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Chen et al.

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(54) **SIMPLIFIED BASIC MEDIA FOR HUMAN PLURIPOTENT CELL CULTURE**

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This patent is subject to a terminal disclaimer.

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C12N 5/0735	(2010.01)
C12N 5/074	(2010.01)

(52) **U.S. Cl.**

CPC ***C12N 5/0606*** (2013.01); ***C12N 5/0696*** (2013.01); *C12N 2500/25* (2013.01); *C12N 2500/38* (2013.01); *C12N 2500/92* (2013.01); *C12N 2500/98* (2013.01); *C12N 2501/115* (2013.01); *C12N 2501/15* (2013.01); *C12N 2501/39* (2013.01); *C12N 2501/727* (2013.01); *C12N 2501/999* (2013.01)

(58) **Field of Classification Search**

CPC C12N 5/0606; C12N 5/0696

See application file for complete search history.

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L5146 Sigma, Lipid Mixture (Undefined).

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(57) **ABSTRACT**

Fully defined media that support pluripotent cell viability, proliferation, cloning, and derivation, as well as methods and compositions including these media are described. Methods for deriving iPS cells from adult individuals under defined, xeno-free conditions are also described.

19 Claims, 16 Drawing Sheets

Figure 1

FIG. 1A

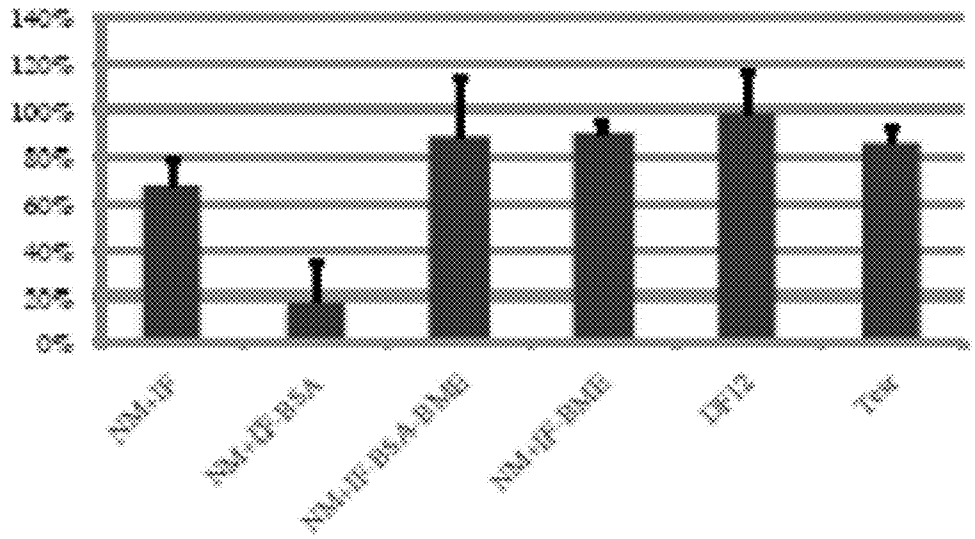


FIG. 1B

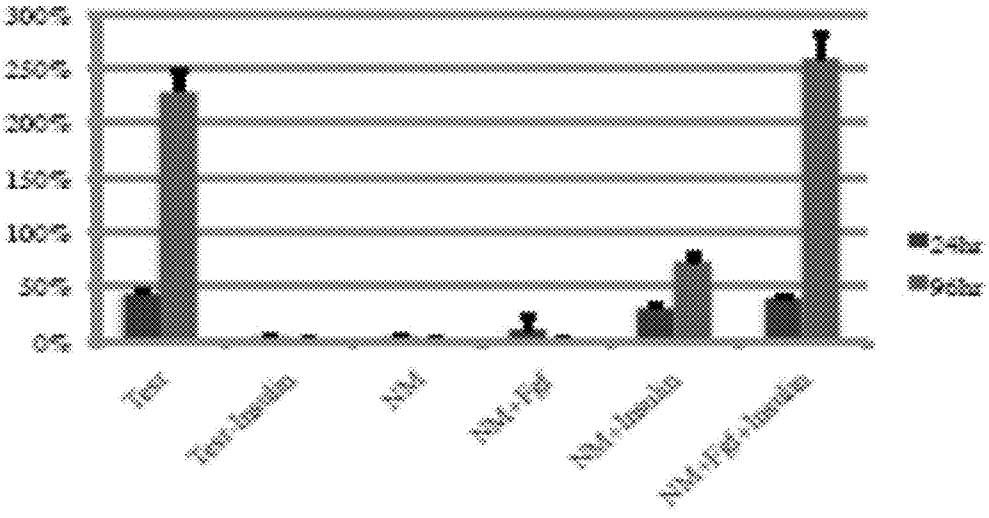


Figure 1
(cont.)

FIG. 1C

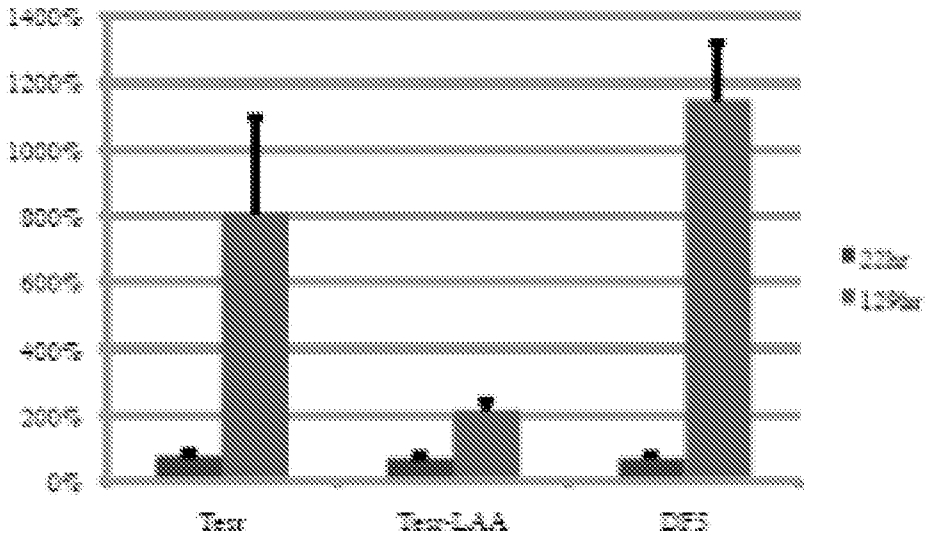


FIG. 1D

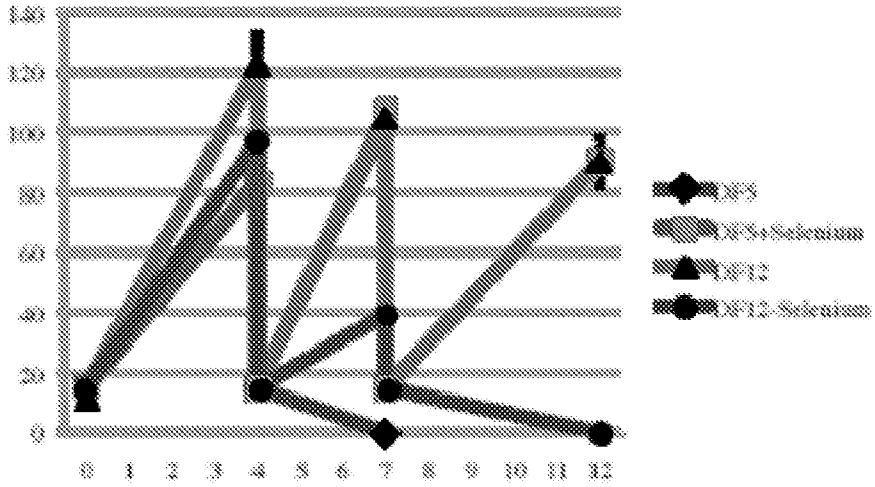


Figure 1
(cont.)

FIG. 1E

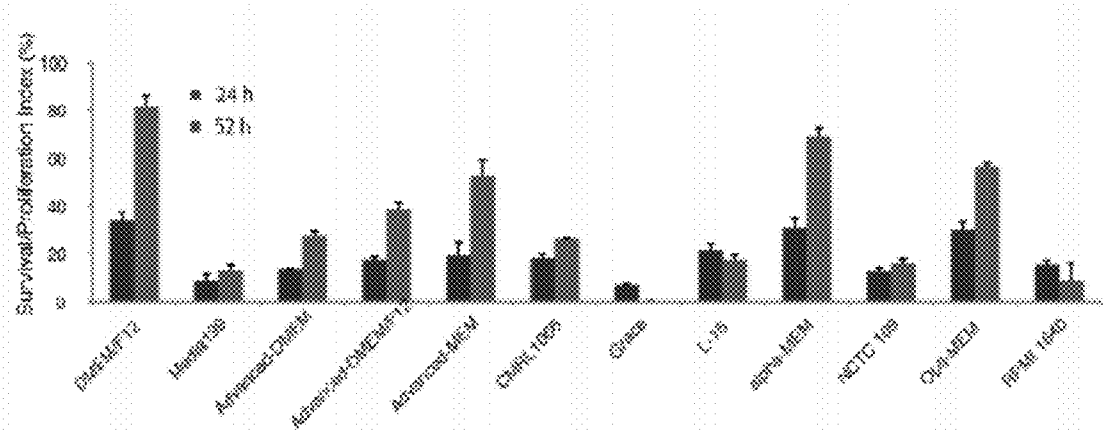
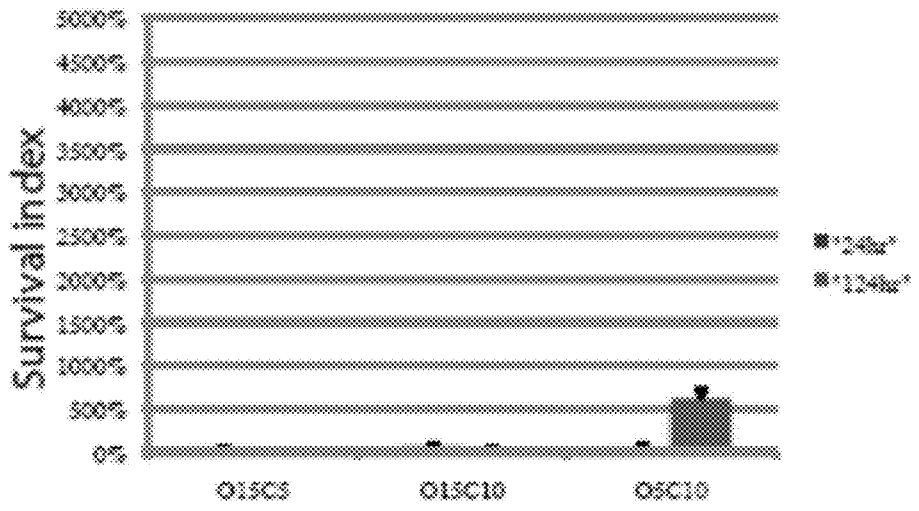


Figure 2

FIG. 2A

TeSR™



DF5S

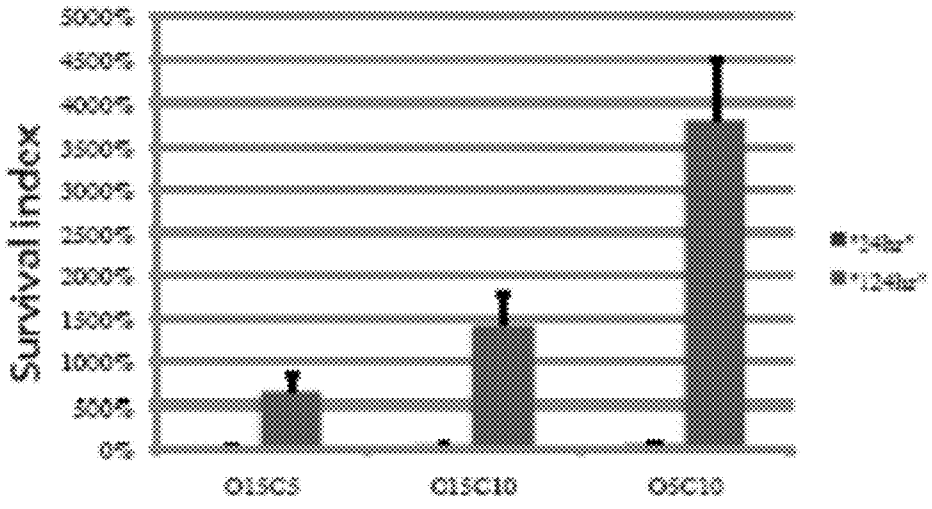


Figure 2
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FIG. 2B

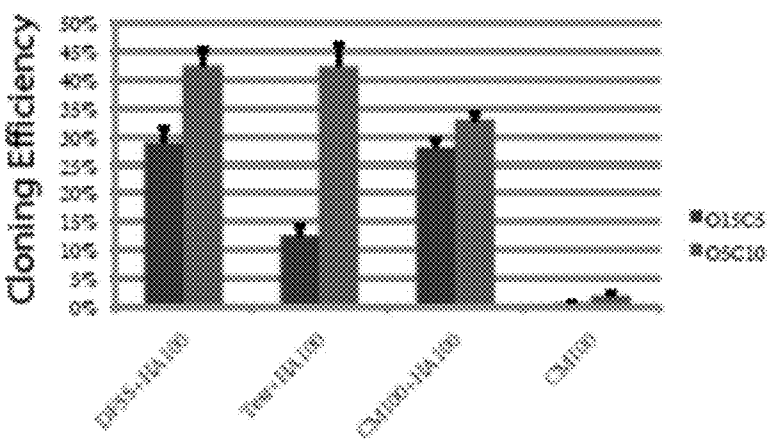

FIG. 2C

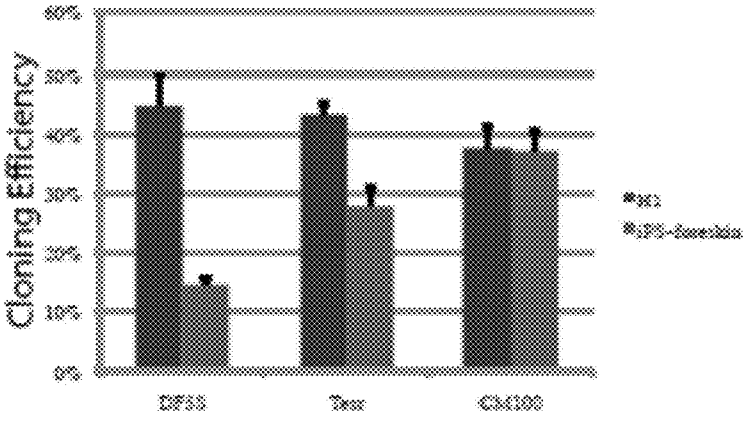

FIG. 2D

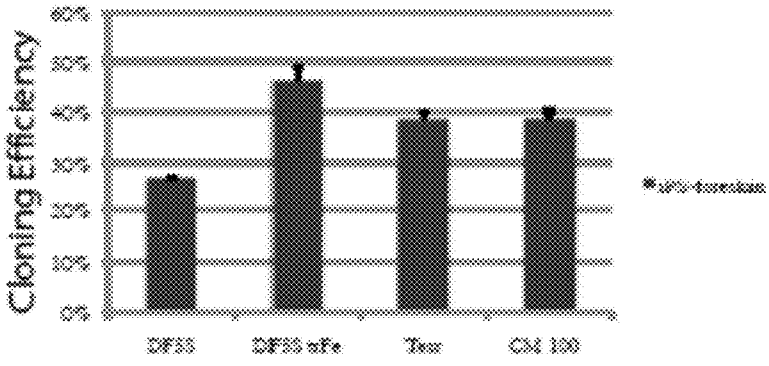

Figure 2
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FIG. 2E

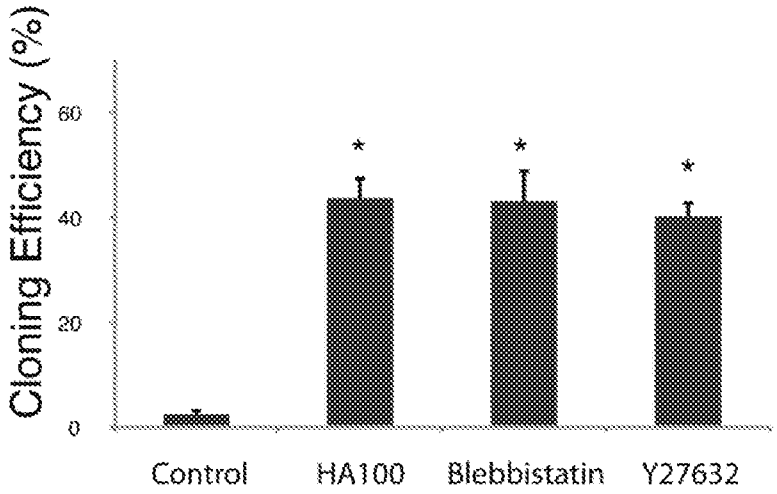

FIG. 2F

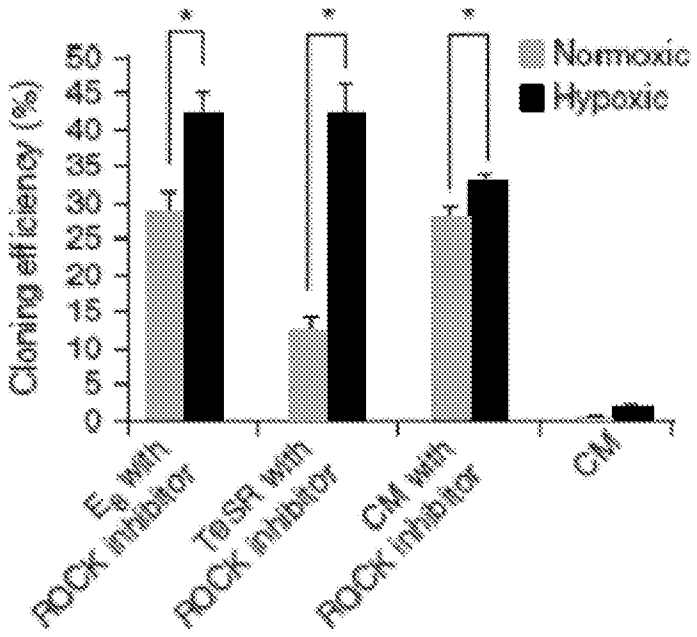

Figure 3
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FIG. 3A

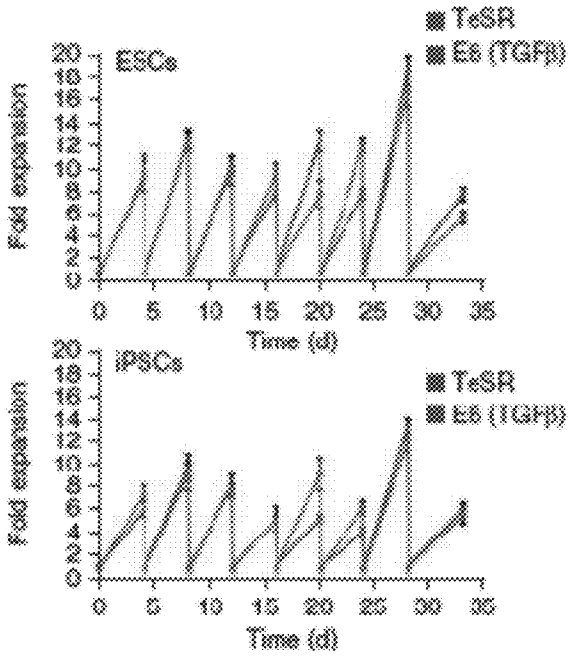

FIG. 3B

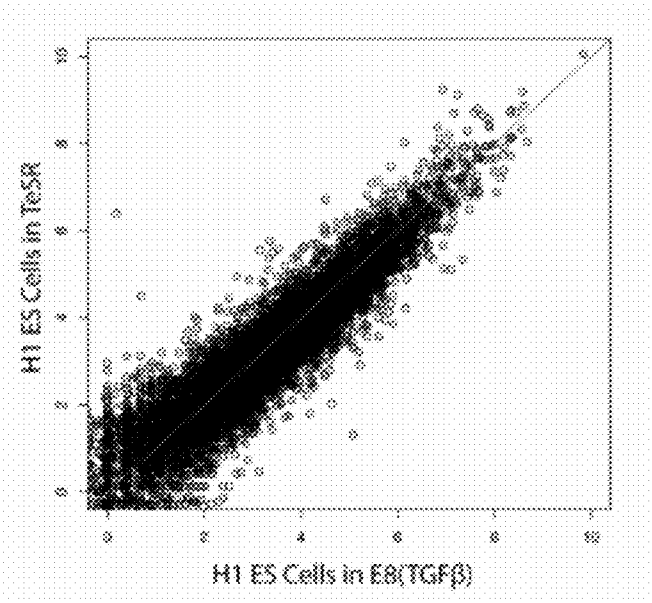

Figure 4

FIG. 4A

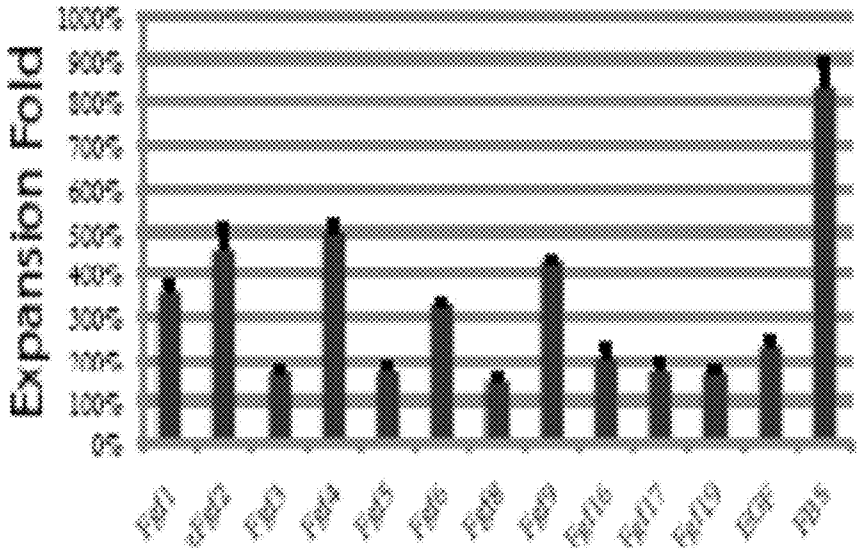

FIG. 4B

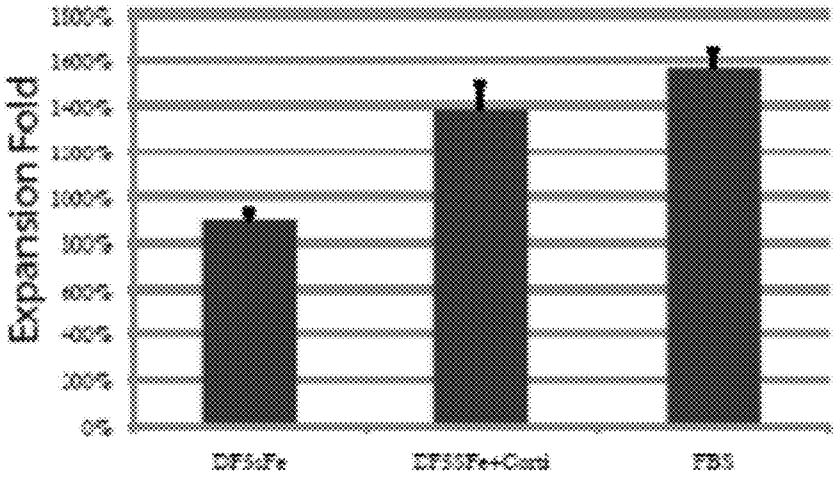

Figure 4
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FIG. 4C

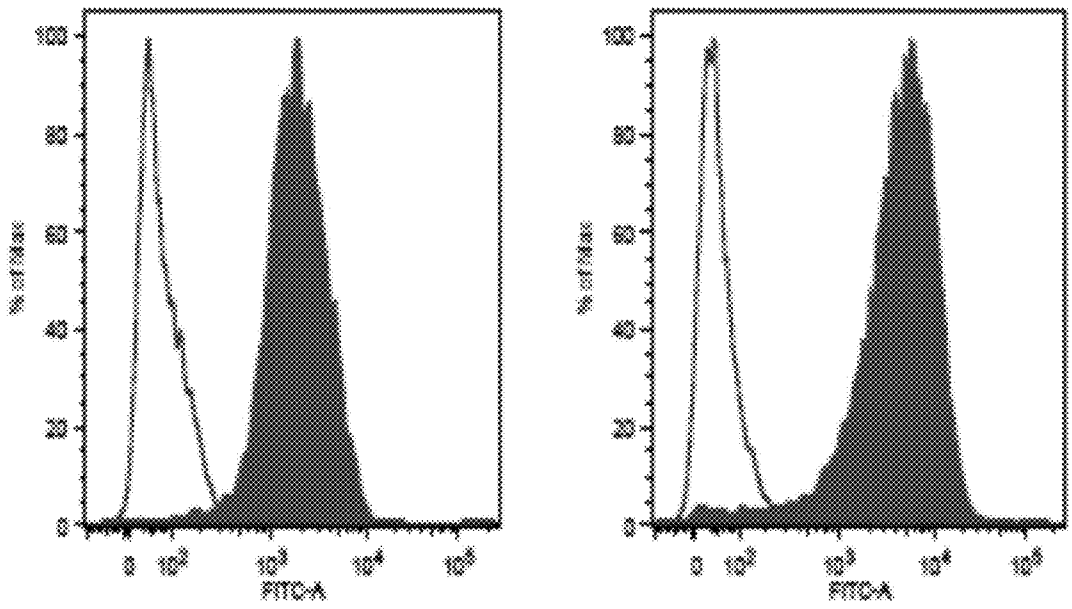

Figure 4
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FIG. 4D

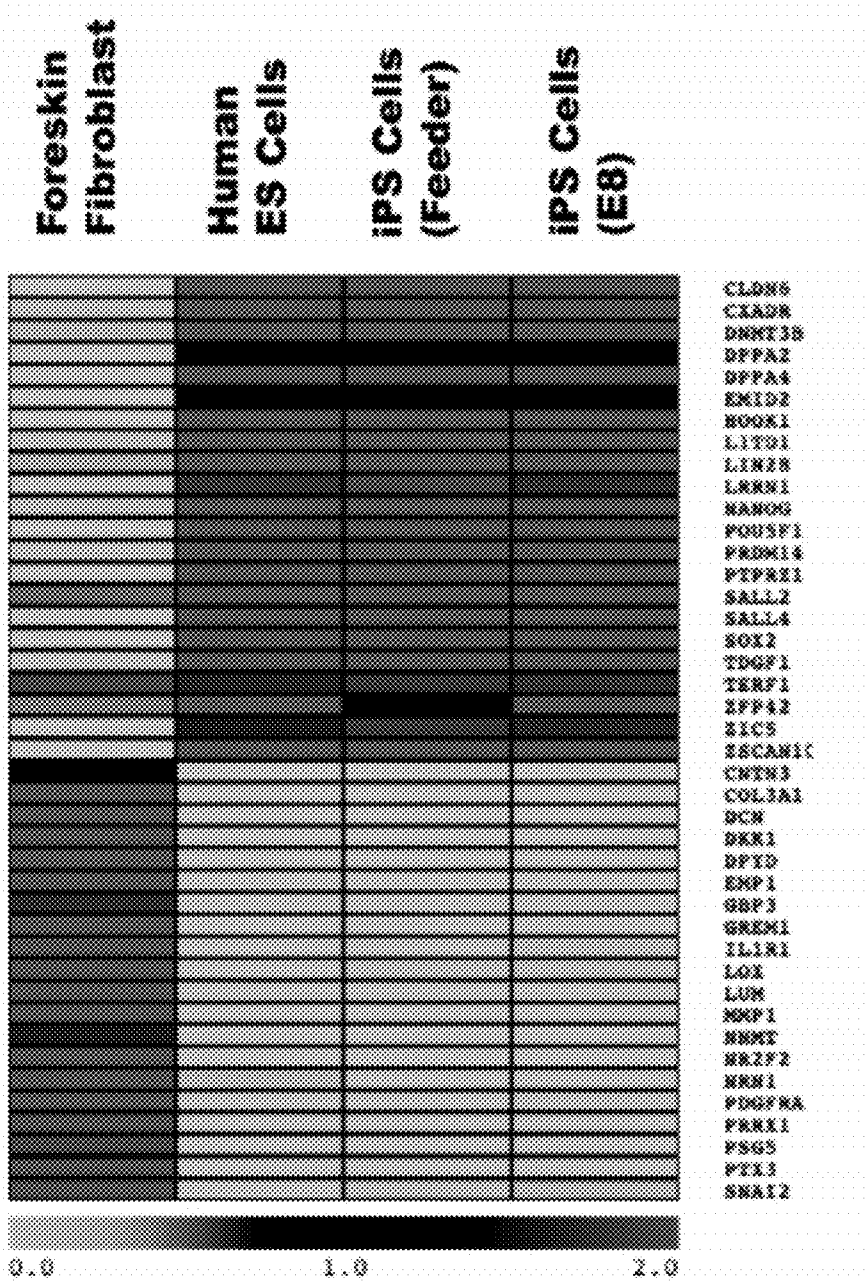

Figure 4
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FIG. 4E

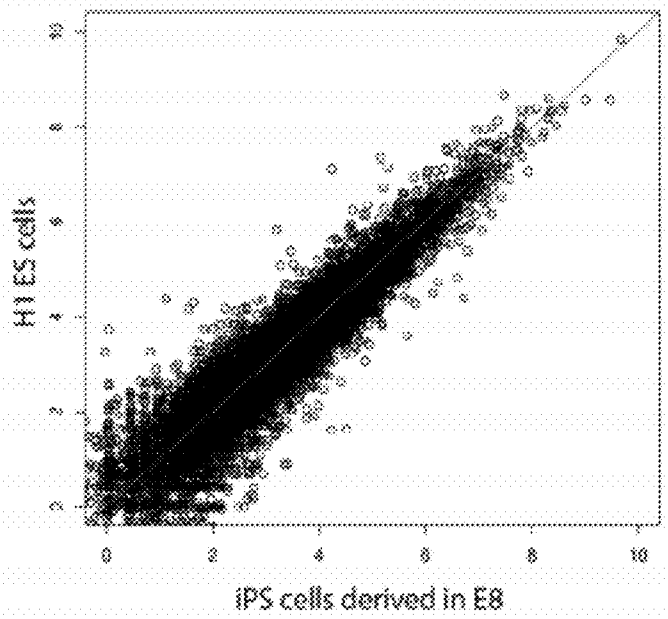

FIG. 4F

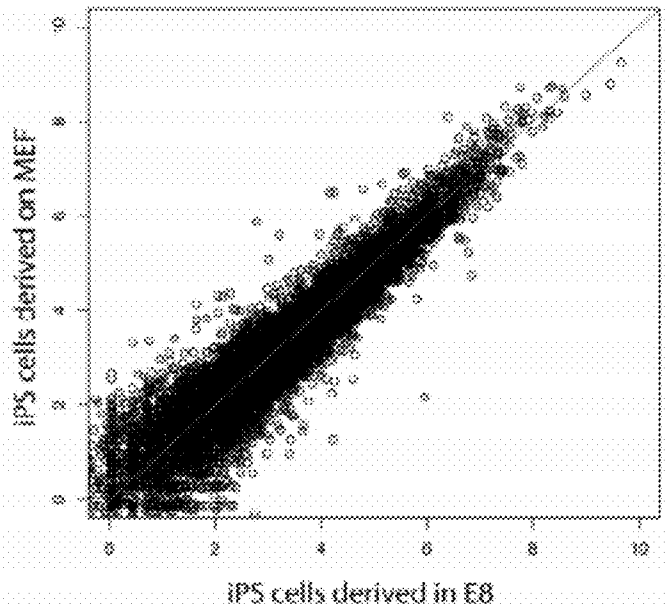

Figure 5

FIG. 5A

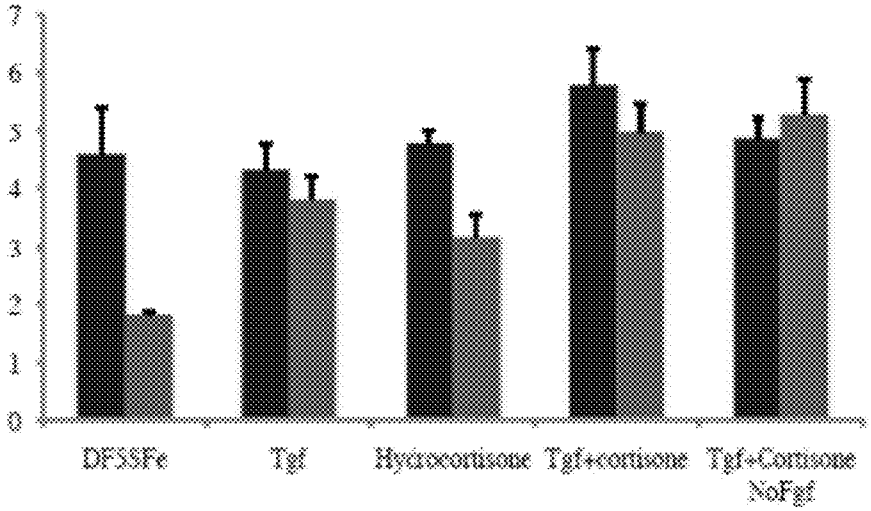

FIG. 5B

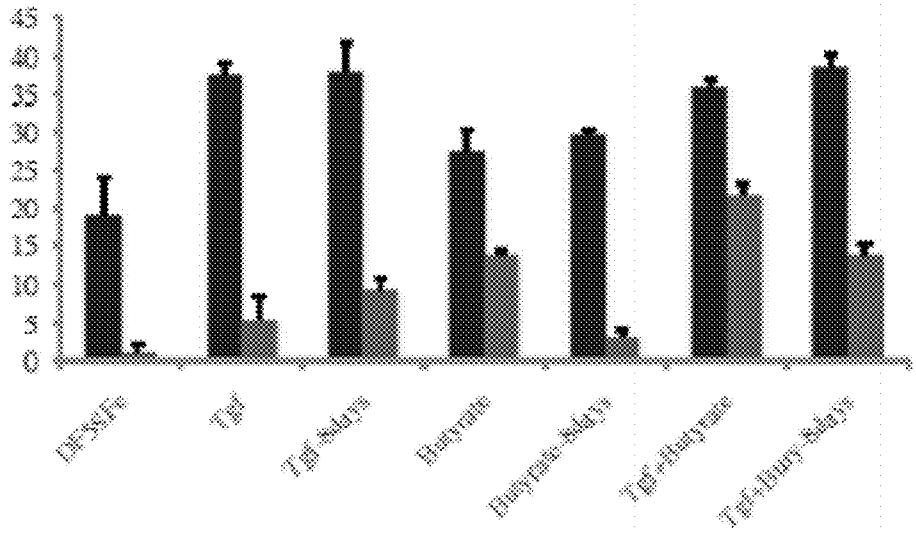

Figure 5
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FIG. 5C

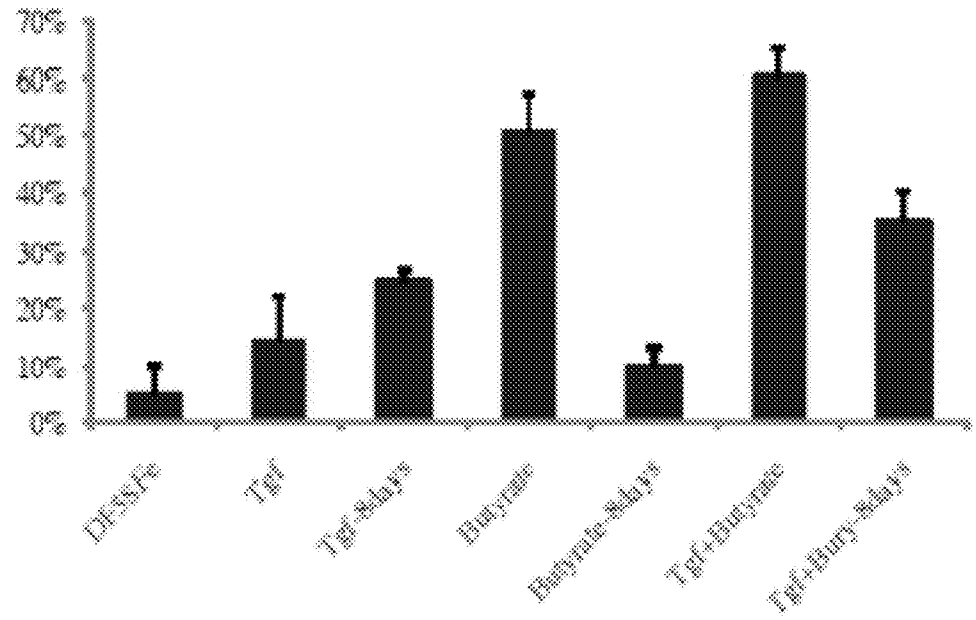

Figure 6

FIG. 6A

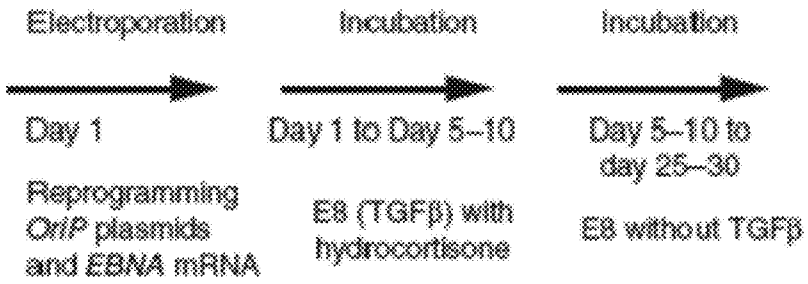

FIG. 6B

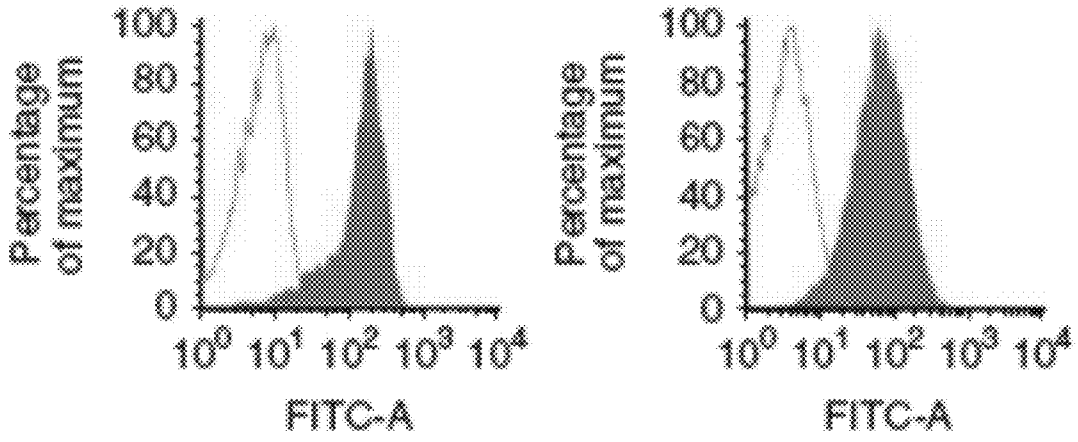

Figure 7

FIG. 7A

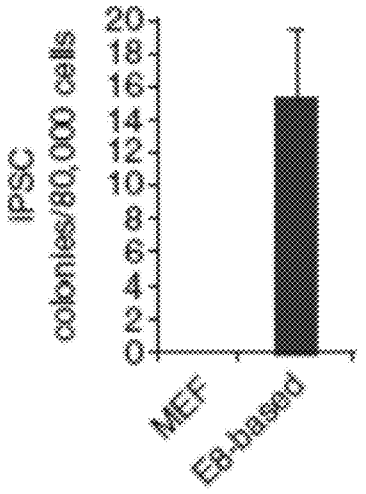

FIG. 7B

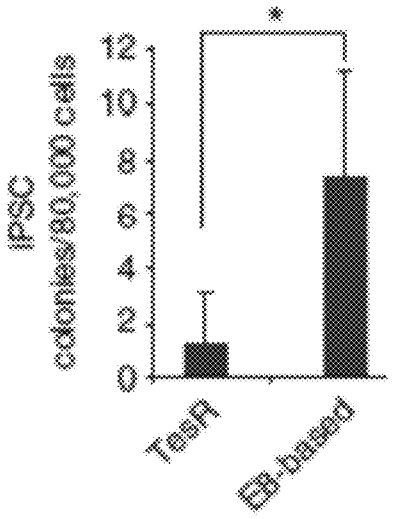

Figure 7
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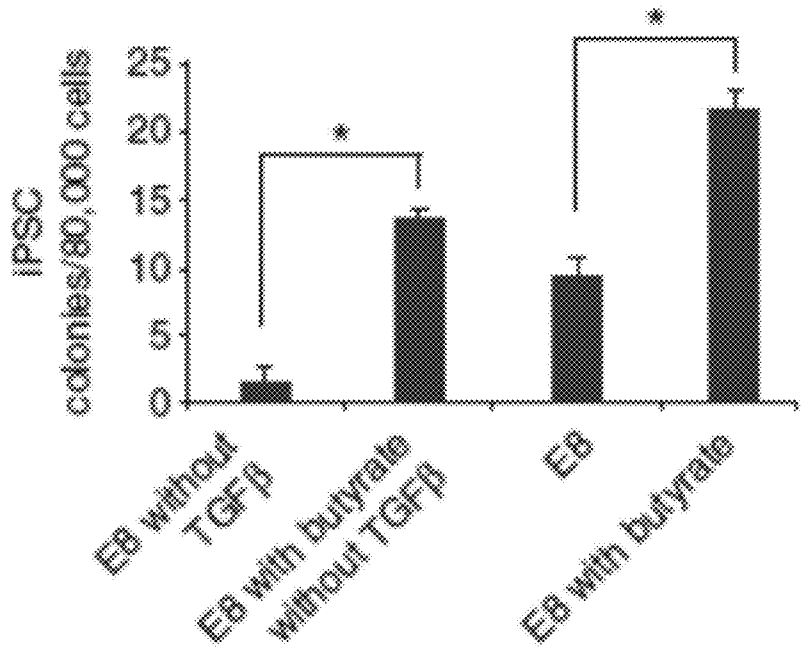
FIG. 7C



SIMPLIFIED BASIC MEDIA FOR HUMAN PLURIPOTENT CELL CULTURE

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under ES017166 awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Ser. No. 61/371,128 filed on Aug. 5, 2010.

BACKGROUND

Pluripotent cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, have the potential to differentiate into cells of all three primary germ layers (Thomson, et al., Science 282, 1145-1147 (1998)). The remarkable developmental potential of pluripotent cells has proven useful for basic research and clinical application. Many basic methods for human pluripotent cell culture, such as growth media, plate coating, and other conditions, have been developed and refined (Ludwig et al., Nat. Biotechnol 24, 185-187 (2006); Ludwig et al., Nat. Methods 3, 637-646 (2006)). For example, while human ES cells were initially cultured in fetal bovine serum (FBS)-containing media on murine embryonic fibroblast (MEF) feeder cells, fully defined media as well as defined protein matrices are now available (Ludwig et al., Nat. Biotechnol 24, 185-187 (2006)).

Over the past ten years, pluripotent cell culture methods have evolved considerably. Several growth media were developed that provide basic nutrients and growth factors for survival and expansion of pluripotent cells and directly determine how cells grow and differentiate. TeSR™ was one of the first defined media that supports pluripotent cell maintenance in an undifferentiated state in the absence of feeder cells or conditioned medium through multiple culture passages (Ludwig et al., Nat. Methods 3, 637-646 (2006); U.S. Pat. No. 7,449,334, each of which is incorporated herein by reference as if set forth in its entirety). TeSR™ contains 18 components in addition to the basal medium DMEM/F12 that itself has 52 components (Table 1).

The variety of different growth media available for pluripotent cell culture contributes to inconsistencies in research findings. The media that are presently used for pluripotent cell derivation and growth, including fully defined media, contain components that can influence pluripotent cells in various ways. Prior to the invention described herein, it was not known how each media component, alone or in combination with other components, affects various pluripotent cell functions such as viability, pluripotency, or differentiation in cell culture.

For example, albumin, the most abundant protein component present in most media, is a lipid carrier and, as such, can affect differentiation or maintenance of pluripotency via its associated lipids. The qualities of albumin and of its associated lipids determine whether it can be used for human pluripotent cell culture. However, albumin quality varies greatly depending on its source, even when produced from a recombinant genetic material, contributing to variations between experiments conducted under otherwise equivalent conditions. Also, while cloned human serum albumin is available, it is seldom used for routine experimentation due to its comparatively high cost.

Efforts to eliminate albumin from the medium have proved unsuccessful. Omission of albumin, or any other growth factor present in TeSR, led to a dramatic decline in human ESC culture performance, such as decreased cell viability, proliferation, and pluripotency (Ludwig et al., Nat. Biotechnol 24, 185-187 (2006)).

To fully exploit the potential of pluripotent cells for drug discovery, testing, and transplantation therapy, derivation and growth of these cells under fully-defined and, ideally, xeno-free, conditions is desirable. There is, thus an unmet need in the art for media free of components that introduce inconsistencies to maintain control over pluripotent cell culture conditions. Specifically, there is a need in the art for pluripotent cell culture media containing only those components that support pluripotent cell functions important for a specific culture objective.

BRIEF SUMMARY

The invention relates generally to media, compositions, and methods for deriving and culturing pluripotent cells, and more particularly, to fully-defined media for pluripotent cells.

In a first aspect, the present invention is summarized as albumin-free media that support viability, growth, and pluripotency of pluripotent cells.

In some embodiments of the first aspect, the medium contains selenium.

In some embodiments of the first aspect, the medium contains NODAL.

In some embodiments of the first aspect, the medium contains transferrin.

In some embodiments of the first aspect, the medium contains transforming growth factor beta (TGF-β).

In some embodiments of the first aspect, the medium contains only water, salts, amino acids, vitamins, a carbon source, insulin, and a fibroblast growth factor (FGF), each in amounts sufficient to support pluripotent stem cell viability.

In some embodiments of the first aspect, the medium contains only water, salts, amino acids, vitamins, a carbon source, insulin, an FGF, selenium, transferrin, and one of TGF-β and NODAL, each in an amount sufficient to support pluripotent stem cell proliferation.

In some embodiments of the first aspect, the medium supports survival after passaging, freezing, proliferation, pluripotency, derivation, and cloning of pluripotent cells.

In some embodiments of the first aspect, the medium is xeno-free.

In a second aspect, the present invention is summarized as a method for culturing pluripotent stem cells in a defined medium. In some embodiments of the second aspect, the medium used to culture pluripotent cells contains only water, salts, amino acids, vitamins, a carbon source, insulin, and an FGF, each in amounts sufficient to support pluripotent cell viability. In some embodiments of the second aspect, the medium used to culture pluripotent cells contains only water, salts, amino acids, vitamins, a carbon source, insulin, an FGF, selenium, transferrin, and one of TGF-β and NODAL, each in an amount sufficient to support pluripotent stem cell proliferation. In some embodiments of the second aspect, the medium contains defined factors that support extended growth, pluripotency, cloning, freezing, or derivation of pluripotent cells. In some embodiments of the second aspect, the medium used to culture pluripotent cells is xeno-free.

In a third aspect, the present invention is directed to an in vitro cell culture composition of pluripotent cells in a medium that is substantially free of β-mercaptoethanol and albumin. In some embodiments of the third aspect, the culture composition is free of fibroblast feeder cells, conditioned medium, and xeno-contamination.

In a fourth aspect, the present invention is summarized as a method for deriving iPS cells from an adult individual under completely defined conditions. The method includes the steps of culturing a somatic cell from an adult individual in a medium containing water, salts, amino acids, vitamins, a carbon source, insulin, and an FGF, all in sufficient amount to maintain viability, and reprogramming the cell in defined conditions such as to derive iPS cells.

In some embodiments of the fourth aspect, the medium contains TGF-β during parts or all of the reprogramming process.

In some embodiments of the fourth aspect, the medium contains butyrate.

In some embodiments of the fourth aspect, the medium contains hydrocortisone.

In some embodiments of the fourth aspect, the medium is xeno-free.

In a fifth aspect, the present invention is summarized as a method for cloning a pluripotent stem cell in an albumin-free medium. The method includes the step of plating pluripotent stem cells at cloning density in an albumin-free medium that supports pluripotent stem cell cloning.

In some embodiments of the fifth aspect, the medium contains a ROCK inhibitor.

In some embodiments of the fifth aspect, the medium contains blebbistatin.

In some embodiments of the fifth aspect, the medium contains only water, salts, amino acids, vitamins, a carbon source, insulin, an FGF, selenium, transferrin, and one of TGF-β and NODAL, each in an amount sufficient to support pluripotent stem cell cloning.

In a sixth aspect, the present invention is summarized as a method of cryopreserving pluripotent stem cells in an albumin-free medium. The method includes the step of freezing pluripotent stem cells in an albumin-free medium.

In some embodiments of the sixth aspect, the medium contains only water, salts, amino acids, vitamins, a carbon source, insulin, an FGF, selenium, transferrin, one of TGF-β and NODAL, and dimethyl sulfoxide (DMSO).

In a seventh aspect, the invention is summarized as an iPS cell derived under albumin-free conditions. iPS cells derived in the absence of albumin are free of endogenous albumin contaminations.

The methods and compositions described herein are useful in a variety of applications for deriving, culturing, and using pluripotent cells. It is an object of the present invention to define short term and long term culture conditions for pluripotent cells limited to factors that support the intended culture objective.

It is another object of the present invention to provide culture conditions for pluripotent cells that maximize percentage of cultured cells in an undifferentiated state.

It is another object of the present invention to provide media that can serve as the platform necessary to examine how various conditions affect pluripotent cells and to compare experiments previously reported in different media backgrounds.

These and other features, objects, and advantages of the present invention will become better understood from the description that follows. In the description, reference is made to the accompanying drawings, which form a part hereof and in which there is shown by way of illustration, not limitation, embodiments of the invention. The description of preferred embodiments is not intended to limit the invention to cover all modifications, equivalents and alternatives. Reference should therefore be made to the claims recited herein for interpreting the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be better understood and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

FIG. **1**A-E illustrate media elements for human ES cell survival and self-renewal in culture. FIG. **1**A illustrates 24 hour survival indices for individualized cells plated in the various media. Media abbreviations are as listed in Table 1. The presence of insulin and fibroblast growth factor (IF), bovine serum albumin (BSA), beta-mecaptoethanol (BME) is indicated by "+" and absence is indicated by "−." FIG. **1**B illustrates 24 hour or 96 hour survival indices for individualized cells plated in the various media. The addition of insulin and fibroblast growth factor (FGF) is indicated by "+" and removal is indicated by "−." FIG. **1**C illustrates 24 hour or 129 hour survival indices for individualized cells cultured in TeSR™ medium with Vitamin C (TeSR), TeSR™ medium without Vitamin C (TeSR™-LAA), or DF5 medium. FIG. **1**D illustrates cell proliferation after each of three passages in DF5, DF5 with added Selenium (DF5+Selenium), DF12, or DF12 from which Selenium had been removed (DF12-Selenium). FIG. **1**E illustrates a comparative analysis of twelve different base media.

FIG. **2** A-F illustrate optimization of human ES cell and iPS cell culture conditions with DF5S. FIG. **2**A shows survival indices for individualized cells that were seeded at low density (~1,500 cells/cm^2) in either DF5S (bottom) or TeSR™ (top) and cultured at different O_2 and CO_2 concentrations (O15C5: 15% O_2 and 5% CO_2; O15C10: 15% O_2 and 10% CO_2; 05C10: 5% O_2 and 10% CO_2). Cell survival was examined at 24 hours and 124 hours. FIG. **2**B shows the cloning efficiency of H1 cells cultured in various media in the presence (+HA100) or absence of the small molecule HA100 (CM100: conditioned media with 100 ng/ml FGF). FIG. **2**C shows the cloning efficiency of H1 cells and iPS cells derived from foreskin fibroblasts in various media. FIG. **2**D shows the cloning efficiency of iPS cells derived from foreskin fibroblasts in various media. DF5S trFe indicates DF5S media to which holotransferrin was added. FIG. **2**E illustrates the cloning efficiency of H1 cells cultured in various media in the presence of HA100 (10 μM, 24 hours), blebbistatin (10 μM, 4 hours), or Y27632 (10 μM, 24 hours), compared to cloning efficiency in the absence of these factors (control). Asterisks indicate $p<0.05$. FIG. **2**F illustrates cloning efficiency of H1 cells in conditioned medium (CM), CM with ROCK inhibitor (HA100), TeSR with ROCK inhibitor, and E8 with ROCK inhibitor in normoxic (dark gray bars) or hypoxic (light gray bars) conditions. Error bars indicate the standard error of the mean; asterisks indicate $p<0.05$.

FIG. **3** A-B illustrate pluripotent cell growth and gene expression in DMEM/F12 supplemented with insulin, transferrin, selenium, L-ascorbic acid, FGF2, and TGF-β or NODAL (referred to herein as "E8 (TGF-β)" and "E8 (NODAL)," respectively). FIG. **3**A illustrates fold expansion of H1 ES cells (top) and iPS cells (bottom) maintained in TeSR™ (dark gray lines) or E8 (TGF-β) (light gray lines). FIG. **3**B illustrates global gene expression of H1 ES cells

grown in E8 (TGF-β) and H1 ES cells grown in TeSR™. RNA of H1 cells maintained in either TeSR or E8 (TGF-β) medium for 3 passages was analyzed by RNA-seq with Illumina Genome Analyzer GAIIX (global gene expression correlation R=0.954 (Spearman Correlation)).

FIG. **4**A-F illustrate iPS cell derivation under defined conditions. FIG. **4**A shows proliferation of foreskin fibroblasts in DF5SFe-based media to which various fibroblast growth factors (FGF) were added, compared to proliferation in FBS-containing media. FIG. **4**B shows fibroblast growth in various media supplemented with hydrocortisone. FIG. **4**C shows expression of the pluripotency markers OCT4 (left) and SSEA4 (right). FIG. **4**D illustrates expression of selected genes by foreskin fibroblasts, hES cells, iPS cells derived on feeder cells (iPS Cells (Feeder)), and iPS cells derived in E8 medium (iPS Cells (E8)). All cells were maintained in E8 (TGF-β) medium prior to RNA analysis, except for fibroblasts, which were maintained in E8 with hydrocortisone. FIG. **4**E illustrates global gene expression of human ES and iPS cells derived in E8 (TGF-β) media (R=0.955). FIG. **4**F global gene expression of iPS cells derived on MEF and iPS cells derived in E8 (TGF-β) media.

FIG. **5**A-C illustrate media improvement for iPS cell derivation. FIG. **5**A shows proliferation of foreskin (dark grey bars) and PRPF8-2 adult fibroblasts (light grey bars) in DF5SFe media supplemented with TGF-β, hydrocortisone, TGF-β and hydrocortisone, or TGF-β and hydrocortisone without FGF. FIG. **5**B illustrates the effect of TGF-β and butyrate on reprogramming of foreskin fibroblasts. Four to five weeks after initial reprogramming transfection, colony numbers for transformed cells and true iPS cells were scored and the ratio of iPS colonies to non-iPS cell colonies was calculated.

FIG. **6**A-B illustrate derivation of iPS cells from adult fibroblasts under fully-defined conditions without secondary passage. FIG. **6**A illustrates an example of a reprogramming protocol. FIG. **6**B illustrates expression of the pluripotency markers OCT4 and SSEA4, as determined by flow cytometric analysis of iPSC lines maintained in DMEM/F12 supplemented with insulin, transferrin, selenium, L-ascorbic acid, FGF2, and TGF-β or NODAL ("E8") for 20 passages. Shaded peak: staining with antibodies specific to OCT4 (left) and SSEA4 (right); unshaded peak: mouse IgG control antibody.

FIG. **7**A-C illustrate reprogramming efficiency of human fibroblasts in various media. FIG. **7**A illustrates the number of iPS cell colonies per 80,000 fibroblasts subjected to reprogramming with mouse fibroblast feeder cells (MEF) or in E8-based medium. To improve efficiency, 100 μM sodium butyrate was added to both conditions. FIG. **7**B illustrates the number of iPS cell colonies per 80,000 fibroblasts subjected to reprogramming in TeSR™ or in E8-based medium. FIG. **7**C illustrates the effects of TGF-β and butyrate exposure time on reprogramming efficiency of foreskin fibroblasts under fully-defined conditions. Fibroblasts were reprogrammed in DMEM/F12 supplemented with insulin, transferrin, selenium, L-ascorbic acid, and FGF2 (E8 without TGF-β) or in E8, in the presence or absence of 100 μM butyrate. Reprogramming efficiency for all conditions was analyzed after 30 days after reprogramming. Asterisks indicate $p<0.05$.

While the present invention is susceptible to various modifications and alternative forms, exemplary embodiments thereof are shown by way of example in the drawings and are herein described in detail. It should be understood, however, that the description of exemplary embodiments is not intended to limit the invention to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

DESCRIPTION OF EXEMPLARY EMBODIMENTS

The present invention relates to the inventors' observation that certain media components once thought to be essential to culturing pluripotent cells can be omitted from pluripotent cell culture media formulated to achieve certain culture objectives.

As used herein, the term "pluripotent cell" means a cell capable of differentiating into cells of all three germ layers. Examples of pluripotent cells include embryonic stem cells and induced pluripotent stem (iPS) cells. As used herein, "iPS cells" refer to cells that are substantially genetically identical to their respective differentiated somatic cell of origin and display characteristics similar to higher potency cells, such as ES cells, as described herein. The cells can be obtained by reprogramming non-pluripotent (e.g. multipotent or somatic) cells.

The invention relates to new media free of factors not essential for a particular culture objective. Examples of culture objectives include, but are not limited to, cell survival, passaging, proliferation, pluripotency, cloning, and iPS cell derivation. Specifically, the invention relates to albumin-free media.

As a point of clarification, "passaging" and "cloning" are distinct methods. "Passaging" describes the process of dividing cells that have been cultivated in a culture vessel up to a certain density into aggregates, which are then placed into new culture vessels. These aggregates can contain any number of cells, typically between 100 to 1,000 cells, which readily initiate growth in culture. In contrast, "cloning" refers to initiating clonal colonies by growing human ES cell colonies from single individual ES cells. As used herein, "cloning efficiency" means the number of individualized cells that form new cell colonies divided by the number of individualized cells plated in culture. Cloning efficiency varies considerably depending on culture conditions. For example, the cloning efficiency of human ES cells under defined and xeno-free conditions on MATRIGEL® is very low (i.e., less than about 0.1%), while cloning efficiency of these cells cultured with fibroblast-conditioned medium, while still low (i.e., less than about 2%), is high enough to initiate clonal ES cell colonies.

Certain media components presently used can be damaging to the cultured cells or induce differentiation. For example, β-mercaptoethanol can damage and even kill cultured pluripotent cells. Serum media additives, such as bovine serum albumin (BSA) or fetal calf serum (FCS), can induce differentiation of cultured pluripotent cells. Also, commercially available serum components can differ significantly in their composition, even when supplied from the same source, introducing unpredictable culture variability. The media described herein are substantially free of damaging, differentiating, and undefined factors present in most conventional pluripotent cell culture media. The disclosed media have been successfully used for various culture objectives, such as supporting short term pluripotent cells viability, e.g., 24 hrs, short term proliferation, e.g., 4-5 days, maintaining pluripotent cells for extended culture periods, e.g., more than 25 passages in 3 months, and to derive iPS cells from both fetal and adult fibroblasts with lentiviral and episomal vectors.

New minimal media specifically tailored for certain cell culture objectives were developed. Various media components, such as salts, vitamins, glucose sources, minerals, and

amino acids were tested, alone or in combination, to determine their individual effect on viability, proliferation, or pluripotency. A new survival assay was developed and used to determine which components are essential for pluripotent cell survival after dissociation. New media were tested for their ability to support proliferation and sustain pluripotency. These media were also used in cloning assays to determine how each medium affects single cells and their cloning efficiency. A complete list of ingredients for each new medium described herein is set forth in Table 1 (light and dark shaded fields indicate presence of a component in the medium, checkered fields indicate interchangeable components, clear fields indicate absence of a component in the medium).

TABLE 1

Media compositions.

Components	GF	NM	DF5	DF5S	DF5SFe	DF12	TeSR	DF5S + TGF-β	DF5S + NODAL	DF5S + HCort	E8
DMEM/F12											
NaHCO3											
L-Ascorbic Acid											
Selenium											
Transferrin											
Glutathione											
L-Glutamine											
Defined lipids											
Thiamine											
Trace elements B											
Trace elements C											
BME											
BSA											
Insulin											
FGF2											
TGF-β											
Pipecolic acid											
LiCl											
GABA											
H2O											
Nodal											
Hydrocortisone											

The various media described herein can be prepared from the basic ingredients. Alternatively, one of skill in the art appreciates the advantageous efficiency of using a basal media as starting material to prepare the disclosed new media. The term "basal medium" as used herein means a medium that supports growth of certain single-celled organisms and cells that do not require special media additives. Typical basal medium components are known in the art and include salts, amino acids, vitamins, and a carbon source (e.g., glucose). Other components that do not change the basic characteristic of the medium but are otherwise desirable can also be included, such as the pH indicator phenol red. For example, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) is a basal medium commonly used to make suitable growth media for mammalian cell culture. A complete list of ingredients of DMEM/F12 is set forth in Table 2.

TABLE 2

DMEM: F-12 Medium Formulation (ATCC Catalog No. 30-2006).

Inorganic Salts (g/liter)	Amino Acids (g/liter)	Vitamins (g/liter)	Other (g/liter)
CaCl2 (anhydrous) 0.11665	L-Alanine 0.00445	D-Biotin 0.00000365	D-Glucose 3.15100
CuSO4 (anhydrous) 0.0000008	L-Arginine•HCl 0.14750	Choline Chloride 0.00898	HEPES 3.57480
Fe(NO3)3•9H2O 0.00005	L-Asparagine•H2O 0.00750	Folic Acid 0.00265	Hypoxanthine 0.00239
FeSO4•7H2O 0.000417	L-Aspartic Acid 0.00665	myo-Inositol 0.01261	Linoleic Acid 0.000044
MgSO4 (anhydrous) 0.08495	L-Cystine•HCl•H2O 0.01756	Niacinamide 0.00202	Phenol Red, Sodium Salt 0.00810
KCl 0.3118	L-Cystine•2HCl 0.03129	D-Pantothenic Acid 0.00224	Putrescine•2HCl 0.00008
NaHCO3 1.20000	L-Glutamic Acid 0.00735	Pyridoxine•HCl 0.00203	Pyruvic Acid•Na 0.05500
NaCl 7.00000	L-Glutamine 0.36510	Riboflavin 0.00022	DL-Thioctic Acid 0.000105
Na2HPO4 (anhydrous) 0.07100	Glycine 0.01875	Thiamine•HCl 0.00217	Thymidine 0.000365
NaH2PO4•H2O 0.06250	L-Histidine•HCl•H2O 0.03148	Vitamin B-12 0.00068	
ZnSO4•7H2O 0.000432	L-Isoleucine 0.05437		
	L-Leucine 0.05895		
	L-Lysine•HCl 0.09135		
	L-Methionine 0.01724		
	L-Phenylalanine 0.03548		
	L-Proline 0.01725		

TABLE 2-continued

DMEM: F-12 Medium Formulation (ATCC Catalog No. 30-2006).			
Inorganic Salts (g/liter)	Amino Acids (g/liter)	Vitamins (g/liter)	Other (g/liter)
	L-Serine 0.02625 L-Threonine 0.05355 L-Tryptophan 0.00902 L-Tyrosine•2Na•2H2O 0.05582 L-Valine 0.05285		

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein.

In describing the embodiments and claiming the invention, the following terminology will be used in accordance with the definitions set out below.

As used herein, "about" means within 5% of a stated concentration range or within 5% of a stated time frame.

As used herein, "essentially serum-free" means that a medium does not contain serum or serum replacement, or that it contains essentially no serum or serum replacement. For example, an essentially serum-free medium can contain less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% serum, wherein the culturing capacity of the medium is still observed.

The term "defined culture medium" or "defined medium," as used herein, means that the identity and quantity of each medium ingredient is known.

As used herein, "a medium consisting essentially of" means a medium that contains the specified ingredients and, optionally, other ingredients that do not materially affect its basic characteristics.

As used herein, "effective amount" means an amount of an agent sufficient to evoke a specified cellular effect according to the present invention.

As used herein, "viability" means the state of being viable. Pluripotent cells that are viable attach to the cell plate surface and do not stain with the dye propidium iodide absent membrane disruption. Short term viability relates to the first 24 hours after plating the cells in culture. Typically, the cells do not proliferate in that time.

As used herein, "short term growth" means cell proliferation for 4-5 days in culture.

As used herein, "extended growth" means growth for at least five passages. Typically, media are tested for their ability to support pluripotent cell growth for more than twenty passages (approximately 2-3 months).

As used herein, "long-term culture" means more than 15 passages (approximately two months in culture).

As used herein, "pluripotency" means a cell's ability to differentiate into cells of all three germ layers.

As used herein, "cloning" means a process of initiating a cell culture from a starting culture, ideally, from a single pluripotent cell or at least from very few cells. Culture conditions that permit clonal culture of undifferentiated pluripotent cells may be the most demanding conditions of all of those required in normal pluripotent cell culture and proliferation.

As used herein, "iPS cell derivation" means reprogramming a cell that is not pluripotent to become pluripotent.

As used herein, "xeno-free" means cell culture conditions free of any cell or cell product of species other than that of the cultured cell.

As used herein, "normoxic condition" means conditions with about 20% oxygen.

As used herein, "hypoxic condition" means conditions with less than about 20% oxygen, e.g., about 5% oxygen.

The invention will be more fully understood upon consideration of the following non-limiting Examples.

EXAMPLES

Example 1

Pluripotent Cell Survival Assay

Five-hundred micro liter of various test media was loaded into each well of 12-well plates prior to the addition of cells. Adherent pluripotent cells were dissociated with TrypLE (Invitrogen) for 5 minutes or until fully detached from the culture plates. TrypLE was neutralized by adding an equal volume of media to the culture. The cells were counted, washed, and resuspended in fresh media at a concentration of 300,000 to 1,000,000 cells/ml. Approximately 100 μl of this cell solution was added into each well of the 12-well plates and the cells were incubated at 37° C. with 5% O_2 and 10% CO_2. Cells were again dissociated at various time points using 0.4 ml TrypLE, which was subsequently neutralized with equal volumes of 10% FBS in DMEM. The cells were counted by flow cytometry. 5000 count bright beads were added to each sample as internal control (approximately 200 beads were counted for each sample). All experiments were performed in triplicates.

Example 2

Growth Factors for Survival and Short Term Growth

TeSR medium contains six growth factors, in addition to those present in the basal medium, fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), γ-aminobutyric acid (GABA), pipecolic acid, lithium chloride (LiCl), and insulin (Table 1). A basic nutrient medium (NM) was created containing all TeSR™ components with the exception of these six growth factors. About 2×10^5 H1 ES cells were dissociated and plated on Matrigel. The survival index was determined after 24 h. NM alone could not support cell survival after dissociation. The addition of insulin to NM resulted in cell survival similar to that observed with TeSR™, but did not support cell growth (FIG. **1**A). The addition of both insulin (20 ug/ml) and FGF2 (100 ng/ml) supported cell survival and additionally led to cell growth in 96 h that was comparable to that observed using TeSR™ medium (FIG. **1**B). Thus, NM supplemented with FGF and Insulin supports human ES cell culture. Twelve different basal nutrient media

supplemented as described above were able to support cell survival and growth (FIG. 1E).

Example 3

L-Ascorbic Acid Supports Short-Term Proliferation

NM contains 11 nutritional components, i.e., DMEM/F12, trace elements B, trace elements C, L-ascorbic acid, thiamine, selenium, L-glutamine, BSA, BME, sodium bicarbonate ($NaHCO_3$), and transferrin (Table 1). DMEM/F12 serves as basal medium and $NaHCO_3$ is used to modify the pH. To determine which other nutritional components were essential when insulin and FGF were present, each factor was added individually to DMEM/F12, $NaHCO_3$, insulin, and FGF. None of the nutritional factors were essential for survival after passaging, but L-ascorbic acid (64 mg/L) was necessary for cell proliferation after passaging (FIG. 1C). L-ascorbic acid, known as Vitamin C, is a major antioxidant and cofactor of several enzymes. Hydroxyproline could partially substitute for L-ascorbic acid. Human ES cells plated in DMEM/F12, $NaHCO_3$, L-ascorbic acid, insulin, and FGF (Defined Factors 5, "DF5," Table 1) maintained similar morphology as human ES cells plated into TeSR.

Example 4

Media Components for Extended Passage

DF5 supported cell growth for only one passage. After the second passage, cells attached poorly and eventually died (FIG. 1C). Cells could be passaged in NM+Insulin+FGF (data not shown) and DF12 (FIG. 1D, Table 1), suggesting that one or more factor present in NM+Insulin+FGF and DF12 is important for extended passage. Each nutritional factor present in NM was added individually to DF5 to determine its ability to support cell expansion after multiple passages. Addition of selenium alone was sufficient to support cell proliferation through multiple passages (FIG. 1D, DF5+Selenium, "DF5S," Table 1).

DF5S was used to expand H1 cells. Cells grown in DF5S were more prone to differentiate than cells grown in TeSR™. However, H1 cells could be grown for several weeks (more than 15 passages), during which the cells maintained human ES cell morphology and high levels of OCT4 expression (FIG. 1E, FIG. 1F). H1 cells grown in DF5S to which either NODAL (100 ng/ml) or TGF-β (2 ng/ml) was added expressed significantly higher levels of NANOG mRNA, compared to H1 cells cultured in DF5S. DF5S+NODAL also supported pluripotency of the two tested human iPS cell lines, as determined by high expression of the pluripotency marker OCT4. All cells (hES cells and iPS cells) grown in DF5S with either NODAL or TGF-β maintained a normal karyotype after long-term passage.

Example 5

Hypoxia Improves Cell Growth and Cloning

H1 cells grew faster in the DF5S medium compared to cells grown in TeSR™ (FIGS. 1C and 2A). To optimize pluripotent cell growth conditions, cells were grown in DF5S with varying osmolarity, pH, oxygen level, and CO_2 level. To increase assay sensitivity only 5,000 cells were seeded in each well and analyzed for survival (24 h) and proliferation (124 h). The greatest improvements were noted when O_2 and CO_2 levels were varied. Ordinary culture conditions use oxygen at ~15% and CO_2 at 5% (O15C5). Higher CO_2 often led to slightly higher survival after 24 hours. Lower oxygen levels increased cell growth in both DF5S and TeSR™. Oxygen at 15% with CO_2 at 10% (O15C10), and oxygen at 5% with CO_2 at 10% (O5C10) increased cell survival (FIG. 2A). Cells failed to thrive at higher O_2 levels (O15C5 and O15C10), while they proliferated at lower oxygen levels (O5C10) (FIG. 2A). Cells in DF5S grew faster than those grown in TeSR™, and grew fastest at 5% O_2 and 10% CO_2 (FIG. 2A). Further decreases in oxygen level to 2% reduced cell growth compared to 5% O_2.

To determine cloning efficiency at various oxygen and CO_2 concentrations, 500 cells were seeded into each well. Even at low oxygen, cloning efficiency was too low (<2%) to determine effects of various conditions on cloning. HA100, a ROCK inhibitor known to increase cloning efficiency, was used to increase cloning efficiency for testing oxygen and CO_2 concentrations. Conditioned medium (CM), known to be the best medium for cloning, was used as control. The addition of HA100 significantly improved cloning efficiency in CM at both O5C10 and O15C5 and cloning efficiency was higher at the O5C10 than O15C5 (FIG. 2B). Cloning efficiency of cells in DF5S was comparable to that of cells in CM under both conditions (FIG. 2B).

Because of the positive impact of hypoxia on cell survival, some of the subsequent examples employ hypoxic conditions when cells were maintained at low density. However, when cells were not cultured at low cell density, experiments were conducted under both normoxic and hypoxic conditions (FIG. 2B).

Example 6

Improved iPS Cell Cloning Efficiency

To determine how DF5S affects cloning efficiency, two iPS cell lines were grown in DF5S and plated at cloning density (approximately 500 cells per 12-well plate well) in the presence of HA100. The cloning efficiency of iPS cells grown in DF5S was lower than that of iPS cells grown in either TeSR™ or CM (FIG. 2C), suggesting that a factor that enhances cloning efficiency is present in TeSR™ medium, but absent from DF5S. To identify such factor, individual TeSR™ components were added individually to DF5S and tested for effect on cloning efficiency. The addition of holo-transferrin to DF5S (DF5SFe) resulted in cloning efficiency comparable to that using TeSR™ (FIG. 2D). Transferrin also lead to noticeable improvement of cloning efficiency of H1 cells in DF5S medium.

The ROCK inhibitors HA100 and Y27632, and blebbistatin in DMEM/F12 supplemented with insulin, trasferrin, selenium, L-ascorbic acid, FGFs, and TGF-β (or NODAL; "E8"), increased cloning efficiency of H1 cells (FIG. 2E), which was further increased by the addition of transferrin and by culture under hypoxic conditions (FIG. 2F)). The cells maintained a normal karyotype after more than 25 passages.

Example 7

NODAL and TGF-β Support Long-Term Maintenance of H1 and iPS Cell Pluripotency in Albumin-Free Media

As described in Example 3, human pluripotent cells, such as H1, H9, and iPS cells, could be grown and passaged over 15 times in DF5S but were prone to differentiate, such that extra care is needed to sustain pluripotency in DF5S. Because pluripotency could be maintained more easily in TeSR™,

growth factors present in TeSR™ were added individually to DF5SFe used to grow H1 cells that were previously cultured in DF5S without differentiating to identify factors supporting long-term pluripotency. Cells were passaged approximately one day after reaching confluency, facilitating cell differentiation, and Oct4 expression, assessed by flow cytometry, was used as indicator of pluripotency.

Human pluripotent cells grown in DF5SFe elongated and lined up along each other, resembling a "spindle" shape just prior to differentiation. This phenotype is often observed at the onset of neural differentiation that is usually suppressed by the TGF-β/BMP pathway. Thus, recombinant proteins of the TGF-β pathway were tested for their ability to support long-term pluripotency. DF5SFe supplemented with NODAL ("E8 (NODAL)") used at TeSR™ concentration sustained high Oct4 expression. DF5SFe supplemented with TGF-β ("E8 (TGF-β)") used at TeSR™ concentration (0.6 ng/ml) supported low levels of Oct4 expression but was able to maintain high Oct4 expression when used at higher concentration (1 ng/ml).

Human ES cell lines, such as H1 and H9 have a culture history that includes exposure to various complex culture components, such as FBS, feeder cells, and knockout serum replacer. Exposure to these components could conceivably create dependency on these components and, consequently, alter cellular response to simplified media. Culture history might play a lesser role for iPS cells, derived from reprogrammed somatic cells, as derivation conditions are less complex. Therefore, different factors were tested with two original lentiviral iPS cell lines (Yu, et al., Science 318:1917 (2007)) grown in DF5SFe. Cells were transferred from MEF plates directly into DF5SFe medium for one passage and then passaged into various growth factor conditions. The addition of either TGF-β (2 ng/ml) or NODAL (100 ng/ml) to DF5SFe ("E8 (TGF-β)" and "E8 (NODAL)," respectively) supported long-term pluripotency of iPS cells. Pluripotency surface markers SSEA4, SSEA3, Tra-1-60, and Tra-1-81 were also expressed. Cells with normal karyotypes were continuously maintained for more than 20 passages. The cells were capable of forming teratomas 5-7 weeks after injection into severe combined immunodeficient (SCID) mice.

E8 (TGF-β) and E8 (NODAL) supported pluripotency of every pluripotent cell line tested, i.e., two human ES cell lines (H1 and H9) and five iPSC lines for more than 25 passages (approximately 3 months) with no sign of differentiation (FIG. **3**). H1 ES cells grown in E8 media have a similar gene expression profile compared to H1 ES cells grown in TeSR™ (FIG. **3**B).

Example 8

Derivation of iPS Cells in Albumin-Free Media

Available reprogramming protocols include incubation of the cells in PBS in the first several days after viral transduction or electroporation, prior to switching the cells to UM100 (U.S. Pat. No. 7,439,064, incorporated herein as if set forth in its entirety) or CM. The simplified media described in previous Examples were tested for their ability to support reprogramming. ES-derived somatic cells could be reprogrammed efficiently in DF5S medium using lentivirus or episomal vectors with or without an initial 2 day culture in FBS-containing media. However, DF5S did not support reprogramming of primary foreskin cells using Nanog, Oct4, Sox2 and Lin28. DF5SFe supported reprogramming of foreskin and adult cells on Matrigel or MEFs using improved lentivirus (Ebert et al., Nature 457(7227):277-280 (2009), incorporated herein by reference as if set forth in its entirety) when the cells were initially incubated in FBS-containing medium. While DF5SFe was as effective as CM in supporting reprogramming, initial exposure to FBS appeared important for reprogramming.

Foreskin cells grow significantly slower in DF5SFe than in FBS media. To determine growth factors that can help primary foreskin cell growth, individual growth factors contained in FBS were tested. The FGF family of growth factors has several members, one or more of which is commonly used for fibroblast culture. DF5SFe contains 100 ng/ml zebrafish recombinant FGF2. Each FGF family member was tested for its ability to support foreskin cell growth. Foreskin cells were aliquoted into the well of culture plates and incubated for 24 hours in DF5SFe minus FGF. Individual FGF types were added at 100 ng/ml for 96 h. FGF1, zFGF2, FGF4, FGF6, and FGF9 supported foreskin cell growth most effectively, but none supported cell growth as well as FBS-containing media (FIG. **4**A). To identify if a non-FGF family member growth factor could promote foreskin cell growth comparable to that seen with FBS, several known fibroblast growth-promoting factors were tested. Hydrocortisone (FIG. **4**B), its derivatives, and dexamethasone added to DF5SFe to replace FBS improved cell growth significantly. DF5SFe+hydrocortisone ("DF5SFeC") also improved iPS cell cloning efficiency.

To determine if DF5S-based media can be used for viral-free iPS cell derivation, foreskin cells were reprogrammed using a viral-free episomal vector, as described in Yu et al., Science 324:797 (2009), incorporated herein by reference as if set forth in its entirety, at hypoxic conditions (O5C10). Plasmid combinations #4 (pEP4EP2SCK2MEN2L and pEP4EO2SET2K, Table 3), #6 (pEP4EO2SEN2L, pEP4EO2SET2K and pEP4EO2SEM2K, Table 3), and #19 (pEP4EO2SEN2K, pEP4EO2SET2K, and pCEP4-M2L, Table 3) were used, and 2 clones were isolated from 10^6 cells after secondary passage.

TABLE 3

Reprogramming vector components and vector combinations

Component	Abbr.	Source	SEQ ID NO	Accession # or sequence
OCT4	O	hESC	1	NM_002701
SOX2	S	hESC	2	NM_003106
NANOG	N	hESC	3	NM_024865
LIN28	L	hESC	4	NM_024674
c-Myc	M	hESC	5	NM_002467
KLF4	K	hESC	6	NM_004235
SV40 T	T	pBABE-puro SV40 LT p	7	EF579667
TERT	TERT	pBABE-hygro-hTERT	8	NM_198253
IRES2	2	pIRES2EGFP	9	—
CMV	C		10	—
EF1α	E		11	—

Vector Combinations

Combination Number	Plasmids	Components
4	pEP4EP2SCK2MEN2L	pEP4-EF1α-OCT4-IRES2-SOX2-CMV-KLF4-IRES2-c-Myc-EF1α-NANOG-IRES2-LN28
	pEP4EO2SET2K	pEP4-EF1α-OCT4-IRES2-SOX2-EF1α-SV40T-IRES2-KLF4
6	pEP4EO2SEN2L	pEP4-EF1α-OCT4-IRES2-SOX2-EF1α-NANOG-IRES2-LN28
	pEP4EO2SET2K	pEP4-EF1α-OCT4-IRES2-SOX2-EF1α-SV40T-IRES2-KLF4
	pEP4EO2SEM2K	pEP4-EF1α-OCT4-IRES2-SOX2-EF1α-c-Myc-IRES2-KLF4

TABLE 3-continued

Reprogramming vector components and vector combinations		
19	pEP4EO2SE N2K	pEP4-EF1α-OCT4-IRES2-SOX2-EF1α-NANOG-IRES2-KLF4
	pEP4EO2SE T2K	pEP4-EF1α-OCT4-IRES2-SOX2-EF1α-SV40T-IRES2-KLF4
	pCEP4-M2L	pCEP4-CMV-c-Myc-IRES2-LN28

Plasmid combinations #6 and #19 were used for the reprogramming. In order to enhance the plasmid entry into the nucleus, ENBA mRNA was electroporated along with plasmid DNA. Around one million cells were transferred onto two 6-well plates in DF5SFeC for 5 days. Medium was then switched to DF5SFe for another 18-25 days. Cells of some of the wells were passaged for a second time using a 1:6 ratio at different time points. Plasmid combination #19 generated more colonies than plasmid combination #6, but most of them did not resemble typical human ES cell morphology. After approximately 25 days, human ES cell-like colonies appeared on the primary plate for both plasmid combinations, with an estimated 24 reprogrammed cells per million foreskin cells using plasmid combination #19 and 8 reprogrammed cells per million foreskin cells using plasmid combination #6. The number of human ES cell-like colonies significantly increased after the secondary passage plates, with an estimated >500/million foreskin cells for each plasmid combination. The increase in the iPS cell colonies on secondary passage plates are likely due to the split of iPS cells on the primary plates. In some instances, primary plates did not have any colonies resembling typical human ES cell morphology, but many iPS cells appeared after secondary passage, suggesting that some iPS cells could not be identified, possibly because they were mixed with somatic cells.

Cells of the iPS cell colonies derived in DF5SFe started to differentiate after only two passages. Six iPS cell colonies were picked from the primary plate and transferred directly into Nodal-containing DF5SFeN (E8 (NODAL)). These cells could be maintained in E8 (Nodal) for more than 15 passages, maintaining their ES cell-like morphology similar to that observed using TeSR™. The cells had normal karyotypes, expressed Oct4 and SSEA4 (FIG. **4**C), and formed teratomas in SCID mice 5-7 weeks after injection.

Foreskin fibroblasts were also reprogrammed in E8 medium. Global gene expression of iPS cells derived in E8 medium was similar to that of H1 cells (FIGS. **4**D and E) or iPS cells derived on feeder cells (FIGS. **4**D and F). Pluripotency markers were highly expressed in both ES and iPS cells, while fibroblast specific marker genes were not expressed (FIG. **4**D). Also, iPS cell could be derived in E8 media using various strategies, e.g., using lentiviral or episomal vectors.

Example 9

Derivation of iPS Cells from Patient Cell Lines in Albumin-Free Media

To determine if cells from adult donors could be reprogrammed using viral-free episomal vectors in the simplified media, two million cells of the patient cell lines OAT or PRPT8 were electroporated with plasmid combinations #4 or #6, along with EBNA mRNA, and transferred onto two 10 cm plates. To maximize reprogramming, FBS-containing media was used for the first 6 days. Cells were kept at O15C5 to match regular adult cell maintenance conditions. Medium was then switched to DF5SFe for another 14-21 days. The cells of one plate were passaged at a 1:2 ratio at different time points. Plasmid combination #6 generated more colonies (approximately 5 per one million cells) than #4, but most of the cells did not resemble typical human ES cell morphology. After approximately 22 days, human ES cell-like colonies appeared on the primary plate for plasmid combination #4. Many more human ES cell-like colonies appeared on the secondary passage plates when plasmid combination #6 was used, with an estimate of approximately 40 colonies per million cells. No iPS cells were produced when using plasmid combination #4. The iPS cell colonies emerged in the middle of other densely-populated cells on the primary plate and could not grow beyond their boundary. However, colonies on the secondary plates expanded to large sizes suitable for colony isolation. Colonies were picked and directly transferred into TeSR™, and 32 picked colonies survived and displayed ES-cell morphology. Genetic analysis confirmed that these colonies were derived from the OAT cell line and exhibited a normal karyotype.

To improve adult cell reprogramming efficiency, TGF-β was added to the reprogramming media. iPS clones were not increased significantly, however, the total number of colonies increased significantly. When TGF-β was removed from the media at the time of hydrocortisone removal, the number of iPS cell colonies increased significantly, suggesting that TGF-β supports reprogramming in the first few days of the process.

Many seemingly non-iPS clones can generate iPS clones after secondary passage, suggesting that iPS cell derivation might be inhibited by surrounding cells. Several reagents were tested for their ability to overcome this effect. Butyrate improved reprogramming efficiency. An approximately 10-fold increase in reprogramming efficiency of foreskin cells was observed when both TGF-β and butyrate were added to the media (FIG. **5**B). TGF-β appeared to exhibit its positive effects during early stages of reprogramming, while butyrate had a positive role in the later stage. TGF-β addition led to increased numbers of colonies during reprogramming, but the number of true iPS cell colonies remained low. Butyrate did not increase the number of colonies, but improved the ratio of true iPS cell to non-iPS cell colonies significantly (FIG. **5**C).

Using TGF-β and butyrate enabled successful reprogramming of somatic cells from an adult individual under completely defined conditions using the episomal vector system. iPS cells were derived from three independent adult somatic cell lines (OAT, GRC M1-29, and PRPF8-2) at an efficiency of 1-100 out of 1×10^6 PRPF8-2 cells and 1 out of 100,000 cells (GRC1-29).

Example 10

Derivation of iPS Cells from an Adult Individual in Completely Defined Conditions

A biopsy was taken from the skin of a male adult donor, washed several times with Hank's Buffered Salt Solution (HBSS) containing antibiotics and antimycotic agents, and incubated in 2 ml of 0.25% trypsin/EDTA (Table 4) or TrypLE select at 4° C. overnight. The sample was rinsed three times, using trypsin inhibitor (Table 4) after the second rinse. The dermis and epidermis were separated using sterile forceps. The dermis was cut into small pieces and incubated in 0.75 ml enzyme solution (Table 4) with defined enzymes at room temperature (12-well or 24-well plate) for 3 hours. After approximately 35 minutes, tissue structures started to break down. An equal volume of medium with 10 μg/ml polyvinylpyrrolidone (PVP) was added and the tissue was mechani-

cally dissociated by pipetting up and down about 10 times. The sample was centrifuged at 400 g for 10 minutes at room temperature and washed twice with fresh media/PVP. The supernatant was discarded, the pellet resuspended in 3 ml of complete medium, and 1 ml of the cell suspension was transferred into wells of 6-well plates coated with 3 μg/well vitronectin. The plates were incubated with 5% CO_2 at 37° C. and the medium was changed every day. Fibroblasts adhered to the plates while nonadherent cells and debris were removed when the medium was changed.

TABLE 4

Reagents and procedures for specimen digestion.

Trypsin/EDTA	Enzyme solution	Trypsin inhibitor
TrypZean 1x (Sigma) TrypLE animal free 0.05%~0.25% (Invitrogen)	HEPES containing RPMI supplemented with 1 mM sodium pyruvate, 1.0 mg/ml Collagenase, 150 units/ml Hyaluronidase, and 140 units/ml DNase I (Roche)	10 mg/ml Trypsin inhibitor (from *Glycine max*) in Water or PBS

After 20 days, reprogramming plasmids were introduced into the fibroblasts using electroporation. Within the next 25 days, multiple iPS colonies emerged and were picked for further analysis. Reprogramming efficiency was about 10 out of 1 million electroporated fibroblasts, without secondary passaging. iPS cells were further passaged to isolate vector-free cell lines.

Example 11

Derivation of iPS Cells from an Adult Individual in Albumin-Free Media Without Secondary Passage

Adult fibroblasts were reprogrammed in E8 (DMEM/F12 supplemented with insulin, transferrin, selenium, L-ascorbic acid, FGF2, and TGF-β (or NODAL)) following the general protocol illustrated in FIG. **6**A. Reprogrammed iPS cell lines maintained in E8 for more than 20 passages continued to express pluripotency markers OCT4 and SSEA4 (FIG. **6**B).

E8 medium significantly enhanced reprogramming efficiency compared to reprogramming efficiencies using mouse fibroblast feeder cells (MEF) (FIG. **7**A) or TeSR™ (FIG. **7**B). Butyrate (100 μM) further enhanced reprogramming efficiency in the presence of TGF-β (E8) or in the absence of TGF-β (E8 without of TGF-β, i.e., DF5SFe) (FIG. **7**C).

Example 12

Cryopreservation of Pluripotent Stem Cells in an Albumin-Free Media

Pluripotent cells were cultured in 6-well plates in E8 medium, essentially as described above. The culture medium was aspirated from each well and the cells were washed twice with 1.0 mL EDTA/PBS (0.5 mM EDTA in PBS, osmalority 340). The cells were then incubated at 37° C. in EDTA/PBS for 5 minutes. The PBS/EDTA was removed, and the cells were rinsed swiftly with 1 ml of E8 medium. The cells were then resuspended in an equal volume of 20% dimethyl sulfoxide (DMSO) and E8 medium (final concentration: 10% DMSO in E8 medium), aliquoted into cryogenic vials, and frozen at −80° C. using a CRYOBOX™. The cells were subsequently moved into a liquid nitrogen tank.

The invention has been described in connection with what are presently considered to be the most practical and preferred embodiments. However, the present invention has been presented by way of illustration and is not intended to be limited to the disclosed embodiments. Accordingly, those skilled in the art will realize that the invention is intended to encompass all modifications and alternative arrangements within the spirit and scope of the invention as set forth in the appended claims.

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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<213> ORGANISM: Simian virus 40

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The invention claimed is:

1. A defined, albumin-free medium free of any component obtained from a non-human animal, the medium comprising water, salts, amino acids, vitamins, glucose, insulin, an FGF, selenium, transferrin, and one of TGF-β and NODAL, each in an amount sufficient to support human pluripotent stem cell proliferation.

2. A method for culturing human pluripotent stem cells, the method comprising the steps of:

placing pluripotent stem cells on a matrix; and

contacting the cells with a defined, albumin-free medium free of any component obtained from a non-human animal, the medium comprising water, salts, amino acids, vitamins, glucose, insulin, an FGF, selenium, transferrin, and one of TGF-β and NODAL, each in an amount sufficient to support human pluripotent stem cell proliferation.

3. The method of claim **2**, wherein the matrix comprises laminin.

4. The method of claim **2**, wherein the matrix comprises vitronectin.

5. The method of claim **2**, wherein the cells are contacted with the medium under hypoxic conditions.

6. The method of claim **2**, wherein the pluripotent stem cells are embryonic stem cells.

7. The method of claim **2**, wherein the pluripotent stem cells are induced pluripotent stem cells.

8. A method for cloning a human pluripotent stem cell, the method comprising the step of:

plating human pluripotent stem cells at cloning density in a defined albumin-free medium free of any component obtained from a non-human animal, the medium comprising water, salts, amino acids, vitamins, glucose, insulin, an FGF, selenium, transferrin, and one of TGF-β and NODAL, each in an amount sufficient to support human pluripotent stem cell cloning.

9. The method of claim **8**, wherein the medium further comprises a ROCK inhibitor.

10. The method of claim **9**, wherein the ROCK inhibitor is selected from the group consisting of HA100 and Y27632.

11. The method of claim **8**, wherein the medium further comprises blebbistatin.

12. A method of cryopreserving human pluripotent stem cells, the method comprising the step of:

freezing human pluripotent stem cells in a defined, albumin-free medium free of any component obtained from a non-human animal, the medium comprising water, salts, amino acids, vitamins, glucose, insulin, an FGF, selenium, transferrin, and one of TGF-β and NODAL, each in an amount sufficient to support human pluripotent stem cell proliferation.

13. The defined, albumin-free medium of claim **1**, consisting essentially of: water, salts, amino acids, vitamins, glucose, insulin, an FGF, selenium, transferrin, and one of TGF-β and NODAL.

14. The defined, albumin-free medium of claim **1**, wherein the medium comprises TGF-β.

15. The defined, albumin-free medium of claim **1**, wherein the medium comprises NODAL.

16. The defined, albumin-free medium of claim **1**, further comprising a ROCK inhibitor.

17. The defined, albumin-free medium of claim **16**, wherein the ROCK inhibitor is selected from the group consisting of HA100 and Y27632.

18. The defined, albumin-free medium of claim **1**, further comprising blebbistatin.

19. The defined, albumin-free medium of claim **1**, consisting of: water, salts, amino acids, vitamins, glucose, insulin, an FGF, selenium, transferrin, and one of TGF-β and NODAL.

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