

DESCRIPTION

METHOD FOR PRODUCING THREE-DIMENSIONAL SKIN TISSUE, AND THREE-DIMENSIONAL SKIN TISSUE

Technical Field

[0001]

The present invention relates to a technique for producing a three-dimensional skin tissue from stem cells.

Background Art

[0002]

The development of skin organoid preparation techniques is underway for applications as a composition for skin transplantation and as screening tools for active ingredients of pharmaceuticals or cosmetics.

So far, there have been proposed techniques of producing an organoid similar to an epithelial structure in a living body using undifferentiated cells or mutated cells (Non Patent Literature 1), an organoid having both an epithelium and a stroma (Non Patent Literature 2), an organoid having a vascular network inside (Non Patent Literature 3, Non Patent Literature 4, and Patent Literature 1), and the like.

In particular, Non Patent Literature 5 and Patent Literature 2 propose techniques for producing a skin organoid having a multilayer structure of an epithelial

layer and a dermal layer.

[0003]

In addition, Non Patent Literature 6 discloses a technique of incising a spherical skin organoid in which an epidermal layer is formed inside, obtained by the method of Non Patent Literature 5, and culturing the skin organoid on a cell culture insert.

Citation List

Patent Literature

[0004]

Patent Literature 1: WO 2013/047639 A

Patent Literature 2: WO 2017/070506 A

Non Patent Literature

[0005]

Non Patent Literature 1: Sato T et al., Nature 459, pp262-265, 2009

Non Patent Literature 2: Spence JR et al., Nature 470, pp105-109, 2011

Non Patent Literature 3: Takebe T et al., Nature 499, pp481-484, 2013

Non Patent Literature 4: Takebe T et al., Cell Stem Cell 16, pp556-565, 2015

Non Patent Literature 5: J Lee et al., Nature 582, pp399-404, 2020

Non Patent Literature 6: Song-yi J et la., iScience.

25(10), 105150, 2022

Summary of Invention

Technical Problem

[0006]

In light of the foregoing prior arts, an object of the present invention is to provide a novel technique for producing a stratified three-dimensional skin tissue in which an epidermal layer and a dermal layer are layered, from stem cells.

Solution to Problem

[0007]

The present invention that achieves the above object, and preferred embodiments thereof are as follows.

[1] A method for producing a three-dimensional skin tissue, the method including a skin tissue formation process of producing a three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered, wherein

the skin tissue formation process includes a three-dimensional culture process of disposing a three-dimensional culture obtained by culturing stem cells in suspension on a culture solution-permeable membrane of a

culture vessel provided with the culture solution-permeable membrane, without incision, and culturing the three-dimensional culture to obtain a stratified three-dimensional skin tissue in which at least a dermal layer and an epidermal layer are layered.

[0008]

A three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered can be easily obtained by culturing a three-dimensional culture obtained by culturing stem cells on a membrane without incision.

[0009]

[2] The method for producing a three-dimensional skin tissue according to [1], wherein the three-dimensional culture process is performed 1 to 20 days after starting differentiation induction of the stem cells.

By adopting the above configuration, it is possible to promote the formation of a stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered.

[0010]

[3] The method for producing a three-dimensional skin tissue according to [1] or [2], wherein in the three-dimensional culture process, air-liquid interface culture and/or two-layer liquid-liquid culture in which different culture solutions are filled above and below across the

membrane are performed.

By adopting the above configuration, it is possible to efficiently obtain a three-dimensional skin tissue having a layered structure of a dermal layer and an epidermal layer.

[0011]

[4] The method for producing a three-dimensional skin tissue according to [3], wherein in the two-layer liquid-liquid culture, a medium for epithelial cells is filled above the membrane, and an upper part of the three-dimensional culture is brought into contact with the medium for epithelial cells and cultured.

By adopting the above configuration, it is possible to efficiently obtain a three-dimensional skin tissue having a layered structure of a dermal layer and an epidermal layer through the promotion of differentiation of cells constituting the epidermal layer in the upper part of the three-dimensional culture.

[0012]

[5] The method for producing a three-dimensional skin tissue according to any one of [1] to [4], wherein in the three-dimensional culture process, the three-dimensional skin tissue in which the epidermal layer is layered above the dermal layer is obtained.

In the three-dimensional skin tissue of this

configuration, the epidermal layer is formed above the dermal layer, and the epidermal layer is in contact with the outside. Thus, the three-dimensional skin tissue of the above configuration can be suitably used as a clinical or test tool.

[0013]

[6] The method for producing a three-dimensional skin tissue according to any one of [1] to [5], wherein the skin tissue formation process includes:

a first induction process of culturing a spheroid containing stem cells in a first differentiation medium containing one or more factors selected from an antagonist of transforming growth factor β (TGF β) signaling, an agonist of fibroblast growth factor (FGF) signaling, and an agonist of bone morphogenetic protein (BMP) signaling, and inducing formation of non-neural ectodermal epithelium; and

a process of culturing the three-dimensional culture that has undergone the first induction process in a second differentiation medium containing an agonist of fibroblast growth factor (FGF) signaling and/or an antagonist of bone morphogenetic protein (BMP) signaling, and inducing the three-dimensional culture to have a higher order structure.

By adopting the above configuration, it is possible to induce differentiation of the culture into a skin tissue in the skin tissue formation process.

[0014]

[7] The method for producing a three-dimensional skin tissue according to any one of [1] to [6], wherein in the culture vessel, the membrane is coated with an extracellular matrix component.

By adopting the above configuration, it is possible to promote the culture to form the layer structure of the dermal layer and the epidermal layer.

[0015]

[8] The method for producing a three-dimensional skin tissue according to any one of [1] to [7], wherein the three-dimensional skin tissue is derived from a human.

[0016]

[9] A three-dimensional skin tissue produced by the method for producing a three-dimensional skin tissue according to any one of [1] to [8].

[0017]

[10] The method for producing a three-dimensional skin tissue according to any one of [1] to [8], wherein in the three-dimensional culture process, the three-dimensional culture disposed on the membrane has a maximum diameter of 2 mm or less.

[0018]

[11] The method for producing a three-dimensional skin tissue according to any one of [1] to [8] and [10],

wherein in the three-dimensional culture process, the three-dimensional culture disposed on the membrane does not have a hair follicle.

[0019]

[12] The method for producing a three-dimensional skin tissue according to any one of [1] to [8] and [10] to [11], wherein in the three-dimensional culture disposed on the membrane during the three-dimensional culture process, mesenchymal cells are undifferentiated or a layer containing the mesenchymal cells has a thickness of 200 μm or less.

[0020]

[13] The method for producing a three-dimensional skin tissue according to any one of [1] to [8] and [10] to [12], wherein the three-dimensional skin tissue obtained in the three-dimensional culture process has a hair follicle.

[0021]

[14] A three-dimensional skin tissue produced by the method for producing a three-dimensional skin tissue according to any one of [1] to [8] and [10] to [13].

[15] The three-dimensional skin tissue according to [14], having a hair follicle.

Advantageous Effects of Invention

[0022]

According to the present invention, it is possible to produce a stratified three-dimensional skin tissue in which an epidermal layer and a dermal layer are layered.

Brief Description of Drawings

[0023]

Fig. 1 illustrates a preferred embodiment of a process flow of a method for producing a three-dimensional skin tissue of the present invention.

Fig. 2 schematically illustrates a state of performing air-liquid interface culture in a three-dimensional culture process S24.

Fig. 3 schematically illustrates a state of performing two-layer liquid-liquid culture in a three-dimensional culture process S24.

Fig. 4 is a fluorescent photograph showing the result of immunohistochemical staining of a three-dimensional skin tissue on day 27 from the start of induction in Example 1 of Test Example 1. The scale bar represents 200 μm .

Fig. 5 is a fluorescent photograph showing the result of immunohistochemical staining of a three-dimensional skin tissue having hair follicles in Test Example 2. Fig. 5(a) illustrates Example 10 on Day 116 from the start of induction, Fig. 5(b) illustrates Example

11 on Day 116 from the start of induction, and Fig. 5(c) illustrates Example 12 on Day 117 from the start of induction. The scale bars represent 100 μm . The dashed lines indicate the position of culture insert membrane, and * indicates the position of hair follicles.

Fig. 6 is a fluorescent photograph after immunohistochemical staining showing the process of inducing differentiation of spheroids into skin tissues in Test Example 3. The scale bars represent 100 μm .

Description of Embodiments

[0024]

The present invention is directed to producing a three-dimensional skin tissue from stem cells. Hereinafter, an embodiment of the present invention will be described. Note that the present invention is not limited to the embodiments described below, and can be appropriately modified within the scope of the present invention.

[0025]

In the present embodiment, the method for producing a three-dimensional skin tissue of the present invention broadly includes a spheroid formation process S1 of obtaining a spheroid of stem cells, and a skin tissue formation process S2 of culturing the spheroid to form a

three-dimensional skin tissue. The skin tissue formation process S2 includes a process of culturing the spheroid using an induction medium (a first induction process S21 of culturing in a first differentiation medium, optionally a second induction process S22 of culturing in a second differentiation medium, and optionally a third induction process S23 of inducing a more advanced structure); and a three-dimensional culture process S24 of forming a stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered.

[0026]

Fig. 1 illustrates four preferred embodiments of the method for culturing a three-dimensional skin tissue of the present invention.

[0027]

In the first embodiment, a spheroid formation process S1, a first induction process S21, a second induction process S22, and a three-dimensional culture process S24 are sequentially performed (Fig. 1(a)). The three-dimensional culture process S24 in the first embodiment includes an induction step S241 of culturing in the second differentiation medium and a maturation step S242 of inducing a higher order structure in which a dermal layer and an epidermal layer are layered.

[0028]

In the second embodiment, a spheroid formation process S1, a first induction process S21, a second induction process S22, and a three-dimensional culture process S24 are sequentially performed (Fig. 1(b)). The three-dimensional culture process S24 in the second embodiment includes the maturation step S242.

[0029]

In the third embodiment, a spheroid formation process S1, a first induction process S21, a second induction process S22, a third induction process S23, and a three-dimensional culture process S24 are sequentially performed (Fig. 1(c)). The three-dimensional culture process S24 in the third embodiment includes the maturation step S242.

[0030]

In the fourth embodiment, a spheroid formation process S1, a first induction process S21, and a three-dimensional culture process S24 are sequentially performed (Fig. 1(d)). The three-dimensional culture process S24 in the fourth embodiment includes the induction step S241 and the maturation step S242.

[0031]

It should be noted that, in these embodiments, examples including a spheroid formation process S1 are illustrated; however, the present invention may also be

carried out starting from the skin tissue formation process S2 using pre-generated spheroids of stem cells.

[0032]

Hereinafter, stem cells to be used in the present invention, a method for culturing the stem cells, and a preferred embodiment of each process will be described.

[0033]

<1> Stem cells and method for culturing the same

Examples of the stem cells used in the present invention include pluripotent stem cells such as induced pluripotent stem cells (iPS cells) and embryonic stem cells (ES cells), and somatic stem cells.

[0034]

The origin of the stem cells is not particularly limited, and for example, cells of mammals such as human, monkey, mouse, rat, guinea pig, rabbit, cat, dog, horse, cow, sheep, goat, and pig can be used.

[0035]

The three-dimensional skin tissue produced by the method of the present invention is effective for clinical application to humans and application as an evaluation tool for human pharmaceuticals, cosmetics, and the like. Thus, in the present invention, it is preferable to use human stem cells.

When the three-dimensional skin tissue is used for

clinical use, the stem cells may be autologous cells or allogeneic cells, but autologous cells are preferably used.

[0036]

iPS cells are artificial stem cells derived from somatic cells, which can be produced by introducing certain nuclear reprogramming factors into somatic cells in the form of a nucleic acid or protein encoding the factor or by elevating the expression of endogenous mRNA and/or protein of the factor by an agent, and have properties almost equivalent to those of ES cells, for example, pluripotency of differentiation and proliferation capability by self-replication (K. Takahashi and S. Yamanaka (2006), *Cell*, 126: 663-676, K. Takahashi et al. (2007), *Cell*, 131: 861-872, J. Yu et al. (2007), *Science*, 318: 1917-1920, M. Nakagawa et al. (2008), *Nat. Biotechnol.*, 26: 101-106).

[0037]

The nuclear reprogramming factors are not particularly limited as long as it is a gene specifically expressed in ES cells or a gene that plays an important role in maintaining undifferentiation of ES cells or a gene product thereof, and examples thereof include Oct3/4, Klf4, Klf1, Klf2, Klf5, Sox2, Sox1, Sox3, Sox15, Sox17, Sox18, c-Myc, L-Myc, N-Myc, TERT, SV40 large T antigen, HPV16 E6, HPV16 E7, Bmi1, Lin28, Lin28b, Nanog, Esrrb, or Esrrg. These nuclear reprogramming factors may be used in

combination during the establishment of iPS cells. For example, it is a combination including at least one, preferably two or more of the nuclear reprogramming factors, and more preferably a combination including three or more of the nuclear reprogramming factors.

[0038]

In the practice of the present invention, a human iPS cell line established as a cell line may be used. For example, a human iPS cell line selected from ChiPSC7, ChiPSC11, ChiPSC12, ChiPSC19, ChiPSC20, ChiPSC21, ChiPSC22, ChiPSC23, 201B7, 201B7-Ff, 253G1, 253G4, 1201C1, 1205D1, 1210B2, and 836B3 can be cultured in the method of the present invention. The human iPS cell line described above is available from Cellartis, iPS Academia Japan, Inc., or Center for iPS Cell Research and Application of Kyoto University.

[0039]

ES cells are stem cells established from an inner cell mass of an early embryo (for example, blastocyst) of a mammal such as a human or a mouse, and having pluripotency and proliferation capability by self-replication. ES cells were discovered in mice in 1981 (M. J. Evans and M. H. Kaufman (1981), Nature 292: 154-156), and ES cell lines were subsequently established in primates such as humans and monkeys.

[0040]

ES cells can be established by removing an inner cell mass from a blastocyst of a fertilized egg of a subject animal and culturing the inner cell mass on a feeder of fibroblasts. In addition, the maintenance of cells by subculture can be performed using a medium to which a substance such as LIF or bFGF is added. Methods for establishing and maintaining human and monkey ES cells are described, for example, in H. Suemori et al. (2006), *Biochem. Biophys. Res. Commun.*, 345:926-932, H. Kawasaki et al. (2002), *Proc. Natl. Acad. Sci. USA*, 99: 1580-1585, and the like. In addition, some research institutions distribute ES cells. For example, KhES-1, KhES-2 and KhES-3, which are human ES cell lines, are available from Institute for Life and Medical Sciences, Kyoto University (Kyoto, Japan).

[0041]

Examples of the somatic stem cells include mesenchymal stem cells, hematopoietic stem cells, neural stem cells, bone marrow stem cells, and germ stem cells. Among somatic stem cells, mesenchymal stem cells are preferred. The term "mesenchymal stem cells" broadly refers to a population of stem cells or progenitor cells thereof capable of differentiating into all or some of mesenchymal cells such as osteoblasts, chondroblasts, and

adipoblasts. More specific examples of the mesenchymal stem cells include bone marrow-derived mesenchymal stem cells, umbilical cord matrix-derived mesenchymal stem cells, adipose tissue-derived mesenchymal stem cells, and hair follicle mesenchymal stem cells.

[0042]

As these stem cells, those obtained or established primarily may be used, or those commercially available may be used.

[0043]

The method for culturing stem cells before subjecting them to the spheroid formation process S1 is not particularly limited as long as they can be cultured while maintaining their differentiation potency. The stem cells are preferably cultured by an adhesion culture method. The stem cells may be adherent and cultured on the culture surface on which feeder cells such as MEF cells are laid, but it is preferable to perform adherent culture under feeder-free conditions.

[0044]

When adhesion culture of stem cells is performed under feeder-free conditions, it is preferable to coat the culture surface with various culture substrates. Examples of the culture substrate include extracellular matrices such as laminin, collagen, fibronectin, vitronectin,

Matrigel, fibrin, and thrombin; amino acid polymers such as poly-L-lysine and poly-D-lysine; and fragments thereof, and one or more of these may be selected and used.

Among the culture substrates, laminin and a fragment thereof are preferred.

[0045]

As the laminin and a fragment thereof, it is preferable to use laminin 511 (laminin composed of $\alpha 5$ chain, $\beta 1$ chain, and $\gamma 1$ chain) and a fragment thereof. Laminin is a major cell adhesion molecule of the basement membrane, is a heterotrimer composed of three subunit chains of an α chain, a β chain, and a γ chain, and is a large glycoprotein having a molecular weight of about 800,000 Da. Laminin refers to a heterotrimeric molecule in which three subunit chains associate on the C-terminal side to form a coiled coil structure and are stabilized by a disulfide bond. Thus, laminin 511 means laminin in which the α chain is $\alpha 5$, the β chain is $\beta 1$, and the γ chain is $\gamma 1$.

[0046]

The laminin may be a variant, and the variant is not particularly limited as long as it is a variant having an integrin binding activity. The laminin is preferably derived from human. The laminin and a fragment thereof preferably exhibit a dissociation constant of 10 nM or less

in binding activity to integrin $\alpha 6 \beta 1$. It is preferable to use a commercially available product as the laminin or a fragment thereof.

[0047]

Examples of the laminin fragment include an E8 fragment (Reference 2: Ido H, et al, J Biol Chem. 2007, 282, 11144-11154) which is a fragment obtained by digesting Laminin 511 with elastase (also referred to as Laminin 511E8 fragment or Laminin 511E8), and a recombinant human laminin 511E8 fragment expressed from a genetically modified silkworm cocoon.

Among the laminin and a fragment thereof, a laminin fragment is preferable, a laminin 511 fragment is more preferable, a laminin 511E8 fragment is further preferable, and a human-derived fragment is still more preferable.

[0048]

Examples of a cell culture vessel coated with the culture substrate include, but are not limited to, a flask, a dish, a petri dish, a bottle, and a plate. The material of the cell culture vessel is not particularly limited, but is preferably one or more selected from synthetic resins (preferably plastics) such as a styrene-based resin (polystyrene, a styrene copolymer, or the like), polycarbonate, a polyolefin-based resin (polyethylene, polypropylene, polyester, an ethylene copolymer, or the

like), a (meth)acrylic resin, a silicone resin, an amino resin, a fluororesin, and a polyimide resin, and a glass substrate.

[0049]

The culture medium used for culturing stem cells is also not limited. For example, StemFit(TM) AK02N (Ajinomoto Co., Inc.), StemSure(TM) hPSC (FUJIFILM Wako Pure Chemical Corporation), mTeSR(TM) 1 (STEMCELL Technologies Inc.), TeSR(TM)-E6 (STEMCELL Technologies Inc.), TeSR(TM)-E8 (STEMCELL Technologies Inc.), StemFlex(TM) (Thermo Fisher Scientific Inc.), Essential6(TM) Medium (Thermo Fisher Scientific Inc.), Essential8(TM) Medium (Thermo Fisher Scientific Inc.), and Essential8(TM) Flex Medium (Thermo Fisher Scientific Inc.) are preferably used. In addition, antibiotics such as Penicillin-Streptomycin (Thermo Fisher Scientific Inc.) and normocin(TM) (Invivogen Inc.) can be added to these media as necessary.

Furthermore, it is also possible to use a self-made culture medium suitable for pluripotent stem cell culture by appropriately adding a growth factor such as FGF, an antibiotic described above, various proteins such as HSA and BSA, and the like to a general cell culture basal medium such as α MEM and DMEM.

[0050]

StemFit(TM) AK03N (Ajinomoto Co., Inc.) is preferred when coated with iMatrix, Essential8(TM) Flex Medium (Thermo Fisher Scientific Inc.) is preferred when coated with Vitronectin, and mTeSR(TM) (STEMCELL Technologies Inc.) is preferred when coated with Matrigel.

[0051]

As an example of the medium on feeder cells such as MEF cells, Dulbecco's modified Eagle's medium/F12 medium containing a knockout serum alternative, L-glutamine, a non-essential amino acid, 2-mercaptoethanol, b-FGF, and the like can be used.

[0052]

<2> Spheroid formation process S1

Specific embodiments of the spheroid formation process S1 are not limited as long as a spheroid of stem cells can be formed. The spheroid formation process S1 is preferably performed by culturing stem cells in suspension.

[0053]

The suspension culture means that cells are grown in suspension in a medium. The means for suspension culture is not particularly limited, and examples thereof include stirring culture, static culture, or a combination of stirring culture and static culture. It is also possible to use a microcarrier as necessary in the static culture.

[0054]

The stirring culture is culture in a state where cells are suspended in a culture medium, rather than being attached to the surface of a culture substrate (vessel). As a method of stirring culture, there are a method of culturing a culture solution while stirring the culture solution with a stirring bar, a stirring blade, or the like, and a method of culturing the culture solution by indirectly flowing the culture solution inside by driving a culture vessel itself, and examples of the former method include a culture method using a spinner flask.

[0055]

The culture using a microcarrier is a culture in a state in which cells are attached to the surface of the microcarrier. As the microcarrier, one composed of a synthetic polymer or a natural polymer can be used. The microcarrier may or may not be coated with an adhesive substrate such as collagen or laminin, but is preferably coated. When using a microcarrier to form a spheroid, it is preferable to perform static culture described later.

[0056]

Examples of the method of static culture include a method of seeding stem cells on a U-shaped bottom 96-well plate for spheroid formation (for example, #174925, Nunclon Sphera: Thermo Fisher Scientific Inc.). The culture substrate for spheroid formation used for the static

culture may have a structure in which the cells do not adhere, the bottom has a U-shape or the like, and the seeded cells naturally gather at the bottom. The shape of the bottom is not limited to the U-shape, and any shape such as a V-shape, an M-shape, and a flat surface can be used. In addition, the plate is not limited to 96-well plates.

[0057]

When a U-shaped bottom plate is used, centrifugation may be performed after seeding the stem cells. The centrifugal force causes stem cells to gather at the bottom of the plate, promoting aggregation.

[0058]

When the culture for maintaining stem cells, which is performed before the spheroid formation process S1, is performed by adhesion culture, the stem cells are peeled from the culture surface using an arbitrary peeling agent such as trypsin or Accutase, and then subjected to the spheroid formation process S1.

[0059]

The amount of the culture medium and the number of stem cells used in the spheroid formation process S1 are not limited, and can be appropriately designed. For example, when using a U-shaped bottom 96-well plate for spheroid formation, the medium amount is preferably 50 to

200 μ l/well, more preferably 80 to 120 μ l/well, and stem cells are seeded at preferably 1000 to 6000 cells/well, more preferably 2000 to 5000 cells/well.

[0060]

The number of live cells may be adjusted so that the number of live cells becomes the number suitable for spheroid formation described above by separately counting live cells and dead cells. The distinction between live cells and dead cells can be easily performed by using a staining reagent specific to live cells or dead cells. Trypan blue can be used as a nuclear staining reagent specific for dead cells.

[0061]

The culture period in the spheroid formation process S1 is not limited as long as it is a period sufficient for spheroid formation. The culture period can be, for example, 1 to 10 days, preferably 1 to 4 days.

[0062]

As the culture medium used in the spheroid formation process S1, the culture medium for stem cell culture described above can be used. More specifically, Essential 8(TM) Flex Medium (Thermo Fisher Scientific Inc.) is preferably used.

[0063]

In the spheroid formation process S1, a component

for promoting spheroid formation may be added to the medium. Examples of the component for promoting spheroid formation include Rho-kinase (ROCK) inhibitors. The ROCK inhibitors are known to have the effect of protecting single cells and small aggregates of cells (see, e.g., Watanabe K, et al., "A ROCK inhibitor permits survival of dissociated human embryonic stem cells", Nat. Biotechnol. 25: 681-686 (2007)).

[0064]

Examples of the ROCK inhibitors that can be suitably used in the spheroid formation process S1 include, but are not limited to, (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoliny)lsulfonyl]homopiperazine dihydrochloride (unofficial name: H-1152), 1-(5-isoquinolinesulfonyl)piperazine hydrochloride (unofficial name: HA-100), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (unofficial name: H-7), 1-(5-isoquinolinesulfonyl)-3-methylpiperazine (unofficial name: isoH-7), N-2-(methylamino)ethyl-5-isoquinoline-sulfonamide dihydrochloride (unofficial name: H-8), N-(2-aminoethyl)-5-isoquinolinesulfonamide dihydrochloride (unofficial name: H-9), N-[2-(p-bromo-cinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (unofficial name: H-89), N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (unofficial name: H-1004), 1-(5-

isoquinolinesulfonyl)homopiperazine dihydrochloride
(unofficial name: H-1077), (S)-(+) -2-methyl-4-glycyl-1-(4-methylisoquinolinyl-5-sulfonyl)homopiperazine
dihydrochloride (unofficial name: glycyl H-1152) and (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (unofficial name: Y-27632).

[0065]

The concentration of the ROCK inhibitor in the medium is preferably 1 to 20 μ M, more preferably 3 to 15 μ M, and further preferably 5 to 12 μ M.

[0066]

<3> Skin tissue formation process S2

The skin tissue formation process S2 is a process of inducing differentiation of stem cells to form a three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered, from a spheroid of stem cells. The skin tissue formation process S2 will be described below. The first day of the skin tissue formation process S2, that is, the day on which differentiation induction is started is defined as "day 0", and used as the reference point for describing the culture period or the like.

[0067]

In the present embodiment, the skin tissue formation process S2 includes a process of inducing differentiation

of spheroid of stem cells (first induction process S21, second induction process S22, and third induction process S23), and a three-dimensional culture process S24 of obtaining a stratified three-dimensional skin tissue in which at least a dermal layer and an epidermal layer are layered, from the three-dimensional culture having undergone these induction processes.

In the present embodiment, the start date of the first induction process S21 in the skin tissue formation process S2 corresponds to "day 0".

[0068]

In the process of differentiation induction, the conditions for differentiation induction are different in the first induction process S21, the second induction process S22, and the third induction process S23. Each process will be described below.

[0069]

<3-1> First induction process S21

The first induction process S21 is a process of inducing the formation of the non-neural ectodermal epithelium. More specifically, the culture is performed in a first differentiation medium containing one or more factors selected from an antagonist of transforming growth factor β (TGF β) signaling, an agonist of fibroblast growth factor (FGF) signaling, and an agonist of bone

morphogenetic protein (BMP) signaling.

[0070]

More specifically, the culture is performed in a first differentiation medium containing an antagonist of TGF β signaling and an agonist of FGF signaling, optionally an agonist of bone morphogenetic protein (BMP) signaling.

[0071]

In one embodiment, from day 0, a spheroid of stem cells is cultured in a first differentiation medium containing all of an antagonist of TGF β signaling, an agonist of BMP signaling, and an agonist of FGF signaling.

[0072]

In another embodiment, a first-1 differentiation medium and a first-2 differentiation medium are used as the first differentiation medium. Specifically, in the present embodiment, the spheroid of stem cells is cultured in a first-1 differentiation medium containing an antagonist of TGF β signaling and an agonist of FGF signaling on day 0, and the spheroid is cultured in a first-2 differentiation medium additionally containing an agonist of BMP signaling at any timing from day 0 to day 1.

[0073]

The first differentiation medium can be prepared by adding the above-described factors to a medium suitable for culturing the above-described stem cells as a base. The

base of the first differentiation medium is not particularly limited as long as it is a medium suitable for culturing stem cells, but Essential6 (TM) Medium (Thermo Fisher Scientific Inc.) can be particularly suitably exemplified.

[0074]

Examples of the antagonist of TGF β signaling include small molecule compounds such as SB431542 (4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazole-2-yl]-benzamide), SB525334 (6-[2-tert-butyl-5-(6-methylpyridine-2-yl)-1H-imidazole-4-yl]-quinoxaline), SD-208 (3-(6-methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide), LDN-193189 (4-[6-[4-(1-piperazinyl)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]-quinoline hydrochloride), E-616452 (2-(3-(6-methylpyridine-2-yl)-1H-pyrazole-4-yl)-1,5-naphthyridine), LY2157299 (2-(6-methylpyridine-2-yl)-3-[6-amido-quinoline-4-yl]-5,6-dihydro-4H-pyrrolo[1,2-b]pyrazole), and LY2109761 (4-[5,6-dihydro-2-(2-pyridinyl)-4H-pyrrolo[1,2-b]pyrazol-3-yl]-7-[2-(4-morpholinyl) ethoxy]-quinoline), RNAi nucleic acids specific for TGF β receptors, and anti-TGF β antibodies. In the present invention, SB431542 can be particularly suitably exemplified.

[0075]

When the small molecule compound is used as the

antagonist of TGF β signaling, the concentration of the small molecule compound in the medium in the first induction process S21 is preferably 1 to 50 μ M, more preferably 3 to 30 μ M, and further preferably 5 to 20 μ M.

[0076]

Examples of the agonist of BMP signaling include one or more selected from the group consisting of BMP4, BMP2, and BMP7, and a conjugate or fragment thereof. In particular, BMP4 or a conjugate or fragment thereof can be suitably exemplified.

[0077]

The concentration of the agonist of BMP signaling described above in the first differentiation medium in the first induction process S21 is preferably 0 to 30 ng/ml, more preferably 0.1 to 20 ng/ml, further preferably 0.5 to 10 ng/ml, still more preferably 1 to 7 ng/ml, and even more preferably 2 to 6 ng/ml.

[0078]

Examples of the agonist of FGF signaling include proteins belonging to the FGF family or conjugates or fragments thereof. In the present invention, bFGF, FGF4, FGF9, and the like can be suitably exemplified as the proteins belonging to the FGF family, and among them, bFGF can be particularly suitably exemplified.

[0079]

The concentration of the agonist of FGF signaling described above in the first differentiation medium in the first induction process S21 is preferably 0.1 to 20 ng/ml, more preferably 1 to 10 ng/ml, and further preferably 2 to 6 ng/ml.

[0080]

An extracellular matrix component may be further added to the first differentiation medium. The extracellular matrix component may be a basement membrane extract (BME). Specific examples of the extracellular matrix component include laminin, collagen IV, entactin (nidogen), and heparan sulfate proteoglycan.

As a reagent containing these extracellular matrix components, Matrigel (BD Biosciences, Corning Incorporated, or the like) can be used.

[0081]

In the present invention, Matrigel refers to a soluble preparation obtained from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. For the preparation of Matrigel, DMEM is preferably used, and for example, DMEM (containing 1 g/L of glucose) can be suitably used.

[0082]

The concentration of the extracellular matrix component in the first differentiation medium is preferably 0.1 to 5 mass%, more preferably 0.3 to 3 mass%, and still

more preferably 0.5 to 2 mass%.

[0083]

In the first induction process S21, the spheroid formed in the spheroid formation process S1 is cultured in suspension in the first differentiation medium. A specific aspect of the suspension culture is not particularly limited, but it is preferable to perform the first induction process S21 in the static culture described above.

[0084]

In a preferred embodiment, the first differentiation medium is dispensed into a 96-well U-shaped bottom plate, preferably at 50 to 200 μ l/well, more preferably at 80 to 150 μ l/well, and one spheroid is cultured individually in each well.

[0085]

The duration of the first induction process S21 is preferably 1 day or more, more preferably 2 days or more, and further preferably 3 days or more. By performing the first induction process S21 in the above duration, the non-neural ectodermal epithelium can be formed more effectively.

The duration of the first induction process S21 is also preferably 7 days or less, more preferably 5 days or less, and further preferably 4 days or less. The duration

is sufficient for the formation of non-neural ectodermal epithelium.

In a particularly preferred embodiment, the duration of the first induction process S21 is 3 days.

[0086]

During the first induction process S21, replacement of the first differentiation medium is optional. When the first induction process S21 is performed in the above period, replacement of the first differentiation medium is unnecessary.

[0087]

<3-2> Second induction process S22

The second induction process S22 is a process of inducing the three-dimensional culture having undergone the first induction process S21 to form a higher order structure. In the second induction process S22, it is preferable to culture the three-dimensional culture in suspension. In the second induction process S22, the culture is preferably performed using a second differentiation medium containing an agonist of fibroblast growth factor (FGF) signaling and/or an antagonist of bone morphogenetic protein (BMP) signaling.

[0088]

In one embodiment, the second differentiation medium contains an antagonist of BMP signaling, and optionally an

agonist of FGF signaling.

[0089]

In another embodiment, the second differentiation medium contains an agonist of FGF signaling and an antagonist of BMP signaling. According to this embodiment, the formation efficiency of the three-dimensional skin tissue can be improved.

[0090]

Examples of the agonist of FGF signaling used in the second induction process S22 include proteins belonging to the FGF family described above or conjugates or fragments thereof. In the present invention, bFGF, FGF4, FGF9, and the like can be suitably exemplified as the proteins belonging to the FGF family, and among them, bFGF can be particularly suitably exemplified.

The agonists of FGF signaling used in the first induction process S21 and the second induction process S22 are preferably the same, and bFGF is more preferably used in both processes.

[0091]

The concentration of the agonist of FGF signaling described above in the second differentiation medium is preferably 1 to 1000 ng/ml, more preferably 10 to 800 ng/ml, further preferably 50 to 500 ng/ml, and still more preferably 100 to 300 ng/ml.

[0092]

Examples of the antagonist of BMP signaling include proteinaceous inhibitors such as Chordin, Noggin, Follistatin, Dorsomorphin (6-[4-(2-piperidine-1-yl-ethoxy)phenyl]-3-pyridine-4-yl-pyrazolo[1,5-a]pyrimidine), its derivatives (P. B. Yu et al. (2007), Circulation, 116: II_60; P. B. Yu et al. (2008), Nat. Chem. Biol., 4: 33-41; J. Hao et al. (2008), PLoS ONE, 3(8): e2904) and LDN-193189 (4-(6-(4-(piperazine-1-yl)phenyl)pyrazolo[1,5-a]pyrimidine-3-yl)quinoline). The antagonist of BMP signaling is preferably LDN-193189.

[0093]

The concentration of the antagonist of BMP signaling in the second differentiation medium is preferably 0 to 4000 nM, more preferably 50 to 3000 nM, further preferably 100 to 2000 nM, and still more preferably 500 to 1500 nM.

[0094]

The second differentiation medium can be prepared by adding the above-described factors to a medium suitable for culturing stem cells described above as a base. The base of the second differentiation medium is not particularly limited, but Essential6(TM) Medium (Thermo Fisher Scientific Inc.) can be particularly suitably exemplified.

[0095]

In the second induction process S22, all the first

differentiation medium used in the first induction process S21 may be removed and replaced with the second differentiation medium.

[0096]

In one embodiment, the second induction process S22 is started by further adding the second differentiation medium to the first differentiation medium in which suspension culture is performed in the first induction process S21.

[0097]

In this case, on the end date of the first induction process S21 (the start date of the second induction process S22), the second differentiation medium is added in an amount of preferably 0.01 to 1 times, more preferably 0.05 to 0.5 times, still more preferably 0.1 to 0.3 times, and still more preferably 0.15 to 0.25 times the amount of the first differentiation medium.

[0098]

In a more preferred embodiment, the second induction process S22 is started by further adding the second differentiation medium to the first differentiation medium in which suspension culture is performed in the first induction process S21. Then, during the second induction process S22, the three-dimensional culture is cultured while a fresh medium not containing an agonist of FGF

signaling and an antagonist of BMP signaling is added to the mixed medium or a part or all of the mixed medium is replaced with the fresh medium.

In such an embodiment, the concentration of components contained in the second differentiation medium is changed stepwise during the second induction process S22. By performing the second induction process S22 so that the medium composition is changed stepwise in this manner, the formation efficiency of the three-dimensional skin tissue can be improved.

[0099]

The duration of the second induction process S22 is preferably 1 day or more, and can be 3 days or more, 4 days or more, or 5 days or more. In another embodiment, the duration of the second induction process S22 can be preferably 7 days or more.

By performing the second induction process S22 in this duration, the formation of the stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered can be promoted in the subsequent three-dimensional culture process S24.

[0100]

The upper limit of the duration of the second induction process S22 is not limited, and can be, for example, 14 days or less, more preferably 10 days or less,

and further preferably 9 days or less. The upper limit of the duration of the second induction process S22 can be 5 days or less, 4 days or less, or 3 days or less.

By performing the second induction process S22 in the above duration, the formation of the stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered can be promoted in the subsequent three-dimensional culture process S24.

[0101]

Here, in the first embodiment of the present invention (Fig. 1(a)), the duration of the second induction process S22 is preferably 1 to 14 days, more preferably 1 to 10 days, further preferably 1 to 8 days, still more preferably 1 to 5 days, even more preferably 1 to 4 days, and further more preferably 1 to 3 days.

In the second embodiment and the third embodiment (Figs. 1(b) to 1(c)), the duration of the second induction process S22 is preferably 3 to 14 days, more preferably 4 to 10 days, further preferably 5 to 10 days, and still more preferably 7 to 9 days.

By performing the second induction process S22 in the above duration, the formation of the stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered can be promoted in the subsequent three-dimensional culture process S24.

[0102]

In one embodiment (for example, the fourth embodiment), the three-dimensional culture process S24 can be performed after the first induction process S21 without performing the second induction process S22.

[0103]

<3-3> Third induction process S23

The skin tissue formation process S2 may optionally include a third induction process S23 for further maturing the three-dimensional culture having undergone the second induction process S22. Specifically, the third embodiment (Fig. 1(c)) of the present invention corresponds to this case.

[0104]

In the third induction process S23, culture is preferably performed in suspension.

The specific form of the suspension culture is not limited, and a form in which the organoid is cultured in suspension in a culture vessel such as a flask, a dish, a petri dish, a bottle, or a plate, all of which are filled with a maturation medium, can be preferably exemplified. The culture vessel preferably has a low adhesion surface.

[0105]

The third induction process S23 may be performed while the culture vessel is allowed to stand, or may be

performed while the culture solution is shaken on a shaker. Examples of the shaker include an orbital shaker that shakes in a two-dimensional orbit. The shaking speed of the orbital shaker is, for example, 50 to 80 rpm.

[0106]

As the maturation medium used in the third induction process S23, any cell culture medium can be used without limitation. For example, Advanced DMEM/F-12 (Thermo Fisher Scientific Inc.), Neurobalsal(TM) MEDIUM (Thermo Fisher Scientific Inc.), or a mixed medium thereof can be suitably exemplified.

[0107]

The duration of the third induction process S23 is preferably 3 days or more, more preferably 4 days or more, further preferably 5 days or more, and still more preferably 6 days or more.

[0108]

The upper limit of the duration of the third induction process S23 is preferably 8 days or less, and more preferably 7 days or less.

[0109]

The duration of the third induction process S23 is preferably 3 to 8 days, more preferably 4 to 8 days, further preferably 5 to 8 days, and still more preferably 6 to 7 days.

[0110]

An extracellular matrix component may be further added to the medium used in the third induction process S23. The extracellular matrix component may be a basement membrane extract (BME). Specific examples of the extracellular matrix component include laminin, collagen IV, entactin (nidogen), and heparan sulfate proteoglycan.

As a reagent containing these extracellular matrix components, Matrigel (BD Biosciences, Corning Incorporated, or the like) can be used.

[0111]

The concentration of the extracellular matrix component in the medium used in the third induction process S23 is preferably 0.05 to 5 mass%, more preferably 0.1 to 2 mass%, and further preferably 0.3 to 1 mass%.

[0112]

<3-4> Three-dimensional culture process S24

The three-dimensional culture process S24 is a process of culturing the three-dimensional culture obtained by suspension culture to obtain a stratified three-dimensional skin tissue in which at least a dermal layer and an epidermal layer are layered.

[0113]

The three-dimensional culture process S24 is preferably started after the differentiation induction in

the first differentiation medium by the first induction process S21.

[0114]

The three-dimensional culture process S24 is preferably started 1 to 20 days, more preferably 1 to 18 days, further preferably 3 to 18 days, still more preferably 3 to 15 days, and particularly preferably 4 to 12 days after starting differentiation induction. It is also preferable that the three-dimensional culture process S24 is started 5 to 20 days, more preferably 5 to 18 days, and further preferably 6 to 18 days after starting differentiation induction.

Then, in one embodiment, the three-dimensional culture process S24 is started at a time point at which preferably 1 day or more, more preferably 2 days or more, and further preferably 3 days or more have elapsed from the start date of differentiation induction. Then, in one embodiment, the three-dimensional culture process S24 is started at a time point at which preferably 20 days or less, more preferably 18 days or less, further preferably 15 days or less, and still more preferably 12 days or less have elapsed from the start date of differentiation induction.

[0115]

That is, in a preferred embodiment, it is preferable

that the three-dimensional culture process S24 is started on preferably days 1 to 20, more preferably days 3 to 18, further preferably days 3 to 15, and particularly preferably days 4 to 12 from the start date of differentiation induction.

[0116]

By setting the start time of differentiation induction as described above, it is possible to promote the formation of a three-dimensional skin tissue having a structure in which an epidermal layer is layered above a dermal layer and at least the epidermal layer is open to the outside. Furthermore, hair follicle formation in the three-dimensional skin tissue can be promoted.

In the present invention, the "structure in which at least the epidermal layer is open to the outside" refers to a form in which the epidermal layer is exposed to the outside, not a "form in which the epidermal layer is enclosed in the dermal layer as the dermal layer surrounds the epidermal layer".

[0117]

For example, in the first embodiment (Fig. 1(a)), the three-dimensional culture process S24 is started on days 5 to 9, more preferably on days 5 to 8, and further preferably on days 6 to 7 from the start date of induction. In a preferred embodiment, the three-dimensional culture

process S24 may be started on days 3 to 12, more preferably on days 4 to 11 from the start date of induction.

By adopting such configuration, it is possible to promote the formation of a stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered. It is also possible to promote the formation of a three-dimensional skin tissue having a structure in which an epidermal layer is layered above a dermal layer and at least the epidermal layer is open to the outside.

[0118]

In the second embodiment (Fig. 1(b)), the three-dimensional culture process S24 may be started on days 10 to 13, more preferably on days 12 to 13 from the start date of induction. In the third embodiment (Fig. 1(c)), it may be started on days 15 to 19, more preferably on days 17 to 18 from the start date of induction. In the fourth embodiment (Fig. 1(d)), it may be started on days 1 to 3, more preferably on days 2 to 3 from the start date of induction.

[0119]

In the three-dimensional culture process S24, preferably, the three-dimensional culture after the first induction process S21, the second induction process S22, or the third induction process S23 is disposed on a culture solution-permeable membrane of a culture vessel provided

with the culture solution-permeable membrane, without incision.

[0120]

In the present invention, a three-dimensional culture having a three-dimensional shape is disposed on a membrane of the culture vessel. That is, the present invention does not involve disposing individually separated cells on a membrane. In a preferred form, it is preferable that the three-dimensional culture is disposed on the membrane while maintaining the three-dimensional shape without performing incision and a treatment of separating cells before being disposed on the membrane.

The treatment of separating cells in the three-dimensional culture is, for example, a treatment of decomposing components of the extracellular matrix of the three-dimensional culture, and specifically, a treatment of the three-dimensional culture with a protease and/or a collagenase (collagen degradation enzyme), and further a treatment with a protease and a collagenase.

In the present invention in which the three-dimensional culture is disposed on a membrane, it is possible to save time and effort such as enzyme treatment for separating cells, and it is possible to more easily obtain a stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered. In

addition, by adopting such a form, it is possible to promote the formation of a three-dimensional skin tissue having a structure in which an epidermal layer is layered above a dermal layer and at least the epidermal layer is open to the outside, and having a hair follicle.

[0121]

The differentiation state of cells and tissues is not particularly limited as long as the three-dimensional culture disposed on the membrane is a culture having a three-dimensional shape obtained through suspension culture of stem cells. For example, in the three-dimensional culture, the cells forming the dermal layer and the cells forming the epidermal layer may each be in an undifferentiated state, or the dermal layer and the epidermal layer may be already formed. When the dermal layer and the epidermal layer are already formed, the three-dimensional culture may have a form including the epidermal layer on the inner side and the dermal layer on the outer side.

In one embodiment, the three-dimensional culture disposed on the membrane may have a spherical form in which only epithelial cells are differentiated, or may have a spherical form in which a layer containing mesenchymal cells is formed so as to surround a layer containing epithelial cells, and the layer containing epithelial cells

is enclosed in the layer containing mesenchymal cells.

[0122]

In the three-dimensional culture obtained through the first induction process S21, non-neural ectodermal epithelium is formed on the surface layer of the culture. That is, in one embodiment, in the three-dimensional culture process S24, the three-dimensional culture in which the non-neural ectodermal epithelium is formed is preferably disposed on the membrane. In addition, the three-dimensional culture disposed on the membrane may be a three-dimensional culture containing one or more, preferably two or more, more preferably three or more, further preferably four or more, and still more preferably all five cells selected from non-neural ectodermal epithelium, epidermal progenitor cells, cranial placode, neuroectodermal cells, and mesenchymal cells.

[0123]

In another embodiment, in the three-dimensional culture process S24, a three-dimensional culture containing epidermal keratinocytes and/or fibroblasts can be disposed on the membrane.

[0124]

In one embodiment, the three-dimensional culture disposed on the membrane preferably does not have a hair follicle. That is, the three-dimensional culture disposed

on the membrane is preferably one in which hair follicles are not differentiated.

By using an immature three-dimensional culture in which hair follicles are not differentiated, it is possible to easily obtain a three-dimensional skin tissue having a structure in which an epidermal layer is layered above a dermal layer and at least the epidermal layer is open to the outside, only by culturing the three-dimensional culture on a membrane.

[0125]

In one embodiment, the three-dimensional culture disposed on the membrane is preferably spherical in shape. The spherical three-dimensional culture may have a substantially spherical shape formed by aggregation of cells.

When a spherical three-dimensional culture is used, the maximum diameter of the three-dimensional culture is preferably 2 mm or less, more preferably 1.5 mm or less, further preferably 1.3 mm or less, and still more preferably 1 mm or less.

By adopting such configuration, it is possible to promote the formation of a three-dimensional skin tissue having a structure in which an epidermal layer is layered above a dermal layer and at least the epidermal layer is open to the outside, only by culturing the three-

dimensional culture on a membrane. Furthermore, hair follicle formation in the three-dimensional skin tissue can be promoted.

The maximum diameter of the three-dimensional culture can be measured by image analysis.

[0126]

When a spherical three-dimensional culture is used, the lower limit value of the maximum diameter of the three-dimensional culture is not particularly limited, but may be 100 μm or more or 300 μm or more.

[0127]

In one embodiment, in the three-dimensional culture disposed on the membrane, it is preferable that mesenchymal cells are undifferentiated or a layer containing the mesenchymal cells has a thickness of not more than a certain value. The thickness of the layer having mesenchymal cells is preferably 200 μm or less, more preferably 180 μm or less, further preferably 150 μm or less, still more preferably 130 μm or less, even more preferably 100 μm or less, further more preferably 90 μm or less, still further more preferably 80 μm or less, even further more preferably 70 μm or less, yet more preferably 60 μm or less, yet further more preferably 50 μm or less, and particularly preferably 40 μm or less.

By adopting such configuration, it is possible to

promote the formation of a three-dimensional skin tissue having a structure in which an epidermal layer is layered above a dermal layer and at least the epidermal layer is open to the outside, only by culturing the three-dimensional culture on a membrane. Furthermore, hair follicle formation in the three-dimensional skin tissue can be promoted.

In one embodiment, the mesenchymal cells are cranial neural crest cells (CNCC).

[0128]

The number of three-dimensional cultures to be disposed on the membrane is not particularly limited, and for example, one or a plurality of three-dimensional cultures obtained through the first induction process S21 may be disposed per space separated by a culture vessel (for example, per well in the case of a multi-well plate). When a plurality of three-dimensional cultures is disposed, 2 or more, 5 or more, or 10 or more three-dimensional cultures may be disposed per space (per well).

[0129]

The membrane provided in the culture vessel used in the three-dimensional culture process S24 is a culture solution-permeable membrane, and a porous membrane can be preferably used as the membrane. Preferred examples of the culture vessel provided with such a membrane include a

culture vessel in which a transwell insert or a cell culture insert having a porous membrane is placed on a cell culture plate (for example, a 24-well plate). The culture vessel itself may be provided with a porous membrane.

[0130]

The porous membrane provided in the culture vessel has a pore size of preferably 0.1 to 8 μm , more preferably 0.4 to 5 μm , and further preferably 0.4 to 3 μm .

[0131]

The thickness of the membrane used in the present invention is not particularly limited, and can be, for example, 10 to 1000 μm . The thickness of the membrane can also be adjusted by the thickness of a coating with an extracellular matrix component or the like described later.

[0132]

The material of the membrane provided in the culture vessel is not particularly limited, but hydrophilic polytetrafluoroethylene (polytetrafluoroethylene, PTFE), cellulose mixed ester, polycarbonate, polyethylene terephthalate, and the like can be appropriately adopted.

[0133]

The membrane provided in the culture vessel may be a membrane having micropores composed of an extracellular matrix component. In the membrane composed of the extracellular matrix component, the extracellular matrix

component forms a network structure. The extracellular matrix component constituting the membrane is preferably collagen, more preferably collagen I, and further preferably atelocollagen obtained from collagen I. Preferable examples of such a culture vessel include a culture vessel in which a highly permeable FibColl (registered trademark) atelocollagen insert for 24-well plates (KOKEN CO., LTD.) is placed on a cell culture plate.

[0134]

The membrane provided in the culture vessel is preferably coated with an extracellular matrix component. The extracellular matrix component to be used is preferably collagen, and more preferably collagen I.

By culturing on the membrane coated with the extracellular matrix component, it is possible to promote the formation of a stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered. It is also possible to promote the formation of a three-dimensional skin tissue having a structure in which an epidermal layer is layered above a dermal layer and at least the epidermal layer is open to the outside.

[0135]

When collagen is used as the extracellular matrix component, the collagen is preferably gelled.

[0136]

In the membrane provided in the culture vessel, feeder cells may be laid on the membrane. As the feeder cells, it is preferable to use adipose-derived mesenchymal stem cells.

[0137]

Further, in the three-dimensional culture process S24, it is preferable to fill a medium below the membrane and culture the three-dimensional culture on the membrane. That is, it is preferable that the medium filled below the membrane and the three-dimensional culture are brought into contact with each other through the membrane, and the medium is supplied to the three-dimensional culture through the membrane. By adopting such configuration, it is possible to promote the formation of a stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered.

[0138]

It is sufficient that the three-dimensional culture is maintained in a state of being disposed on the membrane. That is, the three-dimensional culture may adhere to the membrane, or may not completely adhere to the membrane and may float off the membrane by pipetting or shaking.

When feeder cells are laid on the membrane, it is sufficient that the three-dimensional culture is maintained in a state of being disposed on the feeder cells on the

membrane.

[0139]

In the three-dimensional culture process S24, a culture environment for inducing epithelial cell differentiation is preferably established above the membrane. In a preferred embodiment, in the three-dimensional culture process S24, air-liquid interface culture and/or two-layer liquid-liquid culture is performed on the three-dimensional culture. Preferably, in the three-dimensional culture process S24, air-liquid interface culture is performed.

By adopting such configuration, it is possible to efficiently obtain a three-dimensional skin tissue having a layered structure of a dermal layer and an epidermal layer.

[0140]

Fig. 2 is a view schematically illustrating a state of air-liquid interface culture, and Fig. 3 is a view schematically illustrating two-layer liquid-liquid culture. Figs. 2 and 3 show a state (a) in which three-dimensional culture C obtained in the suspension culture process is disposed on cell culture insert 11 installed in the cell of cell culture plate 10, and a state (b) in which the culture is continued and dermal layer L1 and epidermal layer L2 are formed.

[0141]

In the air-liquid interface culture, the culture is performed across the membrane M by adding culture solution below, while adding no culture medium above (Fig. 2). Specifically, after the three-dimensional culture C obtained by suspension culture is disposed on the membrane M, the culture medium on the membrane M is removed, the upper part of the three-dimensional culture is exposed to the gas phase, and the lower part is cultured in contact with the culture solution.

By adopting the configuration, the differentiation of cells constituting the epidermal layer L2 is promoted in the upper part of the three-dimensional culture C, and a three-dimensional skin tissue having a layer structure of the dermal layer L1 and the epidermal layer L2 can be efficiently obtained.

[0142]

The culture solution to be used is not particularly limited as long as it is a medium suitable for maturation of the three-dimensional culture C, but the maturation medium used in the third induction process S23 can be preferably exemplified. An extracellular matrix component may be added to the maturation medium. The preferred form of the extracellular matrix component to be added is the same as the medium used in the third induction process S23, and specifically, Matrigel can be used.

[0143]

The two-layer liquid-liquid culture is a method in which culture is performed by filling different culture solutions above and below across the membrane M (Fig. 3). In the two-layer liquid-liquid culture, a medium for epithelial cells is filled above the membrane M, and an upper part of the three-dimensional culture C is brought into contact with the medium for epithelial cells and cultured.

By adopting the configuration, the differentiation of cells constituting the epidermal layer L2 is promoted in the upper part of the three-dimensional culture C, and a three-dimensional skin tissue having a layer structure of the dermal layer L1 and the epidermal layer L2 can be efficiently obtained.

[0144]

The medium for epithelial cells to be used is not particularly limited, and examples thereof include, but are not limited to, Keratinocyte serum free medium (KSFM, Thermo Fisher Scientific Inc.), EpiLife(TM) Medium (Gibco), keratinocyte growth medium, minimum essential medium (MEM) containing about 5 to 20% fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), RPMI1640 medium, 199 medium, and F12 medium. In addition, an appropriate component (calcium, growth factors, or the like) may be

appropriately added in the culture of epithelial cells.

[0145]

It is preferable to fill the maturation medium below the membrane M. A preferred embodiment of the maturation medium is the same as the air-liquid interface culture described above.

[0146]

In the present invention, the process of culturing using the maturation medium in the three-dimensional culture process S24 is also referred to as the maturation step S242. In the maturation step S242, an embodiment may be employed in which culture is started in a medium containing an extracellular matrix component, and then culture is performed in a medium not containing an extracellular matrix component.

[0147]

Here, in a preferred embodiment, the maturation step S242 includes both a process of culturing in a maturation medium to which an extracellular matrix component is added and a step of culturing in a maturation medium to which an extracellular matrix component is not added.

In the case of such an embodiment, the maturation step S242 is started with the medium containing the extracellular matrix component, and after 1 to 4 days, the culture is continued while replacing all or a part of the

existing medium with the medium not containing the extracellular matrix component, and then replacing all or a part of the existing medium with the medium not containing the extracellular matrix component every 1 to 4 days.

[0148]

In the maturation step S242 of the third embodiment (Fig. 1(c)), an embodiment may be employed in which a process of culturing in a maturation medium to which an extracellular matrix component is not added is included and a process of culturing in a maturation medium to which an extracellular matrix component is added is not included.

[0149]

In addition, the three-dimensional culture process S24 is preferably started in the middle of differentiation induction in the second differentiation medium after differentiation induction in the first differentiation medium. In addition, the three-dimensional culture process S24 may be started after differentiation induction in the first differentiation medium and includes a differentiation induction process in the second differentiation medium.

In such configuration, for example, when the duration of the second induction process S22 is less than 6 to 10 days in the first embodiment (Fig. 1(a)) and the three-dimensional culture to be used at the start of the three-dimensional culture process S24 is immature, or when

the second induction process S22 is not performed in the fourth embodiment (Fig. 1(d)), in the culture at the start of the three-dimensional culture process S24, it is preferable to perform the process of culturing using the second differentiation medium used in the second induction process S22 (induction step S241) before culturing in the maturation medium.

In such a case, it is preferable that the culture medium filled below the membrane in the air-liquid interface culture or the two-layer liquid-liquid culture in the induction step S241 is used as the second differentiation medium.

[0150]

When the induction step S241 is performed in the three-dimensional culture process S24, the duration of the induction step S241 can be appropriately changed according to the differentiation state of the culture.

In the present invention, the total culture period of the culture using the second differentiation medium in the second induction process S22 and the induction step S241 is preferably 2 days or more, more preferably 5 days or more, and further preferably 7 days or more. In addition, the total culture period of the culture using the second differentiation medium in the second induction process S22 and the induction step S241 can be, for

example, 14 days or less, preferably 13 days or less, more preferably 10 days or less, and further preferably 9 days or less. Specifically, the total culture period of the culture using the second differentiation medium in the second induction process S22 and the induction step S241 can be, for example, 2 to 14 days, preferably 5 to 13 days, more preferably 5 to 10 days, further preferably 7 to 10 days, and still more preferably 7 to 9 days.

[0151]

In another embodiment, the three-dimensional culture process S24 may be started after differentiation induction in the second differentiation medium. In such an embodiment, for example, in the second embodiment (Fig. 1(b)), the three-dimensional culture process S24 can be started after the second induction process S22, and can have a form in which a culture process with the second differentiation medium is not included.

[0152]

Further, the three-dimensional culture process S24 may be started in the middle of culture in the maturation medium after differentiation induction in the second differentiation medium. In such a case, the three-dimensional culture process S24 is preferably started after the completion of the culture in the maturation medium to which an extracellular matrix component is added.

[0153]

In the three-dimensional culture process S24, the three-dimensional culture may be cultured by only one of the air-liquid interface culture and the two-layer liquid-liquid culture, or the two culture methods may be performed in any order.

[0154]

The duration of the three-dimensional culture process S24 is preferably 8 days or more, more preferably 10 days or more, further preferably 14 days or more, and still more preferably 20 days or more.

When a three-dimensional skin tissue having a hair follicle is obtained, the duration of the three-dimensional culture process S24 is preferably 90 days or more, more preferably 100 days or more, and further preferably 105 days or more.

[0155]

The upper limit of the duration of the three-dimensional culture process S24 is not particularly limited, but may be 200 days or less, 150 days or less, 100 days or less, or 80 days or less.

The duration of the three-dimensional culture process S24 can be, for example, 8 to 200 days, 10 to 150 days, 14 to 100 days, or 20 to 80 days.

[0156]

By performing the three-dimensional culture process S24 in the above duration, the formation of a stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered can be promoted.

[0157]

In the three-dimensional culture process S24, unlike Non Patent Literature 6, the three-dimensional culture is cultured on a membrane without incision before the three-dimensional culture has a skin tissue structure differentiated to a higher order after the induction is started. Thus, the procedure for producing the three-dimensional skin tissue can be simplified, and the culture period until the desired three-dimensional skin tissue is obtained can be shortened.

Further, since the present invention including the three-dimensional culture process S24 can be obtained by a simple method without incising the culture, it can be suitably applied to industrial production such as mass culture.

[0158]

In the skin tissue formation process S2, as described above, culture is performed using the second differentiation medium in the second induction process S22 and/or the induction step S241.

In one embodiment, the culture with the second

differentiation medium in the second induction process S22 and the induction step S241 can have the following forms.

[0159]

For example, in an embodiment in which the culture with the second differentiation medium is performed for 2 days or more, a fresh medium not containing an agonist of FGF signaling and an antagonist of BMP signaling may be added in an amount of preferably 0.3 to 1 times, more preferably 0.4 to 0.8 times, and further preferably 0.5 to 0.7 times the amount of the existing differentiation medium 2 to 3 days after the start date of the culture with the second differentiation medium.

Thereafter, at an interval of 1 to 3 days, preferably 30 to 70%, more preferably 40 to 60% of the existing differentiation medium is replaced with the fresh medium, and culture with the second differentiation medium is continued.

[0160]

In the culture with the second differentiation medium, the three-dimensional culture is cultured in a medium containing an antagonist of BMP signaling at a concentration of preferably 1 to 1000 nM, more preferably 5 to 500 nM, and further preferably 10 to 250 nM.

When culture with the second differentiation medium is performed while changing the concentration stepwise, the

concentration can be changed within the above concentration range.

[0161]

In the culture with the second differentiation medium, the three-dimensional culture is cultured in a medium containing an agonist of FGF signaling at a concentration of preferably 0.5 to 250 ng/ml, more preferably 2 to 150 ng/ml, and further preferably 5 to 75 ng/ml.

When culture with the second differentiation medium is performed while changing the concentration stepwise, the concentration can be changed within the above concentration range.

[0162]

The agonist of FGF signaling is preferably adjusted so that the concentration in the culture medium in the culture with the second differentiation medium in the second induction process S22 and/or the induction step S241 is higher than the concentration in the culture medium in the first induction process S21. Specifically, the concentration of the agonist of FGF signaling in the culture medium in the culture with the second differentiation medium in the second induction process S22 and/or the induction step S241 is preferably 1.1 to 20 times, more preferably 2 to 15 times the concentration in

the culture medium in the first induction process S21.

When the second induction process S22 and/or the induction step S241 is performed while changing the concentration of the agonist of FGF signaling stepwise, adjustment may be performed so as to satisfy the above conditions at least at the start time of the second induction process S22.

[0163]

As described above, in the second induction process S22 and/or the induction step S241, the concentration of the second differentiation medium is adjusted stepwise, thereby the formation of a stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered can be promoted.

[0164]

In the induction step S241 in the three-dimensional culture process S24, the culture with the second differentiation medium may be started by adding the second differentiation medium to the first differentiation medium after the culture with the first differentiation medium. That is, the medium used in induction step S241 may contain both the factors contained in the first differentiation medium and the factors contained in the second differentiation medium. A preferred embodiment of such a form is the same as the "embodiment in which the second

induction process S22 is started by further adding the second differentiation medium to the first differentiation medium in which suspension culture is performed in the first induction process S21" in the second induction process S22 described above.

[0165]

In the present invention, in the three-dimensional culture process S24, the three-dimensional culture obtained by culturing the spheroid of stem cells may be cultured in a medium containing an agonist of Wnt signaling, but can also be cultured in a medium containing no agonists of Wnt signaling.

In the method described in Non Patent Literature 6, by culturing in a medium to which an agonist of Wnt signaling is added, the size of the spherical skin organoid to be obtained is further increased, and the skin organoid can be easily incised. On the other hand, in the present invention, a three-dimensional culture obtained by culturing a spheroid of stem cells is disposed on a membrane without incision. Therefore, in the present invention, a desired stratified three-dimensional skin tissue can be formed on a membrane without including the process of culturing in a medium containing an agonist of Wnt signaling as shown in Non Patent Literature 6.

Examples of the agonist of Wnt signaling include a

GSK3 β inhibitor, specifically CHIR9902.

[0166]

<Three-dimensional skin tissue>

According to the present invention, it is possible to produce a stratified three-dimensional skin tissue in which an epidermal layer and a dermal layer are layered. The present invention also pertains to the three-dimensional skin tissue itself produced by the production method described above. A preferred embodiment of the three-dimensional skin tissue of the present invention, which can be produced by the production method described above, will be described below.

[0167]

In a preferred embodiment, the three-dimensional skin tissue of the present invention is such that the epidermal layer is in direct contact with the dermal layer. Since the three-dimensional skin tissue of such an embodiment has a structure close to the skin structure of a living body, it is more preferred for application as a clinical or test tool.

[0168]

In a preferred embodiment, the three-dimensional skin tissue of the present invention has a structure in which an epidermal layer is layered above a dermal layer.

The three-dimensional skin tissue having a normal

multilayer structure as described above is preferred for clinical application and application as a test tool.

[0169]

Furthermore, it is preferable that the three-dimensional skin tissue of the present invention is preferably in a form in which the epidermal layer is layered above the dermal layer and at least the epidermal layer is open to the outside.

The three-dimensional skin tissue in this form has a structure in which an epidermal layer is formed on the upper part of the membrane and the epidermal layer is exposed to the outside. Thus, the obtained three-dimensional skin tissue can be used as a biomaterial or a test tool as it is.

The three-dimensional skin tissue in this form is distinguished from a spherical form in which the epidermal layer is enclosed in the dermal layer by the dermal layer surrounding the epidermal layer.

[0170]

The epidermal layer of the three-dimensional skin tissue of the present invention can contain epidermal keratinocytes. The dermal layer of the three-dimensional skin tissue of the present invention can contain skin fibroblasts.

[0171]

In a preferred embodiment, the three-dimensional skin tissue of the present invention may contain one or more cells selected from the group consisting of mesenchymal stem cells, dermal papilla cells, dermal sheath cells, and follicular epidermal stem cells.

[0172]

In a preferred embodiment, the three-dimensional skin tissue of the present invention has a hair follicle. That is, in a preferred embodiment, the three-dimensional skin tissue is a hair follicle organoid. It is preferable that the hair follicle forms a hair bulb having dermal papilla cells in the dermal layer.

[0173]

<Application>

The three-dimensional skin tissue of the present invention can be clinically applied as a skin composition for transplantation.

That is, the present invention also pertains to a skin composition for transplantation containing the three-dimensional skin tissue described above.

[0174]

In addition, the three-dimensional skin tissue of the present invention can be applied as a test tool.

Specifically, by applying a test substance to the three-dimensional skin tissue of the present invention and

observing its anatomical and molecular biological reactions, it is possible to screen an active ingredient of pharmaceuticals and cosmetics. Examples of the method for applying a test substance to the three-dimensional skin tissue include application and injection.

That is, the present invention also pertains to a method for screening an active ingredient of a pharmaceutical product or a cosmetic product, in which a test substance is applied to the three-dimensional skin tissue described above.

Examples

[0175]

Hereinafter, the present invention will be described in more detail with reference to Examples, but the technical scope of the present invention is not limited to the following Examples.

[0176]

1. Test Example 1

<Culture of cell line>

As stem cells to be used in the test, Cellartis human iPS cell line (passages 21-47) purchased from Takara Bio Inc. was prepared.

[0177]

The cell line was tested for mycoplasma before the

experiment, and was confirmed to be negative. Cells were cultured on 6-well plates coated with vitronectin recombinant human protein (Invitrogen Corporation) at a concentration of 0.5 µg/cm².

[0178]

Stem cells were maintained in Essential8(TM) Flex (Thermo Fisher Scientific Inc.) medium (hereinafter, referred to as an E8 medium) supplemented with 100 µg/ml Normocin. The medium was supplemented every other day or every day, depending on the degree of confluence of the cells. Cells were passaged at about 80% confluence (usually every 4 to 5 days).

[0179]

CTS(TM) TrypLE(TM) Select Enzyme (hereinafter, TrypLE Select; Thermo Fisher Scientific Inc.) was used for cell detachment in cell passaging.

[0180]

Upon passaging, E8 medium containing 10 µM Y27632 (ROCK inhibitor, apoptosis inhibitor; Sengent) was used, and 24 hours after passaging, the medium was re-supplemented with a fresh E8 medium containing no Y27632.

[0181]

<Spheroid formation process>

Stem cells were detached from the culture dishes using TrypLE Select. After detachment, the stem cells were

collected as a suspension of single cells in E8 medium containing 10 μ M Y27632 (hereinafter, referred to as E8-10Y). Trypan blue (Thermo Fisher Scientific Inc.) was used at a ratio of 1 : 1 (Trypan blue: cell suspension) to distinguish between dead cells and live cells, and the number of live cells was measured.

[0182]

The appropriate number of live cells required for the experiment was transferred to E8 medium containing 20 μ M Y27632 (hereinafter, referred to as E8-20Y). The E8-20Y cell suspension was dispensed into a 96-well U-shaped bottom plate (Thermo Fisher Scientific Inc.). At this time, the cells were distributed so as to be 100 μ l/well and 3500 cells/well.

[0183]

The plate into which the cells were dispensed was centrifuged at 110 g for 6 minutes to promote aggregation of the cells. After 24 hours of culture, fresh 100 μ l of E8 medium was added to each well to dilute Y27632, thereby promoting cell proliferation and aggregate growth.

[0184]

<Skin tissue formation process>

In the skin tissue formation process, conditions such as the start time of the three-dimensional culture process and the culture form were investigated (Examples 1

to 9).

[0185]

[Example 1]

(First induction process)

Differentiation induction of the spheroid of stem cells obtained in the spheroid formation process was started. Hereinafter, the start date of differentiation induction (the start date of the skin tissue formation process (first induction process)) will be described as "day 0".

[0186]

On day 0, to start differentiation, all spheroids were collected individually, transferred to a new 96-well U-shaped bottom plate filled with 100 μ l of differentiation medium per well, and non-neuroectoderm formation was started by culturing the spheroid in suspension.

The differentiation medium was based on Essential6(TM) (hereinafter, referred to as "E6 medium") and contains 2% Matrigel (Corning Incorporated), 10 μ M SB431542 (Stemgent Inc.), 4 ng/ml bFGF (hereinafter, FGF; PeproTech Inc.) and 5.0 ng/ml BMP4 (PeproTech Inc.).

[0187]

(Second induction process)

On day 3, to induce the formation of head neural crest like cells (CNC cells), E6 medium containing 1 μ M

LDN-193189 (BMP inhibitors, Stemgent Inc.) and 250 ng/ml FGF was added in an amount of 25 μ l per well, finally to an amount of 125 μ l per well.

[0188]

(Three-dimensional culture process)

On day 6, 75 μ l of fresh E6 medium per well was added to a 96-well U-shaped bottom plate and 200 μ l of the resulting medium was transferred to a 24-well plate. In addition, 200 μ l of a new E6 medium to which Matrigel, SB431542, FGF, BMP4 and LDN-193189 were added was prepared so as to have the same composition, and added to a 24-well plate, and the amount of the medium per well was set to 400 μ l (400 μ l of medium contains 1% Matrigel, 5 μ M SB431542, 2.5 ng/ml BMP4, 125 nM LDN-193189 and 33.25 ng/ml FGF). Further, a culture insert (Millicell, diameter 12 mm; a membrane having a pore size of 3 μ m and made of polycarbonate, hereinafter referred to as "CI") was placed. A membrane coated with collagen was used for CI.

[0189]

The collagen-coated CI was obtained by placing 50 μ L each of a collagen solution gelled by adding 1 N NaOH, which was 0.023 times that of collagen, to Collagen I rat tail (Corning Incorporated) diluted to 2 mg/mL with PBS onto the CI, and allowing the mixture to stand at 37°C and 5%CO₂ for 30 minutes.

[0190]

Next, the culture obtained in the second induction process was placed one by one on the CI, and the medium on the CI was removed. Thereafter, the culture medium was not added above the CI, and air-liquid interface culture was performed in a state where the lower part of the culture was in contact with the culture medium through the porous membrane of the CI.

[0191]

Half of the medium was replaced on days 8 and 10 (200 μ l of spent medium was removed and 200 μ l of fresh E6 medium was added).

[0192]

On day 12, the entire amount of the used medium was removed, and 400 μ l of maturation medium containing 1% Matrigel (Corning Incorporated) was added to each well, and air-liquid interface culture was continued.

[0193]

The maturation medium was based on a 1 : 1 mixed medium of Advanced DMEM/F-12 (Thermo Fisher Scientific Inc.) and Neurobasal(TM) medium (Thermo Fisher Scientific Inc.) supplemented with 1 x GlutaMax(TM) (Thermo Fisher Scientific Inc.), 0.5 x B-27 minus vitamin A (Thermo Fisher Scientific Inc.) and 0.5 x N-2 (Thermo Fisher Scientific Inc.), and contains 0.1 mM 2-mercaptoethanol (Thermo Fisher

Scientific Inc.) and 100 µg/ml normocin.

[0194]

On day 15, half of the spent medium was replaced with a fresh maturation medium containing 1% Matrigel (200 µl of spent medium was removed and 200 µl of fresh medium was added).

From day 18, every three days, half of the medium was replaced with a fresh maturation medium containing no Matrigel, and air-liquid interface culture was continued.

[0195]

[Example 2]

The same procedure as in Example 1 was performed except that in the three-dimensional culture process, two-layer liquid-liquid culture was performed instead of air-liquid interface culture.

[0196]

In the two-layer liquid-liquid culture, the medium on CI was removed, and then Keratinocyte serum free medium (KSFM, Thermo Fisher Scientific Inc.) was added to the CI. That is, the culture was performed in a state in which KSFM was filled above the CI membrane and E6 medium (having the same composition as that used in the air-liquid interface culture of Example 1) was filled below the CI membrane.

[0197]

Medium exchange was performed on days 8 and 10.

Below the CI, 200 μ l, half the volume, of the spent medium was removed and 200 μ l of fresh E6 medium was added. Above the CI, all spent medium was removed and 200 μ l of KSFM was added to the upper part of each CI.

[0198]

On day 12, the total amount of the spent medium was removed, and for each well, 400 μ l of the maturation medium containing 1% Matrigel was added to the lower part of the CI and 200 μ l of KSFM was added to the upper part of the CI, and the two-layer liquid-liquid culture was continued.

[0199]

On day 15, the spent medium was replaced. Below the CI, 200 μ l, half the volume, of the spent medium was removed and 200 μ l of fresh maturation medium containing 1% Matrigel was added. Above the CI, all spent medium was removed and 200 μ l of KSFM was added to the upper part of each CI.

[0200]

From day 18, the medium was changed every three days. In the medium exchange, half of the medium below the CI was exchanged with a fresh maturation medium containing no Matrigel, while the entire volume of the spent medium above the CI was exchanged, and the two-layer liquid-liquid culture was continued.

[0201]

[Example 3]

In the three-dimensional culture process, the same procedure as in Example 2 was performed except that the start time of the three-dimensional culture process on CI was changed from day 6 to day 12 of the start of induction. The following describes the changes.

[0202]

(Second induction process)

The procedure up to day 6 was the same as in Examples 1 and 2.

On day 6, 75 μ l of fresh E6 medium was added to bring the final volume to 200 μ l.

Half of the medium was replaced on days 8 and 10 (100 μ l of spent medium was removed and 100 μ l of fresh E6 medium was added).

[0203]

(Three-dimensional culture process)

On day 12, the total amount of the spent medium was removed, and for each well, 400 μ l of the maturation medium containing 1% Matrigel was added below the CI and 200 μ l of KSFM was added above the CI, and the two-layer liquid-liquid culture was performed.

The procedure of the two-layer liquid-liquid culture was the same as the procedure of Example 2 after day 12.

[0204]

[Example 4]

In the three-dimensional culture process, the start time of the three-dimensional culture process on CI was changed from day 12 to day 18 after the start of induction. The procedure up to day 12 was the same as in Example 3. The following describes the changes.

[0205]

(Third induction process)

On day 12, to induce self-assembly, all cultures were transferred to a maturation medium containing 1% Matrigel filled into individual wells of a 24-well low tack plate (Thermo Fisher Scientific Inc.) by 500 μ l each.

[0206]

To keep the medium circulation constant in suspension culture of the organoids in the maturation medium, the 24-well plate was placed on an orbital shaker shaken at 65 rpm in an incubator at 5%CO₂ 37°C.

[0207]

On day 15, half of the spent medium was replaced with a fresh maturation medium containing 1% Matrigel (250 μ l of spent medium was removed and 250 μ l of fresh medium was added).

[0208]

(Three-dimensional culture process)

On day 18, half of the medium was replaced with a

fresh maturation medium containing no Matrigel, and then 400 μ l of the replaced medium was added per well to a 24-well plate, and a collagen-coated CI was placed.

Subsequently, the culture obtained in the third induction process was placed on the CI one by one per well, and air-liquid interface culture was performed in the same manner as in Example 1.

[0209]

[Example 5]

The same procedure as in Example 4 was performed except that in the three-dimensional culture process, two-layer liquid-liquid culture was performed instead of air-liquid interface culture.

[0210]

Specifically, on day 18, half of the medium was replaced with a fresh maturation medium containing no Matrigel, and for each well, 400 μ l of the replaced medium was added below the CI. One culture was placed on the CI per well, 200 μ l of KSFM was added above the CI, and two-layer liquid-liquid culture was performed. After that, the medium was changed every three days. The method of medium exchange was the same as the procedure of Example 2.

[0211]

[Examples 6 to 9]

Examples 6 to 8, which were the same as Examples 1

to 3 except that CIs without collagen coating were used, were performed. In addition, Example 9 in which air-liquid interface culture was performed using CI without collagen coating in the three-dimensional culture process in Example 3 was also performed. Table 1 shows a list of culture conditions in Examples 1 to 9. "Timing of start of three-dimensional culture process" was described by the number of days from the start date of differentiation induction (start date of differentiation induction was set to day 0).

[0212]

[Table 1]

Table 1

No.	Example 1	Example 2	Example 3	Example 4	Example 5	Example 6	Example 7	Example 8	Example 9
Timing of start of three-dimensional culture process	Day 6	Day 6	Day 12	Day 18	Day 18	Day 6	Day 6	Day 12	Day 12
Presence or absence of collagen on CI	Presence	Presence	Presence	Presence	Presence	Absence	Absence	Absence	Absence
Culture form	Air-liquid interface	Two-layer liquid-liquid culture	Two-layer liquid-liquid culture	Air-liquid interface	Two-layer liquid-liquid culture	Air-liquid interface	Two-layer liquid-liquid culture	Two-layer liquid-liquid culture	Air-liquid interface

[0213]

<Immunostaining>

The skin tissues of Examples 1 to 9 were subjected to immunohistochemical staining for the following markers (primary antibodies) on day 27 from the start of induction.

- E-cadherin (marker for intercellular adhesion

molecules in epithelial cells)

- PDGFR α (marker for mesenchymal cells)

[0214]

Immunostaining was performed according to the following procedure.

Using the obtained skin tissue, a frozen section was cut in parallel with the vertical direction of CI by a conventional method, so as to include both the upper and lower regions of the skin tissue, and the section was washed with PBS-T. Thereafter, blocking was performed by adding 10% Goat Serum, and then a primary antibody (see the above) was reacted at 4°C. After the reaction, washing with PBS-T was performed, and secondary antibodies (Alexa488 Antimouse and Alexa647 Antirabbit) were reacted at room temperature. The skin tissue after the reaction was washed with PBS and mounted on a slide using ProLong Gold Antifade Mountant with DAPI (manufactured by Invitrogen Corporation).

For the obtained skin tissue, a stained image of the skin tissue was captured using a fluorescence microscope (manufactured by Keyence Corporation).

[0215]

<Results>

In the three-dimensional cultures of Examples 1 to 9, a state in which the dermal layer and the epidermal

layer were layered was observed. That is, by culturing an undeveloped three-dimensional culture on a porous membrane such as CI without incision, a stratified three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered could be obtained.

[0216]

In addition, as shown in Fig. 4, it was observed in Example 1 that an epidermal layer expressing E-cadherin was layered on a dermal layer expressing PDGFR α . In addition, the skin tissue obtained in Example 1 exhibited a form in which the epidermal layer was not embedded within the tissue but was instead flatly layered above the dermal layer.

[0217]

2. Test Example 2

After <Culture of cell line> and <Spheroid formation process> were performed in the same manner as in Test Example 1, Examples 10 to 12 were performed by the following procedure.

[0218]

[Example 10]

As in Example 1, the first induction process was performed on the start date of induction, and then the second induction process was performed on day 3 from the start date of induction.

Next, on day 4 from the start of induction, 10 cultures obtained in the second induction process per well were allowed to stand on the CI. In Example 10, FibColl (registered trademark) high-permeability atelocollagen insert for 24-well plates (KOKEN CO., LTD.) was used as CI, and collagen coating of CI was not performed.

Subsequent culturing was performed according to the same procedure as in Example 1, and a three-dimensional skin tissue of Example 10 was obtained.

[0219]

[Example 11]

In Example 11, the same atelocollagen insert as in Example 10 was used as CI, and 10 cultures obtained in the second induction process were placed per well. A three-dimensional skin tissue of Example 11 was obtained by the same procedure as in Example 1 except for the type of CI and the number of cultures allowed to stand.

[0220]

[Example 12]

A three-dimensional skin tissue of Example 12 was obtained by culturing in the same procedure as in Example 9.

[0221]

<Immunostaining>

The skin tissues of Examples 10 to 12 were subjected

to immunohistochemical staining using the following primary and secondary antibodies. Immunohistochemical staining was performed on day 116 from the start of induction in Examples 10 and 11, and on day 117 from the start of induction in Example 12. Immunostaining was performed according to the same procedure as in Test Example 1.

[0222]

- Markers for intercellular adhesion molecules in epithelial cells (Examples 10 to 11)

Primary antibody: E-cadherin, secondary antibody:
Alexa488 Antimouse

- Markers for mesenchymal cells (Examples 10 to 12)

Primary antibody: PDGFR α , secondary antibody:
Alexa647 Antirabbit

- Markers for dermal papilla cells

Primary antibody: SOX2 (Examples 10 to 12),
secondary antibody: Alexa555 Antirat (Examples 10 to 11),
Alexa488 Antimouse (Example 12)

[0223]

As shown in Fig. 5, in the three-dimensional skin tissue of Examples 10 to 12, a dermal layer in which PDGFR α was expressed and an epidermal layer in which E-cadherin was expressed were layered in this order above the membrane indicated by a dashed line. In addition, it was confirmed that hair follicles were formed at the position of * in the

three-dimensional skin tissue.

From the results, it was revealed that when culture on CI is started in an immature state up to about day 12 from the start of induction, the epidermal layer is layered above the dermal layer so that the epidermal layer is opened to the outside, and a stratified three-dimensional skin tissue having hair follicles is formed.

[0224]

3. Test Example 3

The progress of inducing differentiation of the spheroids obtained in the <Spheroid formation process> was confirmed by immunohistochemical staining. Specifically, the cultures on day 3, day 5, day 6, day 8, day 10, day 12, day 15, and day 18 from the start date of induction were subjected to immunohistochemical staining using the following antibodies. The culture was cultured in the same procedure as in Examples 4 and 5 described above, and staining was performed without transferring to CI at day 18 from the start of induction. The procedure of immunohistochemical staining was the same as in Test Example 1.

[0225]

- Markers for intercellular adhesion molecules in epithelial cells

Primary antibody: E-cadherin, secondary antibody:

Alexa488 Antimouse

- Markers for mesenchymal cells

Primary antibody: PDGFR α , secondary antibody:

Alexa647 Antirabbit

[0226]

Fig. 6 shows the results of immunohistochemical staining of each marker for each induction day. On day 3 from the start of induction, fluorescence of E-cadherin was confirmed. Then, over 10 days from the start of induction, it was observed that the culture grew and the outer coat of the aggregates became clear.

On day 12 from the start of induction, fluorescence of PDGFR α was confirmed outside the layer containing epithelial cells, and a layer containing mesenchymal cells was formed so as to surround the layer containing epithelial cells.

On day 15 from the start of induction, the thickness of the layer containing mesenchymal cells increased. On day 18 from the start of induction, the layer containing mesenchymal cells was thicker than on day 15 from the start of induction. At day 18 from the start of induction, formation of hair follicles was not confirmed.

[0227]

Based on the results of Test Examples 1 to 3, it can be said that in order to obtain a three-dimensional skin

tissue in which an epidermal layer is layered above a dermal layer and the epidermal layer is open to the outside, particularly one having a hair follicle, it is important to transfer and culture the three-dimensional culture onto the CI at an immature stage where the number of days has not elapsed from the start of induction and differentiation has not progressed.

Industrial Applicability

[0228]

The present invention can be applied to a skin tissue preparation technique useful as a clinical or screening tool.

Reference Signs List

[0229]

- 10 Cell culture plate
- 11 Cell culture insert
- M Membrane
- C Three-dimensional culture
- L1 Dermal layer
- L2 Epidermal layer

CLAIMS

Claim 1.

A method for producing a three-dimensional skin tissue, the method comprising a skin tissue formation process of producing a three-dimensional skin tissue in which a dermal layer and an epidermal layer are layered, wherein

the skin tissue formation process includes a three-dimensional culture process of disposing a three-dimensional culture obtained by culturing stem cells in suspension on a culture solution-permeable membrane of a culture vessel provided with the culture solution-permeable membrane, without incision, and culturing the three-dimensional culture to obtain a stratified three-dimensional skin tissue in which at least a dermal layer and an epidermal layer are layered.

Claim 2.

The method for producing a three-dimensional skin tissue according to claim 1, wherein the three-dimensional culture process is performed 1 to 20 days after starting differentiation induction of the stem cells.

Claim 3.

The method for producing a three-dimensional skin tissue according to claim 1 or 2, wherein in the three-dimensional culture process, air-liquid interface culture

and/or two-layer liquid-liquid culture in which different culture solutions are filled above and below across the membrane are performed.

Claim 4.

The method for producing a three-dimensional skin tissue according to claim 3, wherein in the two-layer liquid-liquid culture, a medium for epithelial cells is filled above the membrane, and an upper part of the three-dimensional culture is brought into contact with the medium for epithelial cells and cultured.

Claim 5.

The method for producing a three-dimensional skin tissue according to any one of claims 1 to 4, wherein in the three-dimensional culture process, the three-dimensional skin tissue in which the epidermal layer is layered above the dermal layer is obtained.

Claim 6.

The method for producing a three-dimensional skin tissue according to any one of claims 1 to 5, wherein the skin tissue formation process includes:

a first induction process of culturing a spheroid containing stem cells in a first differentiation medium containing one or more factors selected from an antagonist of transforming growth factor β (TGF β) signaling, an agonist of fibroblast growth factor (FGF) signaling, and an

agonist of bone morphogenetic protein (BMP) signaling, and inducing formation of non-neural ectodermal epithelium; and

a process of culturing the three-dimensional culture that has undergone the first induction process in a second differentiation medium containing an agonist of fibroblast growth factor (FGF) signaling and/or an antagonist of bone morphogenetic protein (BMP) signaling, and inducing the three-dimensional culture to have a higher order structure.

Claim 7.

The method for producing a three-dimensional skin tissue according to any one of claims 1 to 6, wherein in the three-dimensional culture process, the three-dimensional culture disposed on the membrane has a maximum diameter of 2 mm or less.

Claim 8.

The method for producing a three-dimensional skin tissue according to any one of claims 1 to 7, wherein in the three-dimensional culture process, the three-dimensional culture disposed on the membrane does not have a hair follicle.

Claim 9.

The method for producing a three-dimensional skin tissue according to any one of claims 1 to 8, wherein in the three-dimensional culture disposed on the membrane during the three-dimensional culture process, mesenchymal

cells are undifferentiated or a layer containing the mesenchymal cells has a thickness of 200 μm or less.

Claim 10.

The method for producing a three-dimensional skin tissue according to any one of claims 1 to 9, wherein the three-dimensional skin tissue is derived from a human.

Claim 11.

The method for producing a three-dimensional skin tissue according to any one of claims 1 to 10, wherein the three-dimensional skin tissue obtained in the three-dimensional culture process has a hair follicle.

Claim 12.

A three-dimensional skin tissue produced by the method for producing a three-dimensional skin tissue according to any one of claims 1 to 11.