

【DESCRIPTION】

【Invention Title】

METHOD FOR MASS PROLIFERATION OF STEM CELLS WITHOUT USING HYDROGEL

5 **【Technical Field】**

The present invention relates to an induced pluripotent stem cell mass proliferation method without using a hydrogel. More specifically, the present invention relates to a method for producing induced pluripotent stem cells from somatic cells and mass proliferating induced pluripotent stem cells using a 3D cell
10 culture plate without using a hydrogel.

【Background Art】

A process of returning differentiated somatic cells to cells in an undifferentiated state (for example, stem cells) refers to reprogramming. Induced pluripotent stem cells (iPSCs) are also called reverse-differentiated stem cells and
15 reverse-differentiated pluripotent stem cells, and reprogramming is the conversion of somatic cells into stem cells using reprogramming factors (Oct4, Klf4, Sox2, c-Myc, and the like) (Non-Patent Documents 1 and 2).

Induced pluripotent stem cells have a wide range of applications such as cell therapy agents, biotissue engineering, new drug development, toxicology, and
20 precision medicine. These applications require culturing large amounts of high-efficiency and high-quality induced pluripotent stem cells and embryonic stem cells, and requires uniform-sized stem cells at the same efficiency, but in fact, research on this mass production technique is inadequate. Further, the efficiency of reprogramming induced pluripotent stem cells has also been quite low to date.

Recently, many studies have been conducted on methods for clinical application, in other words, methods in which foreign genes are not integrated, in other words, DNA integration is not achieved, and among them, an attempt is made to produce induced pluripotent stem cells by a method such as an episomal vector, but this series of
5 production methods have a problem in that the efficiency of the 3D culture method and the reprogramming efficiency of somatic cells are also low.

Moreover, for induced pluripotent stem cell reprogramming, the bottom of a 2D cell culture plate is coated with a hydrogel and used, but colonies are rarely formed, and even though colonies are formed after cell reprogramming, the colonies
10 are not easily separated, which acts as a barrier not only to research purposes, but also to commercialization. In addition, since it is almost impossible to produce a large number of clones in the case of existing methods, it is difficult to stably secure clones in a state reprogrammed into induced pluripotent stem cells by the existing methods. Furthermore, there is also a need for a platform capable of easily
15 screening induced pluripotent stem cells.

Meanwhile, Korean Patent No. 10-1756901 (Patent Document 1) discloses a cell culture chip capable of culturing 3D tissue cells. In the cell culture chip of Patent Document 1, a first culture part, a second culture part, and the third culture part are formed in each layer, and the degree of cell growth progress can be
20 confirmed in each layer. However, the cell culture chip of Patent Document 1 has a problem in that spheroids and/or organoids cannot be obtained in high yield.

Further, there is a case where the pipetting work of replacing a culture solution during cell culture is performed, and in the case of a Corning spheroid microplate capable of 3D cell culture, spheroids or organoids in cell culture are
25 affected, so that there is a problem which is not good for the cell culture environment

because there is a case where the spheroids or organoids are sucked up or the positions thereof are changed during the pipetting work.

Matrigel (product name of BD Bioscience) is a protein complex extracted from sarcoma cells of Engelbreth-Holm-Swarm (EHS) mice, and contains an
5 extracellular matrix (ECM) such as a laminin, collagen and a heparan sulfate proteoglycan, and a growth factor such as a fibroblast growth factor (FGF), an epiderma growth factor (EGF), an insulin-like growth factor (IGF), transforming growth factor-beta (TGF- β), and a platelet-derived growth factor (PDGF). The complex which forms Matrigel is utilized as a substrate for cell culture by providing
10 a complex extracellular environment found in many tissues.

Since Matrigel is derived from mouse sarcoma, there is a high risk of transferring an immunogen and a pathogen. In addition, although Matrigel is used for cell growth and tissue formation, there is also criticism that there is a big problem with cell reproducibility because Matrigel is such a complex material. It is also
15 unclear whether Matrigel simply acts as a passive 3D scaffold which provides a physical support for growing spheroids, or whether Matrigel actively affects spheroid formation by providing a biologically essential element. Furthermore, Matrigel is also expensive. Therefore, although Matrigel is a material which has contributed to the development of the cell culture technology field, there is also a fact that the
20 development of the technology field is hindered by Matrigel.

Thus, the present inventors have conducted continuous studies on a technology of mass-culturing stem cells by enhancing the reprogramming efficiency of stem cells without using a hydrogel, thereby completing the present invention.

[Related Art Documents]

25 [Patent Document]

1. Korean Patent No. 10-1756901

[Non-Patent Documents]

1. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663-676.

5 2. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131:861-872.

【Disclosure】

【Technical Problem】

10 An object of the present invention is to provide a stem cell proliferation method.

However, a technical problem to be achieved by the present invention is not limited to the aforementioned problems, and other problems that are not mentioned may be clearly understood by those skilled in the art from the following description.

15 **【Technical Solution】**

To achieve the object, the present invention provides a stem cell proliferation method, the method including:

- i) culturing cells;
- ii) preparing a hydrogel-free 3D cell culture plate for producing stem cells;
- 20 iii) reprogramming the cultured cells into stem cells in the hydrogel-free 3D cell culture plate;
- iv) forming a spheroid of the reprogrammed stem cells;
- v) preparing a hydrogel-free 3D cell culture plate for subculturing; and
- vi) subculturing the spheroid in the hydrogel-free 3D cell culture plate once

or more by separating the spheroid,

wherein the 3D cell culture plate includes:

a well plate including a plurality of main wells and a plurality of sub wells formed at lower portions of the main wells to be injected with a cell culture solution and including recessed parts on a bottom surface thereof; and a connector for large-
5 capacity and high-speed high content screening (HCS), which supports the well plate, and

the connector for high content screening (HCS) includes a base equipped with a fixing means so as to be attached to and detached from a lower end of the well
10 plate and a cover positioned on an upper portion of the well plate to be coupled to the base, the main well has a step formed so as to be tapered at a predetermined site, and the step has an inclination angle (θ) ranging from 10 to 60° with respect to a wall of the main well.

The cells may be somatic cells or stem cells.

15 The somatic cells may be fibroblasts, but are not limited thereto, and any somatic cells known in the art can be used.

The stem cells may be one or more selected from the group consisting of adult stem cells, embryonic stem cells, mesenchymal stem cells, adipose-derived stem cells, hematopoietic stem cells, cord blood stem cells, and induced pluripotent
20 stem cells, and are not limited thereto, and any can be used as long as the stem cells are known in the art.

The cells may be cultured in a general 2D well plate, a 3D cell culture plate, or a 3D plate according to the present invention.

The hydrogel may be an extracellular matrix-based hydrogel.

25 The extracellular matrix-based hydrogel may be Matrigel (product name).

The subculturing may be subculturing for 1 to 20 generations, but is not limited thereto.

In step vi), the spheroid may be separated into single cells and the single cells are subcultured once or more.

5 The sub well of the 3D cell culture plate may have an inclined surface formed so as to taper toward the recessed part, the sub wells may have an upper end diameter ranging from 3.0 to 4.5 mm, the recessed parts may have an upper end diameter ranging from 0.45 to 1.5 mm, an inclined surface (θ_2) between the sub well and the recessed part may range from 40 to 50°, and a length ratio of the diameter of the sub
10 wells to the diameter of the recessed parts may range from 1:0.1 to 0.5.

The main wells of the 3D cell culture plate may have an individual volume ranging from 100 to 300 μ l, the recessed parts may have an individual volume ranging from 20 to 50 μ l, and an individual volume ratio of the main well to the recessed part may be 1 : 0.1 to 0.5 on average.

15 The main well includes a space part between the step and the sub well, the space part may have a height (a_h) ranging from 2.0 to 3.0 mm on average, the sub well may have a height (b_h) from 1.0 to 2.0 mm on average, and a height ratio ($a_h:b_h$) of the space part to the sub well may range from 1:0.3 to 1.

The somatic cells may be seeded in the sub wells of the cell culture plate at
20 100 to 1000 cells/well.

Hereinafter, the present invention will be described in detail.

Since the present invention may be modified in various forms and include various exemplary embodiments, specific exemplary embodiments will be illustrated in the drawings and described in detail in the Detailed Description.

25 However, the description is not intended to limit the present invention to the

specific embodiments, and it is to be understood that all the changes, equivalents and substitutions included in the idea and technical scope of the present invention are included in the present invention. When it is determined that the detailed description of the related publicly known art in describing the present invention may obscure the gist of the present invention, the detailed description thereof will be omitted.

The terms used in the present application are used only to describe specific embodiments, and are not intended to limit the present invention. Singular expressions include plural expressions unless the context clearly indicates otherwise.

In the present invention, the term “include” or “have” is intended to indicate the presence of the characteristic, number, step, operation, constituent element, part or any combination thereof described in the specification, and should be understood that the presence or addition possibility of one or more other characteristics or numbers, steps, operations, constituent elements, parts or any combination thereof is not pre-excluded.

In general, when cells, spheroids, organoids, and the like are cultured, a hydrogel is used to provide the role of an extracellular matrix. In general, when induced pluripotent stem cells are reprogrammed using a 2D plate or a 3D cell culture plate, the cell culture plate is coated with an extracellular matrix-based hydrogel (for example, Matrigel) and used.

However, the present invention provides a method of producing induced pluripotent stem cells using a 3D cell culture plate which does not include a hydrogel. A specific description on the 3D cell culture plate of the present invention is as follows.

In an exemplary embodiment, the present invention uses a 3D cell culture

plate including:

a well plate including a plurality of main wells and a plurality of sub wells formed at lower portions of the main wells to be injected with a cell culture solution and including recessed parts on a bottom surface thereof; and

5 a connector for large-capacity and high-speed high content screening (HCS), which supports the well plate,

wherein the connector for high content screening (HCS) includes a base equipped with a fixing means so as to be attached to and detached from a lower end of the well plate and a cover positioned on an upper portion of the well plate to be
10 coupled to the base,

the main well has a step formed so as to be tapered at a predetermined site, and the step has an inclination angle (θ) ranging from 10 to 60° with respect to a wall of the main well.

A 96-well plate in the related art has a problem in that it takes a lot of time
15 and costs because experiments and analyses should be performed several times or more in order to evaluate the efficacy of a drug in high yield. Furthermore, there is a case where the pipetting work of replacing a culture solution during cell culture is often performed, and in the case of a Corning spheroid microplate in the related art, spheroids or organoids in cell culture are affected, so that there was a problem which
20 is not good for the cell culture environment because there is a case where spheroids or organoids are sucked up or the positions thereof are changed during the pipetting work.

Therefore, the present invention has been made in an effort to solve the above-described problems, and provides a cell culture plate capable of manufacturing
25 spheroids/organoids in high yield by including a plurality of sub wells in a plurality

of main wells in a well plate, and capable of uniformly capturing images in the well plate by including a connector for large-capacity high-speed high content screening (HCS), which supports the well plate to reduce a tolerance when a large-capacity and high-speed image is captured. Furthermore, the present invention provides a cell culture plate capable of minimizing the effects of the pipetting work during replacement of a medium on cells to be cultured by the step of the main well.

Hereinafter, preferred exemplary embodiments of the present invention will be described in detail with reference to the accompanying drawings. Prior to the description, terms or words used in the specification and the claims should not be interpreted as being limited to a general or dictionary meaning and should be interpreted as a meaning and a concept which conform to the technical spirit of the present invention based on a principle that an inventor can appropriately define a concept of a term in order to describe his/her own invention by the best method.

Accordingly, since the exemplary embodiments described in the present specification and the configurations illustrated in the drawings are only the most preferred exemplary embodiments of the present invention and do not represent all of the technical spirit of the present invention, it is to be understood that various equivalents and modified examples, which may replace the exemplary embodiments and the configurations, are possible at the time of filing the present application.

FIG. 1A is a front view of a cell culture plate according to an exemplary embodiment of the present invention, FIG. 1B is a cross-sectional view of the cell culture plate according to an exemplary embodiment of the present invention, FIG. 2 is a view illustrating a main well formed in the cell culture plate according to an exemplary embodiment of the present invention, and FIG. 3 is a view illustrating a well plate, a base and a cover of the cell culture plate according to an exemplary

embodiment of the present invention ((A) a cover, (B) a base, and (C) a fixing means of a microplate and a base).

Hereinafter, a cell culture plate according to an exemplary embodiment of the present invention will be described in detail with reference to FIGS. 1 to 3.

5 As illustrated in FIGS. 1 to 3, a cell culture plate 10 according to an exemplary embodiment of the present invention includes a well plate 100 including a plurality of main wells 110 and a plurality of sub wells 120 formed at lower portions of the main wells 110 to be injected with a cell culture solution and including recessed parts 121 on a bottom surface thereof; and a connector 200 for large-
10 capacity and high-speed high content screening (HCS), which supports the well plate 100.

First, the well plate 100 according to an exemplary embodiment of the present invention will be described in detail.

The well plate 100 is made into a plate shape that is plastic injection-molded
15 through a mold. In order to manufacture a mold for plastic injection as described above, the main well 110 has a repeating pattern as a well structure such that the unit cost of production can be reduced and the size can be easily increased using fine machining. Therefore, cells are easily mass-produced, and the cells can be transformed into various sizes according to the user's requirements and used.

20 A plurality of the main wells 110 is formed in the well plate 100, and each main well 110 includes a step 101. The step 101 is formed at a predetermined site of the main well 110, and more specifically, the step 101 may be formed at a position which is 1/3 to 1/2 of a total length of the main well 110, and the step 101 may be formed at a position which is 1/3 to 1/2 of the total length from the lower end of the
25 main well 110.

In the related art, there is a case where the pipetting work of replacing a culture solution during cell culture is performed, and in this case, spheroids or organoids in cell culture are affected, so that there is a problem which is not good for the cell culture because there is a case where the spheroids or organoids are sucked up or the positions thereof are changed during the pipetting work, but the step 101 is provided to prevent this problem.

The step 101 may be a space to which a pipette is applied, and specifically, may have an inclination angle (θ) ranging from 10 to 60° with respect to a wall of the main well 110. Alternatively, the step 101 may have an inclination angle ranging from 20 to 50°, preferably ranging from 30 to 45°. When the inclination angle of the step 101 is less than 10°, the inclination angle within the main well 110 is so small that the space to which a pipette can be applied is not sufficient, and as a result, when the culture solution in the main well 110 is sucked up, the pipette may slide inside the sub well 120, causing spheroids or organoids to be sucked up, or the positions thereof, and the like to be changed. Furthermore, when the inclination angle (θ) exceeds 60°, a space to which a pipette can be applied is provided, but the inclination angle of the step 101 is so large that it may be difficult to sufficiently suck up the culture solution, and when cells are seeded on the sub well 120, a problem in that cells are seeded on the step 101 without entering all the sub wells 120 may occur. Therefore, it is desirable to have an inclination angle in the above-described range.

Meanwhile, the main well according to an exemplary embodiment of the present invention may include a space part 130 between the step 101 and a sub well 120 to be described below. Specifically, the space part 130 is a space into which a culture solution is injected, and is a space in which cells inside the sub well 120 can share the same culture solution.

More specifically, the space part 130 may have a height (a_h) ranging from 2.0 to 3.0 mm on average, or ranging from 2.2 to 2.8 mm, or ranging from 2.3 to 2.7 mm on average. Furthermore, the sub well 120 may have a height (b_h) ranging from 1.0 to 2.0 mm on average, or ranging from 1.2 to 1.8 mm on average.

5 For example, the space part 130 may have a height (a_h) of 2.5 mm on average, and the sub well may have a height (b_h) of 1.5 mm on average.

In this case, a height ratio ($a_h:b_h$) of the space part to the sub well may range from 1:0.3 to 1, and more specifically, a height ratio ($a_h:b_h$) of the space part to the sub well 120 may be 1:0.4 to 0.9 or 1:0.5 to 0.8. When a ratio of the height of the
10 sub well 120 to the height of the space part is less than 1:0.3, the cells in culture may escape from the inside even with a small force during the exchange of the media of the sub well 120, and when a ratio of the height of the sub well 120 to the height of the space part exceeds 1:1, the culture solution required for the cells is not sufficiently converted, so that cell death may be induced. Therefore, it is preferred
15 that the space part 130 and the sub well 120 have the above-described height range and height ratio.

Next, the sub wells 120 are formed at lower portion of each of the main wells 110 and include recessed parts 121 on a bottom surface thereof. As a particular aspect, the sub well 120 may include a plurality of recessed parts at lower portions of
20 the main well 110.

The sub wells 120 included at the lower portion of the main well 110 have the same size and shape, thereby enabling spheroids and organoids to be produced under uniform conditions.

The sub well 120 may have an inclined surface formed so as to taper toward
25 the recessed part 121. Specifically, the horizontal area of the upper portion of the

sub well 120 may become smaller as it descends in the vertical direction. For example, the upper portion of the sub well 120 may be formed in an inverted pyramid shape. In the illustrated exemplary embodiment, the upper portion of the sub well 120 may be formed in a shape such as a pyramid shape or a funnel shape in which the horizontal area of the upper portion of the sub well 120 becomes smaller as it descends in the vertical direction.

In particular, the cell culture plate may produce a large amount of spheroids or organoids under uniform conditions by including a plurality of the sub wells 120 so as to have the same size and shape.

As a particular aspect, one main well 110 can include 4 to 25 sub wells 120 of the same size, and the entire microplate 100 may include 96 to 1,728 sub wells 120. Accordingly, the size can be controlled in the same precise manner.

Furthermore, the sub well 120 includes a recessed part 121, and a space is formed in the lower portion of the recessed part such that 3D spheroids or an organoids can be cultured in the recessed part 121. Specifically, the recessed part 121 may be in the form of the letter 'U', 'V', or 'I', and for example, the recessed part 121 may be in the form of the letter 'U'.

The sub well 120 may have an upper end diameter ranging from 3.0 to 4.5 mm, or ranging from 3.5 to 4.3 mm, or 4 mm on average. Furthermore, the recessed part 121 may have an upper end diameter of 0.45 to 1.5 mm, or 0.5 to 1.0 mm or 0.5 mm on average.

Furthermore, a length ratio of the diameter of the sub well 120 to the diameter of the recessed part 121 may range from 1:0.1 to 0.5, and preferably, a length ratio of the diameter of the sub well 120 to the diameter of the recessed part 121 may be 1:0.12.

When the upper end diameter of the recessed part 121 is less than 0.1 compared to the upper end diameter 1 of the sub well 120, a cell culture space of the recessed part 121 cannot be sufficiently provided, which may cause a problem in that cells escape even with a small force during the replacement of the culture solution, and when the upper end diameter of the recessed part 121 is exceeds 0.5 compared to the upper end diameter 1 of the sub well 120, a sufficient culture solution required for cells cannot be replaced, which may cause a problem in that it is difficult to stably culture cells.

Meanwhile, an inclination surface between the sub well 120 and the recessed part 121 may have an inclination angle (θ_2) of 40 to 50°, 42 to 48°, 43 to 47°, or an inclination angle (θ_2) of 45° on average, with respect to a wall of the main well.

The above-described sub well 120 has an advantage in that cells can be cultured at 100 to 1000 cells/well or less, and the spheroid size can be stably controlled.

Further, the main well 110 according to an exemplary embodiment of the present invention has an individual volume ranging from 100 to 300 μl , the recessed part 121 has an individual volume ranging from 20 to 50 μl , and an individual volume ratio of the main well 110 to the recessed part 121 is characterized by being 1:0.07 to 0.5 on average. Preferably, the main well according to an exemplary embodiment has an individual volume ranging from 250 to 300 μl , the recessed part has an individual volume ranging from 25 to 35 μl , and an individual volume ratio of the main well 110 to the recessed part 121 may be 1:0.11 on average.

Specifically, when the main well 110 has an individual volume less than 100 μl , a problem in that a sufficient culture solution cannot be accommodated during cell culture may occur, and when the individual volume exceeds 300 μl , culture

efficiency may be reduced.

Furthermore, the recessed part 121 is a space in which cells are substantially cultured, and when the volume is less than 20 μ l, the cell culture space is not sufficient, which may cause a problem in that cells escape, and when the volume
5 exceeds 50 μ l, a problem in that it is difficult to stably culture cells and the like may occur. Therefore, it is preferred that the main well 110 and the recessed part 121 have volumes in the above-described ranges.

Due to the above-mentioned configuration of the cell culture plate of the present invention, reprogramming into induced pluripotent stem cells occurs at high
10 efficiency without including a hydrogel, that is, without coating the cell culture plate with a hydrogel, and the spheroid is also formed well after the reprogramming.

Further, when the spheroid is separated and subcultured in the cell culture plate of the present invention, the spheroid is formed very well. Specifically, hundreds to thousands of monoclonal induced pluripotent stem cells may be
15 produced by separating the spheroid into single cells and subculturing the single cells.

The cell culture plate 10 according to an exemplary embodiment of the present invention includes a connector 200 for large-capacity and high-speed high content screening (HCS), which supports the well plate 100. Herein, the connector
20 200 for large-capacity and high-speed high content screening (HCS) refers to a connector 200 which is attached to a high content screening (HCS) system, and specifically, the connector may refer to a base 210 and a cover 220 in the present invention.

More specifically, the connector for large-capacity and high-speed high content screening (HCS) includes the base 210 equipped with fixing means 140 and
25 240 so as to be attached to and detached from a lower end of the well plate 100 and a

cover 220 positioned on an upper portion of the well plate 100 to be coupled to the base 210. Moreover, the upper end of the base 210 and the lower end of the well plate 100 are characterized by including fixing means 140 and 240 that can be fixed so as to be attached to and detached from each other.

5 In this case, the base includes a convex part 240 for supporting the well plate 100, and the well plate 100 may include a concave part 140 facing the convex part 240 of the base 210. The well plate 100 may be fixed by the fixing means to uniformly capture images during screening.

The base may be formed of a polyethylene, polypropylene, polystyrene, 10 polyethylene terephthalate, polyamide, polyester, polyvinyl chloride, polyurethane, polycarbonate, polyvinylidene chloride, polytetrafluoroethylene, polyether ether ketone or polyetherimide material, but is not limited thereto.

The well plate may be formed of a polydimethylsilicone, high-fat modified silicone, methylchlorophenyl silicone, alkyl-modified silicone, methylphenylsilicone, 15 silicone polyester, or amino-modified silicone material, but is not limited thereto.

Meanwhile, when induced pluripotent stem cells are formed in the cell culture plate 10 of the present invention, Matrigel need not be used.

FIG. 4 illustrates a comparison of a method of producing induced pluripotent stem cells using a 2D cell culture plate that uses Matrigel and a method of producing 20 induced pluripotent stem cells using a 3D cell culture plate that does not require Matrigel according to the present invention. After somatic cells (fibroblasts) are cultured, induced pluripotent stem cells are produced by transfecting the fibroblasts with an episomal vector by electroporation to induce reprogramming. In the case of 2D Matrigel culture, the process of collecting a colony of induced pluripotent stem 25 cells is complicated, and the yield is low. However, when the 3D culture plate of

the present invention is used, there is no Matrigel, so that a number of single cells reprogrammed into induced pluripotent stem cells gather to form a spheroid, which is a 3D spherical cell aggregate. This spheroid can be easily separated from a 3D cell culture plate, and can be sub-cultured (FIG. 7D). That is, reprogramming efficiency is very high.

Further, as previously described, for the 3D cell culture plate used in the present invention, one main well 110 can include 4 to 25 sub wells 120 of the same size, and the entire microplate 100 may include 96 to 1,728 sub wells 120. Accordingly, it is possible to mass-produce induced pluripotent stem cells and spheroids thereof whose sizes can be controlled in the same precise manner.

FIG. 10 schematically illustrates the method of mass proliferating induced pluripotent stem cell spheroids obtained in the step of reprogramming induced pluripotent stem cells. It can be seen that compared to the 2D cell culture plate (coated with Matrigel), the induced pluripotent stem cell proliferation rate of the present invention is very high. In addition, when spheroids are separated into single cells and the single cells are plated again, and then subcultured, hundreds to thousands of uniformly sized monoclonal spheroids are produced, so that an induced pluripotent stem cell spheroid bank may also be produced.

【Advantageous Effects】

According to the production method of the present invention, it is possible to mass-proliferate stem cells with enhanced reprogramming efficiency without the need for a hydrogel. Further, a monoclonal stem cell spheroid bank can be produced.

【Description of Drawings】

FIG. 1A is a front view of a cell culture plate according to an exemplary embodiment of the present invention, and FIG. 1B is a cross-sectional view of the cell culture plate according to an exemplary embodiment of the present invention.

FIG. 2 is a view illustrating, in detail, a main well formed in the cell culture
5 plate according to an exemplary embodiment of the present invention.

FIG. 3 is a view illustrating a well plate, a base, and a cover of the cell culture plate according to an exemplary embodiment of the present invention ((A) a cover, (B) a base, and (C) a fixing means of a microplate and a base).

FIG. 4A schematically illustrates processes of producing induced pluripotent
10 stem cells according to an exemplary embodiment of the present invention and a comparative example, and FIG. 4B is a set of images illustrating the generation of induced pluripotent stem cells according to an exemplary embodiment of the present invention and a comparative example (In all of the drawings below FIG. 4, for the convenience of description, the 3D cell culture plate of the present invention is not
15 accurately displayed, but is displayed in a U shape for convenience.)

FIG. 5 is a set of images of an exemplary embodiment (3D iPSC) of the present invention and a comparative example (2D iPSC).

FIG. 6 is an alkaline phosphatase (AP) stained image of an exemplary
20 embodiment (3D sph-iPSC) of the present invention and a comparative example (2D Matrigel).

FIG. 7A is an AP image (D4, D9, D15, D21) over time, FIG. 7B compares the number of colonies, FIG. 7C is an E-cadherin expression result, and FIG. 7D illustrates the process of forming a spheroid of iPSCs.

FIG. 8A is a result of showing the size distribution of spheroids (colonies) as
25 a result of 3D culture in the related art and culture according to an exemplary

embodiment of the present invention, and FIG. 8B is the expression result of a reprogramming factor (pluripotency marker).

FIG. 9 illustrates the expression results of pluripotency markers of iPSCs according to an exemplary embodiment of the present invention.

5 FIG. 10 is a schematic view illustrating the method of proliferating induced pluripotent stem cells according to an exemplary embodiment of the present invention and the comparative example.

FIG. 11 is an image illustrating the process of proliferating induced pluripotent stem cells according to an exemplary embodiment of the present invention over time.

FIG. 12 is a graph showing that the size is uniformly distributed when the induced pluripotent stem cells are cultured according to an exemplary embodiment of the present invention.

FIG. 13 is a graph comparing the proliferation efficiencies of an exemplary embodiment (3D cell culture) of the present invention and a comparative example (2D cell culture).

FIGS. 14 and 15 illustrate the results of pluripotency marker expression over the induced pluripotent stem cell proliferation time.

FIG. 16 schematically illustrates the process of production, proliferation and differentiation of induced pluripotent stem cells according to the present invention.

【Modes of the Invention】

Since the present invention may be modified into various forms and include various exemplary embodiments, specific exemplary embodiments will be illustrated in the drawings and described in detail in the Detailed Description. However, the description is not intended to limit the present invention to the specific exemplary

embodiments, and it is to be understood that all the changes, equivalents, and substitutions belonging to the spirit and technical scope of the present invention are included in the present invention. When it is determined that the detailed description of the related publicly known art in describing the present invention may obscure the gist of the present invention, the detailed description thereof will be omitted.

[Examples]

Example 1. Experimental methods

1-1: Culture of fibroblasts and production of induced pluripotent stem cells

The German federal authorities/RKI: AZ 1710-79-1-4-41 E01 (F134), which is a human fibroblast line, was cultured in a DMEM containing 10% FBS (fetal bovine serum, Invitrogen, USA) and 1 mM L-glutamine (Invitrogen, USA) in a 35 mm or 100 mm Petri dish. The cultured fibroblasts were reprogrammed by being transfected (NeonTM transfection system) with an episomal iPSC reprogramming vector (EP5TM kit: Cat. No. A16960. Invitrogen, Carlsbad, CA, USA) by electroporation. The electroporation was performed under the conditions of 1,650 V, 10 ms, and 3 pulses.

As illustrated in FIG. 4A, the transfected fibroblasts were inoculated in a 3D cell culture plate of the present invention (without Matrigel, the Example), a 2D 12-well plate (coated with Matrigel, Comparative Example 1) and a commercialized product Addgene (Comparative Example 2, coated with Matrigel, not illustrated in FIG. 4A), and cultured in an N2B27 medium (containing bFGF). After the fibroblasts were cultured for 15 days, the medium was replaced with an Essential 8TM medium. After 15 days, the number of colonies in the Example and the

Comparative Examples were confirmed by plating 3D iPSC of the Example (3D cell culture plate) on a 12-well plate which is a 2D plate.

1-2: Reprogramming efficiency analysis of fibroblasts

According to the alkaline phosphatase staining kit manual (System Biosciences, USA), reprogrammed cells were washed twice with PBS, fixed with 4% paraformaldehyde, then stained with Blue-color AP solution, washed twice with PBS, and then it was observed under an optical microscope whether the colonies were stained. The number of stained colonies was counted and quantified.

Images of the cultured cells in the Example and the Comparative Examples were captured, and the sizes of cell spheres were compared. Spheroids were subjected to imaging by an automated plate device, and in this case, the device was allowed to perform imaging by automatically focusing. Image size analysis was performed using a macro program of a program called ImageJ (related to FIGS. 5, 6 and 7).

1-3: Optimization of 3D culture method of induced pluripotent stem cells

Images of the 3D induced pluripotent stem cells cultured in the Example and the Comparative Examples were captured, and accordingly, the sizes of the cell spheres were compared and measured (FIG. 8A). An external inspection company (Cell Bio CEFO, Korea) was commissioned to test the results of the images, and this test was performed as a blind test.

1-4: Immunostaining

Reprogrammed cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes. After the fixed cells were reacted with PBS containing 1% BSA and 0.5% Triton X-100 at room temperature for 1 hour, the cells were treated with each of primary antibodies Oct4 (1:100, SantaCruz, CA, USA), Sox2

(1:100, Cell Signaling, Danvers, MA, USA), Nanog (1:200, Cosmo Bio, Koto-Ku, Japan), and E-cadherin (1:200, abcam), and reacted with FITC-conjugated goat anti-rabbit IgG or anti-mouse IgG (1:100, Invitrogen, Carlsbad, CA) as a secondary antibody. Fluorescent images were analyzed under a fluorescence microscope
5 (Olympus, Shinjuku, Tokyo, Japan). DAPI was used as a nuclear staining solution.

1-5: Verification of efficiency of 3D mass culture of stem cells or induced pluripotent stem cells

Induced pluripotent stem cells were seeded with different numbers of cells, and size comparisons were performed by date. It was verified that by putting 0.1,
10 0.3, 0.5, 1, 2, 4×10^5 cells, respectively into the multi-well corresponding to the example, the cell size was maintained and the cell number is constantly increased regardless of the number of cells (related to FIGS. 12 and 13).

As a result of comparing the increases in these cell numbers, it was confirmed that as a result of comparing the numbers of 2D cultured cells at the same period, the
15 number of cells was increased by $22.9 \pm 4.33\%$.

1-6: qPCR

Total RNA was extracted from fibroblasts and reprogrammed cells using an RNA minikit (Qiagen, Inc.), and then converted to cDNA using the Accupower RT mix reagent (Bioneer Corp., Seoul, Korea). qPCR was performed using Real-time
20 PCR FastStart Essential DNA Green Master Mix (Roche, Indianapolis, IN, USA). The primer sequences used in the present invention are as follows in Table 1.

[Table 1]

Genes	Primer sequences (5'-3')	
hCOL1A1	forward	ATGACTATGAGTATGGGGAAGCA
	reverse	TGGGTCCCTCTGTTACACTTT
hOCT4	forward	AATTTGTTCCCTGCAGTGCCC
	reverse	AGACCCAGCAGCCTCAAAAT
hNANOG	forward	GGATCCAGCTTGTCCCCAAA
	reverse	TGCGACACTCTTCTCTGCAG
hSOX2	forward	CGGAAAACCAAGACGCTCAT
	reverse	GTTTCATGTGCGCGTAACTGT
hLIN28	forward	TTCGGCTTCCTGTCCATGAC
	reverse	CCGCCTCTCACTCCCAATAC

Example 2. Confirmation of reprogramming efficiency

Referring to FIG. 4B, it can be seen that in the case of 2D culture, a small amount of colonies begin to be formed only at D15. After iPSC reprogramming was induced up to D15, 3D iPSCs were plated on a 2D plate, and the number of colonies in Comparative Example 1 and Example 1 was compared by plating 3D iPSCs on a 2D plate, and as a result, the difference in the number of colonies formed was large. It can be seen that the iPSC reprogramming yield of the Example is high because the cells that are well differentiated into iPSCs forming a colony. Referring to FIGS. 5 and 7B, it can be seen that the difference in the number of colonies is very large between the 2D culture (Comparative Example 1) and the 3D culture (the Example). Referring to FIG. 6, it can be seen that as a result of alkaline phosphatase (AP) staining, the reprogramming efficiency is very high in 3D iPSC spheroids (the Example, 3D sph-iPCSs). Further, referring to FIGS. 6 and 7A, when 2D Matrigel (Comparative Example 1) and 3D iPSC spheroids (the Example, 3D sph-iPCSs) are compared with each other, the images appear uniform and clear, showing that the 3D cell culture plate of the present invention is capable of large-

scale image analysis.

Referring to FIG. 7C, it can be seen that the reprogramming efficiency in the 3D cell culture plate is very good. In addition, referring to FIG. 7D, it can be seen that since the present invention does not use Matrigel, a large number of single cells
5 reprogrammed into iPSCs gather to form a spheroid, which is a spherical cell aggregate, and these spheroids can be easily separated from the 3D cell culture plate and re-plated. That is, reprogramming efficiency is very high.

FIG. 8 compares the 3D culture in the related art of Comparative Example 2 with the 3D culture of the Example of the present invention (SpheroidFilm in FIG.
10 8B). The 3D culture in the related art is not uniform in size and has a relatively low expression level of oct4. However, the present invention is very uniform in size (99.45%) and has a very high reprogramming factor expression level. That is, the present invention is effective in stem cell culture even when compared to the 3D culture in the related art, and can increase the efficiency of reprogramming somatic
15 cells into induced pluripotent stem cells. Furthermore, a uniform size means that standardized induced pluripotent stem cells and stem cells can be three-dimensionally mass-produced in the form of a spheroid.

Example 3. Analysis of characteristics of stem cells

Referring to FIG. 9, it can be seen that the iPSCs produced according to the
20 present invention have very high expression of pluripotency markers.

Example 4. Confirmation of mass-proliferation of stem cells

Referring to FIGS. 10 and 11, it can be seen that when the iPSCs produced by the present invention are subcultured, the iPSCs can be mass-proliferated. FIG. 13 is a graph comparing a proliferation efficiency when induced pluripotent stem cells
25 reprogrammed in the 2D 12-well plate (coated with Matrigel, Comparative Example

1) are subcultured in a 2D Matrigel-coated plate with a proliferation efficiency when the induced pluripotent stem cells reprogrammed according to the example of the present invention are subcultured in the 3D subculture plate of the present invention. Referring to FIG. 13, it can be seen that in the case of the present invention, the proliferation efficiency is increased by about 23 times. When making a determination in combination with the previous results in FIG. 12, it can be concluded that it is possible to mass-proliferate cells with a uniform size. Referring to FIGS. 14 and 15, it can be seen that the mass-proliferated iPSCs also have very high expression of pluripotency markers.

FIG. 16 schematically illustrates the present invention. When somatic cells are reprogrammed into induced pluripotent stem cells at high efficiency using the 3D cell culture plate of the present invention without using a hydrogel and spheroids of the reprogrammed induced pluripotent stem cells are separated and subcultured, the induced pluripotent stem cells may be mass-proliferated.

iPSCs are generated and proliferate in wells of the cell culture plate of the present invention. The iPSCs can also be stored as they are, and a medium can also be frozen at once. Since the well size can be adjusted without using a hydrogel such as Matrigel, a small amount of medium is used, and thus, the culture is economical. Moreover, the mass-proliferated induced pluripotent stem cells can be differentiated into various cells.

Although a specific part of the present invention has been described in detail, it will be obvious to those skilled in the art that such a specific description is just a preferred embodiment and the scope of the present invention is not limited thereby. Accordingly, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

【Description of Reference Numerals and Symbols】

	100: Well plate
	101: Step
	110: Main well
5	120: Sub well
	121: Recessed part
	130: Space part
	140: Concave part
	200: Connector for large-capacity and high-speed HCS
10	210: Base
	220: Cover
	240: Convex part

【CLAIMS】

【Claim 1】

A method of proliferating stem cells, the method comprising:

- i) culturing cells;
- 5 ii) preparing a hydrogel-free 3D cell culture plate for producing stem cells;
- iii) reprogramming the cultured cells into stem cells in the hydrogel-free 3D cell culture plate;
- iv) forming a spheroid of the reprogrammed stem cells;
- v) preparing a hydrogel-free 3D cell culture plate for subculturing; and
- 10 vi) subculturing the spheroid in the hydrogel-free 3D cell culture plate once or more by separating the spheroid,

wherein the 3D cell culture plate comprises:

a well plate comprising a plurality of main wells and a plurality of sub wells formed at lower portions of the main wells to be injected with a cell culture solution
15 and comprising recessed parts on a bottom surface thereof; and a connector for large-capacity and high-speed high content screening (HCS), which supports the well plate, and

the connector for high content screening (HCS) comprises a base equipped with a fixing means so as to be attached to and detached from a lower end of the well
20 plate and a cover positioned on an upper portion of the well plate to be coupled to the base,

the main well has a step formed so as to be tapered at a predetermined site, and the step has an inclination angle (θ) ranging from 10 to 60° with respect to a wall of the main well.

【Claim 2】

The method of claim 1, wherein the cells are somatic cells or stem cells.

【Claim 3】

5 The method of claim 2, wherein the stem cells are one or more selected from the group consisting of adult stem cells, embryonic stem cells, mesenchymal stem cells, adipose-derived stem cells, hematopoietic stem cells, cord blood stem cells, and induced pluripotent stem cells.

【Claim 4】

10 The method of claim 1, wherein the hydrogel is an extracellular matrix-based hydrogel.

【Claim 5】

The method of claim 4, wherein the extracellular matrix-based hydrogel is Matrigel.

【Claim 6】

15 The method of claim 1, wherein the subculturing is subculturing for 1 to 20 generations.

【Claim 7】

The method of claim 1, wherein in step vi), the spheroid is separated into single cells and the single cells are subcultured once or more.

20 **【Claim 8】**

The method of claim 1, wherein the sub well has an inclined surface formed so as to taper toward the recessed part,

the sub wells have an upper end diameter ranging from 3.0 to 4.5 mm,

the recessed parts have an upper end diameter ranging from 0.45 to 1.5 mm,

an inclined surface (θ_2) between the sub well and the recessed part ranges from 40 to 50°, and

a length ratio of the diameter of the sub wells to the diameter of the recessed parts ranges from 1:0.1 to 0.5.

5 **【Claim 9】**

The method of claim 1, wherein the main well has an individual volume ranging from 100 to 300 μl ,

the recessed part has an individual volume ranging from 20 to 50 μl , and

an individual volume ratio of the main well to the recessed part is 1:0.1 to 0.5

10 on average.

【Claim 10】

The method of claim 1, wherein the main well comprises a space part between the step and the sub well,

the space part has a height (a_h) ranging from 2.0 to 3.0 mm on average,

15 the sub well has a height (b_h) ranging from 1.0 to 2.0 mm on average, and

a height ratio ($a_h:b_h$) of the space part to the sub well ranges from 1:0.3 to 1.

【Claim 11】

The method of claim 1, wherein the somatic cells are seeded in the sub wells of the cell culture plate at 100 to 1000 cells/well.

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