[52] In the present invention it was successfully shown that it is possible to transfer PSCs from an adherent cell culture ("starter culture") to a continuous suspension culture in a bioreactor. It was surprisingly discovered that (a) the addition of an inhibitor of Rho-associated protein kinase (ROCK) prior to cell dissociation increases cell viability and yield and also helps maintaining the pluripotency of the PSCs (see e.g. Example 4) and (b) that PSCs can be cultured and expanded in a medium still comprising the cell dissociation reagent after diluting the cell dissociating reagent (see e.g. Examples 1, 2 and 3). Additionally, it was surprisingly found that (c) a chelating agent such as a solution comprising EDTA (ethylenediaminetetraacetate) can be used in dissociating aggregates of pluripotent stem cells (PSCs) (see Examples 1 and 2). Example 5 underlines that the present invention can be scaled up without further modifications by a factor of more than 30. The invention therefore allows for the automated cultivation of the PSCs in a closed system and thus reduces the number of manual operations such as the transfer of the PSCs out of the bioreactor into a centrifuge during the passaging of the cells. The method of the present invention thus is easier, faster and less expensive than conventional culture systems and allows further automatization of PSC production. Since the method of the invention can, as mentioned above, be carried out in a closed system, it has the further advantage that is ideally suited for establishing a GMP compliant manufacturing process for stem cells.

[53] Before the continuous and automated expansion of PSCs in a bioreactor can be started, PSCs preferably have to be transferred to the bioreactor (see also Fig. 1). It is within the knowledge of a person skilled in the art to culture PSCs in adherent culture. E.g., PSCs may be cultured in T25/T75 culture flasks coated with 0.9 µg/cm² biolaminin 521-MX or other proteins of the ECM in a culture medium suitable for PSCs such as iPSC-Brew medium. For the start of the suspension culture, PSCs from adherent culture may be used. The cell may be dissociated from the flasks by a cell dissociation agent such as EDTA and transferred to the culture medium comprising a ROCKi. Preferably, cell aggregates consisting of 2 to 10 cells are present. These dissociated PSCs may then be used to inoculate a bioreactor. A preferred cell concentration at the beginning of the method of the invention is about 2.5×10^5 cells/ml. The cells are then cultured in suspension under continuous agitation to avoid sedimentation and/or adherence to the bottom of the bioreactor of the PSCs.

[54] After inoculation, the cells preferably are cultured in the cell culture medium comprising the ROCKi for about 2 days to allow formation of cell aggregates. After formation of cell aggregates, the medium may be changed to a cell culture medium free of a ROCKi, or in other words, a cell culture medium that has not been supplemented or comprises a ROCKi.

CLAIMS

1. A method of expanding pluripotent stem cells (PSC) in suspension culture in a bioreactor, the method comprising
   (i) adding an inhibitor of ROCK (ROCKi) to aggregates of pluripotent stem cells being cultivated in suspension in the bioreactor;
   (ii) adding a cell dissociation agent, thereby dissociating aggregates of the pluripotent stem cells, wherein the cell dissociation agent is a chelating agent;
   (iii) diluting the cell dissociation agent added in step (ii) by adding an excess volume of culture medium sufficient to decrease the concentration of the cell dissociation agent to a concentration at which cell aggregates can form again, wherein the culture medium added comprises a ROCKi; (iv) culturing of the mixture obtained in step (iii) under suitable conditions that allow the expansion of the PSCs: and
   (v) exchanging the medium to a medium free of the ROCKi.

2. The method of claim 1, wherein the chelating agent is selected from the group consisting of ethylenediaminetetraacetate (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), iminodisuccinic acid (IDS), polyaspartic acid, ethylenediamine-N,N′-disuccinic acid (EDDS), citrate, citric acid, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), methylglycinediacetic acid (MGDA) and combinations thereof.

3. The method of claim 2, wherein the cell dissociation reagent is EDTA.

4. The method of claim 3, wherein the final concentration of EDTA in step (ii) is at least 100 µM EDTA, in a range of 100 to 1000 µM EDTA, in a range of 250 to 750 µM EDTA, in a range of 400 to 600 µM EDTA or is 500 µM EDTA, preferably 500 µM EDTA.

5. The method of any one of claims 3 or 4, wherein the concentration of EDTA in step (iii) after adding the excess volume of culture medium is 100 µM or less, 95 µM or less, 90 µM or less, 80 µM or less, 70 µM or less, in a range of 100 to 1 µM EDTA, or in a range of 90 to 1 µM EDTA.

6. The method of any one of the preceding claims, wherein the excess volume exceeds the volume of the cell dissociation agent by at least 5 times.

7. The method of any one of the preceding claims, wherein step (iv) is performed for 1 to 3 days, preferably 2 days.

8. The method of claim 1, wherein step (v) starts 1 to 3 days, preferably 2 days, after step (iii).

9. The method of any one of the preceding claims, wherein the ROCKi is selected from the group consisting of AS1892802, fasudil hydrochloride, GSK 269962, GSK 429286, H 1152, HA 1100, OXA 06, RKI 1447, SB 772077B, SR 3677, TC-S 7001, thiazovivin, Y27632 and combinations thereof.

10. The method of any one of the preceding claims, wherein the ROCKi is Y27632.

11. The method of claim 12, wherein Y27632 is added to a final concentration of 10 µM.

12. The method of any one of the preceding claims, wherein the ROCKi is added in step (i) 4 hours or less prior to step (ii).

13. The method of any one of the preceding claims, wherein the addition of an excess volume of the culture medium in step (iii) results in a cell number of $1x10^5$ to $1x10^6$ cells/ml, 1.5 to $7.5x10^5$ cells/ml, $2x10^5$ to $5x10^5$ cells/ml, $2x10^5$ to $3x10^5$ cells/ml or $2.5x10^5$ cells/ml in the culture medium.

14. The method of any one of the preceding claims, wherein the culture medium is selected from the group consisting of IPS-Brew, E8, StemFlex, mTeSR1, and PluriSTEM.

15. The method of claim 14, wherein the culture medium is IPS-Brew.

16. The method of any one of the preceding claims, wherein the culture medium in steps (i) and (iii) is identical.

17. The method of any one of the preceding claims, wherein the temperature of the culture medium is 0 to 50 °C, 35 to 40 °C, 36 to 38 °C or 37 °C, preferably 37 °C.

18. The method of any one of the preceding claims, wherein steps (i) to (iv) or (i) to (v) are repeated once, twice, 3 times, 4 times, 5 times, at least 5 times, or at least 10 times.

19. The method of any one of the preceding claims, wherein the pluripotent stem cells are

selected from the group consisting of induced pluripotent stem cells (iPSC), embryonic stem cells (ESC), parthenogenetic stem cells (pPSC) and nuclear transfer derived PSCs (ntPSC).

20. The method of claim 18, wherein the PSCs maintain their pluripotency after each repetition of steps (i) to (iv) or (i) to (v).

21. The method of any one of the preceding claims, wherein the pluripotent stem cells are TC-1133 cells.

22. The method of any one of the preceding claims, wherein the aggregates in step (ii) have an average diameter of 180 µm to 250 µm, preferably 200 µm to 250 µm, most preferably 200 µm.

23. The method of any one of the preceding claims, wherein the aggregates are dissociated in step (ii) for at least 1 min, at least 2 min, at least 3 min, at least 5 min, at least 10 min, for 1 to 20 min, for 10 to 20 min, for 10 to 15 min or for up to 15 min, preferably for 15 min.

24. The method of any one of the preceding claims, wherein steps (i)-(v) are performed in a closed system.

25. An *in vitro* PSC suspension culture obtained by the method of any one of claims 1 to 24, wherein at least a portion of the PSC in the suspension culture are in the form of aggregates of PSC, and wherein the aggregates of PSC have an average diameter of 150 µm to 300 µm, preferably of 180 µm to 250 µm, most preferably 200 µm.