FORMULATIONS AND METHODS FOR CULTURING EMBRYONIC STEM CELLS

FIELD OF THE INVENTION

The present invention relates to xeno-free formulations for use in the maintenance and derivation of embryonic stem cells, such as human embryonic stem cells.

BACKGROUND OF THE INVENTION

Human embryonic stem cells (hESCs) are pluripotent cells that have the potential to differentiate into all cell types of a human body. Human ESCs are of great therapeutic interest because they are capable of indefinite proliferation in culture and are thus capable of supplying cells and tissues for replacement of failing or defective human tissue. There are high expectations that, in the future, human ESCs will be proliferated and directed to differentiate into specific cell types, which can be transplanted into human bodies for therapeutic purposes.

Embryonic stem cells are difficult to maintain in culture because they tend to follow their natural cell fate and spontaneously differentiate. Most culture conditions result in some level of unwanted differentiation. Stem cells differentiate as a result of many intrinsic and extrinsic factors, including growth factors, extracellular matrix molecules and components, environmental stressors and direct cell-to-cell interactions. Long-term proliferative capacity, pluripotent developmental potential after prolonged culture and karyotypic stability are the key features with respect to the utility of stem cell cultures.

The undifferentiated stage of hESCs can be monitored by judging the morphological characteristics of the cells. Undifferentiated hESCs have a characteristic morphology with very small and compact cells. While some differentiated cells usually appear at the margin of colonies of hESCs, an optimal culture method provides growth support with minimal amount of differentiated cells. There are several biochemical markers that are used to track the status of undifferentiated stage of hESCs such as the transcription factor Oct4 and Nanog as well as cell surface markers TRA-1-60, TRA-1-81, SSEA-3/4. These markers are lost when hESCs begin to differentiate to any cell lineage.

Basic techniques to create and culture hESCs have been described. There are, however, limitations and drawbacks to many of the procedures currently used to culture hESCs. Embryonic stem cells have typically been

derived and proliferated in culture medium containing animal serum (especially fetal bovine serum) or other animal derived products to permit the desired proliferation during such culturing. The presence of animal derived products in hESC culture media has several problems. Firstly, animal derived products may contain toxic proteins or immunogens that evoke an immune response in the recipient and thus lead to rejection upon transplantation (Martin et al., Nat Med. 2005 Feb;11(2):228-32). Secondly, the use of animal products increases the risk of contamination by animal pathogens, such as viruses, mycoplasma and prions, which can pose a serious health risk in cell therapy and other clinical applications (Healy et al., Adv Drug Deliv Rev. 2005 Dec 12;57(13):1981-8). In fact government agencies are increasingly regulating, discouraging and even forbidding the use of cell culture media containing animal derived products, which may contain such pathogens.

To overcome the drawbacks of the use of serum or animal extracts, a number of serum-free media have been developed. Price et al. disclose in US Patent Publication 2002/0076747 a serum replacement, Knockout™ SR medium (Invitrogen, Carlsbad, CA), frequently used in hESC culture. This formulation, however, contains animal derived products, such as bovine serum albumin, and hence is not completely free of xeno-derived components. Several xeno-free serum replacements and media are currently available (X-Vivo 10, X-Vivo 20, SSS, Lipumin, Serex, Plasmanate, SR3). These serum replacements often are specifically formulated to support the culture of a single cell type. Furthermore, Thomson et al. disclose in US Patent Publication 2006/0084168 a serum- and xeno-free cell culture medium, which allegedly support the growth of embryonic stem cells in culture.

Unfortunately, Rajala et al. demonstrate in Hum. Reprod., 2007, 22(5):1231-1238, that all the above-mentioned formulations permit the cultivation of hESCs only for a few passages during an adaptation phase to a new medium without severe differentiation, followed by rapid differentiation upon subsequent passages.

Several feeder-free culture methods have been developed for hESCs. Many of these feeder-free methods utilize animal derived components. In addition, these methods suffer from inadequate reproducibility and currently are unable for long-term maintenance of undifferentiated hESCs with normal karyotype. Feeder-free cultures with enzymatic passaging may also be so demanding for the hESCs that they become more prone to abnormalities.

Because of these problems associated with currently known culture media for hESCs, there is a great need for a defined xeno-free culture medium that reproducibly supports robust growth of hESCs for long-term without substantial differentiation while maintaining pluripotency and normal cell karyotype, and which is compatible with the expected regulatory guidelines governing clinical safety and efficacy.

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to a xeno-free serum replacement formulation comprising retinol. The serum replacement may further comprise a carrier protein selected from a group consisting of fetuin, α-fetoprotein and combinations thereof. In one embodiment, the serum replacement further comprises albumin, preferably human serum albumin.

In one embodiment, the serum replacement comprises at least one lipid or lipid derivative. Preferably, said lipid is selected from the group consisting of lipoproteins such as VLDL, LDL, HDL and cholesterol, phospholipids such as phosphatidylcholine, lysophosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, phosphatidylethanolamine and sphingosine-1-phosphate, and fatty acids such as linoleic acid, conjugated linoleic acid, gamma-linoleic acid, linolenic acid, arachidonic acid, oleic acid, eicosapentaenoic acid, docosahexaenoic acid, palmitic acid, palmitoleic acid, stearic acid, myristic acid and their derivatives (e.g. prostaglandins).

In another embodiment, the serum replacement further comprises at least one amino acid, vitamin, transferrin or transferrin substitute, antioxidant, insulin or insulin substitute, and trace element.

The present invention further relates to a xeno-free cell culture medium comprising a basal medium and a serum replacement according to the present invention. Preferably, said basal medium is selected from the group consisting of KO-DMEM, DMEM, MEM, BME, RPMI 1640, F-10, F-12, aMEM, G-MEM, Iscove's Modified Dulbecco's Medium, HyQ ADCF-Mab and any combinations thereof. In one embodiment, the cell culture medium is supplemented with a fibroblast growth factor, non-essential amino acids, β-mercaptoethanol, L-glutamine and antibiotics.

The present invention also relates to the use of the above-mentioned serum replacement and cell culture medium for culturing stem cell,

preferably embryonic stem cells and more preferably human embryonic stem cells. In one embodiment, said culturing is performed on a feeder cell layer.

The present invention further relates to a method for culturing stem cells, preferably embryonic stem cells and more preferably human embryonic stem cells. The method comprises the steps of contacting said cells with a xeno-free medium according to the present invention and cultivating said cells under conditions suitable for cell culture. In one embodiment, stem cells are cultured on a feeder cell layer.

Furthermore, the present invention relates to a method for initiating a new embryonic stem cell line. The method comprises the steps of providing isolated cells of embryonic origin, contacting said cells with a xeno-free medium according to the present invention, and cultivating said cells under conditions suitable for cell culture. In one embodiment, said cultivation is performed on a feeder cell layer. In a further embodiment, the xeno-free medium according to the present invention is supplemented with laminine, such as human placental laminine, and fibronectin, such as human plasma fibronectin. Preferably, said cells are human embryonic stem cells.

BRIEF DESCRIPTION OF THE DRAWINGS

In the following the invention will be described in greater detail by means of preferred embodiments with reference to the attached drawings, in which

Figures 1A and 1B are light microscopic images of hESC lines during a long-term culture in the culture medium according to the present invention. Figure 1A is a image of HS346 cells, passage 12; figure 1B is a image of HS401 cells, passage 10.

Figures 2A – 2T show light and fluorescent microscopic images of hESCs cultured in different xeno-free culture media or serum replacements unable to maintain undifferentiated growth of the cells. One representative hESC colony after 1 passage in 20% Lipumin (Fig. 2A), in 20% Plasmanate (Fig. 2C), in 40% Plasmanate (Fig. 2E), in 20% SerEx (Fig. 2G), in 20% SR3 (Fig. 2 I), in 20% SSS (Fig. 2K), in X-Vivo 10 (Fig. 2M), in X-Vivo 20 (Fig. 2O), and after 7 passages in TeSR1 (Fig. 2Q) or in control hES medium (Fig. 2S) are shown. The expression of Nanog and SSEA-1 in the corresponding hESC colonies are shown in Figs. 2B, 2D, 2F, 2H, 2J, 2L, 2N, 2P, 2R, and 2T, respectively.

Figures 3A – 3H illustrate hESCs during the adaptation phase in the culture medium according to the present invention or in HEScGRO medium. Fig. 3A represents the adaptation phase 20:80 to HEScGRO medium. Fig. 3B represents the adaptation phase 50:50 to HEScGRO medium. Fig. 3C represents the adaptation phase 80:20 to HEScGRO medium. Fig. 3D represents hESCs after the adaptation phase to HEScGRO medium at passage 1. Fig. 3E represents the adaptation phase 20:80 to the culture medium according to the present invention. Fig. 3F represents the adaptation phase 50:50 to the culture medium according to the present invention. Fig. 3G represents the adaptation phase 80:20 to the culture medium according to the present invention. Fig. 3H represents hESCs after the adaptation phase to the culture medium according to the present invention at passage 1.

Figures 4A – 4F show immunohistochemical stainings of hESC lines after long term culture in the culture medium according to the present invention. Fig 4A shows a staining of HS346 cells, passage 10, with Dapi. Fig. 4B shows a Nanog staining of HS346 cells, passage 10. Fig. 4C shows a SSEA3 staining of HS346 cells, passage 10. Fig. 4D shows a staining of HS401 cells, passage 7, with Dapi. Fig. 4E shows a Nanog staining of HS401 cells, passage 7. Fig. 4 F shows a SSEA3 staining of HS401 cells, passage 7.

Figures 5A and 5B are light microscopic images of new hESC lines 06/015 (passage 6) and 07/046 (passage 51) respectively, after derivation and culture using the culture medium according to the present invention.

Figures 6A, 6B, 6C and 6D show immunohistochemical stainings of new hESC lines 06/015 (passage 6) and 07/046 (passage 44) after derivation and culture using the culture medium according to the present invention. Fig. 6A represents a Nanog staining of 06/015 cells, passage 6, Fig. 6B represents TRA-1-60 staining of 06/015 cells, passage 6, Fig. 6C represents a Nanog staining of 07/046 cells, passage 44, while Fig. 6D represents TRA-1-60 staining of 07/046 cells, passage 44.

Figure 7 is a light microscopic image of a hESC culture in a standard hES medium with increased osmolarity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a serum replacement formulation and to a culture medium comprising said serum replacement. Furthermore, the present invention relates to methods for stem cell culture and maintenance.

Specifically, the invention provides a culture medium for stem cells, preferably human embryonic stem cells (hESCs). Notably, said culture medium supports maintenance and proliferation of embryonic stem cells, such as hESCs, in a substantially undifferentiated state. Advantageously, said culture medium supports maintenance and proliferation of embryonic stem cells, preferably hESCs, over numerous *in vitro* passages. Additionally, the embryonic stem cells cultured in the culture medium according to the present invention are substantially undifferentiated, retain their pluripotency and maintain their genomic integrity. For therapeutic applications, the culture medium according to the invention comprises no components, such as feeder cells, conditioned medium, serum or other medium components, purified from a non-human animal source. More preferably, the culture medium comprises components that are synthesized using recombinant or chemical methods.

The present invention provides a defined xeno-free serum replacement composition that can be used to supplement any suitable basal medium for use in the *in vitro* maintenance and proliferation of stem cells, preferably embryonic stem cells, such as primate (e.g. human) embryonic stem cells. Said serum replacement may be used to supplement both serum-free and not serum-free basal mediums, or any combinations thereof.

The serum replacement according to the present invention is suitable for maintaining and proliferating stem cells in a substantially undifferentiated state, while maintaining both the pluripotency and and the karyotype of the cells, for at least about 20 passages. In other embodiments, the maintenance of stem cells is supported for at least about 30, and preferably at least about 50 passages.

By the term xeno-free it is meant herein that the origin of the reagent is not from a foreign source, i.e. does not contain material of non-human animal origin when human stem cells are to be cultured. Suitable xeno-free sources for culturing human stem cells may include chemical synthesis or synthetic preparations or isolation, preparation or purification of the reagent of interest from bacteria, yeasts, fungi, plants and humans.

By the term serum replacement it is meant herein a composition that may be used to replace animal serum in a final cell culture medium. A conventional serum replacement comprises typically vitamins, albumin, lipids, amino acids, transferrin, antioxidants, insulin and trace elements. The final cell culture medium may further comprise growth factors, non-essential amino

acids, β-mercaptoethanol, L-glutamine and/or antibiotics added directly to the basal medium or further comprised in the serum replacement.

It has now been surprisingly found, that retinol (i.e. vitamin A) plays a crucial role in maintaining stem cells in an undifferentiated state. The effect of different vitamins on the undifferentiated growth of human embryonic stem cells was tested by providing retinol (20 μM), nicotinamide (5 mM and 10 mM) or commercial Vitamin Mix (1%) containing nicotinamide but not retinol (MEM Vitamin Solution (100x), cat. No. 11120-037, provided by Gibco/Invitrogen) to human embryonic stem cells (Table 1). Retinol increased the number of undifferentiated colonies considerably. Nicotinamide had the opposite effect to the embryonic stem cells, promoting their differentiation. Vitamin Mix had no effect on the undifferentiated growth of embryonic stem cells. Undesired results, i.e. differentiation of stem cells, have been previously reported with retinoic acid, a derivative of retinol, e.g. by Schuldiner et al. in Brain Res., 2001, 913(2):201-205, incorporated herein by reference.

TABLE 1. The effect of different vitamins on the undifferentiated growth of human embryonic stem cells

Cell line	Control	Vitamin A 20 μM	Nicotinamide 10 mM	Nicotinamide 5 mM	MEM Vitamin Mix 1%
HS346	0	++	--	-	0
06/015	0	+++	--	-	0

Accordingly, the serum replacement according to the present invention is a xeno-free formulation comprising at least retinol. Typically, retinol is used in a concentration of about 0.1 μM to about 50 μM, preferably about 10 μM to about 40 μM, and more preferably about 20 μM.

The serum replacement may further contain other vitamins such as ascorbic acid, biotin, choline chloride, D-Ca Pantothenate, Folic acid, i-inositol, niacinamide, Pyridoxal, Pyridoxine, Riboflavin, thiamine, Vitamin B 12, Vitamin D2. Typically several vitamins are included in the basal medium and additional vitamin supplementation can be added to the final medium. Suitable concentrations of vitamins in the serum replacement and the final medium according to the present invention, can be readily determined by a skilled person using routine methods well known in the art. Typically, thiamine is used in a concentration of about 9 mg/l, while ascorbic acid is used in a

concentration of about 50 µg/ml in the cell culture medium according to the present invention.

It has also been surprisingly found that fetuin and α-fetoprotein may be used to promote growth of stem cells. Table 2 shows the effect of fetuin and α-fetoprotein on growth rate and size of embryonic stem cell colonies. All formulations shown contained human serum albumin at a concentration of 10 mg/ml. Fetuin was shown to increase the colony size and growth rate the most at a concentration of 0.1 mg/ml and α-fetoprotein at a concentration of 0.05 mg/ml. When fetuin and α-fetoprotein were both included in the formulation, the growth promoting effect was even slightly better than in the formulations including them individually.

TABLE 2. Effect of fetuin and α-fetoprotein on the growth rate and size of the embryonic stem cell colonies (F=Fetuin, A=α-fetoprotein)

	HS346	06/015
Control	0	0
F 0.05 mg/ml	++	++
F 0.10 mg/ml	+++	++
F 0.20 mg/ml	+	+
A 0.05 mg/ml	+++	+++
A 0.10mg/ml	++	+
A 0.20 mg/ml	+	+
A 0.05 mg/ml + F 0.10 mg/ml	+++	+++

Accordingly, the serum replacement according to the present invention may further comprise fetuin, α-fetoprotein and/or any combination thereof. Fetuin and α-fetoprotein are commercially available fetal carrier proteins present at a high plasma concentration in fetal plasma. Fetuin and α-fetoprotein could be used to replace albumin in the serum replacement, but due to their high price it may be feasible to use them in combination with albumin. In one preferred embodiment, the serum replacement comprises about 0.5 mg/ml fetuin and about 0.25 mg/ml α-fetoprotein. In such an embodiment, a basal medium is to be supplemented with 20% serum replacement. In general, a typical final cell culture medium comprises from about 0.01 mg/ml to about 1 mg/ml fetuin and/or α-fetoprotein.

Albumin substitutes suitable for use in the present invention include any compound, which may be used instead of albumin and has essentially similar effects as albumin. Suitable concentration of albumin or albumin substitute in the serum replacement and in the final culture medium according to the present invention, can be readily determined by a skilled person using routine methods well known in the art. Typically, albumins or albumin substitutes are used in the final medium in the range of about 1 mg/ml to about 20 mg/ml, preferably of about 5 mg/ml to about 15 mg/ml. In one embodiment, albumin is present at about 10 mg/ml in the cell culture medium according to the present invention.

The serum replacement according to the present invention further comprises at least one lipid or lipid derivative. Lipids and lipid derivatives suitable for use in the present invention include, but are not limited to lipoproteins such as very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and cholesterol; phospholipids such as phosphatidylcholine, lysophosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, phosphatidylethanolamine and sphingosine-1-phosphate; fatty acids such as linoleic acid, conjugated linoleic acid, gamma-linoleic acid, linolenic acid, arachidonic acid, oleic acid, eicosapentaenoic acid, docosahexaenoic acid, palmitic acid, palmitoleic acid, stearic acid, myristic acid and their derivatives such as prostaglandins.

According to various embodiments of the present invention, the serum replacement may comprise e.g. at least two, at least three or at least four of the lipids or lipid derivatives given above. In one specific embodiment, the serum replacement comprises linoleic acid, arachidonic acid, and oleic acid. In another embodiment the serum replacement further comprises sphingosine-1- phosphate.

A person skilled in the art can readily determine suitable concentrations of lipids and lipid derivatives for use in the present invention using standard methods known in the art. In some embodiments, sphingosine-1-phosphate is used in a concentration to result in a typical range of 1-20 μM sphingosine-1-phosphate in the final cell culture medium according to the present invention.

In one embodiment, the serum replacement comprises retinol, fetuin, α-fetoprotein, at least one lipid or lipid derivative and at least one ingredient, preferably free of endotoxins, selected from the group consisting of

albumins, albumin substitutes, amino acids, vitamins, transferrins, transferrin substitutes, antioxidants, insulin or insulin substitutes, trace elements, and growth factors. Such ingredients are present in the serum replacement composition in a concentration sufficient to support the proliferation of stems cells in a substantially undifferentiated state, while maintaining both the pluripotency and the karyotype of the cells.

Amino acids suitable for use in the present invention include, but are not limited to amino acids, such as glycine, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L-hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, and their D-forms and derivatives. Suitable concentrations of amino acids can be readily determined by a skilled person using routine methods well known in the art. Typical concentration ranges are presented in Table 3. The serum replacement according to the present invention may contain additional non-essential amino acids, such as L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycine,L-proline, L-serine, and their D-forms and derivatives. Such additional non-essential amino acids may be included in the serum replacement or added directly to the final cell culture medium according to the present invention. Non-essential amino acids may be provided as a commercially available mixture, such as MEM non-essential amino acids (NEAA) provided by Invitrogen. Typically, the concentration of said mixture in the final medium according to the present invention is about 1%.

L-glutamine is preferably added to the cell culture medium according to the present invention as a stabilized, dipeptide form of L-glutamine such as Glutamax (Invitrogen, 2 mM). When desired, L-glutamine may be included in the serum replacement according to the present invention.

Transferrins are involved in iron delivery to cells, controlling free iron concentration in biological fluids and preventing iron-mediated free radical toxicity. Suitable transferrin substitutes for use in the present invention include any compound which may be used instead of transferrin and has essentially similar effects as transferrin. Such substitutes include, but are not limited to, iron salts and chelates (e.g., ferric citrate chelate or ferrous sulfate). Suitable concentrations of transferrin or transferrin substitute in the serum replacement and the final medium according to the present invention, can be readily determined by a skilled person using routine methods well known in the art. Typically, suitable range of transferrin or transferrin substitute in the final

medium according to the present invention is about 1 µg/ml to about 1000 µg/ml, preferably about 5 µg/ml to about 100 µg/ml, and more preferably, about 5 µg/ml to about 10 µg/ml. In one embodiment, transferrin is present at about 8 µg/ml in the cell culture medium according to the present invention.

Antioxidants suitable for use in the present invention include, but are not limited to glutathione and ascorbic acid. Suitable concentrations of antioxidants in the serum replacement and the final medium according to the present invention can be readily determined by a skilled person using routine methods well known in the art. According to one embodiment, glutathione is present at 1,5 µg/ml and ascorbic acid is present at 50 µg/ml in the cell culture medium according to the present invention.

Insulin substitutes suitable for use in the present invention include any compound, which may be used instead of insulin and has essentially similar effects as insulin. Suitable concentration of insulin or insulin substitute in the serum replacement and the final medium according to the present invention can be readily determined by a skilled person using routine methods well known in the art. Typically, suitable range of insulin in the final medium is about 1 µg/ml to about 1000 µg/ml, preferably about 1 µg/ml to about 100 µg/ml, more preferably about 50 µg/ml to about 15 µg/ml. In one embodiment, insulin is present at about 10 µg/ml.

Trace elements suitable for use in the present invention include, but are not limited to Mn^{2+}, Si^{4+}, Mo^{6+}, V^{5+}, Ni^{2+}, Sn^{2+}, Al^{3+}, Ag^{+}, Ba^{2+}, Br^{-}, Cd^{2+}, Co^{2+}, Cr^{3+}, F^{-}, Ge^{4+}, I^{-}, Rb^{+}, Zr^{4+} and Se^{4+} and salts thereof. Suitable concentrations of trace elements or salts thereof can be readily determined by a skilled person using routine methods known in the art. Commercially available trace element compositions such as Trace Elements B and C provided by CellGro Mediatech Inc. may also be used. When desired, trace elements Cu^{2+} and/or Zn^{2+} may be included e.g. in the form of a commercially available Trace Element A composition provided by CellGro Mediatech Inc.

Furthermore, the present inventors have shown that lithium chloride may be harmful for embryonic stem cells resulting in differentiation thereof. Thus, in a specific embodiment, the serum replacement is devoid lithium chloride.

Growth factors suitable for use in the present invention include fibroblast growth factors (FGFs) such as basic FGF (bFGF or FGF-2). Suitable range of FGF in final medium according to the present invention is about 1

ng/ml to about 1000 ng/ml, preferably about 2 ng/ml to about 100 ng/ml, and more preferably about 4 ng/ml to about 20 ng/ml. In one embodiment, FGF is present at about 8 ng/ml. While FGF is preferably used, other materials, such as certain synthetic small peptides (e.g. produced by recombinant DNA variants or mutants) designed to activate fibroblast growth factor receptors, may be used instead of FGF. Growth factors may be included in the serum replacement according to the present invention or they may be added separately to the final cell culture medium according to the present invention.

Antibiotics can also be used, to avoid contamination of the serum replacement or the medium according to the present invention. Suitable antibiotics or combinations thereof, as well as suitable concentrations are apparent to a person skilled in the art. However, if the medium is to be used in the culture of cells for clinical applications one might want to avoid the use of antibiotics.

Furthermore, β-mercaptoethanol may be included in the serum replacement according to the present invention or it may be added separately into the final culture medium according to the present invention. Typically, the final concentration of β-mercaptoethanol is about 0.1 mM in the culture medium.

In obvious embodiments of the present invention, any of the components of the serum replacement described above may be added directly into a basal medium to provide a final cell culture medium instead of being provided in the serum replacement according to the present invention.

The present invention further provides a defined xeno-free culture medium for the *in vitro* maintenance and proliferation of stem cells, preferably embryonic stem cells. Said culture medium comprises a basal medium and a serum replacement composition set forth herein. Suitable basal media for use in the present invention include, but are not limited to KnockOut Dulbecco's Modified Eagle's Medium (KO-DMEM), Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, a Minimal Essential Medium (aMEM), Glasgow's Minimal Essential Medium (G-MEM), Iscove's Modified Dulbecco's Medium and HyQ ADCF-MAb (HyClone) and any combinations thereof. According to one preferred embodiment the basal medium is KO-DMEM. The term “basal medium” refers to any medium which is capable of supporting growth of ES cells, and in general supplies standard inorganic salts, vitamins, glucose, a

buffer system and essential amino acids. Preferably the basal medium can be supplemented with about 1g/L to about 3.7 g/L sodium bicarbonate. Preferably, the basal medium is supplemented with about 2.2 g/L sodium bicarbonate.

The osmolarity of the culture affects to the success and vitality of stem cell cultures. Osmolarity, measured in milli-osmoles, is a measure of the number of dissolved particles in a solution, which is a measurement of the osmotic pressure that a solution will generate. Normal human serum has an osmolarity of about 290 milli-osmoles. Media for *in vitro* culture of other mammalian cells vary in osmolarity, but some media have an osmolarity as high as 330 milli-osmoles. Preferably, the osmolarity of the medium according to the present invention is between about 280 and about 330 mOsmol. However, osmolarity of the medium can be as low as about 260 mOsmol and as high as about 340 mOsmol. In one embodiment according to the present invention, hESCs are grown in an osmolarity of about 320-330 milli-osmoles.

According to one embodiment, lipids, albumin, amino acids, vitamins, transferrin, antioxidants, insulin, and trace elements are included in the serum replacement, while growth factors, non-essential amino acids, β-mercaptoethanol, L-glutamine and antibiotics are added directly to the cell culture medium. Final composition of one preferred culture medium is exemplified in Table 3.

TABLE 3. Final composition of one preferred culture medium according to the present invention.

Ingredients marked with an asterisk are provided in the form of a serum replacement according to the present invention.

Ingredient	Concentration in one preferred culture medium (mg/l)	Typical concentration range (mg/ml)
Fatty acids*		
Linoleic acid	1	0-1000
Arachidonic acid	1	0-1000
Oleic acid	1	0-1000
Sphingosine-1-phosphate	10 µM	0-20 µM

Amino acids*		
Glycine	53	0-200
L-histidine	183	0-250
L-isoleucine	615	0-700
L-methionine	44	0-200
L-phenylalanine	336	0-400
L-proline	600	0-1000
L-hydroxyproline	15	0-100
L-serine	162	0-250
L-threonine	425	0-500
L-tryptophan	82	0-100
L-tyrosine	84	0-100
L-valine	454	0-500
Vitamins*		
Thiamine	9	0-20
Retinol	20 µM	0-100 µM
Antioxidants*		
Glutathione	1,5	0-20
Ascorbic acid	50	0-200
Proteins		
Human serum albumin*	10000	0-50000
Fetuin*	100	10-1000
α-fetoprotein*	50	10-1000
Insulin*	10	0-200
Transferrin*	8	0-200
FGF	0.008	0.004-0.5
Trace elements*		
$MnSO_4 \cdot H_2O$	0.17	0-10
$Na_2SiO_3 \cdot 9H_2O$	140	0-200
Molybdic acid Ammonium salt	1.24	0-10
NH_4VO_3	0.65	0-10
$NiSO_4 \cdot 6H_2O$	0.13	0-10
$SnCl_2$ (anhydrous)	0.12	0-10
$AlCl_3 \cdot 6H_2O$	1.20	0-10
$AgNO_3$	0.17	0-10

$Ba(C_2H_3O_2)_2$	2.55	0-10
KBr	0.12	0-10
$CdCl_2$	2.28	0-10
$CoCl_2 \cdot 6H_2O$	2.38	0-10
$CrCl_3$ (anhydrous)	0.32	0-10
NaF	4.20	0-10
GeO_2	0.53	0-10
KI	0.17	0-10
RbCl	1.21	0-10
$ZrOCl_2 \cdot 8H_2O$	3.22	0-10
Selenium	0.00001	0.00000-0.1
Other ingredients		
NEAA	1%	0-10%
L-glutamine	2 mM	1-2 mM
β-mercaptoethanol	0.1 mM	0-1 mM
antibiotics	50 U/ml	0-100 U/ml
Basal medium		

The serum replacement or the culture medium according to the present invention may be provided in a liquid or a dry form. Furthermore, they may be provided as any suitable concentrated formulation. As an example, basal medium may be supplemented with 10%, 15% or 20% (vol/vol) serum replacement so as to result in final concentrations of ingredients as given above. When desired, ingredients of the serum replacement or the medium may be divided into compatible subformulations.

The culture medium according to the present invention for embryonic stem cell, preferably hESCs, supports proliferation of embryonic stem cells in a substantially undifferentiated state, while maintaining the potential to differentiate into derivatives of endoderm, mesoderm and ectoderm tissues and maintaining the karyotype of the stem cells, for at least about 20, preferably at least about 30, and more preferably at least about 50 passages.

The culture medium according to the present invention is useful in a plethora of applications. Stem cells may be proliferated in the medium according to the invention, and in some applications differentiated for therapeutic applications. Stem cells cultured in the culture medium according to the present invention may be used to study cell proliferation and

differentiation, including identifying molecules that affect one or both processes; used to screen for drug candidates that affect proliferation, differentiation and/or regeneration; cells genetically modified and used to produce proteins or other molecules.

The present invention thus provides a method for culturing and maintaining stem cells in a xeno-free culture. Said method comprises contacting stem cells with the culture medium according to the present invention, and cultivating said cells under conditions suitable for stem cell culture. Such conditions are apparent to a person skilled in the art.

The compositions and methods according to the present invention are useful in the culturing of stem cells, preferably embryonic stem cells, and more preferably primate embryonic stem cells. Preferably, primate embryonic stem cells that are cultured using this method are hESCs that are true embryonic stem cell lines in that they: (i) are capable of indefinite proliferation in vitro in an undifferentiated state; (ii) are capable of differentiation to derivatives of all three embryonic germ layers (endoderm, mesoderm, and ectoderm), even after prolonged culture; and (iii) maintain a normal karyotype throughout prolonged culture. Embryonic stem cells are, therefore, referred to as being pluripotent.

Stem cells that can be cultured in the medium according to the present invention may be from any animal, preferably mammals and more preferably, primates. Preferred cell types that can be cultured in a substantially undifferentiated state using the defined culture medium of the invention include stem cells derived from humans, monkeys, and apes. With regard to human stem cells, hESCs are preferred. hESCs can be derived from an embryo, preferably from a pre-implantation embryo, such as from a blastula or a morula. Stem cells derived from non-primate mammals, such as mice, rats, horses, sheep, pandas, goats and zebras, can also be cultured in the medium of the invention. While the culture medium is preferably used for culturing embryonic stem cells, it may be used for culturing adult stem cells, such as, but not limited to, hematopoietic stem cells (HSCs). The art is replete with information of both embryonic and adult stem cells. Stem cells, including human embryonic stem cells, cultured in accordance with the present invention can be obtained from any suitable source using any appropriate technique, including, but not limited to, immunosurgery. For example, procedures for isolating and growing human embryonic stem cells are described in U.S. Pat.

No. 6,090,622. Procedures for obtaining Rhesus monkey and other non-human primate embryonic stem cells are described in U.S. Pat. No. 5,843,78 and international patent publication WO 96/22362. In addition, methods for isolating Rhesus monkey embryonic stem cells are described by Thomson et al., (1995, Proc. Natl. Acad. Sci. USA, 92:7844- 7848).

The present invention further provides a method for derivation, or initiation, of new embryonic stem cell lines. The method comprises the steps of providing isolated cells of embryonic origin, contacting said cells with a xeno-free medium according to the embodiments of the present invention, and cultivating said cells under conditions suitable for cell culture. In one embodiment, the medium is supplemented with laminine, such as human placental laminine, and fibronectin, such as human plasma fibronectin. Preferably, laminine and fibronectin are used in a concentration of 5 µg/ml.

The compositions and methods according to the present invention may optionally be used for culturing and/or initiating stem cell lines on a feeder cell layer. Suitable feeder cells include but are not limited to fibroblasts, such as human foreskin fibroblasts, e.g. CRL-2429 (ATCC, Mananas, USA).

In some embodiments, the compositions and methods according to the present invention are used for feeder cell-free culture of stem cells.

Example 1. Human ESC cultured in a xeno-free culture media according to the present invention remain morphologically undifferentiated.

Three hESC lines HS237, HS346 and HS401 (Hovatta *et al.*, Hum Reprod. 2003 Jul;18(7):1404-9, Inzunza *et al.*, Stem Cells. 2005 Apr;23(4):544-9) were initially derived and cultured in a standard hES medium (disclosed in US 2002/0076747) containing 80% (vol/vol) KnockOut DMEM (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 20% (vol/vol) KnockOut Serum Replacement (ko-SR, Invitrogen), 2 mM Glutamax (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 0.1 mM MEM non-essential amino acids (Cambrex Bio Science), 50 U penicillin/ml-50 µg streptomycin/ml (Cambrex Bio Science) and 8 ng/ml recombinant human basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN, USA). Commercially available human foreskin fibroblast cells (CRL-2429, ATCC, Mananas, USA) were used as feeder cells.

Human ESC were gradually adapted to test culture conditions using an increasing proportion of the culture medium according to the present

invention (with ratios of said culture medium to the standard hES media at 20:80, 50:50, 80:20) up to 100% during four weeks of culture. The culture medium according to one embodiment of the present invention contained bFGF (8 ng/ml; R&D Systems), human serum albumin (10 mg/ml; Sigma or Vitrolife), insulin (10 ug/ml; Invitrogen), transferrin (8 ug/ml; Sigma), Glutathione (1.5 µg/ml, Sigma), Thiamine hydrochloride (9 µg/ml, Sigma), Ascorbic acid (50 µg/ml, Sigma), Amino acids (as listed in Table 3), Trace elements B and C (1:1000, Cellgro, Herndon, VA, USA), linoleic acid (1 µg/ml, Sigma), arachidonic acid (1 µg/ml, Cayman Chemicals), oleic acid (1 µg/ml, Cayman Chemicals), retinol (20 µM, Sigma), sphingosine-1 phosphate (10 µM, Sigma) in KODMEM, further supplemented with 2 mM Glutamax, 0.1 mM MEM non-essential amino acids and 0.1 mM β -mercaptoethanol. All medium components were synthetic, recombinant or of human origin. Osmolarity was adjusted to 320-330 mOsm/Kg with 5 M NaCl. Cells were mechanically passaged every 6-8 days to new mitotically inactivated feeder cells.

To determine whether hESCs grown in the culture medium according to the present invention were maintained in an undifferentiated state, the morphology of the cells was examined after every passage. Human embryonic stem cell line HS237 was maintained in the culture medium according to the present invention at least for 23 passages, HS346 for at least 15 passages and HS401 for at least 17 passages. The morphology of hESC lines remained undifferentiated after long-term culture in the culture medium according to the present invention (Fig. 1).

Example 2. Comparison of a culture medium according to the present invention to HesGro and other commercially available xeno-free serum replacements.

In order to test different culture conditions and the suitability of the culture conditions for long-term maintenance of human ESCs, an evaluation assay was performed in which hESCs were cultured under different xeno-free test conditions. The test conditions, cell lines and passage numbers employed are listed in Table 4. Human ESCs were gradually adapted to different test media using an increasing proportion of test media (with ratios of test media to hES media at 20:80, 50:50, 80:20) up to 100% test media during the four weeks of culture. The differentiation was first judged by morphology and then confirmed by immunofluoresence analysis. The hESC colonies grown in the

commercially available culture media (Lipumin, SerEx, SSS, SR3, TeSR1, Plasmanate, X-vivo10, X-vivo 20 and human serum) showed an increased expression of a marker common to the differentiated hESC (SSEA-1, 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and were negative to a marker common to the undifferentiated hESCs (Nanog, 1:200, Santa Cruz Biotechnology) (Figure 2). Human ESC line HS237 cultured in hES medium was used as a control in immunofluoresence analysis (Figure 2).

The culture medium according to the present invention was also compared to a xeno-free commercially available proprietary HEScGRO medium (Chemicon) developed for hESCs. HEScGRO medium was unable to maintain undifferentiated state of hESCs. The differentiation began already during the adaptation phase with hESCs cultured with HEScGRO medium. (Figure 3). The results clearly showed that HEScGRO medium is not able to maintain the undifferentiated growth of hESCs. Only the culture medium according to the present invention of the xeno-free culture media tested was able to maintain the undifferentiated growth of hESCs on human feeder cells.

To confirm that hESCs grown in the culture medium according to the present invention for long term remain undifferentiated, the expression of stem cell markers Nanog (1:200, Santa Cruz Biotechnology), SSEA3 (1:200, Santa Cruz Biotechnology) was examined (Figure 4).

Table 4: The test conditions, cell lines and passage numbers employed

Test reagent	**Cell line and starting passage**	**Medium composition [a]**
Control hES medium	HS181 p62 HS237 p59, p74 HS293 p49 HS306 p50	80% ko-DMEM; 20% ko-SR
Lipumin™ 10x	HS181 p62 HS237 p59, p74 HS293 p49	80/90% ko-DMEM; 10/20% Lipumin
Plasmanate	HS181 p62 HS237 p74	80/60% ko-DMEM; 20/40% Plasmanate
SerEx 10x	HS181 p62 HS237 p59	80/90% ko-DMEM; 10/20% SerEx

	HS293 p49	
Serum Substitute Supplement SSS	HS181 p62 HS237 p59, p74 HS293 p49	80/90% ko-DMEM; 10/20% SSS
SR3	HS181 p60 HS237 p61 HS293 p42	80/90% ko-DMEM; 10/20% SR3
TeSR1	HS237 p74 HS181 p62	DMEM/F12; 16.5 mg/ml HSA; 108 µg/ml transferrin; 196 µg/ml insulin; 6 mg/L thiamine HCl; 41.5 mg/L LiCl; 2 mg/L glutathione; 50 mg/L L-ascorbic acid; 1:1000 trace elements B and C solution; 0.1 mg/ml GABA; 0.02 mg/L sodium selenite; 0.127 µg/ml pipecolic acid; 0.6 ng/ml TGF-β1; 1:500 chemically defined lipid concentrate
X-Vivo 10	HS237 p59 HS293 p49	100% X-vivo10; 0.12 ng/ml TGFβ1
X-Vivo 20	HS181 p60 HS237 p61	100% X-vivo20
HEScGRO	HS346 p68-p71 HS401 p77-p80	100% HEScGRO
Medium of the present invention	HS237 p80-p103 HS346 p67-p81 HS401 p75-p91	As described in example 1

[a]In all other cases except HEScGRO, the test medium is supplemented with 2 mM Glutamax, 0.1 mM β-mercaptoethanol, 0.1 mM MEM

non-essential amino acids, 50 U penicillin/ml-50 µg streptomycin/ml, and 8 ng/ml bFGF. Appreviations: Ko-DMEM, KnockOut Dulbecco's modified Eagle medium; ko-SR, KnockOut Serum Replacement; DMEM/F12, Dulbecco's modified Eagle medium: F12 Nutrient mixture; HSA, human serum albumin; LiCl, litium chloride; GABA, γ-aminobutyric acid; TGF-β1, Transforming growth factor- β1.

Example 3. Characterization of pluripotency (RT-PCR) and karyotyping during long-term culture of several hESC lines.

To confirm that hESCs cultured in the medium according to the present invention still maintain their pluripotency *in vitro*, embryoid body formation and differentiation assays of HS237, HS346 and HS401 cells were performed. Subsequently, the embryoid bodies (EBs) continued to differentiate on plates for at least 20 days. The EBs were formed by mechanically dissecting hESC colonies and transferring the resulted pieces onto a culture dish without feeder cells. The EBs were cultured in the culture medium according to the present invention without bFGF for at least 20 days before the isolation of RNA. The hESC cultured in a standard hES medium were used as a control and samples were prepared similarly.

Total RNA was isolated from EBs using RNeasy mini kit (Qiagen, Valencia, CA, USA). The RNA extraction was performed according to the manufacturer instructions. Complementary DNA (cDNA) was synthesized from 50 ng of total RNA using Sensiscript Reverse Transcription Kit (Qiagen) according to manufacturer instructions. The expression of markers characteristic of ectoderm (neurofilament 68KD), endoderm (α-fetoprotein) and mesoderm (α-cardiac actin) development in EBs were determined using RT-PCR primers (Proligo, Sigma). Glyseraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control. The negative control contained sterilized water instead of cDNA template. The PCR reactions were carried out in the Eppendorf Mastercycler as follows: denaturation at 95 °C for 3 minutes and 40 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 minute, followed by final extension at 72 °C for 5 minutes. The PCR products were analyzed with electrophoresis on 1.5 % agarose gel containing 0.4 µg/ml ethidium bromide (Sigma) and DNA standard (MassRulerTM DNA Ladder Mix, Fermentas). In all hESC lines the EBs contained cells from three different lineages (Table 5). Hence, the culture

medium according to the present invention was sufficient to maintain the pluripotency of hESCs.

Table 5: RT-PCR analysis of embryoid bodies differentiated from HS237, HS346 and HS401 lines cultured in the culture medium according to the present invention.

Embryonic layer	Gene	HS237	HS346	HS401
ectoderm	neurofilament 68KD	+	+	+
endoderm	α-fetoprotein	+	+	+
mesoderm	α-cardiac actin	+	+	+
	GAPDH	+	+	+

No major translocations or other chromosomal changes were observed in karyotyping of the hESCs. Thus, hESCs cultured in the culture medium according to the present invention maintain their genomic integrity.

Example 4. Derivation of new hESC lines using a culture medium according to the present invention.

Using the culture medium according to the present invention, we have been able to derive new hESC line from surplus bad quality human embryo donated for stem cell research. A prior and informed consent was obtained from the donors of the embryos used in the derivation of new embryonic stem cell lines. Furthermore, Regea, Institute for Regenerative Medicine, University of Tampere, Finland has the approval of the Ethical Committee of Pirkanmaa Hospital District to derive and culture hESC lines.

This media as described in example 1 highly supported the derivation of new hESC lines. In addition this medium enabled the derivation procedure without any immunosurgery methods e.g using mechanical isolation of cells from embryo. Moreover, this medium enabled the derivation procedure using human fibroblasts cultured without animal-derived media thus suitable for production of hESC for clinical applications under GMP-standards and without any trace of animal-derived components. At derivation procedure, the media can be supplemented with 5 µg/ml human placental laminine and

human plasma fibronectin to increase attachment of cells during derivation process.

To determine whether new hESC lines were growing in the culture medium according to the present invention and maintained undifferentiated state, the morphology of the cells was examined after every passage (Figure 5). The new hESC lines derived using said culture medium were characterized by immunocytochemical staining with several markers specific for undifferentiated hESC (Figure 6) and pluripotency of the lines was determined with *in vitro* embryoid body formation as described above. In addition, the derived new hESC lines were determined to have maintained normal karyotype for 06/015 cells at passage 16 and for 07/046 cells at passages 20 and 44.

The composition of the formulation of the present invention was further optimized as described above in Table 2. It was found that fetuin and α-fetoprotein may be used to promote growth of stem cells. Fetuin was shown to increase the colony size and growth rate the most at a concentration of 0.1 mg/ml and α-fetoprotein at a concentration of 0.05 mg/ml. When fetuin and α-fetoprotein were both included in the formulation, the growth promoting effect was even slightly better than in the formulations including them individually.

Example 5. Characterization of the effect of osmolarity on hESCs.

To further demonstrate that the osmolarity of the medium for culturing hESCs should be less than 350 mOsm/kg, hESCs were cultured and monitored in the standard hES medium. Osmolarity of the medium was raised to 350 mOsm/kg with 5 M NaCl. The proliferation of hESCs decreased rapidly and excessive differentiation was observed. hESCs were maintained in hES medium with osmolarity of 350 mOsm/kg for 4 passages. HESCs showed reduced proliferation and excessive differentiation after 3 passages (Figure 7). On the other hand, hESCs cultured in an osmolarity of 326 mOsm/kg remained undifferentiated.

It will be obvious to a person skilled in the art that, as the technology advances, the inventive concept can be implemented in various ways. The invention and its embodiments are not limited to the examples described above but may vary within the scope of the claims.

All references cited are included herein by reference.

CLAIMS

1. A xeno-free serum replacement comprising retinol.

2. The serum replacement according to claim 1, further comprising a carrier protein selected from a group consisting of fetuin, α-fetoprotein and combinations thereof.

3. The serum replacement according to claim 2, further comprising serum albumin.

4. The serum replacement according to any one of claims 1-3, further comprising a lipid selected from the group consisting of lipoproteins, phospholipids and fatty acids.

5. The serum replacement according to claim 4, wherein said lipoproteins are selected from the group consisting of VLDL, LDL, HDL and cholesterol.

6. The serum replacement according to claim 4, wherein said phospholipids are selected from the group consisting of phosphatidylcholine, lysophosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, phosphatidylethanolamine and sphingosine-1-phosphate.

7. The serum replacement according to claim 4, wherein said fatty acids are selected from the group consisting of linoleic acid, conjugated linoleic acid, gamma-linoleic acid, linolenic acid, arachidonic acid, oleic acid, eicosapentaenoic acid, docosahexaenoic acid, palmitic acid, palmitoleic acid, stearic acid, myristic acid and their derivatives.

8. The serum replacement according to claim 7, wherein said derivative is a prostaglandin.

9. The serum replacement according to any of the preceding claims, further comprising at least one amino acid, transferrin or transferrin substitute, antioxidant, insulin or insulin substitute, and trace element.

10. The serum replacement according to claim 9, wherein said amino acid is selected from the group consisting of glycine, L-histidine, L-proline, L-hydroxyproline, L-serine, L-threonine, L-valine, L-isoleucine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine and D-isoforms and derivatives thereof.

11. The serum replacement according to claim 9, wherein said antioxidant is selected from the group consisting of glutathione and ascorbic acid.

12. The serum replacement according to claim 9, wherein said trace element is selected from the group consisting of Mn^{2+}, Si^{4+}, Mo^{6+}, V^{5+}, Ni^{2+}, Sn^{2+}, Al^{3+}, Ag^{+}, Ba^{2+}, Br^{-}, Cd^{2+}, Co^{2+}, Cr^{3+}, F^{-}, Ge^{4+}, I^{-}, Rb^{+}, Zr^{4+} and Se^{4+} and salts thereof.

13. A xeno-free cell culture medium comprising a basal medium and a serum replacement according to any of the preceding claims.

14. The cell culture medium according to claim 13, wherein said basal medium is selected from the group consisting of KO-DMEM, DMEM, MEM, BME, RPMI 1640, F-10, F-12, aMEM, G-MEM, Iscove's Modified Dulbecco's Medium, HyQ ADCF-Mab and any combinations thereof.

15. The cell culture medium according to claim 14 further comprising a fibroblast growth factor, non-essential amino acids, β-mercaptoethanol, L-glutamine and antibiotics.

16. A method for culturing stem cells, said method, comprising

a) contacting said cells with a xeno-free medium according to any one of claims 13 - 15, and

b) cultivating said cells under conditions suitable for cell culture.

17. The method according to claim 16, wherein said cells are embryonic stem cells.

18. The method according to claim 17, wherein said cells are human embryonic stem cells.

19. The method according to any one of claims 16 – 18, wherein said stem cells are cultured on a feeder cell layer.

20. A method for initiating a new embryonic stem cell line comprising

a) providing isolated cells of embryonic origin,

b) contacting said cells with a xeno-free medium according to any one of claims 13 – 15,

c) cultivating said cells under conditions suitable for cell culture.

21. The method according to claim 20, wherein said cultivation is performed on a feeder cell layer.

22. The method according to claim 20, wherein said medium is supplemented with laminine and fibronectin.