obtained by purifying cell aggregates on day 63 after the start of differentiation induction, reaggregating them, treating them with DX for 3, 7, or 14 days after 3 days later, and culturing them up to day 103 after the start of differentiation induction.

[Fig. 22]

Fig. 22A and 22B are diagrams showing the measurement results over time of the amounts of ACTH and GH in the culture supernatants of cell aggregates obtained by purifying cell aggregates on day 63 after the start of differentiation induction, reaggregating them, treating them with DX for 3, 7, or 14 days after 3 days later, and culturing them up to day 103 after the start of differentiation induction. Fig. 22C shows a diagram with the results of a GH stimulation test using GRF and performed using the cell aggregates obtained by purifying cell aggregates on day 63 after the start of differentiation induction, reaggregating them, treating them with DX for 14 days after 3 days later, and culturing them up to day 103 after the start of differentiation induction. Fig. 22D shows a diagram with the results of a GH suppressing test using somatostatin and performed using the cell aggregates obtained by purifying cell aggregates on day 63 after the start of differentiation induction, reaggregating them, treating them with DX for 14 days after 3 days later, and culturing them up to day 103 after the start of differentiation induction.

[Description of Embodiments]

[0011]

[Production method of cell aggregate containing pituitary hormone-producing cell]

In the present specification, the definitions of the terms are as follows. The “stem cell” means an undifferentiated cell having differentiation potency and proliferative capacity (particularly self-renewal competence). The stem cell includes pluripotent stem cell, multipotent stem cell, unipotent stem cell and the like according to the differentiation potency. The “pluripotent stem cell” refers to

[0063]

That "a specific cell does not substantially present" means that the proportion of the specific cells to the total cells in the cell aggregate is not more than 2% or not more than 1%.

[0064]

By confirming the time course of expression of these markers, those of ordinary skill in the art can link these stages to the specific number of differentiation days in a specific differentiation induction method. For example, in the below-mentioned differentiation induction method, cell aggregates around 12 to 30 days after the start of differentiation contain late stage pituitary precursor tissue, cell aggregates around 30 to 60 days after the start of differentiation contain early stage pituitary tissue, and cell aggregates around 60 to 100 days after the start of differentiation contain middle stage pituitary tissue.

[0065]

One embodiment of the cell aggregate derived from a pluripotent stem cell and containing a hypothalamic cell, a pituitary progenitor cell, and a pituitary hormone-producing cell, obtained in step (A), is, for example, a cell aggregate (cell aggregate around 30 to 100 days after the start of differentiation) containing hypothalamic tissue (optionally containing Nkx2.1-expressing cell) and pituitary tissue containing not less than one and not more than 4 types of pituitary hormone-producing cells.

One embodiment of the cell aggregate derived from a pluripotent stem cell and containing a hypothalamic cell, a pituitary progenitor cell, and a pituitary hormone-producing cell, obtained in step (A), is, for example, a cell aggregate (cell aggregate around 30 to 60 days after the start of differentiation) containing hypothalamic tissue (optionally containing Nkx2.1-expressing cell) and the early stage pituitary tissue.

can be set as appropriate. When the suspension culturing in step (1) is performed further in the presence of a Shh signal transduction pathway activating substance, the culture period in the presence of the Shh signal transduction pathway activating substance in step (1) and step (2) is preferably 30 days.

[0153]

In step (2) and the steps thereafter, it is also preferable to add an FGF signal transduction pathway activating substance to the culture environment from the aspect of promoting differentiation into pituitary placode. The FGF signal transduction pathway activating substance is not particularly limited as long as it is a substance capable of enhancing the signal transduction pathway mediated by FGF (fibroblast growth factor). Examples of the FGF signal transduction pathway activating substance include FGF proteins such as FGF1, FGF2 (sometimes referred to as bFGF), FGF3, FGF8, FGF10, and the like, anti-FGF receptor antibody, FGF partial peptide and the like. These substances may be used alone or in combination.

[0154]

FGF2 protein and FGF8 protein are available from, for example, FUJIFILM Wako Pure Chemical Corporation. The FGF signal transduction pathway activating substance preferably contains at least one selected from the group consisting of FGF2, FGF3, FGF8 and FGF10, and variant thereof, more preferably contains FGF2, further preferably contains recombinant human FGF2.

[0155]

The concentration of FGF signal transduction pathway activating substance in the medium can be appropriately determined according to the substances used within a range capable of achieving the aforementioned effects. From the aspects of differentiation into cells constituting pituitary, and promotion of differentiation and survival and

isolation may be seeded in the gel. The cells may be seeded after sorting out specific cell type such as basal cell by using a cell sorter or the like. As a further embodiment of the culture method of embedding in a gel, co-culture with cells other than the pituitary such as fibroblast, mesenchymal cell, vascular cell, and the like can also be performed. Gel-embedding culture as described above can be performed with reference to, for example, Nature 501, 373-379 (2013), Nature, 499, 481-484 (2013), Nat Protoc 14, 518-540 (2019), Genes 2020, 11, 603, and the like.

[0180]

In the third step and the steps thereafter, it is also preferable to perform a culture method in which the cells are physically shaken, for the purpose of improving nutrition and oxygen supply to the cells and improving substance exchange. Examples of such culture method include methods other than static culture, such as shaking culture, rotation culture, stirring culture, and the like. The means for performing shaking culture, rotation culture, stirring culture, and the like are not particularly limited. For example, they can be performed by placing the culture vessel, in which the cells are cultured, on a rotator, shaker, or the like, or by placing the cells in an environment where a stirrer or the like is rotating. Those skilled in the art can appropriately set the parameters such as the speed and the like of shaking culture, rotation culture, and stirring culture within a range that does not cause damage to cells. For example, when shaking culture is performed using a waveform fluctuation 3D shaker (e.g., Mini-Shaker 3D, manufactured by Biosan), for example, the shaking speed range can be set within the range of 5 to 60 rpm, preferably 5 to 40 rpm, more preferably 5 to 20 rpm. When shaking culture is performed using a reciprocating type shaker (e.g., NS-LR, manufactured by AS ONE Corporation), for example, the shaking speed range can be set within the range of 15 to 60 rpm, preferably 15 to 50 rpm, more preferably 15 to 45 rpm.

[0204]

A still another embodiment of the method of the present invention is, for example, the following production method.

A method for producing a cell aggregate containing pituitary hormone-producing cells, which includes the following steps:

(1) a step of separating cells expressing EpCAM from a cell population obtained by dispersing a cell aggregate derived from pluripotent stem cells and containing not less than one and not more than 4 kinds of pituitary hormone-producing cells and containing Nkx2.1-expressing cells in hypothalamic tissue, and

(2) a step of suspension culturing the separated EpCAM-expressing cell for not less than 14 days under conditions that allow the pituitary progenitor cell to mature into a pituitary hormone-producing cell.

[0205]

[Cell aggregate containing pituitary hormone-producing cells]

The cell aggregate containing pituitary hormone-producing cells obtained by the method of the present invention (the cell aggregate of the present invention) has the following characteristics.

(1) The percentage of cells positive for at least one of EpCAM and E-cadherin in the cell aggregate is not less than 80%.

(2) The percentage of neural cells in the cell aggregate is not more than 20%.

(3) The percentage of ACTH-positive cells in the cell aggregate is not less than 5%.

(4) The cell density of the cell aggregate is 3,000 to 20,000 cells/mm^2.

(5) The major axis of the cell aggregate is 200 to 3,000 μm.

(6) On the surface of the cell aggregate, cells positive for at least one of EpCAM and E-cadherin form a plane attachment to each other to form an epithelial structure.

(7) The cell aggregate forms a sponge-like structure.

[0221]

On the surface of the cell aggregate, cells positive for at least one of EpCAM and E-cadherin may form a plane attachment to each other to form an epithelial structure.

[0222]

The “plane adhesion” means that adjacent cells adhere to each other on the surface. More specifically, the plane adhesion means that the proportion of the surface area of one cell that is adhered to the surface of another cell is, for example, not less than 1%, preferably not less than 3%, more preferably not less than 5%, further preferably not less than 10%. In one embodiment, the surface of a cell refers to the surface of a cell membrane. Cell surfaces can be observed by immunostaining for cell adhesion factors (e.g., E-cadherin, EpCAM). An example of a state other than plane adhesion is point adhesion. Point adhesion means that a cell and a cell are adhered at points.

[0223]

The “epithelial structure” refers to a structure characteristically possessed by epithelial tissues, such as the apical surface or base membrane. It can be confirmed by immunostaining using the apical surface and base membrane markers described below.

[0224]

The epithelial tissue becomes polarized, forming an “apical surface” and a “base membrane”. The “base membrane” refers to a 50-100 nm base membrane containing a lot of base membrane markers laminin and type IV collagen in which a basal layer (basal membrane) produced by epithelial cells exists. The “apical surface” refers to a surface (superficial surface) formed on the opposite side to the “base membrane”. Such apical surface can be identified by immunostaining method and the like well known to those skilled in the art, by using antibodies against apical surface markers (e.g., atypical-PKC (hereinafter abbreviated as “aPKC”), E-cadherin, N-cadherin).

with a serum-free medium supplemented with 20% KSR, transferred to a 6-well culture plate containing a new serum-free medium (2 mL) supplemented with 20% KSR, and GRF 2 μg/mL or somatostatin 100 ng/mL was added. After culturing for 24 hr under condition of 37°C, 40% O_2, the culture supernatant was recovered. The ACTH concentration in the collected culture supernatant was measured by the ELISA method used in clinical testing (testing outsourced to SRL, Inc.). As a result, the addition of GRF increased the amount of GH secretion by a little less than two times (Fig. 22C), and the addition of somatostatin decreased the amount of GH secretion by 20% (Fig. 22D). From the above results, it was found that the cell aggregates obtained by DX treatment during long period culture after purification and reaggregation contain functional pituitary hormone-producing cells that have not only GH secretion capacity but also responsiveness.

[Industrial Applicability]

[0254]

The method of the present invention makes it possible to efficiently obtain a cell aggregate containing pituitary hormone-producing cells, by differentiating pluripotent stem cells into cell aggregates containing hypothalamic cells, pituitary progenitor cells, and pituitary hormone producing cell, efficiently separating functional pituitary hormone-producing cells and/or progenitor cells therefor by using EpCAM marker, and further differentiating the cells. In addition, since separated and purified pituitary hormone-producing cells and the like exhibit superior pituitary hormone secretory capacity by physiological stimulation of pituitary hormone secretion, they can be used for the treatment and the like of diseases relating to pituitary gland.

This application is based on a patent application No. 2021-162253 filed in Japan (filing date: September 30, 2021) and a patent application No. 2022-116715 filed in Japan (filing date: July 21, 2022), the contents of which are incorporated in