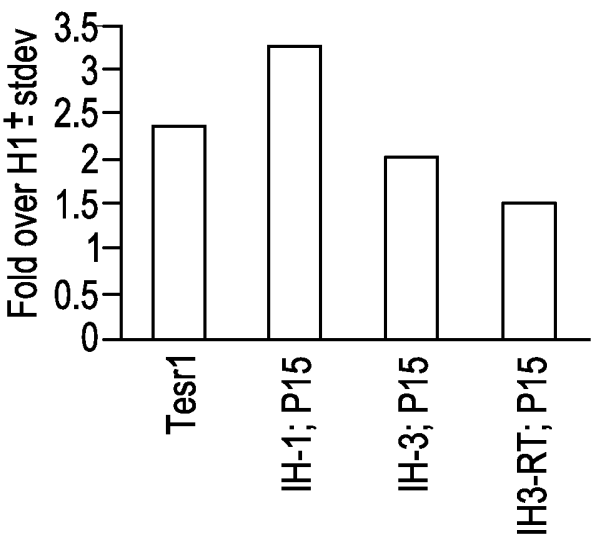


FIG. 12E

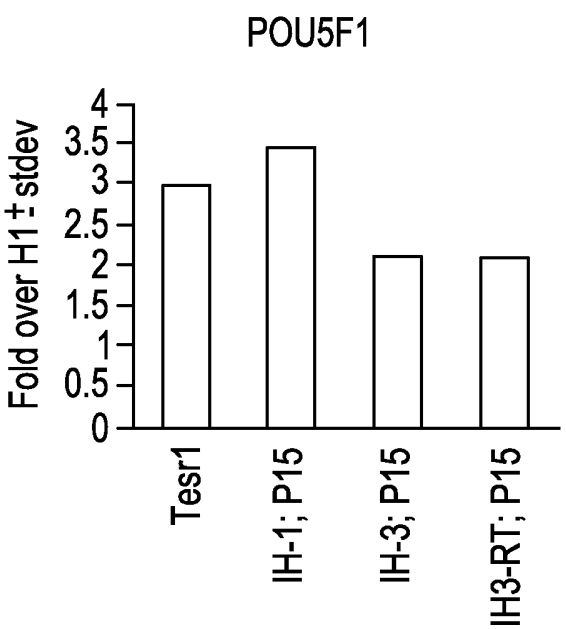


FIG. 12F

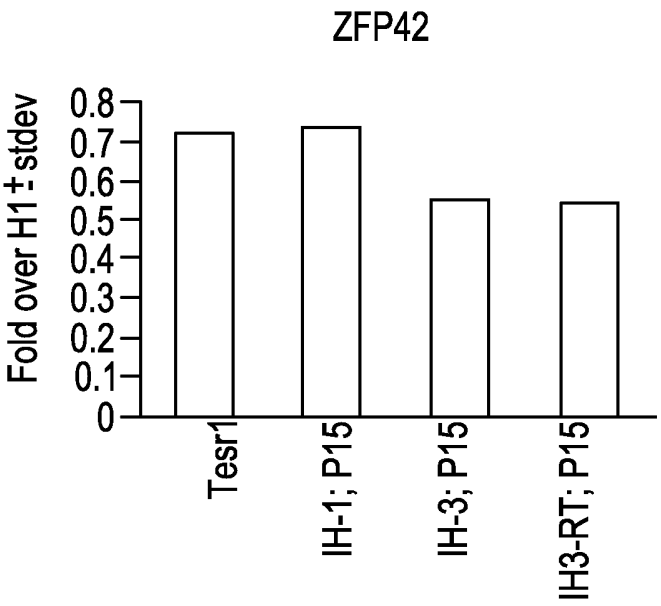


FIG. 13A

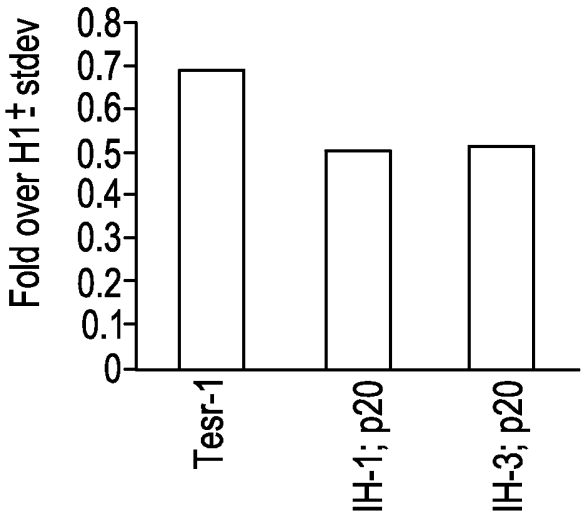


FIG. 13B

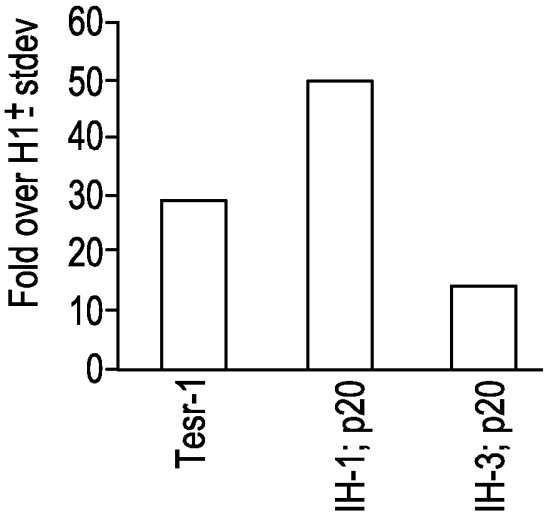


FIG. 13C

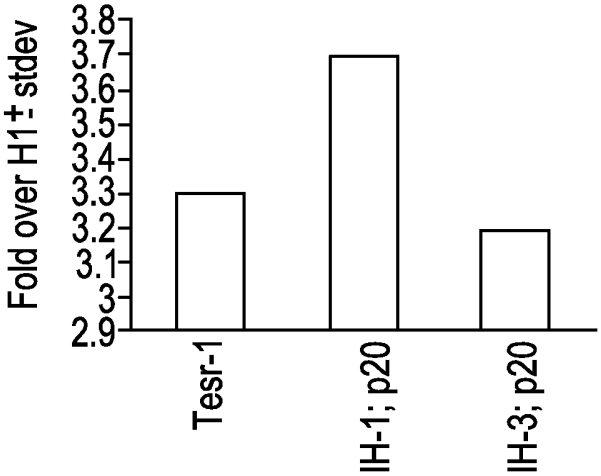


FIG. 13D

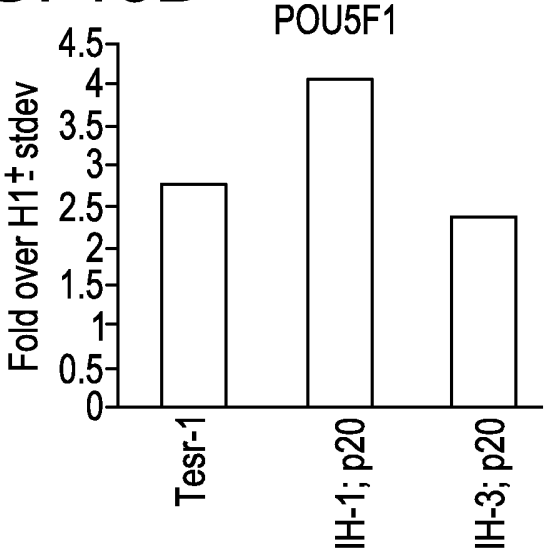


FIG. 13E

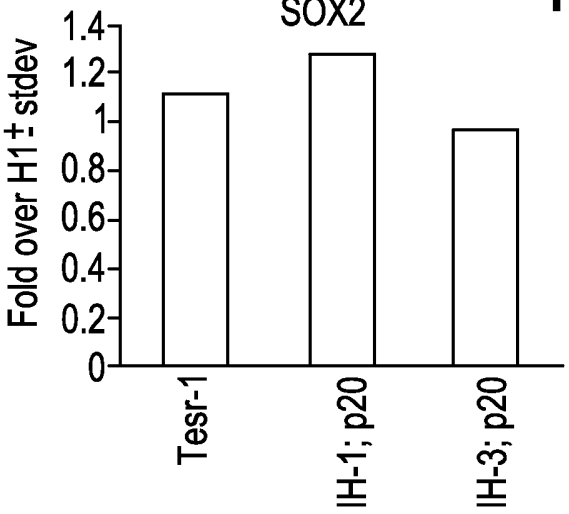


FIG. 13F

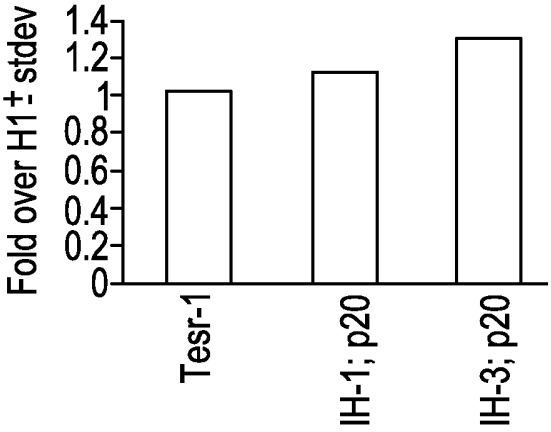


FIG. 14A

Sigma BSA – P0

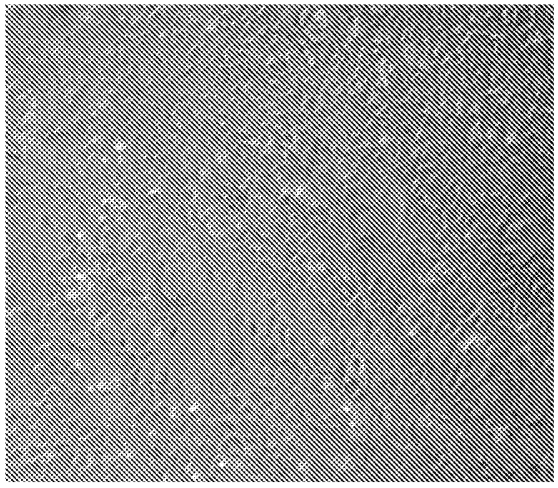


FIG. 14B

Fatty-acid free BSA – P0

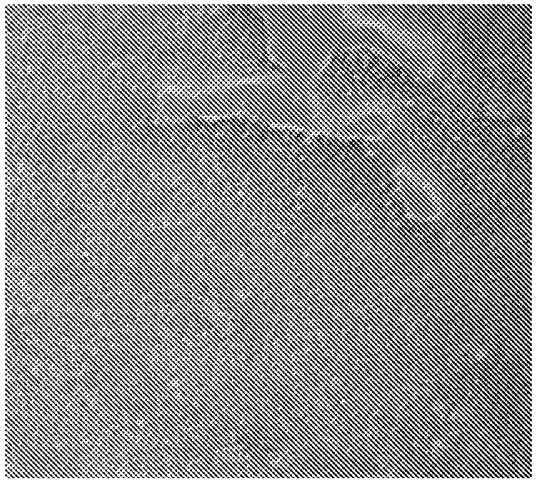


FIG. 15A

Sigma BSA – P3

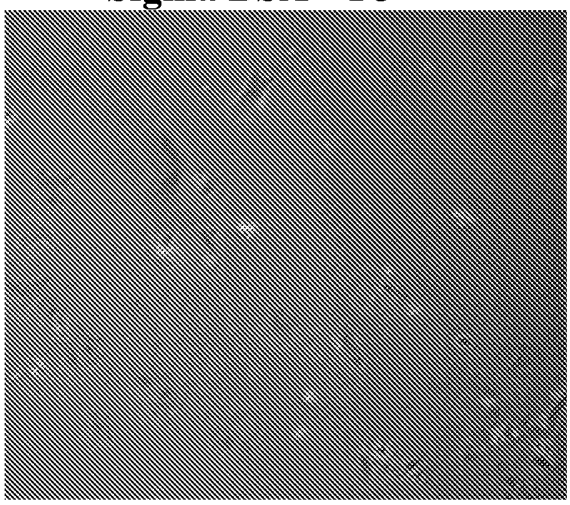


FIG. 15B

Fatty-acid free BSA – P3

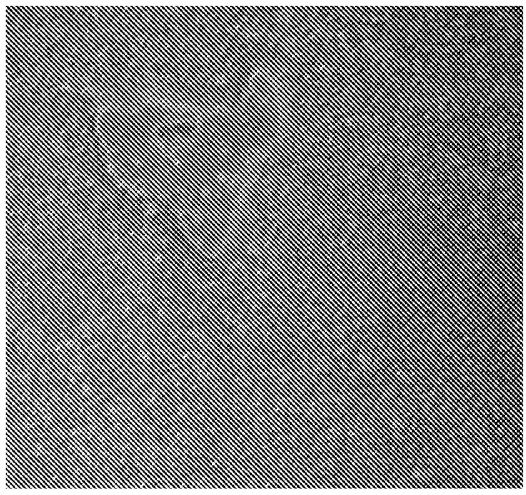


FIG. 16A

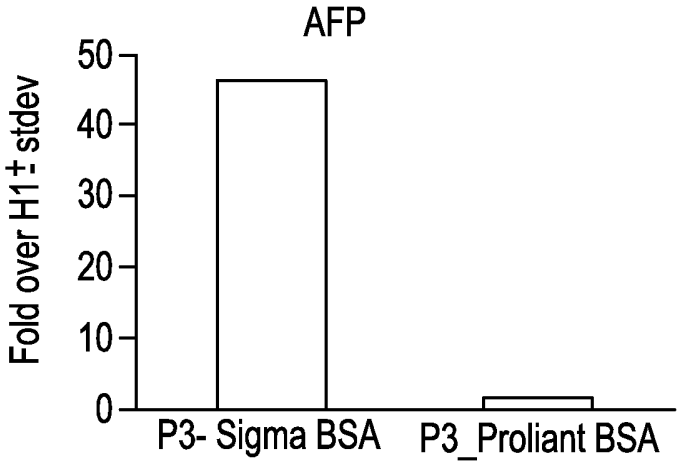


FIG. 16B

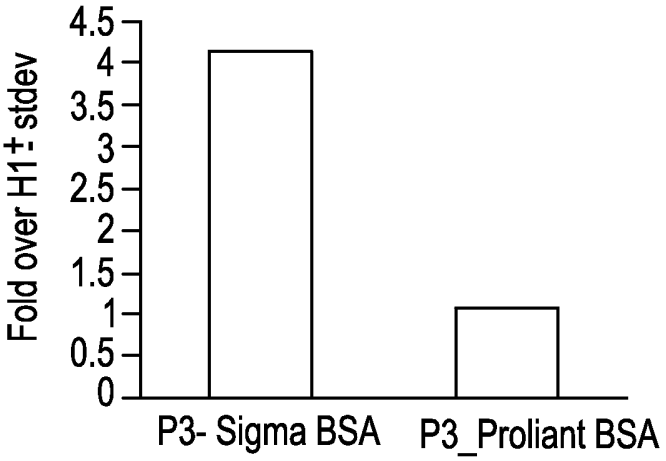


FIG. 16C

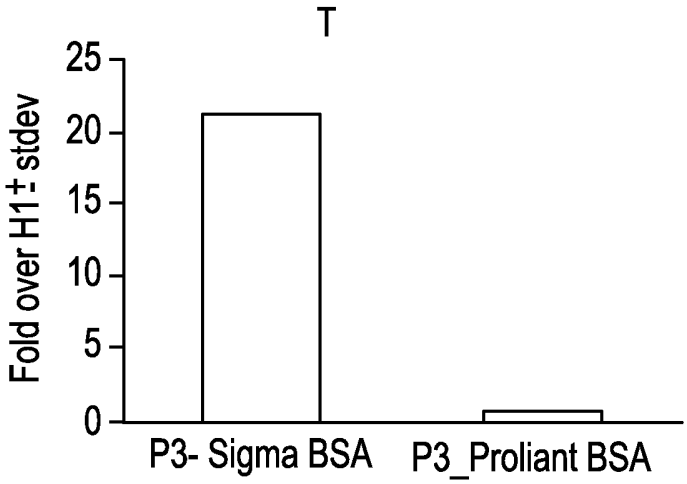


FIG. 17A

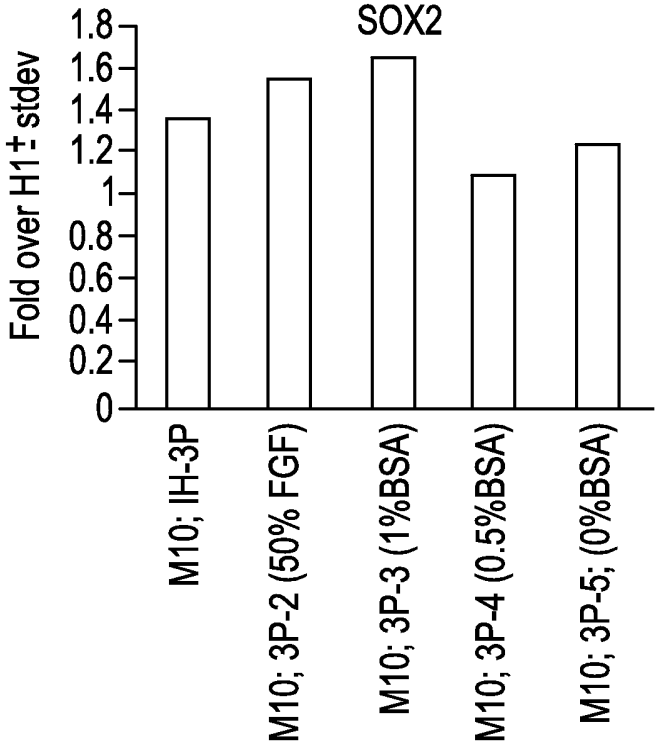


FIG. 17B

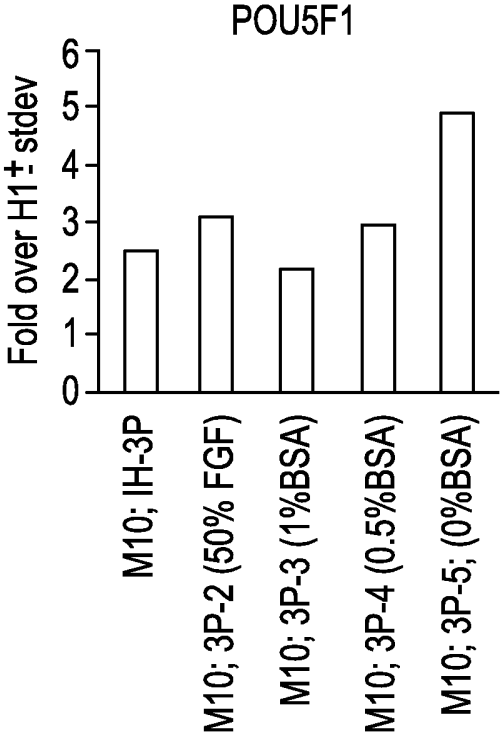


FIG. 17C

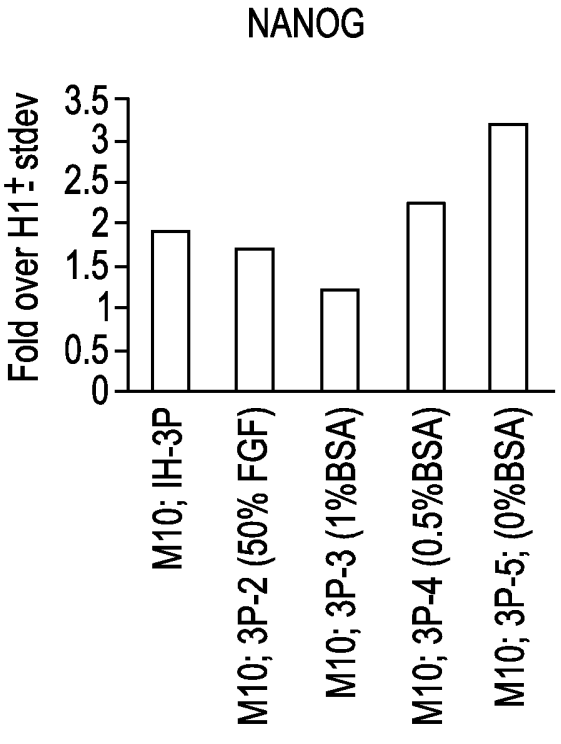


FIG. 17D

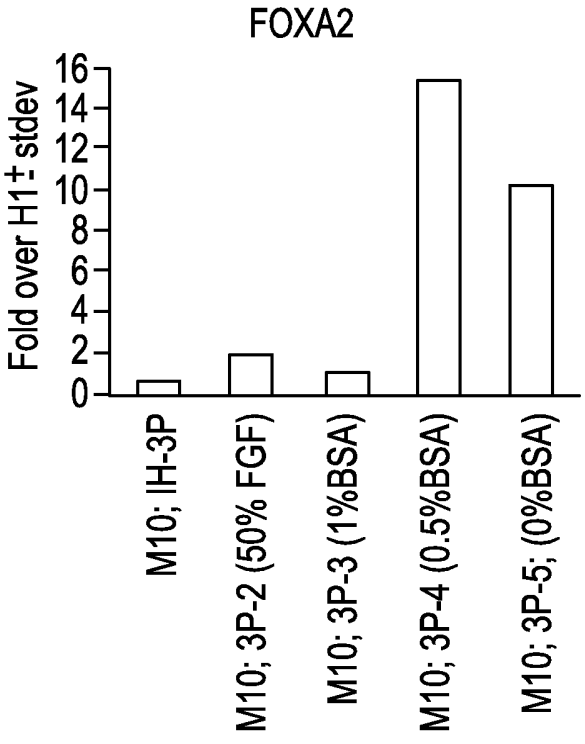


FIG. 18A

IH-3

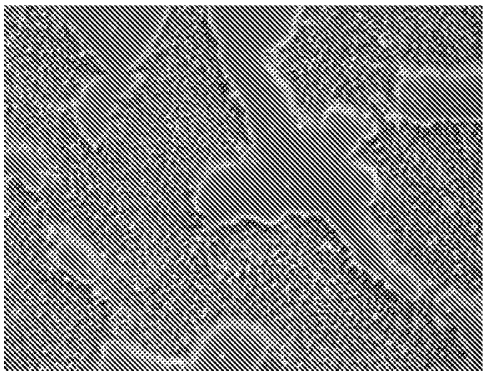


FIG. 18B

IH-3P-2

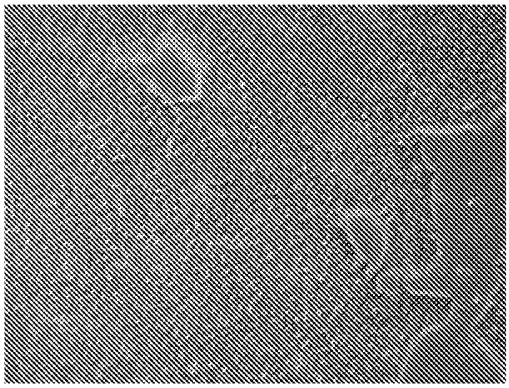


FIG. 18C

IH-3P-3

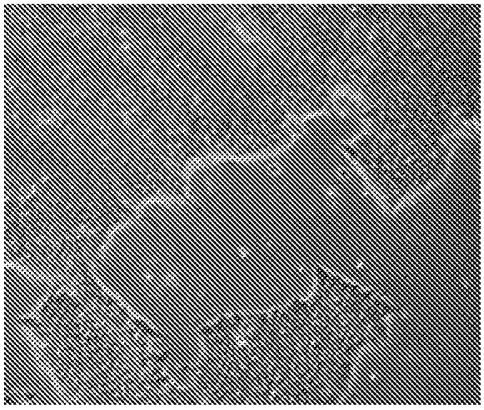


FIG. 18D

IH-3P-4

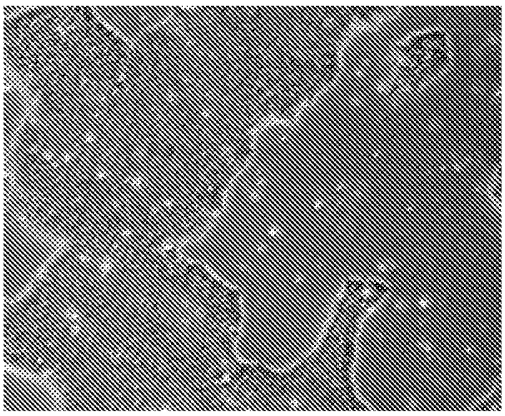
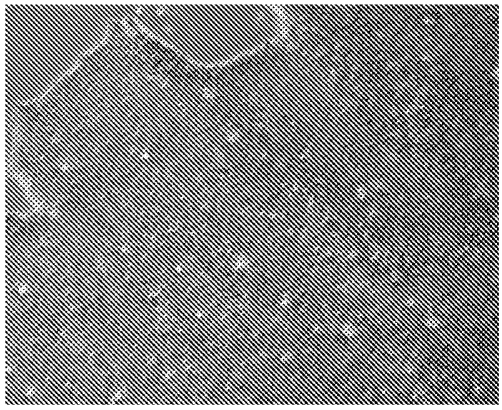


FIG. 18E

IH-3P-5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/029360

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/0735(2010.01)i, C12N 5/02(2006.01)i

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 5/0735; C12Q 1/02; C12N 5/00; C12N 5/06; A61K 35/30; C12N 5/08; C12N 5/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: culture, pluripotent stem cell, DMEM-F12, MCDB-131, etc.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2012-019122 A2 (WISCONSIN ALUMNI RESEARCH FOUNDATION) 9 February 2012 See abstract; paragraphs [0006], [00010], [00040], [00075]-[00078], and [00088]; claims 1 and 2.	1-5,9-17
A	US 2009-0269845 A1 (REZANIA, ALIREZA) 29 October 2009 See abstract; paragraphs [0023]-[0031]; claims 1-21.	1-5,9-17
A	WO 2008-036447 A2 (LIFESCAN) 27 March 2008 See abstract; paragraphs [0018]-[0023].	1-5,9-17
A	US 2010-0255580 A1 (REZANIA, ALIREZA) 7 October 2010 See abstract; claims 1-11.	1-5,9-17
A	US 2011-0229441 A1 (BENCHOUA, ALEXANDRA et al.) 22 September 2011 See abstract; claims 1, 2.	1-5,9-17

☐ Further documents are listed in the continuation of Box C.

☒ 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
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"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
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2013 (26.06.2013)	**27 June 2013 (27.06.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
HEO, Joo Hyung
Telephone No. 82-42-481-8150

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/029360

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-8
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/029360

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012-019122 A2	09.02.2012	WO 2012-019122 A3	03.05.2012
US 2009-0269845 A1	29.10.2009	AU 2009-239442 A1	29.10.2009
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		JP 2010-533500 A	28.10.2010
		KR 10-2010-0042649 A	26.04.2010
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US 2011-0229441 A1	22.09.2011	EP 2356218 A1	17.08.2011
		JP 2012-510805 A	17.05.2012
		WO 2010-063848 A1	10.06.2010

摘要

本发明提供了促进成分确定的培养基中的未分化多能干细胞增殖的方法。具体地，本发明提供了用于培养、维持和扩增多能干细胞的成分确定的细胞培养制剂，其中在成分确定的细胞培养制剂中培养干细胞，使细胞的多能性和核型稳定性维持至少 10 代。还公开的是表达 OCT4、SOX2、NANOG 和 FOXA2 的在成分确定的培养基条件下生长的细胞群体。