

inhibitor, and VEGF.

Here, examples of the TGF β /Smad inhibitor include SB431542, LY2157299, and LY2109761, and SB431542 is preferred. The concentration of SCF is preferably 1 to 100 ng/mL, more
5 preferably 20 to 50 ng/mL. When SB431542 is used, the concentration of the TGF β /Smad inhibitor is preferably 1 to 10 μ M, more preferably 1 to 5 μ M. The concentration of VEGF is preferably 1 to 100 ng/mL, more preferably 50 to 100 ng/mL, similar to the medium (2-1).

10 [0036]

Furthermore, the medium (2-2) may contain a ROCK inhibitor or bFGF. Specific examples and concentrations of each when in use are the same as those described for the medium (2-1).

15 [0037]

The basal medium for the medium (2-2) is not particularly limited, and the same basal medium as described for the medium (2-1) can be used.

[0038]

20 The medium (2-3) is a medium containing SCF and Flt3L. The concentration of SCF is preferably 1 to 100 ng/mL, more preferably 20 to 50 ng/mL. The concentration of Flt3L is preferably 1 to 100 ng/mL, more preferably 20 to 50 ng/mL.
[0039]

25 Furthermore, the medium (2-3) may contain at least one selected from a ROCK inhibitor, interleukin (IL)-3, and IL-7. Examples of the ROCK inhibitor include those mentioned above, and Y27632 is preferred. When Y27632 is used, the concentration of the ROCK inhibitor is preferably 1 to 20 μ M.
30 When IL-3 is added, the concentration is preferably 1 to 100 ng/mL. When IL-7 is added, the concentration is preferably 1 to 100 ng/mL.

[0040]

The basal medium for the medium (2-3) is not particularly
35 limited, and a medium suitable for inducing hematopoietic

and it is more desirable to perform medium exchange by continuous perfusion operation.

[0043]

Therefore, the three-dimensional culture in step (2) is preferably a three-dimensional culture including, for example, (2-1) a culture step using medium (2-1) as the second medium, (2-2) a culture step using medium (2-2) as the second medium, and (2-3) a culture step using medium (2-3) as the second medium. Respective steps (2-1) to (2-3) are preferably performed in this order.

[0044]

In this step (2), as in step (1), the temperature, pH, and dissolved oxygen concentration can be controlled to any desired value for induction into hematopoietic progenitor cells by using a temperature sensor, a pH sensor, a dissolved oxygen sensor, and the like.

[0045]

In the method of the present invention, NK cell populations can be obtained without any particular purification step from step (1) to step (3). Therefore, the cell populations obtained in step (2) may be directly subjected to the next step (3) as they are, or only the hematopoietic progenitor cells may be sorted and then subjected to step (3).

The inventors have confirmed that the desired NK cells can be obtained in step (3) by using either method. Therefore, the method of the present invention may include a step of removing cells other than hematopoietic progenitor cells from the "cell population including hematopoietic progenitor cells" obtained in step (2), but it is desirable to subject the "cell population including hematopoietic progenitor cells" obtained in step (2) directly to step (3).

NK cells are induced by subjecting the thus-obtained "cell population including hematopoietic progenitor cells" to step (3).

[0052]

In addition, when IL-7, Flt3L, a ROCK inhibitor, a GSK3 β inhibitor, or a TGF β R inhibitor is added in step (3), the induction efficiency can be improved by adjusting the timing of adding these factors. For example, IL-7 and Flt3L are preferably removed on or after the 10th day of culture in step (3), more preferably on or after the 20th day. In addition, a ROCK inhibitor, a GSK3 β inhibitor, and a TGF β R inhibitor are preferably added for 4 to 7 days before the end of culture.

10 [0053]

The basal medium to be used in step (3) is not particularly limited. For example, media such as AIM-V Medium (Thermo Fisher Scientific), Stemline (registered trademark) II (Sigma-Aldrich), ALyS505N-0 (Cell Science Institute, Inc.), and Stem Pro-34 SFM (Thermo Fisher Scientific) are used, and AIM-V Medium is preferably used. Furthermore, the medium may contain human serum, fetal bovine serum (FBS), or a serum replacement. The concentration thereof is preferably 1 to 20%, more preferably 1 to 10%.

20 [0054]

In step (3), it is preferable to further perform a step of removing the spheres. The timing of removal may be, for example, before the start of this step, during this step, or at the end of this step, but it is not particularly limited and can be performed at any time.

The spheres are preferably removed using a 20 to 100 μ m cell strainer, and more preferably removed using a 20 to 40 μ m cell strainer.

[0055]

30 In this step (3), as in step (1), the temperature, pH, and dissolved oxygen concentration can be controlled to any desired value for induction into NK cells by using a temperature sensor, a pH sensor, a dissolved oxygen sensor, and the like.

35 [0056]

and the medium exchange was performed manually. As a method for medium exchange, the medium exchange was performed by stopping the stirring of the culture container, standing same for several min, and changing the total amount of the
5 supernatant on days 1, 2, 3, 4, 7, 9, and 12. Samples with a 30 mL culture medium volume were cultured in a rotary stirring container, cultured in a CO₂ incubator, and floating cultured at 5%, 37°C. The culture medium was mixed and cultured at a rotation speed of 55 rpm. Samples with a 250 mL culture medium
10 volume were cultured under similar control as the perfusion culture samples, except that the medium exchange was performed manually.

[0068]

On the 14th day of culture, the spheres were removed from
15 the culture medium to obtain a suspension of floating cells, and the cell surface markers known to be expressed in hematopoietic progenitor cells (CD34, CD43, CD45, CD117) were confirmed by flow cytometry (Fig. 3).

The production efficiency of hematopoietic progenitor
20 cells was compared between the production by perfusion culture and the manual medium exchange. As a result, in this step, the medium exchange that occurs during the process was continuously performed under automatic control, the culture could be performed without moving the culture tank to a sterile
25 environment such as a safety cabinet, and there was no significant difference in the expression of the confirmed markers. Therefore, hematopoietic progenitor cells could be induced by perfusion culture in step (2).

[0069]

30 step (3): Step of inducing hematopoietic stem cell into natural killer cell

NK cells were produced by seeding the hematopoietic progenitor cells produced in step (2) (floating cells containing spheres) into a three-dimensional culture container
35 at a cell density of 1.0×10^5 cells/mL. In addition, subculture