

a Wnt signal transduction pathway inhibiting substance and addition of SAG (10 nM) as an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 12D-F "Condition 2").

•Condition 3. Condition including addition of IWR-1e (3 μ M) as a Wnt signal transduction pathway inhibiting substance and addition of SAG (30 nM) as an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 12G-I "Condition 3").

A cell aggregate was formed by day 2 after the start of suspension culture under Condition 1, Condition 2 and Condition 3 (step 2 completed, and step 3 started).

[0331]

On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.

On day 12 after the start of suspension culture, a 80% medium exchange operation was performed 3 times by using the aforementioned serum-free medium so that the concentration of exogenous IWR-1e would be 3% or less as compared to that before medium exchange. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the aforementioned serum-free medium not containing Y27632, SAG, human recombinant BMP4 and IWR-1e.

The thus-prepared cells on day 20 after the start of

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adhesive 96-well culture plate (PrimeSurface 96V-bottom plate, manufactured by SUMITOMO BAKELITE). Thereafter, the cells were subjected to suspension culture at 37°C, 5% CO₂. As the serum-free medium (gfCDM+KSR) therefor, a serum-free medium which is
5 a 1:1 mixture of F-12 medium and IMDM medium supplemented with 10% KSR, 450 µM 1-monothioglycerol, 1 x Chemically defined lipid concentrate was used. At the time of the start of suspension culture (day 0 after the start of suspension culture, start of step 2), Y27632 (final concentration 20 µM) was added
10 to the above-mentioned serum-free medium and the cells were cultured in a serum-free medium under three conditions of the following Conditions 1 - 3.

•Condition 1. Condition including addition of IWR-1e (3 µM) as a Wnt signal transduction pathway inhibiting substance and no
15 addition of an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 13A-C, "Condition 1").

•Condition 2. Condition including addition of IWR-1e (3 µM) as a Wnt signal transduction pathway inhibiting substance and
20 addition of SAG (10 nM) as an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 13D-F "Condition 2").

•Condition 3. Condition including addition of IWR-1e (3 µM) as
25 a Wnt signal transduction pathway inhibiting substance and addition of SAG (30 nM) as an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 13G-I "Condition 3").

30 A cell aggregate was formed by day 2 after the start of suspension culture under Condition 1, Condition 2 and Condition 3 (step 2 completed, and step 3 started).

[0335]

On day 3 after the start of suspension culture, a medium
35 not containing Y27632 or SAG, containing human

On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.

On day 10 after the start of suspension culture, a 80% medium exchange operation was performed 3 times by using the aforementioned serum-free medium so that the concentration of exogenous IWR-1e would be 3% or less as compared to that before medium exchange. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the aforementioned serum-free medium not containing Y27632, SAG, human recombinant BMP4 and IWR-1e.

The thus-prepared cells on day 20 after the start of suspension culture were subjected to bright field observation using an inverted microscope (manufactured by Nikon Corporation, ECLIPSE Ti). As a result, it was found that a neural tissue was formed.

[0340]

A part of the cells on day 20 after the start of the aforementioned suspension culture was extracted and subjected to a differentiation culture according to the method described in "Nature Communications 6, 6286 (2015)".

The cell aggregate on day 20 after the start of suspension culture were transferred to a 90 mm low adhesive culture dish (manufactured by SUMITOMO BAKELITE CO., LTD.) and cultured in a serum-free medium (DMEM/F12 medium added with 1% N2 supplement) containing a Wnt signal transduction pathway activating substance (CHIR99021, 3 μ M) and an FGF signal

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1: Precondition (Shh) treatment, Fig. 15) in a Stem Fit
(registered trade mark) medium (AK03N; manufactured by
Ajinomoto Co., Inc.) (that is, Sonic hedgehog signal
transduction pathway activating substance was allowed to act
5 for two days before start of differentiation).

The thus-prepared Precondition (Shh)-treated human iPS
cells were treated with the cell dispersion solution by using
TrypLE Select (Life Technologies), further dispersed into
single cells by pipetting operation, and the above human iPS
10 cells dispersed into single cells were suspended in 100 μ l of a
serum-free medium at 1.0×10^4 cells per well of a non-cell-
adhesive 96-well culture plate (PrimeSurface 96V-bottom plate,
manufactured by SUMITOMO BAKELITE). Thereafter, the cells were
subjected to suspension culture at 37°C, 5% CO₂. As the serum-
15 free medium (gfCDM+KSR) therefor, a serum-free medium which is
a 1:1 mixture of F-12 medium and IMDM medium supplemented with
10% KSR, 450 μ M 1-monothioglycerol, 1 x Chemically defined
lipid concentrate was used. At the time of the start of
suspension culture (day 0 after the start of suspension culture,
20 start of step 2), Y27632 (final concentration 20 μ M) was added
to the above-mentioned serum-free medium and the cells were
cultured in a serum-free medium under three conditions of the
following Conditions.

•Condition 1. Condition including addition of IWR-1e (3 μ M) as
25 a Wnt signal transduction pathway inhibiting substance and
addition of SAG (30 nM) as an exogenous Sonic hedgehog signal
transduction pathway activating substance to the aforementioned
serum-free medium at the start of step 2.

A cell aggregate was formed by day 2 after the start of
30 suspension culture under Condition 1, Condition 2 and Condition
3 (step 2 completed, and step 3 started).

[0347]

On day 3 after the start of suspension culture, a medium
not containing Y27632 or SAG, containing human recombinant BMP4
35 (manufactured by R&D) and further containing

a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.

10 •Condition B. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration of exogenous IWR-1e would not change.

15 Further, On day 6 after the start of suspension culture, a half-medium exchange operation was performed using the aforementioned serum-free medium not containing Y27632 or SAG and containing human recombinant BMP4 (manufactured by R&D) and IWR-1e such that the concentration of exogenous human recombinant BMP4 and the concentration of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.

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[0356]

After culturing under two conditions of the above-mentioned Conditions A, B, the cells were further cultured as follows.

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On day 12 after the start of suspension culture, a 80% medium exchange operation was performed 3 times by using the aforementioned serum-free medium so that the concentration of exogenous IWR-1e would be 3% or less as compared to that before medium exchange. Thereafter, a half-medium exchange operation

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above-mentioned serum-free medium and the cells were cultured in a serum-free medium under the following Condition.

•Condition. Condition including addition of IWR-1e (3 μ M) as a Wnt signal transduction pathway inhibiting substance and no addition of an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 16, C, D).

A cell aggregate was formed by day 2 after the start of suspension culture (step 2 completed, and step 3 started).

10 [0360]

Furthermore, the cells were cultured in a serum-free medium under two conditions of the following Conditions C and D.

•Condition C. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.

•Condition D. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration of exogenous IWR-1e would not change.

Further, On day 6 after the start of suspension culture, a half-medium exchange operation was performed using the aforementioned serum-free medium not containing Y27632 or SAG and containing human recombinant BMP4 (manufactured by R&D) and IWR-1e such that the concentration of exogenous human recombinant BMP4 and the concentration of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation

iPS cells were treated with the cell dispersion solution by using TrypLE Select (Life Technologies), further dispersed into single cells by pipetting operation, and the above human iPS cells dispersed into single cells were suspended in 100 μ l of a serum-free medium at 1.0×10^4 cells per well of a non-cell-adhesive 96-well culture plate (PrimeSurface 96V-bottom plate, manufactured by SUMITOMO BAKELITE). Thereafter, the cells were subjected to suspension culture at 37°C, 5% CO₂. As the serum-free medium (gfCDM+KSR) therefor, a serum-free medium which is a 1:1 mixture of F-12 medium and IMDM medium supplemented with 10% KSR, 450 μ M 1-monothioglycerol, 1 x Chemically defined lipid concentrate was used. At the time of the start of suspension culture (day 0 after the start of suspension culture, start of step 2), Y27632 (final concentration 20 μ M) was added to the above-mentioned serum-free medium and the cells were cultured in a serum-free medium under the following Condition.

- Condition 1. Condition including addition of IWR-1e (3 μ M) as a Wnt signal transduction pathway inhibiting substance and no addition of an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 17, A, B).

A cell aggregate was formed by day 2 after the start of suspension culture (step 2 completed, and step 3 started).
[0365]

Furthermore, the cells were cultured in a serum-free medium under two conditions of the following Conditions A, B.

- Condition A. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-

le to avoid change of the concentration of exogenous IWR-1e.

•Condition B. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change. Further, On day 6 after the start of suspension culture, a half-medium exchange operation was performed using the aforementioned serum-free medium not containing Y27632 or SAG and containing human recombinant BMP4 (manufactured by R&D) and IWR-1e such that the concentration of exogenous human recombinant BMP4 and the concentration of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.

[0366]

After culturing under two conditions of the above-mentioned Conditions A, B, the cells were cultured as follows.

On day 10 after the start of suspension culture, a 80% medium exchange operation was performed 3 times by using the aforementioned serum-free medium so that the concentration of exogenous IWR-1e would be 3% or less as compared to that before medium exchange. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the aforementioned serum-free medium not containing Y27632, SAG, human recombinant BMP4 and IWR-1e.

The thus-prepared cells on day 20 after the start of suspension culture were subjected to bright field observation using an inverted microscope (manufactured by Nikon Corporation, ECLIPSE Ti). As a result, it was found that a neural tissue was formed under Condition A and Condition B.

[0367]

Wnt signal transduction pathway inhibiting substance and no addition of an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 18, A, B).

5 A cell aggregate was formed by day 2 after the start of suspension culture (step 2 completed, and step 3 started).
[0370]

Furthermore, the cells were cultured in a serum-free medium under two conditions of the following Conditions A, B.

- 10 •Condition A. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and
15 the concentration (3 μ M) of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.
- 20 •Condition B. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and
25 the concentration (3 μ M) of exogenous IWR-1e would not change. Further, On day 6 after the start of suspension culture, a half-medium exchange operation was performed using the aforementioned serum-free medium not containing Y27632 or SAG and containing human recombinant BMP4 (manufactured by R&D) and
30 IWR-1e such that the concentration of exogenous human recombinant BMP4 and the concentration of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further
35 containing IWR-1e to avoid change of the concentration of

The thus-prepared Precondition (BM*PR*-i 24h+Shh 48h)-treated human iPS cells were treated with the cell dispersion solution by using TrypLE Select (Life Technologies), further dispersed into single cells by pipetting operation, and the
5 above human iPS cells dispersed into single cells were suspended in 100 μ l of a serum-free medium at 1.0×10^4 cells per well of a non-cell-adhesive 96-well culture plate (PrimeSurface 96V-bottom plate, manufactured by SUMITOMO BAKELITE). Thereafter, the cells were subjected to suspension
10 culture at 37°C, 5% CO₂. As the serum-free medium (gfCDM+KSR) therefor, a serum-free medium which is a 1:1 mixture of F-12 medium and IMDM medium supplemented with 10% KSR, 450 μ M 1-monothioglycerol, 1 x Chemically defined lipid concentrate was used. At the time of the start of suspension culture (day 0
15 after the start of suspension culture, start of step 2), Y27632 (final concentration 20 μ M) was added to the above-mentioned serum-free medium and the cells were cultured in the serum-free medium under the following Condition.

•Condition. Condition including addition of IWR-1e (3 μ M) as a
20 Wnt signal transduction pathway inhibiting substance and no addition of an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 19, A, B).

A cell aggregate was formed by day 2 after the start of
25 suspension culture (step 2 completed, and step 3 started).
[0375]

Furthermore, the cells were cultured in a serum-free medium under two conditions of the following Conditions A, B.

•Condition A. On day 3 after the start of suspension culture,
30 a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change.
35 Thereafter, a half-medium exchange operation was performed once

every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.

•Condition B. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change. Further, On day 6 after the start of suspension culture, a half-medium exchange operation was performed using the aforementioned serum-free medium not containing Y27632 or SAG and containing human recombinant BMP4 (manufactured by R&D) and IWR-1e such that the concentration of exogenous human recombinant BMP4 and the concentration of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.

[0376]

The cells were cultured as follows under two conditions of the above-mentioned Conditions A, B.

On day 10 after the start of suspension culture, a 80% medium exchange operation was performed 3 times by using the aforementioned serum-free medium so that the concentration of exogenous IWR-1e would be 3% or less as compared to that before medium exchange. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the aforementioned serum-free medium not containing Y27632, SAG, human recombinant BMP4 and IWR-1e.

The thus-prepared cells on day 20 after the start of suspension culture were subjected to bright field observation using an inverted microscope (manufactured by Nikon Corporation, ECLIPSE Ti). As a result, it was found that a neural tissue

substance, and no addition of an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 20, A, B).

5 A cell aggregate was formed by day 2 after the start of suspension culture (step 2 completed, and step 3 started).
[0380]

Furthermore, the cells were cultured in a serum-free medium under two conditions of the following Conditions A, B.
10 •Condition A. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e and SB431542 was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5
15 nM (55 ng/ml) and the concentrations of exogenous IWR-1e (3 μ M) and SB431542 (5 μ M) would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e and SB431542 to
20 avoid change of the concentration of exogenous IWR-1e and SB431542.

•Condition B. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing
25 IWR-1e and SB431542 was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentrations of exogenous IWR-1e (3 μ M) and SB431542 (5 μ M) would not change. Further, On day 6 after the start of suspension culture, a half-medium exchange
30 operation was performed using the aforementioned serum-free medium not containing Y27632 or SAG and containing human recombinant BMP4 (manufactured by R&D) and IWR-1e and SB431542 such that the concentration of exogenous human recombinant BMP4 and the concentration of exogenous IWR-1e and SB431542 would
35 not change. Thereafter, a half-medium exchange operation was

TrypLE Select (Life Technologies), further dispersed into single cells by pipetting operation, and the above human iPS cells dispersed into single cells were suspended in 100 μ l of a serum-free medium at 1.0×10^4 cells per well of a non-cell-
5 adhesive 96-well culture plate (PrimeSurface 96V-bottom plate, manufactured by SUMITOMO BAKELITE). Thereafter, the cells were subjected to suspension culture at 37°C, 5% CO₂. As the serum-free medium (gfCDM+KSR) therefor, a serum-free medium which is a 1:1 mixture of F-12 medium and IMDM medium supplemented with
10 10% KSR, 450 μ M 1-monothioglycerol, 1 x Chemically defined lipid concentrate was used. At the time of the start of suspension culture (day 0 after the start of suspension culture, start of step 2), Y27632 (final concentration 20 μ M) was added to the above-mentioned serum-free medium, and the cells were
15 cultured in the serum-free medium. A cell aggregate was formed by day 2 after the start of suspension culture.

•Condition. Condition including addition of IWR-1e (3 μ M) as a Wnt signal transduction pathway inhibiting substance and SB431542 (5 μ M) as a TGF β signal transduction pathway
20 inhibiting substance, and no addition of an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 21, A, B).

A cell aggregate was formed by day 2 after the start of
25 suspension culture (step 2 completed, and step 3 started).
[0385]

Furthermore, the cells were cultured in a serum-free medium under two conditions of the following Conditions A, B.

•Condition A. On day 3 after the start of suspension culture,
30 a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e and SB431542 was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentrations of exogenous IWR-1e (3 μ M)
35 and SB431542 (5 μ M) would not change. Thereafter, a half-

medium exchange operation was performed once every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e and SB431542 to avoid change of the concentration of exogenous IWR-1e and
5 SB431542.

•Condition B. On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e and SB431542 was added by 50 μ l such that a final
10 concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentrations of exogenous IWR-1e (3 μ M) and SB431542 (5 μ M) would not change. Further, On day 6 after the start of suspension culture, a half-medium exchange operation was performed using the aforementioned serum-free
15 medium not containing Y27632 or SAG and containing human recombinant BMP4 (manufactured by R&D) and IWR-1e and SB431542 such that the concentration of exogenous human recombinant BMP4 and the concentration of exogenous IWR-1e would not change. Thereafter, a half-medium exchange operation was performed once
20 every 2 - 4 days with the serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e and SB431542.

[0386]

25 The cells cultured under two conditions of the above-mentioned Conditions A, B were cultured as follows.

On day 10 after the start of suspension culture, a 80% medium exchange operation was performed 3 times by using the aforementioned serum-free medium so that the concentration of
30 exogenous IWR-1e and SB431542 would be 3% or less as compared to that before medium exchange. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the aforementioned serum-free medium not containing Y27632, SAG, human recombinant BMP4, IWR-1e and SB431542.

35 The thus-prepared cells on day 20 after the start of

pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 22A, B).

•Condition 2. Condition including addition of IWR-1e (3 μ M) as a Wnt signal transduction pathway inhibiting substance and
5 addition of SAG (10 nM) as an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 22C, D).

A cell aggregate was formed by day 2 after the start of suspension culture under Condition 1 and Condition 2 (step 2
10 completed, and step 3 started).

[0390]

On day 3 after the start of suspension culture, under Condition 1 and Condition 2, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D)
15 and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change.

On day 6 after the start of suspension culture, a 80%
20 medium exchange operation was performed 3 times by using the aforementioned serum-free medium so that the concentration of exogenous IWR-1e would be 3% or less as compared to that before medium exchange. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the aforementioned
25 serum-free medium not containing Y27632, SAG, human recombinant BMP4 and IWR-1e.

[0391]

The thus-prepared cells on day 18 after the start of suspension culture were subjected to bright field observation
30 using an inverted microscope (manufactured by Nikon Corporation, ECLIPSE Ti). As a result, it was found that a neural tissue was formed under Condition 1 and Condition 2.

[0392]

The aggregates on day 18 after the start of the
35 aforementioned suspension culture were fixed with 4% para-

•Condition 2. Condition including addition of IWR-1e (3 μ M) as a Wnt signal transduction pathway inhibiting substance and addition of SAG (10 nM) as an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 23E-H).

A cell aggregate was formed by day 2 after the start of suspension culture under Condition 1 and Condition 2 (step 2 completed, and step 3 started).

[0395]

On day 3 after the start of suspension culture, under Condition 1 and Condition 2, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration (3 μ M) of exogenous IWR-1e would not change.

On day 6 after the start of suspension culture, a 80% medium exchange operation was performed 3 times by using the aforementioned serum-free medium so that the concentration of exogenous IWR-1e would be 3% or less as compared to that before medium exchange. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the aforementioned serum-free medium not containing Y27632, SAG, human recombinant BMP4 and IWR-1e.

[0396]

The thus-prepared cells on day 11 after the start of suspension culture were subjected to bright field observation using an inverted microscope (manufactured by Nikon Corporation, ECLIPSE Ti). As a result, it was found that a neural tissue was formed under Condition 1 and Condition 2.

[0397]

A part of the aggregates on day 11 after the start of the aforementioned suspension culture was extracted and fixed with 4% para-formaldehyde to produce cryosections. These cryosections were immunostained for Chx10 (anti-Chx10 antibody,

under two conditions of the following Conditions 1 and 2.

•Condition 1. Condition including addition of IWR-1e (3 μ M) as a Wnt signal transduction pathway inhibiting substance and no addition of an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 24A, B, "Condition 1").

•Condition 2. Condition including addition of IWR-1e (3 μ M) as a Wnt signal transduction pathway inhibiting substance and addition of SAG (10 nM) as an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 24E, F "Condition 2").

By day 2 after the start of suspension culture, cell aggregates were formed under Condition 1 and Condition 2 (step 2 completed, and step 3 started).

[0407]

On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4 (manufactured by R&D) and further containing IWR-1e was added by 50 μ l such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration of exogenous IWR-1e (3 μ M) would not change. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the aforementioned serum-free medium not containing Y27632, SAG, human recombinant BMP4 and further containing IWR-1e to avoid change of the concentration of exogenous IWR-1e.

On day 12 after the start of suspension culture, a 80% medium exchange operation was performed 3 times by using the aforementioned serum-free medium so that the concentration of exogenous IWR-1e would be 3% or less as compared to that before medium exchange. Thereafter, a half-medium exchange operation was performed once every 2 - 4 days with the aforementioned serum-free medium not containing Y27632, SAG, human recombinant BMP4 and IWR-1e.

adhesive 96-well culture plate (PrimeSurface 96V-bottom plate, manufactured by SUMITOMO BAKELITE). Thereafter, the cells were subjected to suspension culture at 37°C, 5% CO₂. As the serum-free medium (gfCDM+KSR) therefor, a serum-free medium which is
5 a 1:1 mixture of F-12 medium and IMDM medium supplemented with 10% KSR, 450 µM 1-monothioglycerol, 1 x Chemically defined lipid concentrate was used. At the time of the start of suspension culture (day 0 after the start of suspension culture, start of step 2), Y27632 (final concentration 20 µM) was added
10 to the above-mentioned serum-free medium, and the cells were cultured in a serum-free medium under two conditions of the following Conditions 3 and 4.

•Condition 3. Condition including addition of IWR-1e (3 µM) as a Wnt signal transduction pathway inhibiting substance and no
15 addition of an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 24C, D, "Condition 3").

•Condition 4. Condition including addition of IWR-1e (3 µM) as a Wnt signal transduction pathway inhibiting substance and
20 addition of SAG (10 nM) as an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2 (Fig. 24G, H "Condition 4").

By day 2 after the start of suspension culture, cell
25 aggregates were formed under Condition 3 and Condition 4 (step 2 completed, and step 3 started).

[0412]

On day 3 after the start of suspension culture, a medium not containing Y27632 or SAG, containing human recombinant BMP4
30 (manufactured by R&D) and further containing IWR-1e was added by 50 µl such that a final concentration of exogenous human recombinant BMP4 would be 1.5 nM (55 ng/ml) and the concentration of exogenous IWR-1e (3 µM) would not change.

Thereafter, a half-medium exchange operation was performed once
35 every 2 - 4 days with the aforementioned serum-free medium not

subconfluence were feeder-free cultured for one day under conditions including addition of SB431542 (TGF β signal transduction pathway inhibiting substance (TGF β R-i), 5 μ M) and SAG (Sonic hedgehog signal transduction pathway activating substance (Shh), 300 nM) (step 1: Precondition (TGF β R-i+Shh) treatment, Fig. 25 "Precondition: TGF β R-i+Shh") in a Stem Fit (registered trade mark) medium (AK03N; manufactured by Ajinomoto Co., Inc.).

The thus-prepared Precondition (TGF β R-i+Shh)-treated human iPS cells were treated with the cell dispersion solution by using TrypLE Select (Life Technologies), further dispersed into single cells by pipetting operation, and the above human iPS cells dispersed into single cells were suspended in 100 μ l of a serum-free medium at 1.3×10^4 cells per well of a non-cell-adhesive 96-well culture plate (PrimeSurface 96 slit well plate, manufactured by SUMITOMO BAKELITE). Thereafter, the cells were subjected to suspension culture at 37°C, 5% CO₂. As the serum-free medium (gfCDM+KSR) therefor, a serum-free medium which is a 1:1 mixture of F-12 medium and IMDM medium supplemented with 10% KSR, 450 μ M 1-monothioglycerol, 1 x Chemically defined lipid concentrate was used. At the time of the start of suspension culture (day 0 after the start of suspension culture, start of step 2), Y27632 (final concentration 20 μ M) was added to the above-mentioned serum-free medium and the cells were cultured in a serum-free medium under the following Condition 2.

•Condition 2. Condition including addition of IWR-1e (3 μ M) as a Wnt signal transduction pathway inhibiting substance and addition of SAG (10 nM) as an exogenous Sonic hedgehog signal transduction pathway activating substance to the aforementioned serum-free medium at the start of step 2

A cell aggregate was formed by day 2 after the start of suspension culture (step 2 completed, and step 3 started).

[0416]

On day 4 after the start of suspension culture, a