CLAIMS

1. An *in vitro* method of producing three-dimensional cell clusters of pluripotent stem cells comprising the steps of:

a. treating pluripotent stem cells cultured in a planar adherent culture with a chelating agent or enzyme to release cell aggregates from the planar adherent culture;
b. transferring the cell aggregates from the planar adherent culture to a dynamic suspension culture in the presence of a Rho-kinase inhibitor;
c. diluting the culture using culture media and a Rho-kinase inhibitor to a concentration of cells from about 1 to about 1.5 million cells/ml; and
d. expanding the suspension of cell aggregates to generate cell clusters, wherein the cell clusters maintain pluripotency.

2. The method of claim 1, wherein the pluripotent stem cells are treated with an enzyme selected from neutral protease or Accutase.

3. The method of claim 2, wherein the enzyme is neutral protease.

4. The method of any one of claims 1 to 3, wherein the treating in step a. does not completely disaggregate the cells to a single cell suspension.

5. The method of any one of claims 1 to 4, wherein the pluripotent stem cells are selected from the group consisting of induced pluripotent stem cells, human umbilical cord tissue-derived cells, parthenotes, human embryonic stem cells (hES) and amniotic fluid derived-cells.

6. The method of claim 5, wherein the cells are H1 hES.

7. The method of any one of claims 1 to 6, wherein the cells in the cell cluster express CD9, SSEA4, TRA-1-60, and TRA-1-81, and lack expression of CXCR4.

8. The method of any one of claims 1 to 7, wherein the pluripotent stem cells are treated with a chelating agent.

9. The method of claim 8, wherein the chelating agent is ethylenediaminetetraacetic acid

(EDTA).

10. A method of differentiating cell clusters of pluripotent stem cells in a dynamically agitated suspension culture system comprising:

a. treating pluripotent stem cells cultured in a planar adherent culture with a chelating agent or enzyme to release cell aggregates from the planar adherent culture;
b. transferring the cell aggregates from the planar adherent culture to a dynamic suspension culture in the presence of a Rho-kinase inhibitor;
c. diluting the culture using culture media and a Rho-kinase inhibitor to a concentration of cells from about 1 to about 1.5 million cells/ml;
d. expanding the suspension of cell aggregates to generate pluripotent cell clusters, wherein the cell clusters express CD9, SSEA4, TRA-1-60, and TRA-1-81, and lack expression of CXCR4; and
e. differentiating the pluripotent cell clusters in a dynamic agitated suspension culture system to generate a gut tube cell population, a neural precursor cell population or a cardiomyocyte precursor population.

11. The method of claim 10, wherein the pluripotent stem cells are selected from the group consisting of induced pluripotent stem cells, human umbilical cord tissue-derived cells, parthenotes, human embryonic stem cells (hES) and amniotic fluid derived-cells.

12. The method of claim 10 or claim 11, wherein the method generates a gut tube cell population which expresses β cell transcription factors.

13. The method of claim 12, wherein the transcription factors are PDX1 and/or NKX6.1.

14. The method of any one of claims 10 to 13, wherein the method comprises differentiating the pluripotent cell clusters in a dynamic agitated suspension culture system to generate a gut tube cell population.

15. The method of any one of claims 10 to 13, wherein the method comprises differentiating the pluripotent cell clusters in a dynamic agitated suspension culture system to generate a neural precursor cell population.

16. The method of any one of claims 10 to 13, wherein the method comprises differentiating the pluripotent cell clusters in a dynamic agitated suspension culture system to generate a cardiomyocyte precursor population.

17. The method of any one of claims 10 to 16, wherein the pluripotent stem cells are treated with a chelating agent.

18. The method of claim 17, wherein the chelating agent is EDTA.

19. The method of any one of claims 10 to 18, wherein the pluripotent stem cells are treated with an enzyme.

20. The method of claim 19, wherein the enzyme is a neutral protease or Accutase.

21. The method of any one of claims 10 to 20, wherein the treating in step a. does not completely disaggregate the cells to a single cell suspension.

22. The method of any one of claims 10 to 21, wherein the step of differentiating comprises culturing at an oxygen range from about hypoxia to about 30% of ambient, a lipid in the range of 0.1% to about 2%, or the combination thereof.

23. The method of claim 14, wherein the method comprises differentiating the three-dimensional pluripotent stem cell clusters to definitive endoderm cells in a culture medium supplemented with either (i) a cyclic aniline-pyridinotriazine and GDF8 or (ii) WNT3A and activin A.

24. The method of claim 23, wherein the cyclic aniline-pyridinotriazine is 14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo[19.3.1.1~2,6- ~.1~8,12.~]heptacosa-1(25),2(27),3,5,8(26),9,11,21,23-non- aen-16-one.

25. The method of any one of claims 1 to 24, wherein the dynamic agitated suspension culture system comprises microcarriers.

26. An *in vitro* method of producing pluripotent stem cell clusters comprising the steps of:

a. treating pluripotent stem cells cultured in a planar adherent culture with an enzymatic or chelating agent at room temperature for a time sufficient to release the pluripotent stem cells as cell aggregates;
b. removing said enzymatic or chelating agent;
c. adding culture media supplemented with a Rho-kinase inhibitor to the cell aggregates
d. transferring the cell aggregates cells to a dynamic suspension culture system; and
e. expanding the suspension of cell aggregates to generate cell clusters, wherein the cells in the cell clusters are pluripotent.

27. The method of claim 26, wherein the method comprises use of chelating agent.

28. The method of claim 27, wherein the chelating agent is EDTA.

29. The method of any one of claims 26 to 28, wherein the method comprises use of an enzymatic agent.

30. The method of claim 29, wherein the enzymatic agent is a neutral protease or Accutase.

31. The method of any one of claims 26 to 30, wherein the pluripotent stem cells are selected from the group consisting of induced pluripotent stem cells, human umbilical cord tissue-derived cells, parthenotes, human embryonic stem cells (hES) and amniotic fluid derived-cells.

32. The method of any one of claims 26 to 31 further comprising differentiating the three-dimensional pluripotent stem cell clusters in the dynamic agitated suspension culture system to generate a gut tube cell population, a neural precursor cell population or a cardiomyocyte precursor population.