(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 December 2024 (26.12.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/263961 A2

(51) International Patent Classification:
C12N 5/00 (2006.01) *C12N 5/071* (2010.01)

(21) International Application Number:
PCT/US2024/035082

(22) International Filing Date:
21 June 2024 (21.06.2024)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/510,087 23 June 2023 (23.06.2023) US
63/582,207 12 September 2023 (12.09.2023) US

(71) Applicant: **CHILDREN'S HOSPITAL MEDICAL CENTER** [US/US]; 3333 Burnet Ave., Cincinnati, Ohio 45229-3039 (US).

(72) Inventors: **KASENDRA, Magdalena**; 792 Foresthill Drive, Crescent Springs, Kentucky 41017 (US). **BOYLIN, Katherine**; 316 Telford Avenue, Oakwood, Ohio 45419 (US).

(74) Agent: **GAURAV, Apurv** et al.; Norton Rose Fulbright US LLP, 98 San Jacinto Boulevard, Suite 1100, Austin, Texas 78701 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH,

(54) **Title:** METHODS OF MATRIX-FREE SUSPENSION CULTURE

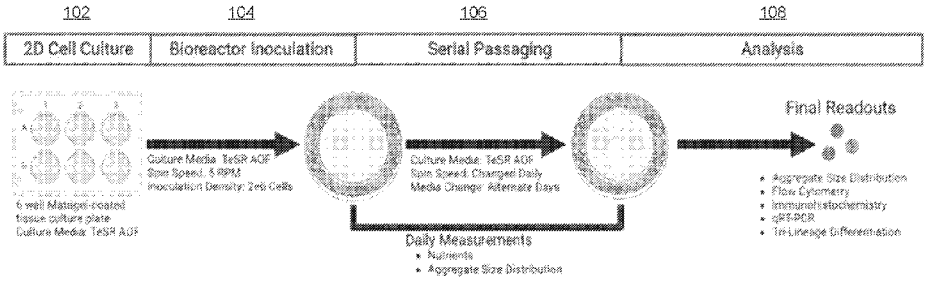


FIG. 1A

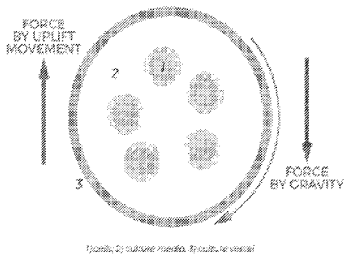


FIG. 1B

(57) **Abstract:** Disclosed herein are methods of utilizing and forming suspension cultures of pluripotent stem cells (PSC), and differentiated cells, spheroids and organoids derived from PSCs (e.g., at industrial efficiency and/or scalability), and compositions comprising the same. These methods may be performed in suspension culture, without the use of basement membrane matrices, during PSC maintenance and expansion, as well as during differentiation of PSCs into differentiated cells and organoids, for example definitive endoderm (DE), hindgut spheroids (HGS), and intestinal organoids (IO). In some aspects, the methods may be xeno-free, and may be performed as per Good Manufacturing Practices (GMP). Also disclosed herein are methods controlling the polarity of epithelial cells in IOs, wherein the apical layer is oriented to the outside of the organoid, or alternatively to the inside of the organoid. Also disclosed are uses of the methods and compositions for transplantation and treatment.

WO 2024/263961 A2

[Continued on next page]

TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

METHODS OF MATRIX-FREE SUSPENSION CULTURE

CROSS REFERENCE

[0001] This application claims priority to U.S. Provisional Patent Application Nos. 63/510,087, filed June 23, 2023, and 63/582,207, filed September 12, 2023, both entitled "METHODS OF MATRIX-FREE SUSPENSION CULTURE," and both of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

[0002] Aspects of the present disclosure relate generally to suspension cultures of pluripotent stem cells (PSCs), PSC aggregates, differentiated cells, spheroids, and organoids derived from the PSCs and PSC aggregates. The presently disclosed methods for making said PSCs, PSC aggregates, differentiated cells, spheroids and organoids may be performed without the use of basement membrane matrices during PSC maintenance and expansion, as well as during differentiation of PSCs and PSC aggregates into differentiated cells and organoids, for example definitive endoderm (DE), hindgut spheroids (HGS), and intestinal organoids (IO). Also disclosed herein are methods controlling the polarity of epithelial cells in IOs, wherein the apical layer is oriented to the outside of the organoid, or alternatively to the inside of the organoid. Furthermore, disclosed herein are compositions comprising such PSCs, differentiated cells, spheroids and organoids. Even further, disclosed herein are method of treatment using such compositions.

BACKGROUND

[0003] Direct differentiation of human intestinal organoids (HIO) from human PSC (hPSC) enables creation of a human intestinal tissue recapitulating the structure, presence of all known intestinal epithelial cell types and function (absorption, mucus and hormone production) of its in vivo counterpart. However, direct differentiation protocols may suffer from high inter-hPSC line, inter-experimental and inter-well variability as they rely on the spontaneous formation and detachment of spheroids at ~Day 7 of culture. In addition, the transition from two-dimensional (e.g., monolayer) culture conditions to three-dimensional (e.g., Matrigel-

embedded) culture conditions can represent a potentially significant bottleneck of HIO manufacture. High variability in addition to low yield of HIOs production and requirement for the use of Matrigel (highly variable animal-derived extracellular matrix) represent potentially significant impediments to the use of HIOs in basic/translational research and potential therapeutic applications. Improved methods of culturing PSC and differentiated cells, spheroids and organoids, and resulting compositions thereof, are needed.

SUMMARY

[0004] Aspects of the present disclosure relate generally to suspension culture methods and compositions of pluripotent stem cells (PSC), three-dimensional PSC aggregates, differentiated cells, spheroids and organoids derived from PSCs. These methods may be performed without the use of xenogeneic basement membrane matrices during PSC maintenance and expansion, as well as during differentiation of PSCs into differentiated cells and organoids, for example definitive endoderm (DE), hindgut spheroids (HGS), and intestinal organoids (IO). In some embodiments, the disclosed methods and compositions utilize a suspension culture for each of the following phases in the manufacture of an organoid from a PSC: maintenance and expansion of PSC, differentiation of PSC into DE, differentiation of DE into spheroids (e.g., hindgut spheroids), and differentiation and maturation of spheroids into an organoid (e.g., an intestinal organoid).

[0005] Disclosed herein are also intermediate and resulting compositions for each of the aforementioned phases. In various embodiments, such compositions are based on three-dimensional expansion of PSCs, PSC aggregates, DE, spheroid, and/or organoids, via a suspension culture.

[0006] Disclosed herein are methods controlling the polarity of epithelial cells in IOs, wherein the apical layer is oriented to the outside of the organoid, or alternatively to the inside of the organoid.

[0007] Exemplary embodiments of the present disclosure are provided in the following numbered embodiments:

[0008] Embodiment 1: A method comprising: (a) inoculating a liquid culture media with PSCs; (b) culturing, in a bioreactor, the liquid culture media that is inoculated with the PSCs such that three-dimensional PSC aggregates form in the liquid culture media, wherein the

culturing in the bioreactor comprises suspending the PSCs in the liquid culture media; (c) passaging the PSCs by: (i) dissociating at least a portion of the three-dimensional PSC aggregates into single cells; and (ii)inoculating a second liquid culture media with the dissociated three-dimensional PSC aggregates of (i) with PSCs.

[0009] Embodiment 2: The method of embodiment 1, wherein the liquid culture media and the second liquid culture media are free of materials of animal or human origin; optionally wherein the liquid culture media and the second liquid culture media are free of any extracellular matrix and/or basement membrane matrix.

[0010] Embodiment 3: The method of any one of the preceding embodiments, wherein the PSCs are passaged when the diameter of a majority of the three-dimensional PSC aggregates formed is not more than 500 μm.

[0011] Embodiment 4: The method of any one of the preceding embodiments, wherein the PSCs are passaged when the diameter of a majority of the three-dimensional PSC aggregates formed is not more than 400 μm.

[0012] Embodiment 5: The method of any one of the preceding embodiments, wherein the PSCs are passaged when the diameter of a majority of the three-dimensional PSC aggregates formed is not more than 300 μm.

[0013] Embodiment 6: The method of any one of the preceding embodiments, wherein at least 80% of the three-dimensional PSC aggregates are dissociated into single cells.

[0014] Embodiment 7: The method of any one of the preceding embodiments, optionally wherein at least 90% of PSC aggregates are dissociated into single cells.

[0015] Embodiment 8: The method of embodiment 1, where the method comprises passaging the PSCs two or more times by culturing the PSCs of inoculated second liquid culture media until additional three-dimensional PSC aggregates form.

[0016] Embodiment 9: The method of any one of the preceding embodiments, wherein the liquid culture media of (a) and/or the second liquid culture media of (c)(ii) is inoculated at a density of about 100,000-220,000 PSCs/ml.

[0017] Embodiment 10: The method of any one of the preceding embodiments, wherein the liquid culture media of (a) and/or the second liquid culture media of (c)(ii) is inoculated at a density of about 180,000-220,000 PSCs/ml.

[0018] Embodiment 11: The method of any one of the preceding embodiments, wherein the passaging occurs after a period of time following the inoculation in (a) that is between about 40-168 hours.

[0019] Embodiment 12: The method of any one of the preceding embodiments, wherein the passaging occurs after a period of time following the inoculation in (a) that is between about 40-84 hours.

[0020] Embodiment 13: The method of any one of the preceding embodiments, wherein the passaging occurs after a period of time following the inoculation in (a) that is between about 66-78 hours.

[0021] Embodiment 14: The method of any one of the preceding embodiments, wherein the method further comprises exchanging a portion of the culture media in the bioreactor of (a) after a period of time following the inoculation in (a) and/or (c)(ii) that is between about 36-60 hours.

[0022] Embodiment 15: The method of any one of the preceding embodiments, wherein the method further comprises exchanging a portion of the culture media in the bioreactor of (a) after a period of time following the inoculation in (a) and/or (c)(ii) that is between about 42-54 hours.

[0023] Embodiment 16: The method of any one of the preceding embodiments, wherein the method further comprises exchanging a portion of the liquid culture media in the bioreactor of (a) after a period of time following the inoculation in (a) and/or (c)(ii), wherein the portion of the liquid culture media exchanged is at least 50% of the liquid culture media in the bioreactor of (a).

[0024] Embodiment 17: The method of any one of the preceding embodiments, the method further comprising, prior to the inoculation in (a): culturing the PSCs on the surface of a substrate; and collecting the PSCs from the surface of the substrate for use in the inoculation of (a) when the PSCs are in a logarithmic growth phase and/or at 35-55% confluency, optionally wherein said collecting comprises dissociating the PSCs prior to the inoculation of (a).

[0025] Embodiment 18: The method of embodiment 17, wherein the PSCs are collected from the surface of the substrate for use in the inoculation of (a) when the PSCs are in a logarithmic growth phase and/or at 40-50% confluency.

[0026] Embodiment 19: The method of any one of the preceding embodiments, wherein the dissociation is chemical, enzymatic and/or mechanical dissociation.

[0027] Embodiment 20: The method of any one of the preceding embodiments, wherein the dissociation is enzymatic, optionally wherein the enzyme comprises a proteolytic and/or a collagenolytic enzyme, optionally wherein the enzyme is Accutase.

[0028] Embodiment 21: The method of any one of the preceding embodiments, wherein the bioreactor of (a) and/or (b) comprises a rotating chamber comprising the liquid culture media, wherein the rotation speed of the rotating chamber is selected such that the number of PSCs in the liquid culture media at (c) is at least 2 or 2.5-fold the number of PSCs used to inoculate the liquid culture media, optionally wherein the number of PSCs in the liquid culture media at (c) is at least 2 or 2.5-fold the number of PSCs used to inoculate the liquid culture media for at least two passages of the PSCs.

[0029] Embodiment 22: The method of any one of the preceding embodiments, wherein the liquid culture media is a serum-free media; optionally, wherein the media comprises recombinant human basic fibroblast growth factor (rh bFGF) and/or recombinant human transforming growth factor β (rh TGFβ).

[0030] Embodiment 23: The method of any one of the preceding embodiments, wherein, following one or more passages, the portion of PSCs expressing Oct4, SSEA1 and TRA 1-60 at levels at least as high as the average expression level of the PSCs used in the inoculation in (a) is at least 85%.

[0031] Embodiment 24: The method of embodiment 23, wherein, following the one or more passages, the portion of PSCs expressing Oct4, SSEA1 and TRA 1-60 at levels at least as high as the average expression level of the PSCs used in the inoculation in (a) is at least 95%.

[0032] Embodiment 25: The method of any one of the preceding embodiments, wherein the PSCs express SOX2 and KLF4.

[0033] Embodiment 26: A method for differentiating PSCs into definitive endoderm (DE) in a three dimensional suspension culture, the method comprising: (d) culturing, in a bioreactor, a liquid culture media inoculated with PSCs; wherein the culturing of the liquid culture media inoculated with PSCs of (d) comprises suspending the PSCs in the liquid culture media; and (e) culturing, in a bioreactor, the PSCs of (d) in liquid definitive endoderm differentiation culture media for a period of time sufficient to differentiate the PSCs into DE,

wherein the culturing of the PSCs of (d) in the liquid definitive endoderm differentiation culture media comprises suspending the PSCs in the liquid definitive endoderm differentiation culture media.

[0034] Embodiment 27: The method of embodiment 26, wherein the liquid culture media or the liquid definitive endoderm differentiation culture is free of materials of animal or human origin; optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

[0035] Embodiment 28: The method of embodiment 26 or 27, wherein the culturing in (d) is for a period of time between about 18-54 hours.

[0036] Embodiment 29: The method of any one of embodiments 26-28, wherein the culturing in (d) is for a period of time between about 24-48 hours.

[0037] Embodiment 30: The method of any one of embodiments 26-29, wherein the liquid culture media inoculated with PSCs cultured in (d) is the PSC inoculated culture media of (c)(ii) of any one of embodiments 1-24.

[0038] Embodiment 31: The method of any one of embodiments 26-30, wherein the period of time sufficient to differentiate the PSCs into DE is a period of time that is between about 48-96 hours.

[0039] Embodiment 32: The method of any one of embodiments 26-31, wherein the period of time sufficient to differentiate the PSCs into DE is a period of time that is between about 60-84 hours.

[0040] Embodiment 33: The method of any one of embodiments 26-32, wherein the period of time sufficient to differentiate the PSCs into DE is a period of time that is between about 66-78 hours.

[0041] Embodiment 34: The method of any one of embodiments 26-33, wherein culturing the PSCs in the liquid definitive endoderm differentiation culture media for a period of time sufficient to differentiate the PSCs into DE comprises culturing the PSCs in a culture media comprising a nodal signaling pathway activator and/or a Wnt signaling pathway activator for a first period, then culturing the PSCs in a culture media comprising the nodal signaling pathway activator and/or the Wnt signaling pathway activator and a serum or serum replacement for a second period, and then culturing the PSCs in a culture media comprising the nodal signaling

pathway activator and/or the Wnt signaling pathway activator and the serum or serum replacement for a third period.

[0042] Embodiment 35: The method of embodiment 34, wherein the culture media in which the PSCs are cultured for the first period further comprises a BMP activator.

[0043] Embodiment 36: The method of embodiment 34 or 35, wherein the culture media in which the PSCs are cultured for the second period, and the culture media in which the PSCs are cultured for the third period, comprise: the nodal signaling pathway activator and/or the Wnt signaling pathway activator and a serum; optionally wherein the serum is FBS.

[0044] Embodiment 37: The method of embodiment 34 or 35, wherein the culture media in which the PSCs are cultured for the second period, and the culture media in which the PSCs are cultured for the third period, comprise: the nodal signaling pathway activator and/or the Wnt signaling pathway activator and a serum replacement; optionally wherein the serum replacement is knockout serum replacement (KSR).

[0045] Embodiment 38: The method of any one of embodiments 34-37, wherein each of the first, second and third period of time is between about 18-30 hours.

[0046] Embodiment 39: The method of any one of embodiments 34-38, wherein each of the first, second and third period of time is between about 20-28 hours.

[0047] Embodiment 40: The method of any one of embodiments 26-39, wherein the efficiency of DE induction is at least about 35%.

[0048] Embodiment 41: The method of any one of embodiments 26-40, wherein the efficiency of DE induction is at least about 45-55%.

[0049] Embodiment 42: The method of any one of embodiments 26-41, wherein the DE expresses Sox17 and FoxA2.

[0050] Embodiment 43: A method for differentiating definitive endoderm (DE) into hindgut spheroids (HGS) in a three dimensional suspension culture, the method comprising: (f) culturing, in a bioreactor, DE in liquid hindgut differentiation culture media for a period of time sufficient to differentiate the DE into HGS, wherein the culturing of the DE comprises suspending the DE in the liquid hindgut differentiation culture media.

[0051] Embodiment 44: The method of embodiment 43, wherein the liquid hindgut differentiation culture media is free of materials of animal or human origin; optionally wherein

the liquid hindgut differentiation culture media is free of any extracellular matrix and/or basement membrane matrix.

[0052] Embodiment 45: The method of embodiment 43, wherein the DE cultured in (f) is the DE of any one of embodiments 25-39.

[0053] Embodiment 46: The method of any one of embodiments 43-45, wherein the period of time sufficient to differentiate the DE into HGS is a period of time that is between about 60-120 hours.

[0054] Embodiment 47: The method of any one of embodiments 43-46, wherein the period of time sufficient to differentiate the DE into HGS is a period of time that is between about 84-108 hours.

[0055] Embodiment 48: The method of any one of embodiments 43-47, wherein the period of time sufficient to differentiate the DE into HGS is a period of time that is between about 90-102 hours.

[0056] Embodiment 49: The method of any one of embodiments 43-48, wherein the liquid hindgut differentiation culture media is changed after a period of time that is between about 20-28 hours.

[0057] Embodiment 50: The method of any one of embodiments 43-49, wherein the liquid hindgut differentiation culture media is changed after a period of time that is between about 22-26 hours.

[0058] Embodiment 51: The method of any one of embodiments 43-50, wherein the liquid hindgut differentiation culture media comprises a Wnt signaling pathway activator, an FGF signaling pathway activator, and optionally FBS.

[0059] Embodiment 52: The method of embodiment 51, wherein the Wnt signaling pathway activator comprises CHIR99021.

[0060] Embodiment 53: The method of embodiment 51 or 52, wherein the FGF signaling pathway activator comprises FGF4.

[0061] Embodiment 54: The method of any one of embodiments 51-53, wherein the FGF signaling pathway activator is FGF4 at a concentration between about 50-750 ng/ml.

[0062] Embodiment 55: The method of any one of any one of embodiments 51-54, wherein the Wnt pathway activator is CHIRON 99021 at a concentration between about 0.5 – 6 μM.

[0063] Embodiment 56: A method for differentiating hindgut spheroid (HGS) into intestinal organoids (IO) in a three dimensional suspension culture, the method comprising:

[0064] (g) culturing, in a bioreactor, HGS in liquid IO maturation culture media for a period of time sufficient to differentiate the HGS into IO, wherein the culturing of the HGS comprises suspending the HGS in the liquid IO maturation culture media.

[0065] Embodiment 57: The method of embodiment 56, wherein the liquid IO maturation culture media is free of materials of animal or human origin; optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

[0066] Embodiment 58: The method of embodiment 56 or 57, wherein the HGS cultured in (g) is the HGS of any one of embodiments 40-52.

[0067] Embodiment 59: The method of any one of embodiments 56-58, wherein the period of time sufficient to differentiate the HGS into IO is a period of time that is between about 12-30 days.

[0068] Embodiment 60: The method of any one of embodiments 56-59, wherein the period of time sufficient to differentiate the HGS into IO is a period of time that is between about 15-28 days.

[0069] Embodiment 61: The method of any one of embodiments 56-60, wherein the IO maturation culture media is changed after a period of time that is between about 24-54 hours.

[0070] Embodiment 62: The method of any one of embodiments 56-61, wherein the IO maturation culture media is changed after a period of time that is between about 46-50 hours.

[0071] Embodiment 63: The method of any one of embodiments 56-62, wherein the IO maturation culture media comprises one or more of EGF, R-spondin, Noggin, Gremlin 1, and/or Epiregulin (EREG).

[0072] Embodiment 64: The method of embodiment 63, wherein the concentration of EGF, R-sponding, Noggin, Gremlin 1, and/or EREG is between about 25-150 ng/ml.

[0073] Embodiment 65: The method of embodiment 63 or 64, wherein the concentration of EGF R-sponding, Noggin, Gremlin 1, and/or EREG is between about 50-100 ng/ml.

[0074] Embodiment 66: The method of any one of embodiments 56-65, wherein the HGS are not dissociated prior to culturing in the IO maturation culture media, wherein epithelial cells of the IO formed have a polarity wherein the apical surface is oriented to the outside of the IO.

[0075] Embodiment 67. A method for differentiating hindgut spheroids (HGS) into intestinal organoids (IO) having an apical-in polarity in a three dimensional suspension culture, the method comprising the method of any one of embodiments 56-66; wherein the method further comprises dissociating at least a portion of the HGS into HGS single cells prior to incubation in the IO maturation culture media; wherein the culturing of the HGS comprises suspending the dissociated HGS single cells and any non-dissociated HGS in the liquid IO maturation culture media; wherein epithelial cells of the IO formed from the dissociated HGS single cells have a polarity wherein the apical surface is oriented to the inside of the IO.

[0076] Embodiment 68. The method of embodiment 67, wherein the liquid IO maturation culture media is free of materials of animal or human origin; optionally wherein the liquid IO maturation culture media is free of any extracellular matrix and/or basement membrane matrix.

[0077] Embodiment 69. The method of embodiment 67 or 68, wherein at least 80% of the HGS are dissociated into single cells.

[0078] Embodiment 70. The method of any one of embodiments 67-69, wherein at least 90% of HGS are dissociated into single cells.

[0079] Embodiment 71. The method of any one of embodiments 67-70, wherein a concentration of dissociated HGS single cells are in the IO maturation culture media, wherein the concentration is between about 0.05x105 - 80x105 dissociated HGS single cells/ml of IO maturation culture media.

[0080] Embodiment 72: The method of any one of embodiments 67-71, wherein a concentration of dissociated HGS single cells are in the IO maturation culture media, wherein the concentration is between about 10x105-80x105 dissociated HGS single cells/ml of IO maturation culture media.

[0081] Embodiment 73: The method of any one of embodiments 67-72, wherein a concentration of dissociated HGS single cells are in the IO maturation culture media, wherein the concentration is between about 20x105-60x105 dissociated HGS single cells/ml of IO maturation culture media.

[0082] Embodiment 74: The method of any one of embodiments 67-73, wherein the dissociation is chemical, enzymatic and/or mechanical dissociation.

[0083] Embodiment 75: The method of any one of embodiments 67-74, wherein the dissociation is enzymatic, optionally wherein the enzyme comprises a proteolytic and/or a collagenolytic enzyme, optionally wherein the enzyme is Accutase.

[0084] Embodiment 76: The method of any one of embodiments 67-75, wherein the method further comprises transplanting the IO into a subject.

[0085] Embodiment 77: The method of embodiment 76, wherein transplanting the IO into the subject comprises transplanting the IO under the kidney capsule of a non-human animal, optionally for a period of time that is between about 6-20 weeks.

[0086] Embodiment 78: The method of embodiment 77, wherein the IO is transplanted under the kidney capsule of a non-human animal for a period of time that is between about 12-20 weeks.

[0087] Embodiment 79: The method of any one of embodiments 77-78, wherein the IO is transplanted under the kidney capsule of a non-human animal for a period of time that is between about 16-20 weeks.

[0088] Embodiment 80: The method of embodiment 76, wherein transplanting the IO into the subject comprises transplanting the IO into an intestinal lumen of the subject for treatment of an intestine of the subject.

[0089] Embodiment 81: The method of any one of embodiments 76-80, wherein the IO is matured in vitro for a period of time prior to transplantation, optionally wherein the period of time is between about 7-28 days.

[0090] Embodiment 82: The method of any one of embodiments 76-81, wherein the IO is matured in vitro for a period of time prior to transplantation that is between about 14-28 days.

[0091] Embodiment 83: The method of any one of embodiments 76-82, wherein the IO is matured in vitro for a period of time prior to transplantation that is between about 21-28 days.

[0092] Embodiment 84: The method of any one of embodiments 26-42, the method further comprising differentiating the DE into a spheroid, optionally wherein the differentiating comprises: (h) culturing, in a bioreactor, the DE in liquid differentiation culture media for a period of time sufficient to differentiate the DE into a spheroid, wherein the culturing of the DE comprises suspending the DE in the liquid differentiation culture media; optionally wherein the spheroid is a foregut or a hindgut spheroid.

[0093] Embodiment 85: The method of embodiment 84, wherein the liquid differentiation culture media is free of materials of animal or human origin; optionally wherein the liquid differentiation culture media is free of any extracellular matrix and/or basement membrane matrix.

[0094] Embodiment 86: The method of embodiment 84 or 85, the method further comprising: (i) culturing, in a bioreactor, the spheroids in liquid organoid maturation culture media for a period of time sufficient to differentiate the spheroids into an organoid, wherein the culturing of the spheroid comprises suspending the spheroid in the liquid organoid maturation culture media; optionally wherein the organoid is selected from the group consisting of a liver, pancreatic, gastric, antral gastric, fundal gastric, intestinal, lung, or colonic organoid.

[0095] Embodiment 87: The method of any one of embodiments 84-86, wherein the liquid organoid maturation culture media is free of materials of animal or human origin; optionally wherein the liquid organoid maturation culture media is free of any extracellular matrix and/or basement membrane matrix.

[0096] Embodiment 88: The method of any one of the preceding embodiments, wherein the PSC is an induced PSC (iPSC) or an embryonic stem cells (ESC).

[0097] Embodiment 89: The method of any one of the preceding embodiments, wherein the PSC is a human PSC, optionally a human iPSC (hiPSC).

[0098] Embodiment 90: A PSC or three-dimensional PSC aggregate made by the method of any one of embodiments 1-25 or 88-89.

[0099] Embodiment 91: DE made by the method of any one of embodiments 26-42 or 88-89.

[0100] Embodiment 92: An HGS made by the method of any one of embodiments 43-552, or 88-89.

[0101] Embodiment 93: An IO made by the method of any one of embodiments 56-89.

[0102] Embodiment 94: An IO having an apical-in polarity, wherein epithelial cells of the IO have a polarity wherein the apical surface is oriented to the inside of the IO, optionally wherein the IO is a human IO (hIO).

[0103] Embodiment 95: The IO having an apical-in polarity made by the method of any one of embodiments 67-89.

[0104] Embodiment 96: A spheroid made by the method of any one of embodiments 84, 85, or -89.

[0105] Embodiment 97: An organoid made by the method of any one of embodiments 86-89.

[0106] Embodiment 98: A method of treatment comprising transplanting the IO of any one of embodiments 93-95, or cells derived therefrom, into an animal, optionally wherein the animal is suffering from a GI disease state; optionally wherein the animal is human.

[0107] Embodiment 99: A method of screening a compound for activity comprising contacting the IO of any one of embodiments 93-95, or cells derived therefrom, with the compound and measuring a response of the IO to the compound.

[0108] Embodiment 100: A method of screening a compound for activity comprising contacting the organoid of embodiment 97, or cells derived therefrom, with the compound and measuring a response of the organoid to the compound.

[0109] Embodiment 101: The method of any one of the preceding embodiments, wherein the method is free of any xenogenic material, optionally wherein the organoids are clinical grade and suitable for transplantation in a human.

[0110] Embodiment 102: The method of any one of the preceding embodiments, wherein the bioreactor of (a), (b), (d), (e), (f), (g), (h), and/or (i) comprises a rotating chamber comprising the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media; wherein the rotating chamber is a cylindric section which is rotated around its longitudinal axis, thereby suspending the PSCs and/or three-dimensional PSC aggregates in the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media; optionally wherein the chamber is oriented such that its longitudinal axis is parallel to the ground.

[0111] Embodiment 103: The method of any one of the preceding embodiments, wherein the bioreactor of (a), (b), (d), (e), (f), (g), (h), and/or (i) comprises a rotating chamber comprising the liquid culture media, the second liquid culture media, the liquid definitive endoderm

differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media in a volume that is between about 5ml to about 50 L.

[0112] Embodiment 104: The method of any one of the preceding embodiments, wherein the bioreactor of (a), (b), (d), (e), (f), (g), (h), and/or (i) comprises a rotating chamber comprising the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media; wherein the rotation of the chamber is between about 3-7 rpm; and optionally wherein the rotational speed is a speed selected to keep the PSCs, the three-dimensional PSC aggregates, the spheroids, and/or the organoids in statical orbit.

[0113] Embodiment 105: The method of any one of the preceding embodiments, wherein the bioreactor of (a), (b), (d), (e), (f), (g), (h), and/or (i) comprises a rotating chamber comprising the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media; wherein the average shear stress on the PSCs, the three-dimensional PSC aggregates, the spheroids, and/or the organoids is less than about 5.0 dynes/cm^2.

[0114] Embodiment 106: The method of any one of the preceding embodiments, wherein the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, the liquid hindgut differentiation culture media, the liquid IO maturation culture media, the liquid differentiation culture media, and/or the liquid organoid maturation culture media comprises an anti-apoptotic agent.

[0115] Embodiment 107: The method of any one of the preceding embodiments, wherein the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, the liquid hindgut differentiation culture media, the liquid IO maturation culture media, the liquid differentiation culture media, and/or the liquid organoid maturation culture media comprises an anti-adhesion agent.

[0116] Embodiment 108: The method of embodiment of any one of the preceding embodiments, wherein the liquid culture media comprises an anti-adhesion agent.

[0117] Embodiment 109: The method of embodiment 108, wherein the anti-adhesion agent is DSS, xantham gum, A-205804, I-CAM1, carboxymethyl cellulose, and/or Neural Organoid Basal Medium 2 (NOBM).

[0118] Embodiment 110: The method of any one of embodiments 108-109, wherein the anti-adhesion agent is DSS at a concentration that is between about 1 μg/ml – 1000 μg/ml of the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, the liquid hindgut differentiation culture media, the liquid IO maturation culture media, the liquid differentiation culture media, and/or the liquid organoid maturation culture media.

[0119] Embodiment 111: A composition for a three-dimensional expansion and maintenance of pluripotent stem cell (PSC) cultures, the composition comprising: a liquid culture media comprising recombinant human basic fibroblast growth factor (rh bFGF), and/or a recombinant human transforming growth factor β (rh TGFβ); and PSCs suspended in the culture media.

[0120] Embodiment 112: The composition of embodiment 111, wherein the liquid culture media is a serum-free media, wherein the liquid culture media is free of materials of animal or human origin; optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

[0121] Embodiment 113: The composition of embodiment 111 or 112, further comprising: an anti-apoptotic agent.

[0122] Embodiment 114: The composition of any one of embodiments 111-113, wherein the PSCs express Oct4, SSEA1, TRA 1-60, Sox 2, and/or TRA-1-81.

[0123] Embodiment 115: The composition of any one of embodiments 111-114, wherein the PSCs express Oct4, SSEA1, TRA 1-60, Sox 2, and TRA-1-81.

[0124] Embodiment 116: The composition of any one of embodiments 111-115, further comprising: an anti-adhesion agent.

[0125] Embodiment 117: The composition of embodiment 116, wherein the anti-adhesion agent is one or both of DSS or xantham gum.

[0126] Embodiment 118: The composition of any one of embodiments 111-117, wherein the PSCs are suspended in the liquid culture media at a density that is about 50,000 – 1,000,000 PSCs/ml of culture media.

[0127] Embodiment 119: The composition of any one of embodiments 111-117, wherein the PSCs are suspended in the liquid culture media at a density that is about 100,000 – 300,000 PSCs/ml of culture media

[0128] Embodiment 120: The composition of any one of embodiments 111-119, wherein the PSCs are suspended in the culture media at a density that is about 180,000 - 220,000 PSCs/ml of culture media.

[0129] Embodiment 121: A composition for differentiating PSCs into definitive endoderm (DE) in a three dimensional suspension culture, the composition comprising: a liquid DE differentiation culture media; and PSCs suspended in the liquid DE differentiation culture media.

[0130] Embodiment 122: The composition of embodiment 121, wherein the liquid DE differentiation culture media is a serum-free media, wherein the liquid DE differentiation culture media is free of materials of animal or human origin; optionally wherein the liquid DE differentiation culture media is free of any extracellular matrix and/or basement membrane matrix.

[0131] Embodiment 123: The composition of embodiment 121 or 122, wherein the PSCs have an average diameter of less than about 500μm.

[0132] Embodiment 124: The composition of any one of embodiments 121-123, wherein the PSCs have an average diameter of less than about 400μm.

[0133] Embodiment 125: The composition of any one of embodiments 121-124, wherein the PSCs have an average diameter of less than about 300μm.

[0134] Embodiment 126: The composition of any one of embodiments 121-125, wherein the liquid DE differentiation culture media comprises a nodal signaling pathway activator and/or a Wnt signaling pathway activator. at a concentration of about 10 to 200 ng/mL of liquid DE differentiation culture media.

[0135] Embodiment 127: The composition of embodiment 126, wherein the nodal signaling pathway activator or the Wnt signaling pathway activator is at a concentration of about 10 to 200 ng/mL of liquid DE differentiation culture media

[0136] Embodiment 128: The composition of any one of embodiments 126-127, wherein the nodal signaling pathway activator or the Wnt signaling pathway activator is at a concentration of about 50 to 150 ng/mL ng/mL of liquid DE differentiation culture media.

[0137] Embodiment 129: The composition of any one of embodiments 126-128, wherein the nodal signaling pathway activator or the Wnt signaling pathway activator at a concentration of about 100 to 200 ng/mL ng/mL of liquid DE differentiation culture media.

[0138] Embodiment 130: The composition of any one of embodiments 126-129, wherein the liquid DE differentiation culture media further comprises serum or a serum replacement at a concentration of about 0% to 20%.

[0139] Embodiment 131: The composition of any one of embodiments 126-130, wherein the liquid DE differentiation culture media further comprises serum or serum replacement at a concentration of about 2% to 5%.

[0140] Embodiment 132: The composition of any one of embodiments 111-131, further comprising: DE differentiated from the PSCs.

[0141] Embodiment 133: The composition of embodiment 132, wherein the DE differentiated from the PSCs expresses Sox17 and/or FoxA2.

[0142] Embodiment 134: The composition of embodiment 132 or 133, wherein the DE differentiated from the PSCs expresses Sox17 and FoxA2.

[0143] Embodiment 135: A composition for differentiating DE into hindgut spheroids (HGS) in a three dimensional suspension culture, the composition comprising: a liquid hindgut differentiation culture media comprising a Wnt signaling pathway activator, an FGF signaling pathway activator, and optionally FBS; and DE suspended in the liquid hindgut differentiation culture media.

[0144] Embodiment 136: The composition of embodiment 135, wherein the liquid hindgut differentiation culture media is free of materials of animal or human origin; optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

[0145] Embodiment 137: The composition of embodiment 135 or 136, wherein the Wnt signaling pathway activator comprises CHIR99021, and wherein the FGF signaling pathway activator comprises FGF4.

[0146] Embodiment 138: The composition of any one of embodiments 135-137, wherein the FGF signaling pathway activator is at a concentration that is at least about 50 ng/ml of the liquid hindgut differentiation culture media.

[0147] Embodiment 139: The composition of any one of embodiments 135-138, wherein the FGF signaling pathway activator is at a concentration that is at least about 500 ng/ml of the liquid hindgut differentiation culture media.

[0148] Embodiment 140: The composition of any one of embodiments 135-139, wherein the Wnt pathway activator is at a concentration that is at least about 0.5 μM of the liquid hindgut differentiation culture media

[0149] Embodiment 141: A composition for differentiating HGS into intestinal organoids (IO) in a three dimensional suspension culture, the composition comprising: a liquid IO maturation culture media comprising EGF; and HGS suspended in the liquid IO maturation culture media.

[0150] Embodiment 142: The composition of embodiment 141, wherein the liquid IO maturation culture media is free of materials of animal or human origin; optionally wherein the liquid IO maturation culture media is free of any extracellular matrix and/or basement membrane matrix.

[0151] Embodiment 143: The composition of embodiment 141 or 142, wherein lumen of the HGS suspended in the liquid IO maturation culture media is exterior facing relative to the liquid IO maturation culture media.

[0152] Embodiment 144: The composition of any one of embodiments 141-143, wherein the concentration of EGF is at least about 25 ng/ml.

[0153] Embodiment 145: The composition of any one of embodiments 141-144, wherein the concentration of EGF is at least about 100 ng/ml.

[0154] Embodiment 146: The composition of any one of embodiments 141-145, wherein at least a portion of the HGS suspended in the liquid IO maturation culture media comprises dissociated HGS single cells.

[0155] Embodiment 147: The composition of embodiment 146, wherein at least 80% of the HGS are dissociated HGS single cells; optionally wherein at least 90% of HGS are dissociated single cells.

[0156] Embodiment 148: The composition of any one of embodiments 146 or 147, wherein a concentration of the dissociated HGS single cells in the liquid IO maturation culture media is in a range that about 0.1x105-80x105 dissociated HGS single cells/ml of liquid IO maturation culture media.

[0157] Embodiment 149: The composition of any one of embodiments 146-148, wherein a concentration of the dissociated HGS single cells in the liquid IO maturation culture media is in a range that is about 20x105-60x105 dissociated HGS single cells/ml of liquid IO maturation culture media.

[0158] Embodiment 150: The composition of any one of embodiments 141-149, further comprising: IO differentiated from the HGS.

[0159] Embodiment 151: The composition of embodiment 150, wherein epithelial cells of the IO formed from the dissociated HGS single cells have a polarity such that an apical surface is oriented to the inside of the IO.

[0160] Embodiment 152: The composition of any one of embodiments 141-151, wherein the HGS express CdX2.

[0161] Embodiment 153: The composition of any one of embodiments 141-152, wherein the HGS expresses FOX-F1 but does not express SOX2.

[0162] Embodiment 154: The composition of embodiments 141-153, wherein the liquid IO maturation culture media further comprises noggin.

[0163] Embodiment 155: A composition comprising: a liquid culture media; and three-dimensional PSC aggregates suspended in the liquid culture media.

[0164] Embodiment 156: The composition of embodiment 155, wherein the liquid culture media is free of materials of animal or human origin; optionally wherein the liquid culture media is free of any extracellular matrix and/or basement membrane matrix.

[0165] Embodiment 157: The composition of embodiment 155 or 156, wherein at least a portion of the three-dimensional PSC aggregates are dissociated as single cells.

[0166] Embodiment 158: The composition of any one of embodiments 155-157, wherein an average size of diameters of the three-dimensional PSC aggregates is less than 400 µm.

[0167] Embodiment 159: The composition of any one of embodiments 155-158, wherein an average size of diameters of the three-dimensional PSC aggregates is less than 350 µm.

[0168] Embodiment 160: The composition of any one of embodiments 155-159, wherein an average size of diameters of the three-dimensional PSC aggregates is less than 300 µm.

[0169] Embodiment 161: The composition of any one of embodiments 155-160, further comprising: an anti-adhesion agent.

[0170] Embodiment 162: The composition of embodiment 161, wherein the anti-adhesion agent is DSS, xantham gum, A-205804, I-CAM1, carboxymethyl cellulose, and/or Neural Organoid Basal Medium 2 (NOBM).

[0171] Embodiment 163: The composition of embodiment 161 or 162, wherein the anti-adhesion agent is at a concentration that is between about 1 μg/ml – 1000 μg/ml of the liquid culture media.

BRIEF DESCRIPTION OF THE DRAWINGS

[0172] In addition to the features described herein, additional features and variations will be readily apparent from the following descriptions of the drawings and exemplary embodiments. It is to be understood that these drawings depict embodiments and are not intended to be limiting in scope.

[0173] FIG. 1A depicts an embodiment of an experimental protocol to explore the impact of various culture conditions on the maintenance and expansion of PSCs.

[0174] FIG. 1B depicts an embodiment of an illustration of the basic principle of the operation of a suspension culture where the chamber is rotated around its longitudinal axis when the longitudinal axis is oriented parallel to the ground. The depicted rotating vessel bioreactor (cell culture systems), rotates continuously to keep the cells, PSC aggregates, spheroids and/or organoids suspended by counterbalancing the gravitation forces, thereby ideally keeping them in statical orbit. As a result, cells grown in the rotating vessel bioreactor experience very low shear forces.

[0175] FIG. 2 depicts the results of an embodiment of a study examining the impact of the inoculation density of PSC in the suspension culture media on the formation of PSC aggregates and cell death at various time points following culture inoculation.

[0176] FIG. 3 depicts the results of an embodiment of a study comparing dissociation reagents' ability to form single cell cultures from PSC aggregates which form viable and homogenous PSC aggregates 24 and 96 hours post-inoculation of the single cells.

[0177] FIGs. 4A-4C depict the results of an embodiment of a study examining how the stage of growth of the PSC two-dimensional culture used to inoculate the suspension culture.

FIG. 4A depicts an embodiment of an iPSC growth curve and two timepoints for harvesting iPSCs for inoculation of the suspension culture: d0 refers to the day at which the PSC line would usually be passaged (80-90% confluency) – note that this day may differ for different PSC lines (usually ranging from 4-6 days post-seeding); d-1 refers to one day before PSC culture reached "passage ready" confluency when monolayer is at 40-50% confluency. FIG. 4B depicts an embodiment of photographs of a starting two-dimensional PSC culture at d-1 and d0, the resulting PSC aggregates at day 4 (d4) of suspension culture inoculated with the d-1 or d0 PSC. FIG. 4C depicts an embodiment of a graph of the PSC aggregate size distribution at d4 of the suspension culture using either d-1 or d0 PSC inoculates.

[0178] FIG. 5 depicts the results of an embodiment of a study comparing how the size of PSC aggregates in a suspension culture at the time of their passaging impacts the yield of PSC aggregates formed after reinoculation. Passaging of PSC aggregates at Day 3 (when the diameter of the majority of aggregates is <400 μm) results in a successful propagation of the culture. Passaging of PSC aggregates at Day 4, when their diameter exceeds 400 μm results in a much lower yield of aggregates.

[0179] FIGs. 6A-6C depict the results of an embodiment of a study comparing how the bioreactor rotation speed impacts the yield of PSC aggregates formed. FIG. 6A depicts an embodiment of PSC aggregates on day 3 (d3) of suspension culture at various bioreactor chamber rotation speeds. FIG. 6B depicts an embodiment of a graph of the PSC aggregate size distribution at d3 of the suspension at various bioreactor chamber rotation speeds. FIG. 6C depicts an embodiment of a chart comparing the output of PSC cells at the first (P1), second (P2) and third (P3) passaging of suspension cultures at various bioreactor chamber rotation speeds.

[0180] FIG. 7 depicts the results of an embodiment based on a study comparing the use of anti-apoptotic agents. As shown in FIG. 7, the use of anti-apoptotic agents increase cell (e.g., PSCs) recovery with each passage. In particular, the anti-apoptotic agent CEPTwas found to provide higher cell recovery than the anti-apoptotic agent ROCKi.

[0181] FIG. 8 depicts the results of an embodiment of a study comparing how culture media, mTeSR 1 (research media) and mTeSR AOF (animal product free media), impacts PSC expansion and maintenance at the first (P1), second (P2) and third (P3) passaging of a suspension culture.

[0182] FIGs. 9A and 9B depict the results of an embodiment of a study comparing how culture media, mTeSR 1 (research media; FIG. 9A) and mTeSR AOF (animal product free media; FIG. 9B), impacts stem cell markers: Oct4, SSEA4 and TRA 1-60.

[0183] FIG. 10 depicts the results of an embodiment of a study comparing how cell line (research-grade, PSC cell line 72.3; clinical-grade PSC cell line FF3 produced under GMP) impact the production of PSC aggregates at the first (P1), second (P2) and third (P3) passaging of a suspension culture.

[0184] FIGs. 11A and 11B depict the results of an embodiment of a study comparing the impact of two-dimensional (2D) culturing (FIG. 11A) to suspension culturing (3D) (FIG. 11B) on the expression of stem cell markers Oct4, SSEA4 and TRA 1-60 in research-grade PSC cell line 72.3.

[0185] FIGs. 12A and 12B depict the results of an embodiment of a study comparing the impact of two-dimensional (2D) culturing (FIG. 12A) to suspension culturing (3D) (FIG. 12B) on the expression of stem cell markers Oct4, SSEA4 and TRA 1-60 in clinical-grade PSC cell line FF3 produced under GMP.

[0186] FIGs. 13A-13B depict the result of an embodiment based on a study investigating the formation of three-dimensional PSC aggregates from PSCs, where the three-dimensional PSC aggregates are able to retain their pluripotency. As shown in the images of FIG. 13A, PSCs are able to form three-dimensional PSC aggregates progressing from Day 1 to Day 4. As shown in FIG. 13B, number of such aggregates increases from 600 in Day 1 to ~1400 in Day 4. In addition, markers of pluripotency, such as OCT4 and SSEA4 are evident in the three-dimensional PSC aggregates in the confocal imaging..

[0187] FIGs. 14A-14B depict the results of an evaluation of pluripotent 3D PSC aggregates across multiple lines and passages. As shown in FIG. 14A, there was a marked formation of three-dimensional PSC aggregates across passages for iPSC line 72.3 and ESC line H1. FIG. 14B shows an increase in cell count as well as an increase in diameter of the PSC aggregates across the passages for both lines. Furthermore, FIG. 14B shows that an expression of pluripotency markers (e.g., OCT4 and SSEA-4) remained at least 90% across all the passages and lines tested for three-dimensional PSC aggregates, similar to the expression in conventional, two-dimensionally grown PSC monolayers.

[0188] FIG. 15 depicts the results an evaluation comparing the pluripotency of PSC lines grown in three-dimensional suspension culture according to methods described herein to PSC lines grown in conventional 2D monolayers. As shown in FIG. 15, based on the higher gene expression of the pluripotency markers OCT4, SOX2, and KLF4, there is an increased pluripotency of PSC lines (H1 and 72.3) grown in three-dimensional suspension culture in comparison to those lines grown in 2D monolayers.

[0189] FIG. 16 depicts an embodiment of an experimental protocol for the matrix-free suspension culture production of HIOs from hiPSCs.

[0190] FIG. 17 depicts the results of an embodiment of a study examining the impact of acclimatization of PSCs to suspension culturing on the production of HIOs at various time points.

[0191] FIGs. 18A and 18B depict the results of an embodiment of a study comparing the efficiency of DE induction in two-dimensional (2D) culturing (FIG. 18A) and suspension culturing (3D) (FIG. 18B) by examining expression of definitive endoderm markers Sox 17 and FoxA2.

[0192] FIG. 19 depicts the results of an embodiment demonstrating the effect of exposure to Activin A at different time points (e.g., at 24 hours, at 48 hours, or at 72 hours post-passaging) to DE induction efficiency in 3D suspension culture. As shown in FIG. 19, 3D PSC aggregates exposed to Activin A at 48 hour post-passaging showed the highest DE differentiation efficiency, based on the expression of DE markers FoxA2 and Sox17.

[0193] FIG. 20 depicts the results of an embodiment demonstrating the effect of 3D PSC aggregate size on intestinal tissue differentiation at the DE stage. As shown in FIG. 20, the size of the 3D PSC aggregates at the time of the exposure to Activin determines DE induction efficiency. Furthermore, FIG. 20 shows that smaller size of the 3D PSC aggregate (e.g., less than 500 μm (e.g., less than about 400 μm (e.g., less than about 350 μm (e.g., less than about 300 μm)))) ensures better DE induction.

[0194] FIG. 21 depicts the results of an embodiment demonstrating the effect of 3D PSC aggregate size on intestinal tissue differentiation at the HGS stage. The effects were demonstrated by the expression of CDX2, which is a marker for intestinal tissue differentiation. As shown in FIG. 21, there is a strong expression of CDX2 at the hindgut stage of differentiation of 3D PSC aggregates with diameters smaller than 300 μm at the time of DE induction. However, there is a weaker and more sparse expression of CDX2 for 3D PSC aggregates having diameters

greater than 300 μm at the time of DE induction. The results further confirm the importance that size of the 3D PSC aggregates has on differentiation efficiency.

[0195] FIG. 22 depicts the results of an embodiment demonstrating the effect of 3D PSC aggregate size on intestinal tissue differentiation at the HIO stage. The effects were demonstrated by the expression of CDX2, a marker for intestinal tissue differentiation. As shown in FIG. 22, differentiation of 3D PSC aggregates having diameters no more than 300 μm resulted in apical-in HIO formation and substantially homogenous expression of CDX2 across generated HIOs. However, differentiation of 3D PSC aggregates having diameters at least 300 μm resulted in mixed apical-out and apical-out structures and epithelial structures with weak or no CDX2 expression.

[0196] FIGs. 23-27 depict the results of an embodiment based on a study investigating the effect of dextran sulfate sodium (DSS), an anti-adhesion agent, mediating the size of 3D PSC aggregates. As previously discussed, 3D PSC aggregates having a smaller size (e.g., diameters of less than about 500 μm (e.g., less than about 400 μm (e.g., less than about 300 μm))) is favorable for intestinal tissue differentiation. The images of FIG. 23 shows the effects of varied concentrations of DSS on the size of 3D PSC aggregates, with a concentration of 10 μg/ml having the highest effect of reducing the size of the 3D PSC aggregates. FIG. 24 further shows that the effects of DSS on reducing aggregate size were noted across different PSC lines (72.3, FF3, H1, and H1 GFP). FIG. 25 further shows that a DSS concentration of 10 μg/ml had the highest reduction in the average diameter across the lines, while also increasing the yield of aggregates being formed. FIGS. 26 and 27 further explore the effect of 10 μg/ml DSS on average size of PSC aggregates across different lines. In particular, FIG. 26 shows that 10 μg/ml of DSS is sufficient to induce smaller diameter of PSC aggregates, causing an average of ~200 μm decrease in mean diameter of PSC aggregates upon DSS treatment, in comparison to untreated controls. FIG. 27 also shows that this effect is consistent across different iPSC and ESC lines, and leads to the shift in the frequency of PSC aggregate size distribution.

[0197] FIG. 28 depicts the results of an embodiment based on a study investigating the effect of DSS on the pluripotency of 3D PSC aggregates, as measured by pluripotency markers SOX2 and OCT4. As shown in the confocal images, DSS had no negative effect on the pluripotency of the 3D PSC aggregates any of the concentrations tested. The study also investigated the effect of DSS on the viability of 3D PSC aggregates, as measured by the release

of a viability marker lactate dehydrogenase (LDH). The study found that DSS at concentrations at or below 1000 μg/ml has no negative effect on the viability of PSC aggregates.

[0198] FIG. 29 depicts the results of an embodiment based on a study investigating the effects of various treatment regimes for applying DSS on the average size, numbers, and pluripotency of 3D PSC aggregates. As shown in FIG. 29, the treatment regimes that were tested included control (i.e., no treatment regime), at inoculation, and throughout. The results show that Treatment with 10 ug/ml of DSS at the time of inoculation is sufficient to maintain PSC aggregates <400 um, while having no negative effect on the PSC aggregate numbers or expression of pluripotency genes. However, prolonged treatment (Throughout) with DSS lead to a decreased OCT4 expression.

[0199] FIG. 30 depicts the results of an embodiment based on a study investigating the effects of DSS on the average size of 3D PSC aggregates across passages. As shown in FIG. 30, the effect of DSS treatment on PSC aggregate size (a decrease in diameter) was maintained across multiple passages.

[0200] FIG. 31 depicts the results of an embodiment based on a study investigating the effects of DSS on the propensity of 3D PSC aggregates to differentiate. The propensity is measured based on differentiation efficiency, which is marked by an expression of markers FOXA2 and SOX17. As shown in FIG. 31, similar differentiation efficiency of 3D PSC aggregates toward differentiation to DE was found, as noted by the expression of Sox17 and FoxA2 in the presence and absence of DSS, demonstrating that there is no negative effect of DSS treatment on cells propensity to differentiation.

[0201] FIG. 32 depicts the results of an embodiment of an experiment demonstrating the development of well-patterned HIOs in suspension culture having either an apical-out epithelial cell polarity or an apical-in epithelial polarity.

[0202] FIG. 33 depicts the results of an embodiment based on the development of well-patterned HIOs in 3D suspension culture. The successful development of correctly patterned HIOs in the suspension culture is confirmed by immunofluorescent staining. The markers CDX2, ZO-1, and Vim1 signify the differentiation into HIOs.

[0203] FIGs. 34A and 34B depict the results of an embodiment of an experiment demonstrating the *in vivo* maturation of HIOs developed in suspension culture following transplantation under the kidney capsule of a mouse. FIG. 34A is an embodiment of a

photograph of an HIO 9 weeks post-transplant under the kidney capsule. FIG. 34B is an embodiment of H & E staining of an HIO 9 weeks post-transplant under the kidney capsule.

[0204] FIG. 35 depicts the results of an embodiment of an experiment demonstrating that the polarity of epithelial cells in HIOs developed in suspension culture can be modified. Dissociation of hindgut spheroids at day 7 (+ dissociation) and their reaggregation in suspension culture leads to the apical surface on the inside of the HIO (apical-in). If the DE is not dissociated at day 7 (- dissociation), the result is the apical surface on the outside of the HIO (apical-out).

DETAILED DESCRIPTION

[0205] Aspects of the present disclosure relate generally to suspension culture methods of pluripotent stem cells (PSC), differentiated cells, spheroids and organoids derived from PSCs, and compositions of the same. In some aspects, these methods may be performed and/or may facilitate industrial efficiency and scalability These methods may be performed without the use of basement membrane matrices (e.g., xenogenic basement membrane matrices) during PSC maintenance and expansion, as well as during differentiation of PSCs into differentiated cells and organoids, for example definitive endoderm (DE), hindgut spheroids (HGS), and intestinal organoids (IO). In some aspects, the methods may be xeno-free, and may be performed as per Good Manufacturing Practices (GMP). In some embodiments, the disclosed methods utilize a suspension culture for each of the following phases in the manufacture of an organoid from an PSC: maintenance and expansion of PSC, differentiation of PSC into DE, differentiation of DE into spheroids (e.g., hindgut spheroids), and differentiation and maturation of spheroids into an organoid (e.g., an intestinal organoid). Also disclosed are uses of the methods and compositions for transplantation and treatment.

[0206] In some embodiments of the methods disclosed herein, one or more of the following aspects of current culture protocols are improved: improve the transition from two-dimensional (e.g., monolayer) culture conditions to three-dimensional (e.g., Matrigel-embedded) culture conditions to remove or reduce a potentially significant bottleneck; eliminate the need for the use of extracellular (basement membrane) matrix (e.g. Matrigel) for organoid (e.g., IO) production, thereby reducing variability driven by batch-to-batch variations in extracellular matrix compositions and concentrations, and advance the transition toward xeno-free manufacturing of human organoids; decrease the labor by eliminating the manual plating in

Matrigel domes found in some current organoid culturing methods; eliminate the manual processing around day 14 of some current culturing methods; enable large scale production of organoids; decrease variability in the resulting cell, spheroid, and/or organoid.

[0207] One challenge in using organoids is the difficulty in accessing the apical, or luminal, surface of the epithelium, which is typically enclosed within the organoid interior. Many applications require access to the apical or luminal surface of the organoid, because this is the mucosal surface that normally interfaces with the external environment and thus absorbs nutrients, interacts with GI microbes and uptakes drugs or toxins. The apical surface also secretes mucins, antimicrobial peptides and enzymes that regulate interactions between gut luminal contents and the epithelium. Embodiments disclosed herein relate to a method of suspension culture that enables full control of human organoid epithelial polarity and creation of apical-out organoids.

Expansion and Maintenance of PSCs in Suspension Cultures

[0208] Aspects of the disclosure related to a method for three-dimensional expansion and maintenance of pluripotent stem cell (PSC) in suspension cultures. In some embodiments, PSCs are induced PSCs (iPSCs) or an embryonic stem cells (ESCs). In some embodiments, the PSC is human (hPSC).

[0209] In some embodiments, the PSC is a human iPSC (hiPSC). In some embodiments, the method comprises: (a) inoculating a liquid culture media with PSCs (optionally hPSC or hiPSC); (b) culturing in a bioreactor the liquid culture media that is inoculated with the PSCs such that three-dimensional PSC aggregates form in the liquid culture media, wherein the culturing in the bioreactor comprises suspending the PSCs in the liquid culture media; and (c) passaging the PSCs by: (i) dissociating at least a portion of the three-dimensional PSC aggregates into single cells; and inoculating a second liquid culture media with the dissociated three-dimensional PSC aggregates of i.) with PSCs.

[0210] In some embodiments, the diameter of the majority of PSC aggregates (e.g., the three-dimensional PSC aggregates) formed is, or is not more than, 500, 450, 400, 350, 300, 250 μm, or a range defined by any two of the preceding values; optionally wherein the diameter of the majority of PSC aggregates is not more than 500 μm, not more than 400 μm, not more than 350 μm, or not more than 300 μm.

[0211] In some embodiments the suspension comprises rotation of the chamber in which the culture media is contained, causing rotation of the culture media around an axis that is parallel to the ground.

[0212] In some embodiments, at least 80, 85, 90, 95, 98 or 99% of the PSC aggregates are dissociated into single cells at (c)(i); optionally wherein at least 90% of PSC aggregates are dissociated into single cells at (c)(i).

[0213] In some embodiments, the PSCs may be inoculated to form a density that is, is at least, or is not more than, 50,000, 100,000, 180,000, 200,000, 220,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1,000,000, 1,500,000, or 2,000,000 PSCs/ml of culture media in the bioreactor, or a range defined by any two of the preceding values; in some embodiments, the density is 50,000-1,000,000, 50,000-500,000, 100,000-300,000, 180,000-220,000 or 200,000 PSCs/ml of culture media; in some embodiments, the density is 180,000-220,000 or 200,000 PSCs/ml of culture media.

[0214] In some embodiments, the second liquid culture media with the dissociated three-dimensional PSC aggregates of (i) may be inoculated with PSCs form a density that is, is at least, or is not more than, 50,000, 100,000, 180,000, 200,000, 220,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1,000,000, 1,500,00, or 2,000,000 PSCs/ml of culture media in the bioreactor, or a range defined by any two of the preceding values; in some embodiments, the density is 50,000-1,000,000, 50,000-500,000, 100,000-300,000, 100,000-200,000, 180,000-220,000 or 200,000 PSCs/ml of culture media; in some embodiments, the density is 180,000-220,000 or 200,000 PSCs/ml of culture media.

[0215] In some embodiments, the method comprises passaging the PSCs two or more times by culturing the PSCs of inoculated culture media of (c)ii.) until three-dimensional PSC aggregates form, and repeating (c). In some embodiments, the culture is passaged 2, 3, 4, or more times. In some embodiments, the PSCs maintain expression of markers of stem cells, for example Oct4, SSEA4 and/or TRA 1-60 (e.g., Oct4, SSEA4 and TRA 1-60). In some embodiments, at least 85, 90, or 95% of the cells maintain their status as PSCs as evidence by expression of markers of stem cells, for example Oct4, SSEA4 and/or TRA 1-60 (e.g., Oct4, SSEA4 and TRA 1-60) following at least 2, 3, 4 or more passages. In some embodiments, in (a) (the initial inoculation of the suspension culture) and/or (c)(ii) (re-inoculation of the suspension culture during passaging), the culture media is inoculated at a density of about 100,000-220,000,

about 180,000-220,000, or about 200,000 PSCs/ml of culture media. In some embodiments, the passaging occurs after a period of time following the inoculation in (a) and/or (c)(ii) that is, is at least, or is not more than about 40, 48, 54, 60, 66, 72, 78, 84, 90, 96, or 168 hours, or a range defined by any two of the preceding values; optionally wherein the period of time is about 40-54, 40-84, 60-84, 66-78, or 72 hours. In some embodiments, the PSCs are passaged one or more times (e.g., 3 times), and following one or more passages (e.g., following the third passage) the portion of PSCs expressing Oct4, SSEA1 and/or TRA 1-60 (e.g., Oct4, SSEA4 and TRA 1-60) at levels at least as high as the average expression level of the PSCs used in the initial inoculation of the suspension culture in (a) is, or is at least, 85, 90, 93, 95, 97, 98 or 99%, or a range defined by any two of the preceding values. In some embodiments, the portion of PSCs expressing Oct4, SSEA1 and/or TRA 1-60 (e.g., Oct4, SSEA4 and TRA 1-60) at levels at least as high as the average expression level of the PSCs used in the inoculation in (a) is at least 95%. In some embodiments, the PSCs are passaged one or more times. In some embodiments, the PSCs are passaged at least 3 times. Following one or more passages (e.g., following the third passage) the portion of PSCs expressing Oct4, SSEA1 and/or TRA 1-60 (e.g., Oct4, SSEA4 and TRA 1-60) at levels at least as high as the average expression level of the PSCs used in the initial inoculation of the suspension culture in (a) is, or is at least, 85, 90, 93, 95, 97, 98 or 99%, or a range defined by any two of the preceding values. In some embodiments, the portion of PSCs expressing Oct4, SSEA1 and/or TRA 1-60 (e.g., Oct4, SSEA4 and TRA 1-60) at levels at least as high as the average expression level of the PSCs used in the inoculation in (a) is at least 95%. In some embodiments, the PSCs are passaged one or more times (e.g., at least 3 times), and the PSCs express SOX2 and/or KLF4 (e.g., SOX2 and KLF4).

[0216] In some embodiments, the method further comprises exchanging a portion of the culture media in the bioreactor after a period of time following the inoculation in (a) and/or (c)(ii). In some embodiments, the period of time is, is at least, or is not more than, 36, 42, 48, 54 or 60 hours, or a range defined by any two of the preceding values; optionally wherein the period of time is 42-54, or 48 hours. In some embodiments, the portion of the culture media exchanged is, or is at least, 50, 60, 70, 80, 90, of 100% of the culture media in the bioreactor, or a range defined by any two of the preceding values; optionally wherein the portion is at least 80% or 90% of the culture media in the bioreactor.

[0217] In some embodiments, the PSCs used to inoculate the suspension culture are first cultured on the surface of a substrate, e.g., on the surface of a culture flask (an adherent culture), also referred to herein as a two-dimensional or 2D culture. PSCs cultured on the surface of a substrate such as a culture flask typically form a monolayer of cells on the surface of the substrate. In some embodiments, the method further comprises prior to the inoculation of the suspension culture with PSCS in (a), culturing the PSCs on the surface of a substrate, and collecting the PSCs from the surface of the substrate for use in the inoculation of the suspension culture of (a) when the PSCs are in a logarithmic growth phase and/or at 35-55% or 40-50% confluency. In some embodiments, the collecting comprises dissociating the PSCs prior to the inoculation of the suspension culture in (a). Methods for dissociation of cells in adherent cultures may include but are not limited to chemical, enzymatic and/or mechanical dissociation. In some embodiments, the dissociation is chemical, e.g., via use of EDTA. In some embodiments, the dissociation is enzymatic. In some embodiments, the enzyme used for dissociation comprises a proteolytic and/or a collagenolytic enzyme, for example Accutase.

[0218] Devices for the suspension culturing of cells may include bioreactors. Use of bioreactors for suspension culturing (spinning flask and vertical wheel bioreactors) may result in the generation of variable level of shear stress (e.g. shear stress can vary between 0.1-10 dynes/cm^2 in spinning flask depending on rotation speed, vessel size and shape, and culture medium volume). PSCs, e.g., human PSCs, are sensitive to high shear stress that may cause unexpected cell death and differentiation. In some embodiments, a bioreactor having reduced and/or low shear stress, e.g., relative to traditional suspension culture bioreactors (spinning flask and vertical wheel bioreactors) or a bioreactor that is shear stress-free is used. In some embodiments, the bioreactor having reduced and/or low and/or no shear stress functions by rotating continuously and the uplifting movement from the rotation being counteracted by gravity to suspend cells within the media. In some embodiments, the bioreactor comprises a rotating chamber comprising the suspension culture media. In some embodiments, the chamber is a vessel having the shape of a cylindric section (e.g., shaped like a petri dish, optionally where the depth of the dish is greater than a typical petri dish). In some embodiments, the chamber is rotated around its longitudinal axis, which causes the liquid culture media contained in the chamber to rotate, thereby suspending the cells and aggregates in the liquid culture media. In some embodiments, the chamber is oriented such that its longitudinal axis is parallel to the

ground, such that cells (e.g., individual cells, aggregates, spheroids, organoids, etc.) within the chamber rises on one side of the chamber due to the rotation of the chamber and liquid culture media, and fall on the opposite side due to the force of gravity.

[0219] FIG. 1B depicts an embodiment of an illustration of the operation of a suspension culture where the chamber is rotated around its longitudinal axis (not illustrated, but which is normal to the plane of the illustration, passing through the center of the chamber/vessel) when the longitudinal axis is oriented parallel to the ground. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the volume of culture media in the chamber is, is at least, or is not more than, 5, 10, 20, 30, 40, 50 ml, or a range defined by any of the preceding values. In some embodiments, the volume of culture media in the chamber is 5-10 ml; optionally 10 ml. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the rotation of the chamber is, is at least, or is not more than, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80 rpm, or a range defined by any two of the preceding values. In some embodiments, the rotation is not more than 40 rpm. In some embodiments, the rotational speed is a speed selected to keep the cells and/or aggregates suspended in statical orbit. In some embodiments, the rotation is 3-7 rpm. In some embodiments, the rotation speed of the chamber is selected such that the number of PSCs in the culture at the time of passaging in (c) is least 2 or 2.5-fold the number of PSCs used to inoculate the culture media. In some embodiments, the rotation speed of the chamber is selected such that the number of PSCs in the culture at passaging in (c) is least 2 or 2.5-fold the number of PSCs used to inoculate the culture media for at least two or at least three passages of the PSCs. In some embodiments, the bioreactor is configured such that the average shear stress on the cells and aggregates in the culture media while the culture media is in motion (e.g., the chamber is rotating) is less than 5.0, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, or 0.005 dynes/cm^2. In some embodiments, the average shear stress on the cells and aggregates in the culture media is less than 0.1 dynes/cm^2.

[0220] Culture media may be used for the expansion and maintenance of PSCs. In some embodiments, the culture media is a serum-free media. In some embodiments, the culture media comprises recombinant human basic fibroblast growth factor (rh bFGF) and recombinant human transforming growth factor β (rh TGFβ). In some embodiments, the culture media is free of materials of animal or human origin. In some embodiments, the culture media is free of any

extracellular matrix and/or basement membrane matrix (e.g., Matrigel or similar products). In some aspects, the lack of the extracellular matric and/or basement membrane matrix allows for three-dimensional suspension of cultures. In some embodiments, any of the aforementioned culture media may be liquid culture media.

[0221] Aspects of the disclosure relate to PSCs produced by the suspension culture methods described above and elsewhere herein.

[0222] Aspects of the disclosure include compositions for expansion and maintenance of pluripotent stem cell (PSC) cultures. In various embodiments, the composition comprises: a liquid culture media comprising recombinant human basic fibroblast growth factor (rh bFGF), and/or a recombinant human transforming growth factor β (rh TGFβ); and PSCs suspended in the liquid culture media. In some embodiments, the liquid culture media is a serum-free media, wherein the liquid culture media is free of materials of animal or human origin; optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

[0223] In some embodiments, the composition further comprises an anti-apoptotic agent (e.g., ROCK inhibitor (ROCKi) and/or CEPT).

[0224] In some embodiments, the PSCs express Oct4, SSEA1 and/or TRA 1-60. In at least one embodiment, the PSCs express Oct4, SSEA1 and TRA 1-60. In some embodiments, the PSCs express the PSCs further express Oct4, SSEA1, TRA 1-60, Sox2, and/or TRA-1-81.

[0225] In some embodiments, the composition further comprises an anti-adhesion agent. For example, the anti-adhesion agent may be one or both of DSS or xantham gum. In some embodiments, the PSCs are suspended in the liquid culture media at a density that is about 50,000 – 1,000,000 PSCs/ml of culture media. In some embodiments, the PSCs are suspended in the liquid culture media at a density that is about 100,000 – 300,000 PSCs/ml of culture media. In some embodiments, the PSCs are suspended in the culture media at a density that is about 100,000 - 220,000 PSCs/ml of culture media. In some embodiments, the PSCs are suspended in the culture media at a density that is about 180,000 - 220,000 PSCs/ml of culture media.

Differentiation of Suspension Cultures of PSC into Definitive Endoderm (DE)

[0226] Aspects of the disclosure include methods of differentiating PSCs into definitive endoderm (DE) in a three dimensional suspension culture. In some embodiments, PSCs are induced PSC (iPSC) or an embryonic stem cells (ESC). In some embodiments, the PSC is human

(hPSC). In some embodiments, the PSC is a human iPSC (hiPSC). In some embodiments, the method comprises: (d) culturing in a bioreactor a liquid culture media inoculated with PSCs, wherein the culturing of the PSC inoculated culture media of (d) comprises suspending the PSCs in the liquid culture media; and (e) culturing in a bioreactor, the PSCs of (d) in liquid definitive endoderm differentiation culture media for a period of time sufficient to differentiate the PSCs into DE, wherein the culturing of the PSC inoculated culture media of (d) comprises suspending the PSCs in the liquid definitive endoderm differentiation culture media.

[0227] In some embodiments, the culturing in (d) is for a period of time that is, is at least, is not more than, 18, 24, 30, 36, 42, 48, or 54 hours, or a range defined by any two of the preceding values; optionally wherein the period of time is 18-54 or 24-48 hours.

[0228] In some embodiments of the method, the liquid culture media inoculated with PSCs cultured in (d) is the PSC inoculated culture media of the passaged PSC culture of (c)(ii) described in the methods above, and elsewhere herein. In some embodiments, the differentiation is performed on the PCS suspension culture described above and elsewhere herein, where it is a continuation of any one of the methods described above and elsewhere herein, further comprising differentiating the PSCs of the PSC inoculated culture media of (c)(ii) into DE, optionally wherein the differentiating comprises: (d') culturing the PSCs of the PSC inoculated culture media of (c)(ii) in a bioreactor, wherein the culturing comprises suspending the PSCs in the liquid culture media, and wherein the culturing is for a period of time that is, is at least, is not more than, 18, 24, 30, 36, 42, 48, or 54 hours, or a range defined by any two of the preceding values; optionally wherein the period of time is 18-54 or 24-48 hours; and (e) culturing in a bioreactor the PSCs of (d') in definitive endoderm differentiation culture media for a period of time sufficient to differentiate the PSCs into DE, wherein the culturing of the PSC inoculated culture media of (d') comprises suspending the PSCs in the liquid definitive endoderm differentiation culture media.

[0229] In some embodiments, the liquid culture media and/or the liquid definitive endoderm media is cultured with PSCs at a density that is, is at least, or is not more than, 50,000, 100,000, 180,000, 200,000, 220,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1,000,000, 1,500,00, or 2,000,000 PSCs/ml of culture media, or a range defined by any two of the preceding values; optionally wherein the density is 50,000-1,000,000, 50,000-500,000, 100,000-300,000, 180,000-220,000 or 200,000 PSCs/ml of culture media;

[0230] In some embodiments, the period of time sufficient to differentiate the PSCs into DE is a period of time that is, is at least, is not more than, 48, 54, 60, 66, 72, 78, 84, 90 or 96 hours, or a range defined by any two of the preceding values; optionally wherein the period of time is 60-84, 66-78, or 72 hours. In some embodiments, culturing the PSCs in definitive endoderm differentiation culture media for a period of time sufficient to differentiate the PSCs into DE comprises: culturing the PSCs in culture media comprising a Wnt signaling pathway activator (e.g., CHIR99021) and/or a Nodal signaling pathway activator (e.g., Activin A), and optionally a BMP signaling pathway activator, for a first period; then culturing the PSCs in culture media comprising the Wnt signaling pathway activator and/or the Nodal signaling pathway activator, and optionally a serum (e.g., FBS) or serum replacement (e.g., Knockout Replacement Serum (KRS), HAS, etc.) for a second period, and then culturing the PSCs in culture media comprising the Wnt signaling pathway activator and/or the Nodal signaling pathway activator, and optionally the serum or the serum replacement for a third period. In some embodiments, each of the first, second and third periods of time is independently selected from a period of time that is, is at least, or is not more than, 18, 20, 22, 24, 26, 28 or 30 hours, or a range defined by any two of the preceding values; optionally wherein the first, second and third period of time is 20-28, 22-26, or 24 hours. In some embodiments, the efficiency of DE induction is, or is at least, 35, 40, 45, 50, 55, 60, 70, 80, 90 or 95%, or a range defined by any two of the preceding values; optionally wherein the efficiency of DE induction is at least 45% or at least 50%; optionally wherein the efficiency of DE induction is 45-55%; optionally wherein the efficiency of DE induction is 80-95%. In some embodiments, the DE expresses Sox17 and FoxA2.

[0231] In some embodiments, the pluripotent stem cells are differentiated into definitive endoderm cells by contacting the pluripotent stem cells with Activin A, a BMP signaling pathway activator, or both. In some embodiments, the pluripotent stem cells are contacted with a concentration of Activin A that is, is about, is at least, is at least about, is not more than, or is not more than about, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 ng/mL, or any concentration of Activin A within a range defined by any two of the aforementioned concentrations, for example, 10 to 200 ng/mL, 10 to 100 ng/mL, 100 to 200 ng/mL, or 50 to 150 ng/mL. In some embodiments, the pluripotent stem cells are contacted with Activin A at a concentration of 100 ng/mL, or about 100 ng/mL. In some embodiments, the

pluripotent stem cells are contacted with a concentration of a BMP signaling pathway activator that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 ng/mL, or any concentration of a BMP signaling pathway activator within a range defined by any two of the aforementioned concentrations, for example, 1 to 200 ng/mL, 1 to 100 ng/mL, 1 to 200 ng/mL, 1 to 80 ng/mL, 1 to 30 ng/mL. In some embodiments, the pluripotent stem cells are contacted with a BMP signaling pathway activator at a concentration of 15 ng/mL or about 15 ng/mL.

[0232] In some embodiments, a bioreactor having reduced and/or low shear stress, e.g., relative to traditional suspension culture bioreactors (spinning flask and vertical wheel bioreactors) or a bioreactor that is shear stress-free is used. In some embodiments, the bioreactor having reduced and/or low and/or no shear stress functions by rotating continuously and the uplifting movement from the rotation being counteracted by gravity to suspend cells within the media. In some embodiments, the bioreactor comprises a rotating chamber comprising the suspension culture media. In some embodiments, the chamber is a vessel having the shape of a cylindric section (e.g., shaped like a petri dish, optionally where the depth of the dish is greater than a typical petri dish). In some embodiments, the chamber is rotated around its longitudinal axis, which causes the liquid culture media contained in the chamber to rotate, thereby suspending the cells and aggregates in the liquid culture media. In some embodiments, the chamber is oriented such that its longitudinal axis is parallel to the ground, such that cells (e.g., individual cells, aggregates, spheroids, organoids, etc.) within the chamber rises on one side of the chamber due to the rotation of the chamber and liquid culture media, and fall on the opposite side due to the force of gravity. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the volume of culture media in the chamber is, is at lease, or is not more than, 5, 10, 20, 30, 40, 50 ml, or a range defined by any of the preceding values. In some embodiments, the volume of culture media in the chamber is 5-10 ml; optionally 10 ml. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the rotation of the chamber is, is at least, or is not more than, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80 rpm, or a range defined by any two of the preceding values. In some embodiments, the rotation is not more than 40 rpm. In some embodiments, the rotation is 3-7 rpm. In some embodiments, the rotational speed is a speed selected to keep the

cells and/or aggregates suspended in statical orbit. In some embodiments, the bioreactor is configured such that the average shear stress on the cells and aggregates in the culture media while the culture media is in motion (e.g., the chamber is rotating) is less than 5.0, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, or 0.005 dynes/cm2. In some embodiments, the average shear stress on the cells and aggregates in the culture media is less than 0.1 dynes/cm2.

[0233] In some embodiments, the culture media is free of materials of animal or human origin. In some embodiments, the culture media is free of any extracellular matrix and/or basement membrane matrix (e.g. Matrigel or similar products).

[0234] Aspects of the disclosure include compositions for differentiating PSCs into definitive endoderm (DE) in a three dimensional suspension culture. In some embodiments, the composition comprises: a liquid DE differentiation culture media; and PSCs suspended in the liquid DE differentiation culture media. In some aspects, the liquid DE differentiation culture media is a serum-free media, wherein the liquid DE differentiation culture media is free of materials of animal or human origin; optionally wherein the liquid DE differentiation culture media is free of any extracellular matrix and/or basement membrane matrix. In some embodiments, the PSCs have an average diameter of less than about 400um. In some embodiments, the PSCs have an average diameter of less than about 300um.

[0235] In some embodiments, the liquid DE differentiation culture media comprises Activin A at a concentration of about 10 to 200 ng/mL of liquid DE differentiation culture media. In some embodiment, the liquid DE differentiation culture media comprises Activin A at a concentration of about 50 to 150 ng/mL ng/mL of liquid DE differentiation culture media. In some embodiments, the liquid DE differentiation culture media comprises Activin A at a concentration of about 100 to 200 ng/mL ng/mL of liquid DE differentiation culture media. In another embodiment, the liquid DE differentiation culture media further comprises FBS at a concentration of about 0% to 20%. In some embodiments, the liquid DE differentiation culture media further comprises FBS at a concentration of about 0.2% to 10%. In some aspects, the liquid DE differentiation culture media further comprises FBS at a concentration of about 2% to 5%.

[0236] In some embodiments, the composition further comprises DE differentiated from the PSCs. The DE differentiated from the PSCs may express Sox17 and/or FoxA2. In some embodiments, the DE differentiated from the PSCs expresses Sox17 and FoxA2.

[0237] Aspects of the disclosure relate to DE produced by the suspension culture methods described above and elsewhere herein.

Differentiation of Suspension Cultures of DE into Hindgut Spheroids (HGS)

[0238] Aspects of the disclosure include methods of differentiating DE into hindgut spheroids (HGS) in a three dimensional suspension culture. In some embodiments, the DE are derived from PSCs. In some embodiments, the PSCs are induced PSC (iPSC) or an embryonic stem cells (ESC). In some embodiments, the PSC is human (hPSC). In some embodiments, the PSC is a human iPSC (hiPSC). In some embodiments, the method comprises: (f) culturing in a bioreactor DE in liquid hindgut differentiation culture media for a period of time sufficient to differentiate the DE into HGS, wherein the culturing of the DE comprises suspending the DE in the liquid hindgut differentiation culture media. In some embodiments, the DE cultured in (f) is the DE made by any one of the methods disclosed above and elsewhere herein. In some embodiments, the differentiation is performed on the DE culture described above and elsewhere herein, where it is a continuation of any one of the methods described above and elsewhere herein, further comprising differentiating the DE into HGS, optionally wherein the differentiating comprises: (f) culturing in a bioreactor the DE in liquid hindgut differentiation culture media for a period of time sufficient to differentiate the DE into HGS, wherein the culturing of the DE comprises suspending the DE in the liquid hindgut differentiation culture media.

[0239] In some embodiments, the period of time sufficient to differentiate the DE into HGS is a period of time that is, is at least, is not more than, 60, 66, 72, 78, 84, 90, 96, 102, 108, 114, 120 hours, or a range defined by any two of the preceding values; optionally wherein the period of time is 84-108, 90-102, or 96 hours. In some embodiments, the hindgut differentiation culture media is changed after a period of time that is, is at least, or is not more than, 18, 20, 22, 24, 26, 28 or 30 hours, or a range defined by any two of the preceding values; optionally wherein the first, second and third period of time is 20-28, 22-26, or 24 hours.

[0240] In some embodiments, the hindgut differentiation culture media comprises a Wnt signaling pathway activator, an FGF signaling pathway activator, and optionally FBS. In some embodiments, the hindgut differentiation culture media comprises a Wnt signaling pathway activator, wherein the Wnt signaling pathway activator comprises CHIR99021, an FGF signaling

pathway activator, wherein the FGF signaling pathway activator comprises FGF4, and optionally FBS. In some embodiments, the FGF signaling pathway activator is FGF4, optionally wherein the concentration is, is about, is at least, or is at least about 50 ng/ml, 100 ng/ml, 150 ng/ml, 200 ng/ml, 250 ng/ml, 300 ng/ml, 350 ng/ml, 400 ng/ml, 450 ng/ml, 500 ng/ml, 550 ng/ml, 600 ng/ml, 650 ng/ml, 700 ng/ml, or 750 ng/ml, or a range defined by any two of the preceding values, optionally 50-750 ng/ml, 50-100 ng/ml, or 50-500 ng/ml, or optionally at a concentration of 500 ng/ml. In some embodiments, the Wnt pathway activator is CHIRON 99021, optionally wherein the concentration is, is about, is at least, or is at least about 0.5 μM, 1 μM, 1.5 μM, 2 μM, 2.5 μM , 3 μM, 3.5 μM, 4 μM, 4.5 μM, 5 μM, 5.5 μM, or 6 μM, or a range defined by any two of the preceding values, optionally 0.5 – 6 μM, 0.5-3 μM, 3-6 μM, 2-4 μM, or optionally at a concentration of 3 μM.

[0241] In some embodiments, a bioreactor having reduced and/or low shear stress, e.g., relative to traditional suspension culture bioreactors (spinning flask and vertical wheel bioreactors) or a bioreactor that is shear stress-free is used. In some embodiments, the bioreactor having reduced and/or low and/or no shear stress functions by rotating continuously and the uplifting movement from the rotation being counteracted by gravity to suspend cells within the media. In some embodiments, the bioreactor comprises a rotating chamber comprising the suspension culture media. In some embodiments, the chamber is a vessel having the shape of a cylindric section (e.g., shaped like a petri dish, optionally where the depth of the dish is greater than a typical petri dish). In some embodiments, the chamber is rotated around its longitudinal axis, which causes the liquid culture media contained in the chamber to rotate, thereby suspending the cells and aggregates in the liquid culture media. In some embodiments, the chamber is oriented such that its longitudinal axis is parallel to the ground, such that cells (e.g., individual cells, aggregates, spheroids, organoids, etc.) within the chamber rises on one side of the chamber due to the rotation of the chamber and liquid culture media, and fall on the opposite side due to the force of gravity. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the volume of culture media in the chamber is, is at lease, or is not more than, 5, 10, 20, 30, 40, 50 ml, or a range defined by any of the preceding values. In some embodiments, the volume of culture media in the chamber is 5-10 ml; optionally 10 ml. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the rotation of the chamber is, is at least, or is not more than, 1, 2, 3, 4,

5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80 rpm, or a range defined by any two of the preceding values. In some embodiments, the rotation is not more than 40 rpm. In some embodiments, the rotation is 3-7 rpm. In some embodiments, the bioreactor is configured such that the average shear stress on the cells, aggregates, and/or spheroids in the culture media while the culture media is in motion (e.g., the chamber is rotating) is less than 5.0, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, or 0.005 dynes/cm^2. In some embodiments, the average shear stress on the cells, aggregates, and/or spheroids in the culture media is less than 0.1 dynes/cm^2.

[0242] In some embodiments, the culture media is free of materials of animal or human origin. In some embodiments, the culture media is free of any extracellular matrix and/or basement membrane matrix (e.g. Matrigel or similar products).

[0243] Aspects of the disclosure relate to HSGSs produced by the suspension culture methods described above and elsewhere herein.

[0244] Aspects of the disclosure include compositions for differentiating DE into hindgut spheroids (HGS) in a three dimensional suspension culture. In some embodiments, the composition comprises: a liquid hindgut differentiation culture media comprising a Wnt signaling pathway activator, an FGF signaling pathway activator, and optionally FBS; and DE suspended in the liquid hindgut differentiation culture media. In some embodiments, the liquid hindgut differentiation culture media is free of materials of animal or human origin; optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

[0245] In some embodiments, the Wnt signaling pathway activator comprises CHIR99021, and wherein the FGF signaling pathway activator comprises FGF4. In some embodiments, the FGF signaling pathway activator is at a concentration that is at least about 50 ng/ml of the liquid hindgut differentiation culture media. In some embodiments, the FGF signaling pathway activator is at a concentration that is at least about 500 ng/ml of the liquid hindgut differentiation culture media. In some embodiments, the Wnt pathway activator is at a concentration that is at least about 0.5 μM of the liquid hindgut differentiation culture media.

Differentiation of Suspension Cultures of HGS into Intestinal Organoids (IO)

[0246] Aspects of the disclosure include methods of differentiating HGS into intestinal organoids (IO) in a three dimensional suspension culture. In some embodiments, the HGS are derived from PSCs. In some embodiments, the PSCs are induced PSC (iPSC) or an embryonic

stem cells (ESC). In some embodiments, the PSC is human (hPSC). In some embodiments, the PSC is a human iPSC (hiPSC). In some embodiments, the method comprises: (g) culturing in a bioreactor HGS in liquid IO maturation culture media for a period of time sufficient to differentiate the HGS into IO, wherein the culturing of the HGS comprises suspending the HGS in the liquid IO maturation culture media. In some embodiments, the HGS cultured in (g) is the HGS of any one of the methods described above and elsewhere herein for producing HGS. In some embodiments, the differentiation is performed on the HGS culture described above and elsewhere herein, where it is a continuation of any one of the methods described above and elsewhere herein, further comprising differentiating the HGS into IO, optionally wherein the differentiating comprises: (g) culturing in a bioreactor HGS in liquid IO maturation culture media for a period of time sufficient to differentiate the HGS into IO, wherein the culturing of the HGS comprises suspending the HGS in the liquid IO maturation culture media.

[0247] In some embodiments, the period of time sufficient to differentiate the HGS into IO is a period of time that is, is at least, is not more than, about 12, 13, 14, 15, 16, 17, 18, 19, 20, or 30 days, or a range defined by any two of the preceding values; optionally wherein the period of time is 12-30, 12-20, 15-28, 15-20, or 20 days; optionally wherein the period of time is at least 12, 15, or 20 days. In some embodiments, the IO maturation culture media is changed after a period of time that is, is at least, or is not more than, about 24, 28, 32, 36, 38, 42, 44, 46, 48, 50, 52, or 54 hours, or a range defined by any two of the preceding values. In some embodiments, the first, second and third period of time is about 24-54, 42-54, 46-50, or 48 hours.

[0248] In some embodiments, the IO maturation culture media comprises EGF, R-spondin, Noggin, Gremlin 1, and/or Epiregulin (EREG). In some embodiments, the concentration of EGF, R-spondin, Noggin, Gremlin 1, and/or Epiregulin (EREG) is, is about, is at least, or is at least about 25 ng/ml, 50 ng/ml, 75 ng/ml, 100 ng/ml, 125 ng/ml, 150 ng/ml, 175 ng/ml, or 200 ng/ml, or a range defined by any two of the preceding values, optionally 25-100 ng/ml, 50-150 ng/ml, 100 ng/ml, or optionally is at a concentration of 100 ng/ml.

[0249] In some embodiments, the HGS are not dissociated prior to culturing in the IO maturation culture media, wherein epithelial cells of the IO formed have a polarity wherein the apical surface is oriented to the outside of the IO. In other words, the apical surface faces outward to the medium.

[0250] In some embodiments, a bioreactor having reduced and/or low shear stress, e.g., relative to traditional suspension culture bioreactors (spinning flask and vertical wheel bioreactors) or a bioreactor that is shear stress-free is used. In some embodiments, the bioreactor having reduced and/or low and/or no shear stress functions by rotating continuously and the uplifting movement from the rotation being counteracted by gravity to suspend cells within the media. In some embodiments, the bioreactor comprises a rotating chamber comprising the suspension culture media. In some embodiments, the chamber is a vessel having the shape of a cylindric section (e.g., shaped like a petri dish, optionally where the depth of the dish is greater than a typical petri dish). In some embodiments, the chamber is rotated around its longitudinal axis, which causes the liquid culture media contained in the chamber to rotate, thereby suspending the cells and aggregates in the liquid culture media. In some embodiments, the chamber is oriented such that its longitudinal axis is parallel to the ground, such that cells (e.g., individual cells, aggregates, spheroids, organoids, etc.) within the chamber rises on one side of the chamber due to the rotation of the chamber and liquid culture media, and fall on the opposite side due to the force of gravity. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the volume of culture media in the chamber is, is at lease, or is not more than, 5, 10, 20, 30, 40, 50 ml, or a range defined by any of the preceding values. In some embodiments, the volume of culture media in the chamber is 5-10 ml; optionally 10 ml. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the rotation of the chamber is, is at least, or is not more than, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80 rpm, or a range defined by any two of the preceding values. In some embodiments, the rotation is not more than 40 rpm. In some embodiments, the rotation is 3-7 rpm. In some embodiments, the rotational speed is a speed selected to keep the cells and/or aggregates suspended in statical orbit. In some embodiments, the bioreactor is configured such that the average shear stress on the cells, aggregates, spheroids and/or organoids in the culture media while the culture media is in motion (e.g., the chamber is rotating) is less than 5.0, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, or 0.005 dynes/cm2. In some embodiments, the average shear stress on the cells, aggregates, spheroids and/or organoids in the culture media is less than 0.1 dynes/cm2.

[0251] In some embodiments, the culture media is free of materials of animal or human origin. In some embodiments, the culture media is free of any extracellular matrix and/or basement membrane matrix (e.g. Matrigel or similar products).

[0252] Aspects of the disclosure relate to IO produced by the suspension culture methods described above and elsewhere herein. In some embodiments, the epithelial cells of the IO have a polarity wherein the apical surface is oriented to the outside of the IO (aka, apical-out).

[0253] Aspects of the disclosure relate to compositions for differentiating HGS into intestinal organoids (IO) in a three dimensional suspension culture. In various embodiments, the compositions comprise: a liquid IO maturation culture media comprising EGF; and HGS suspended in the liquid IO maturation culture media. In some embodiments, the liquid IO maturation culture media is free of materials of animal or human origin; optionally wherein the liquid IO maturation culture media is free of any extracellular matrix and/or basement membrane matrix.

[0254] In some embodiments, the lumen of the HGS suspended in the liquid IO maturation culture media is exterior facing relative to the liquid IO maturation culture media.

[0255] In some embodiments, the concentration of EGF is at least about 25 ng/ml. In some aspects, the concentration of EGF is at least about 100 ng/ml.

[0256] In at least one embodiment, at least a portion of the HGS suspended in the liquid IO maturation culture media comprises dissociated HGS single cells. In some embodiments, at least 80% of the HGS are dissociated HGS single cells; optionally at least 90% of HGS are dissociated single cells. In at least one embodiment, a concentration of the dissociated HGS single cells in the liquid IO maturation culture media is in a range that about $0.1x10^5$ - $80x10^5$. In another embodiment, a concentration of the dissociated HGS single cells in the liquid IO maturation culture media is in a range that is about $20x10^5$ - $60x10^5$ dissociated HGS single cells/ml of liquid IO maturation culture media.

[0257] In some embodiments, the composition further comprises: IO differentiated from the HGS. In some aspects, epithelial cells of the IO formed from the dissociated HGS single cells have a polarity such that an apical surface is oriented to the inside of the IO. In some embodiments, the HGS express CdX2. In some aspects, the HGS expresses FOX-F1 but does not express SOX2.

[0258] In some embodiments, the liquid IO maturation culture media further comprises noggin.

Modification of the Polarity of Epithelial Cells in IOs

[0259] Aspects of the disclosure include methods of modifying the polarity of epithelial cells in IOs derived from HGSs in a three dimensional suspension culture. In some embodiments, the HGS are derived from PSCs. In some embodiments, the PSCs are induced PSC (iPSC) or an embryonic stem cells (ESC). In some embodiments, the PSC is human (hPSC). In some embodiments, the PSC is a human iPSC (hiPSC). In some embodiments of the method for differentiating HGS into intestinal organoids (IO) having an apical-in polarity in a three dimensional suspension culture, the method comprises: (g) culturing in a bioreactor HGS in liquid IO maturation culture media for a period of time sufficient to differentiate the HGS into IO, wherein the culturing of the HGS comprises suspending the HGS in the liquid IO maturation culture media, wherein the method further comprises dissociating at least a portion of the HGS into single cells prior to incubation in the IO maturation culture media, wherein the culturing of the HGS comprises suspending the dissociated HGS single cells and any non-dissociated HGS in the liquid IO maturation culture media, wherein epithelial cells of the IO formed from the dissociated HGS single cells have a polarity wherein the apical surface is oriented to the inside of the IO (aka, apical-in). In some embodiments, the dissociated HGS cultured in (g) is the HGS of any one of the methods described above and elsewhere herein for producing HGS. In some embodiments, the differentiation is performed on the HGS culture described above and elsewhere herein, where it is a continuation of any one of the methods described above and elsewhere herein, wherein the method further comprises dissociating at least a portion of the HGS into single cells prior to incubation in the IO maturation culture media, wherein the culturing of the HGS comprises suspending the dissociated HGS single cells and any non-dissociated HGS in the liquid IO maturation culture media, wherein epithelial cells of the IO formed from the dissociated HGS single cells have a polarity wherein the apical surface is oriented to the inside of the IO. In some embodiments, at least 80, 85, 90, 95, 98 or 99% of the HGS are dissociated into single cells; optionally wherein at least 90% of HGS are dissociated into single cells. In some embodiments, a concentration of dissociated HGS single cells are in the IO maturation culture media, wherein the concentration is, is at least, is not more than,

$0.05x10^5$, $0.1x10^5$, $0.5x10^5$, $1x10^5$, $2x10^5$, $4x10^5$, $6x10^5$, $8x10^5$, $16x10^5$, $10x10^5$,$20x10^5$, $40x10^5$, or $80x10^5$ dissociated HGS single cells/ml of IO maturation culture media, or a range defined by any two of the preceding values; optionally wherein the concentration is $0.1x10^5$-$80x10^5$, $1x10^5$-$16x10^5$, or $2x10^5$-$6x10^5$, $10x10^5$-$80x10^5$, $20x10^5$-$60x10^5$ dissociated HGS single cells/ml of IO maturation culture media; optionally wherein the concentration is $40x10^5$ dissociated HGS single cells/ml of IO maturation culture media.

[0260] In some embodiments, the dissociation is chemical, enzymatic and/or mechanical dissociation. In some embodiments, the dissociation is chemical, e.g., EDTA. In some embodiments, the dissociation is enzymatic, optionally wherein the enzyme comprises a proteolytic and/or a collagenolytic enzyme, optionally wherein the enzyme is Accutase.

[0261] In some embodiments, a bioreactor having reduced and/or low shear stress, e.g., relative to traditional suspension culture bioreactors (spinning flask and vertical wheel bioreactors) or a bioreactor that is shear stress-free is used. In some embodiments, the bioreactor having reduced and/or low and/or no shear stress functions by rotating continuously and the uplifting movement from the rotation being counteracted by gravity to suspend cells within the media. In some embodiments, the bioreactor comprises a rotating chamber comprising the suspension culture media. In some embodiments, the chamber is a vessel having the shape of a cylindric section (e.g., shaped like a petri dish, optionally where the depth of the dish is greater than a typical petri dish). In some embodiments, the chamber is rotated around its longitudinal axis, which causes the liquid culture media contained in the chamber to rotate, thereby suspending the cells and aggregates in the liquid culture media. In some embodiments, the chamber is oriented such that its longitudinal axis is parallel to the ground, such that cells (e.g., individual cells, aggregates, spheroids, organoids, etc.) within the chamber rises on one side of the chamber due to the rotation of the chamber and liquid culture media, and fall on the opposite side due to the force of gravity. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the volume of culture media in the chamber is, is at lease, or is not more than, 5, 10, 20, 30, 40, 50 ml, or a range defined by any of the preceding values. In some embodiments, the volume of culture media in the chamber is 5-10 ml; optionally 10 ml. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the rotation of the chamber is, is at least, or is not more than, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80 rpm, or a range defined by any two of the preceding

values. In some embodiments, the rotation is not more than 40 rpm. In some embodiments, the rotation is 3-7 rpm. In some embodiments, the rotational speed is a speed selected to keep the cells, aggregates, spheroids and/or organoids suspended in statical orbit. In some embodiments, the bioreactor is configured such that the average shear stress on the cells, aggregates, spheroids and/or organoids in the culture media while the culture media is in motion (e.g., the chamber is rotating) is less than 5.0, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, or 0.005 dynes/cm2. In some embodiments, the average shear stress on the cells, aggregates, spheroids and/or organoids in the culture media is less than 0.1 dynes/cm2.

[0262] In some embodiments, the culture media is free of materials of animal or human origin. In some embodiments, the culture media is free of any extracellular matrix and/or basement membrane matrix (e.g. Matrigel or similar products).

[0263] Aspects of the disclosure relate to IO produced by the suspension culture methods described above and elsewhere herein. In some embodiments, the epithelial cells of the IO have a polarity wherein the apical surface is oriented to the inside of the IO (aka, apical-in). Aspects of the disclosure relate to IO, wherein the epithelial cells of the IO have a polarity wherein the apical surface is oriented to the inside of the IO (aka, apical-in).

In vivo Maturation of IOs by Implantation Under the Kidney Capsule

[0264] Aspects of the disclosure relate to methods of *in vivo* maturation of IOs by implantation of the IO under the kidney capsule of a subject (e.g., an animal). In some embodiments, the IO is derived from PSCs. In some embodiments, the PSCs are induced PSC (iPSC) or an embryonic stem cells (ESC). In some embodiments, the PSC is human (hPSC). In some embodiments, the PSC is a human iPSC (hiPSC). In some embodiments, the method comprises transplanting an IO under the kidney capsule of a non-human animal, optionally for a period of time that is, is at least, or is not more than, 6, 8, 10, 12, 14, 16, 18, or 20 weeks, or a range defined by any two of the preceding values, optionally, 6-20, 6-14, or 8-12 weeks. In some embodiments, the IO is the IO of any one of the methods described above and elsewhere herein for producing IO. In some embodiments, the IO is matured *in vitro* for a period of time prior to transplantation, optionally wherein the period of time is, is at least, or is not more than, 7, 10, 14, 16, 21, 25, or 28 days, or a range defined by any two of the preceding values, optionally 7-28, 14-28, or 21-28 days.

Implantation of IOs For Intestinal Treatment

[0265] Aspects of the disclosure relate to methods of treatment of an intestine of a subject (e.g., due to intestinal lesions, damages, tissue loss, etc.) by implantation of the IO into the intestinal lumen of the subject (e.g., patient, human, animal, etc.). In some embodiments, the IO is derived from PSCs. In some embodiments, the PSCs are induced PSC (iPSC) or an embryonic stem cells (ESC). In some embodiments, the PSC is human (hPSC). In some embodiments, the PSC is a human iPSC (hiPSC). In some embodiments, the method comprises transplanting an IO into the intestinal lumen, optionally for a period of time that is, is at least, or is not more than, 6, 8, 10, 12, 14, 16, 18, or 20 weeks, or a range defined by any two of the preceding values, optionally, 6-20, 6-14, or 8-12 weeks. In some embodiments, the IO is the IO of any one of the methods described above and elsewhere herein for producing IO. In some embodiments, the IO is matured *in vitro* for a period of time prior to transplantation, optionally wherein the period of time is, is at least, or is not more than, 7, 10, 14, 16, 21, 25, or 28 days, or a range defined by any two of the preceding values, optionally 7-28, 14-28, or 21-28 days.

Production of Spheroids and Organoids by Suspension Cultures

[0266] An aspect of the present disclosure is a method of differentiating the DE into a spheroid, optionally wherein the differentiating comprises: (f) culturing in a bioreactor the DE of any of the methods disclosed above and elsewhere herein in liquid differentiation culture media for a period of time sufficient to differentiate the DE into a spheroid, wherein the culturing of the DE comprises suspending the DE in the liquid differentiation culture media; optionally wherein the spheroid is a foregut or a hindgut spheroid. Differentiation media for differentiating DE into spheroids (e.g., foregut or hindgut) are known in the art, and can be used in the methods disclosed herein.

[0267] An aspect of the present disclosure is a method of differentiating spheroids into organoids. In some embodiments, the method comprises culturing in a bioreactor the spheroids of any of the methods disclosed above and elsewhere herein in liquid organoid maturation culture media for a period of time sufficient to differentiate the spheroids into an organoid, wherein the culturing of the spheroid comprises suspending the spheroid in the liquid organoid maturation culture media; optionally wherein the organoid is selected from the group consisting of a liver, pancreatic, gastric, antral gastric, fundal gastric, intestinal, lung, or colonic organoid.

Differentiation media for differentiating spheroids (e.g., foregut or hindgut) into organoids are known in the art, and can be used in the methods disclosed herein.

[0268] In some embodiments, a bioreactor having reduced and/or low shear stress, e.g., relative to traditional suspension culture bioreactors (spinning flask and vertical wheel bioreactors) or a bioreactor that is shear stress-free is used. In some embodiments, the bioreactor having reduced and/or low and/or no shear stress functions by rotating continuously and the uplifting movement from the rotation being counteracted by gravity to suspend cells within the media. In some embodiments, the bioreactor comprises a rotating chamber comprising the suspension culture media. In some embodiments, the chamber is a vessel having the shape of a cylindric section (e.g., shaped like a petri dish, optionally where the depth of the dish is greater than a typical petri dish). In some embodiments, the chamber is rotated around its longitudinal axis, which causes the liquid culture media contained in the chamber to rotate, thereby suspending the cells and aggregates in the liquid culture media. In some embodiments, the chamber is oriented such that its longitudinal axis is parallel to the ground, such that cells (e.g., individual cells, aggregates, spheroids, organoids, etc.) within the chamber rises on one side of the chamber due to the rotation of the chamber and liquid culture media, and fall on the opposite side due to the force of gravity. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the volume of culture media in the chamber is, is at lease, or is not more than, 5, 10, 20, 30, 40, 50 ml, or a range defined by any of the preceding values. In some embodiments, the volume of culture media in the chamber is about 5-50 ml. In some embodiments, the volume of culture media in the chamber is about 5-10 ml; optionally about 10 ml. In some embodiments, the bioreactor comprises a rotating chamber comprising the culture media, wherein the rotation of the chamber is, is at least, or is not more than, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80 rpm, or a range defined by any two of the preceding values. In some embodiments, the rotation is not more than about 40 rpm. In some embodiments, the rotation is 3-7 rpm. In some embodiments, the rotational speed is a speed selected to keep the cells, aggregates, spheroids and/or organoids suspended in statical orbit. In some embodiments, the bioreactor is configured such that the average shear stress on the cells, aggregates, spheroids and/or organoids in the culture media while the culture media is in motion (e.g., the chamber is rotating) is less than 5.0, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, or 0.005

dynes/cm2. In some embodiments, the average shear stress on the cells, aggregates, spheroids and/or organoids in the culture media is less than 0.1 dynes/cm2.

[0269] In some embodiments, the culture media is free of materials of animal or human origin. In some embodiments, the culture media is free of any extracellular matrix and/or basement membrane matrix (e.g. Matrigel or similar products).

[0270] Aspects of the disclosure relate to spheroids produced by the suspension culture methods described above and elsewhere herein. Aspects of the disclosure relate to organoids produced by the suspension culture methods described above and elsewhere herein.

PSCs for Use In the Methods Disclosed Herein

[0271] In some embodiments of any of the methods disclosed above and elsewhere herein, the PSC is an induced PSC (iPSC) or an embryonic stem cells (ESC). In some embodiments of any of the methods disclosed above and elsewhere herein, the PSC is a human PSC, optionally a human iPSC (hiPSC). Pluripotent stem cells can be derived from any suitable source. In some embodiments of any of the methods disclosed above and elsewhere herein, the source of pluripotent stem cells is a mammalian source, optionally human, non-human primate, rodent, porcine, and bovine.

[0272] In some embodiments, the PSCs, definitive endoderm cells, spheroids, or organoids are genetically modified or edited according to methods known in the art. For example, gene editing using CRISPR nucleases such as Cas9 are explored in PCT Publications WO 2013/176772, WO 2014/093595, WO 2014/093622, WO 2014/093655, WO 2014/093712, WO 2014/093661, WO 2014/204728, WO 2014/204729, WO 2015/071474, WO 2016/115326, WO 2016/141224, WO 2017/023803, and WO 2017/070633, each of which is hereby expressly incorporated by reference in its entirety.

Cells, Aggregates, Spheroids, and Organoids

[0273] An aspect of the disclosure is PSCs or PSC aggregates made by any of the methods disclosed above and elsewhere herein. An aspect of the disclosure is DE made by any of the methods disclosed above and elsewhere herein. An aspect of the disclosure is HGS made by any of the methods disclosed above and elsewhere herein. An aspect of the disclosure is IO made by any of the methods disclosed above and elsewhere herein. An aspect of the disclosure

is an IO having an apical-in polarity, wherein epithelial cells of the IO have a polarity wherein the apical surface is oriented to the inside of the IO, optionally wherein the IO is a human IO (hIO). In some embodiments, the IO having an apical-in polarity is made by any of the methods disclosed above and elsewhere herein.

Methods and Uses

[0274] An aspect of the disclosure is a method of treatment comprising transplanting an IO made by any of the methods disclosed above and elsewhere herein, or cells derived therefrom, into an animal, optionally wherein the animal is suffering from a GI disease state. In some embodiments, the animal is human. An aspect of the disclosure is the use of an IO made by any of the methods disclosed above and elsewhere herein, or cells derived therefrom, in the manufacture of a medicament for treatment of an animal comprising transplanting the IO, or cells derived therefrom, into the animal, optionally wherein the animal is suffering from a GI disease state. In some embodiments, the animal is human.

[0275] An aspect of the disclosure is a method of screening a compound for activity comprising contacting an IO made by any of the methods disclosed above and elsewhere herein, or cells derived therefrom, with the compound and measuring a response of the IO to the compound. In some embodiments, the IO is a model for an intestinal disease, and assessing the effects of the candidate compound or composition on the IO comprises assessing the effects of the candidate compound or composition on the disease. In some embodiments, the IO has been produced from cells derived from a subject. In some embodiments, the cells derived from the subject are induced pluripotent stem cells. In some embodiments, the subject has an intestinal disease.

Terms

[0276] In the following detailed description, reference is made to the accompanying drawings, which form a part hereof. In the drawings, similar symbols typically identify similar components, unless context dictates otherwise. The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. Other embodiments may be utilized, and other changes may be made, without departing from the spirit or scope of the subject matter presented herein. It will be readily understood that the aspects of the present

disclosure, as generally described herein, and illustrated in the Figures, can be arranged, substituted, combined, separated, and designed in a wide variety of different configurations, all of which are explicitly contemplated herein.

[0277] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood when read in light of the instant disclosure by one of ordinary skill in the art to which the present disclosure belongs. For purposes of the present disclosure, the following terms are explained below.

[0278] The disclosure herein uses affirmative language to describe the numerous embodiments. The disclosure also includes embodiments in which subject matter is excluded, in full or in part, such as substances or materials, method steps and conditions, protocols, or procedures.

[0279] The articles "a" and "an" are used herein to refer to one or to more than one (for example, at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[0280] Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises," and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they materially affect the activity or action of the listed elements.

[0281] The terms "individual", "subject", or "patient" as used herein have their plain and ordinary meaning as understood in light of the specification, and mean a human or a non-human mammal, e.g., a dog, a cat, a mouse, a rat, a cow, a sheep, a pig, a goat, a non-human primate, or a bird, e.g., a chicken, as well as any other vertebrate or invertebrate. The term "mammal" is used in its usual biological sense. Thus, it specifically includes, but is not limited to, primates,

including simians (chimpanzees, apes, monkeys) and humans, cattle, horses, sheep, goats, swine, rabbits, dogs, cats, rodents, rats, mice, guinea pigs, or the like.

[0282] The terms "effective amount" or "effective dose" as used herein have their plain and ordinary meaning as understood in light of the specification, and refer to that amount of a recited composition or compound that results in an observable effect. Actual dosage levels of active ingredients in an active composition of the presently disclosed subject matter can be varied so as to administer an amount of the active composition or compound that is effective to achieve the desired response for a particular subject and/or application. The selected dosage level will depend upon a variety of factors including, but not limited to, the activity of the composition, formulation, route of administration, combination with other drugs or treatments, severity of the condition being treated, and the physical condition and prior medical history of the subject being treated. In some embodiments, a minimal dose is administered, and dose is escalated in the absence of dose-limiting toxicity to a minimally effective amount. Determination and adjustment of an effective dose, as well as evaluation of when and how to make such adjustments, are contemplated herein.

[0283] The terms "function" and "functional" as used herein have their plain and ordinary meaning as understood in light of the specification, and refer to a biological, enzymatic, or therapeutic function.

[0284] The term "inhibit" as used herein has its plain and ordinary meaning as understood in light of the specification, and may refer to the reduction or prevention of a biological activity. The reduction can be by a percentage that is, is about, is at least, is at least about, is not more than, or is not more than about, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, or an amount that is within a range defined by any two of the aforementioned values. As used herein, the term "delay" has its plain and ordinary meaning as understood in light of the specification, and refers to a slowing, postponement, or deferment of a biological event, to a time which is later than would otherwise be expected. The delay can be a delay of a percentage that is, is about, is at least, is at least about, is not more than, or is not more than about, 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or an amount within a range defined by any two of the aforementioned values. The terms inhibit and delay may not necessarily indicate a 100% inhibition or delay. A partial inhibition or delay may be realized.

[0285] As used herein, the term "isolated" has its plain and ordinary meaning as understood in light of the specification, and refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from equal to, about, at least, at least about, not more than, or not more than about, 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98%, about 99%, substantially 100%, or 100% of the other components with which they were initially associated (or ranges including and/or spanning the aforementioned values). In some embodiments, isolated agents are, are about, are at least, are at least about, are not more than, or are not more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, substantially 100%, or 100% pure (or ranges including and/or spanning the aforementioned values). As used herein, a substance that is "isolated" may be "pure" (e.g., substantially free of other components). As used herein, the term "isolated cell" may refer to a cell not contained in a multi-cellular organism or tissue.

[0286] As used herein, "in vivo" is given its plain and ordinary meaning as understood in light of the specification and refers to the performance of a method inside living organisms, usually animals, mammals, including humans, and plants, as opposed to a tissue extract or dead organism.

[0287] As used herein, "ex vivo" is given its plain and ordinary meaning as understood in light of the specification and refers to the performance of a method outside a living organism with little alteration of natural conditions.

[0288] As used herein, "in vitro" is given its plain and ordinary meaning as understood in light of the specification and refers to the performance of a method outside of biological conditions, e.g., in a petri dish or test tube.

[0289] The terms "nucleic acid" or "nucleic acid molecule" as used herein have their plain and ordinary meaning as understood in light of the specification, and refer to polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, those that appear in a cell naturally, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action,

and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (e.g., enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, or phosphoramidate. The term "nucleic acid molecule" also includes so-called "peptide nucleic acids," which comprise naturally-occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded. "Oligonucleotide" can be used interchangeable with nucleic acid and can refer to either double stranded or single stranded DNA or RNA. A nucleic acid or nucleic acids can be contained in a nucleic acid vector or nucleic acid construct (e.g. plasmid, virus, retrovirus, lentivirus, bacteriophage, cosmid, fosmid, phagemid, bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC), or human artificial chromosome (HAC)) that can be used for amplification and/or expression of the nucleic acid or nucleic acids in various biological systems. Typically, the vector or construct will also contain elements including but not limited to promoters, enhancers, terminators, inducers, ribosome binding sites, translation initiation sites, start codons, stop codons, polyadenylation signals, origins of replication, cloning sites, multiple cloning sites, restriction enzyme sites, epitopes, reporter genes, selection markers, antibiotic selection markers, targeting sequences, peptide purification tags, or accessory genes, or any combination thereof.

[0290] A nucleic acid or nucleic acid molecule can comprise one or more sequences encoding different peptides, polypeptides, or proteins. These one or more sequences can be joined in the same nucleic acid or nucleic acid molecule adjacently, or with extra nucleic acids in between, e.g. linkers, repeats or restriction enzyme sites, or any other sequence that is, is about,

is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, or 300 bases long, or any length in a range defined by any two of the aforementioned lengths. The term "downstream" on a nucleic acid as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a sequence being after the 3'-end of a previous sequence, on the strand containing the encoding sequence (sense strand) if the nucleic acid is double stranded. The term "upstream" on a nucleic acid as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a sequence being before the 5'-end of a subsequent sequence, on the strand containing the encoding sequence (sense strand) if the nucleic acid is double stranded. The term "grouped" on a nucleic acid as used herein has its plain and ordinary meaning as understood in light of the specification and refers to two or more sequences that occur in proximity either directly or with extra nucleic acids in between, e.g. linkers, repeats, or restriction enzyme sites, or any other sequence that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, or 300 bases long, or any length in a range defined by any two of the aforementioned lengths, but generally not with a sequence in between that encodes for a functioning or catalytic polypeptide, protein, or protein domain.

[0291] The nucleic acids described herein comprise nucleobases. Primary, canonical, natural, or unmodified bases are adenine, cytosine, guanine, thymine, and uracil. Other nucleobases include but are not limited to purines, pyrimidines, modified nucleobases, 5-methylcytosine, pseudouridine, dihydrouridine, inosine, 7-methylguanosine, hypoxanthine, xanthine, 5,6-dihydrouracil, 5-hydroxymethylcytosine, 5-bromouracil, isoguanine, isocytosine, aminoallyl bases, dye-labeled bases, fluorescent bases, or biotin-labeled bases.

[0292] The terms "peptide", "polypeptide", and "protein" as used herein have their plain and ordinary meaning as understood in light of the specification and refer to macromolecules comprised of amino acids linked by peptide bonds. The numerous functions of peptides, polypeptides, and proteins are known in the art, and include but are not limited to enzymes, structure, transport, defense, hormones, or signaling. Peptides, polypeptides, and proteins are often, but not always, produced biologically by a ribosomal complex using a nucleic acid template, although chemical syntheses are also available. By manipulating the nucleic acid

template, peptide, polypeptide, and protein mutations such as substitutions, deletions, truncations, additions, duplications, or fusions of more than one peptide, polypeptide, or protein can be performed. These fusions of more than one peptide, polypeptide, or protein can be joined in the same molecule adjacently, or with extra amino acids in between, e.g. linkers, repeats, epitopes, or tags, or any other sequence that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, or 300 bases long, or any length in a range defined by any two of the aforementioned lengths. The term "downstream" on a polypeptide as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a sequence being after the C-terminus of a previous sequence. The term "upstream" on a polypeptide as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a sequence being before the N-terminus of a subsequent sequence.

[0293] The term "purity" of any given substance, compound, or material as used herein has its plain and ordinary meaning as understood in light of the specification and refers to the actual abundance of the substance, compound, or material relative to the expected abundance. For example, the substance, compound, or material may be at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% pure, including all decimals in between. Purity can be affected by unwanted impurities, including but not limited to nucleic acids, DNA, RNA, nucleotides, proteins, polypeptides, peptides, amino acids, lipids, cell membrane, cell debris, small molecules, degradation products, solvent, carrier, vehicle, or contaminants, or any combination thereof. In some embodiments, the substance, compound, or material is substantially free of host cell proteins, host cell nucleic acids, plasmid DNA, contaminating viruses, proteasomes, host cell culture components, process related components, mycoplasma, pyrogens, bacterial endotoxins, and adventitious agents. Purity can be measured using technologies including but not limited to electrophoresis, SDS-PAGE, capillary electrophoresis, PCR, rtPCR, qPCR, chromatography, liquid chromatography, gas chromatography, thin layer chromatography, enzyme-linked immunosorbent assay (ELISA), spectroscopy, UV-visible spectrometry, infrared spectrometry, mass spectrometry, nuclear magnetic resonance, gravimetry, or titration, or any combination thereof.

[0294] The term "yield" of any given substance, compound, or material as used herein has its plain and ordinary meaning as understood in light of the specification and refers to the actual overall amount of the substance, compound, or material relative to the expected overall amount. For example, the yield of the substance, compound, or material is, is about, is at least, is at least about, is not more than, or is not more than about, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% of the expected overall amount, including all decimals in between. Yield can be affected by the efficiency of a reaction or process, unwanted side reactions, degradation, quality of the input substances, compounds, or materials, or loss of the desired substance, compound, or material during any step of the production.

[0295] The term "intestinal organoid" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to three-dimensional cellular structures that present many properties of the small intestine of an organism. In some embodiments, intestinal organoids relate to those derived from human cells and exhibit the properties of a human small intestine. However, intestinal organoids from other mammals are also encompassed. Intestinal organoids as used herein are derived from pluripotent stem cells (e.g. embryonic stem cells or induced pluripotent stem cells) or an intermediate thereof (e.g. definitive endoderm), where the process of differentiating pluripotent stem cells into definitive endoderm, then hindgut endoderm (which may be in the form of spheroids), and finally to an intestinal organoid results in a cellular structure that has the composition, structure, and function resembling a naturally developed intestine. A significant difference between the intestinal organoids used herein and enteroids, which are cellular structures derived from adult intestinal epithelium, and other so-called organoids produced from non-pluripotent adult intestinal stem cells, is that the intestinal organoids used herein contain both epithelium and mesenchyme. The mesenchyme performs an important supportive role for the epithelium, and greatly enhances the viability and robust function of the intestinal organoid. The intestinal organoids used herein may exhibit a lumen with epithelial villus-like involutions closely resembling normal intestine, and peristaltic behavior. As a result of the differentiation process from pluripotent stem cells, the intestinal organoids used herein also contain specialized intestinal cell types, including enterocytes, Goblet cells, Paneth cells, and enteroendocrine cells. References disclosing embodiments of intestinal organoids suitable for use herein include WO 2011/140441, WO 2016/061464, WO

2018/200481, WO 2020/160371, and WO 2021/030373, each of which are incorporated herein by reference in their entirety.

[0296] The term "colonic organoid" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to three-dimensional cellular structures that present many properties of the colon of an organism. In some embodiments, colonic organoids relate to those derived from human cells and exhibit the properties of a human colon. However, colonic organoids from other mammals are also encompassed. Colonic organoids as used herein are derived from pluripotent stem cells (e.g. embryonic stem cells or induced pluripotent stem cells) or an intermediate thereof (e.g. definitive endoderm), where the process of differentiating pluripotent stem cells into definitive endoderm, then hindgut endoderm (which may be in the form of spheroids), and finally to a colonic organoid results in a structure that has the composition, structure, and function resembling a naturally developed colon. A significant difference between the colonic organoids used herein and colonoids, which are cellular structures derived from adult colon epithelium, and other so-called organoids produced from non-pluripotent adult colon stem cells, is that the colonic organoids used herein contain both epithelium and mesenchyme. The mesenchyme performs an important supportive role for the epithelium, and greatly enhances the viability and robust function of the colonic organoid. The colonic organoids used herein may exhibit a lumen with crypts but substantially free of villus-like structures. As a result of the differentiation process from pluripotent stem cells, the colonic organoids used herein also contain specialized colonic cell types, including a high number of Goblet cells (relative to intestinal organoids) and colonic enteroendocrine cells, but substantially free of Paneth cells. References disclosing embodiments of colonic organoids suitable for use herein include WO 2018/106628, which is incorporated herein by reference in their entirety.

[0297] The terms "fragmentation," "fragmented," "dissociation," and "dissociated" as used herein have their plain and ordinary meanings as understood in light of the specification and refer to the partial or complete fragmentation or dissociation of an organoid or other three-dimensional multicellular structure to produce a population of single cells and viable multicellular structures, fragments, or clumps, without excessively shearing or damaging the cells such that that all or the majority of dissociated organoid comprises intact and healthy cells. Accordingly, "fragmented" and the like does not generally refer to, e.g., non-living subcellular components or fragments of single cells, such as liberated intracellular contents or non-living

vesicles, although these components may be present in embodiments of fragmented organoid compositions by way of natural apoptosis of cells or unintended damage during dissociation of organoids. Fragmentation or dissociation of the organoid may be done in a variety of methods generally known in the art. The process of fragmentation or dissociation may be such that some of the resultant cells are found as small multi-cellular clumps/fragments rather than as single cells. The population of dissociated cells comprising multi-cellular clumps/fragments among single cells is contemplated for use herein. In some embodiments, the dissociated cell populations or compositions are present exclusively as multi-cellular clumps/fragments. In some embodiments, the dissociated cell populations or compositions are present exclusively as single cells without multi-cellular clumps/fragments. In some embodiments, the dissociated cell populations or compositions are predominantly (e.g. greater than 70%, 80%, or 90% of cells) multi-cellular clumps/fragments, with relatively few single cells. In some embodiments, the dissociated cell populations or compositions are present as a mixture of single cells and multi-cellular clumps/fragments.

[0298] The term "enzymatic dissociation" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to fragmentation or dissociation of an organoid or other three-dimensional multicellular structure using the catalytic activity of one or more enzymes. A process generally well known in the art, enzymatic dissociation typically involves the use of proteolytic enzymes (e.g. trypsin), or enzymes specific for other molecules (e.g. hyaluronidase) involved in adherence to surface or intercellular bonds.

[0299] The term "mechanical dissociation" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to fragmentation or dissociation of an organoid or other three-dimensional multicellular structure using a mechanical force. A process generally well known in the art, mechanical dissociation may be accomplished, for example, through trituration through narrow bore channels, where the channels may be in the form of pipettes, needles, microfluidic channels, or the like.

[0300] The term "mucosa" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to the most inner layer of the gastrointestinal tract. The epithelium is the most inner layer of the mucosa, and is where epithelial cells and other specialized cells such as Goblet cells are found. The epithelium also forms the villi structure of

the intestine. The epithelium is surrounded by connective tissue called the lamina propria, and a thin layer of smooth muscle.

[0301] The term "muscularis" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to the muscularis propria of the gastrointestinal tract. The muscularis regulates peristaltic behavior of the intestine and colon, and originates from the mesenchymal layer of the nascent gut tube during development.

[0302] The term "regionality" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to the qualities and features that distinguish one cell type from another. In the context of intestine and colon (and other gastrointestinal organs), both organs originate from the same definitive endoderm but early specification results in the proper development and differentiation of the two organs and constituent cells commensurate with their function. Consequently, intestinal tissue exhibits a different regionality than colon tissue. As shown herein, intestinal and colonic organoids used for engraftment in an intestinal injury model retain their respective qualities even after integration into the cell layers of a different organ (e.g. intestinal organoid into host colon tissue or colonic organoid into host intestinal tissue).

[0303] The term "intestinal barrier" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to the cellular and mucosal barrier that separates the intraluminal contents of the gastrointestinal tract from the surrounding tissue and circulatory system, while still permitting nutrient exchange. This barrier is mediated by the intracellular junctions between the cells of the epithelium. During intestinal damage, this barrier can be disrupted, resulting in abnormal function of the intestine, passage of potentially pathogenic microorganisms or antigens into the body, and leaking of blood and molecules into the lumen.

[0304] As used herein, "pharmaceutically acceptable" has its plain and ordinary meaning as understood in light of the specification and refers to carriers, excipients, and/or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed or that have an acceptable level of toxicity. A "pharmaceutically acceptable" "diluent," "excipient," and/or "carrier" as used herein have their plain and ordinary meaning as understood in light of the specification and are intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying

agents, and the like, compatible with administration to humans, cats, dogs, or other vertebrate hosts. Typically, a pharmaceutically acceptable diluent, excipient, and/or carrier is a diluent, excipient, and/or carrier approved by a regulatory agency of a Federal, a state government, or other regulatory agency, or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, including humans as well as non-human mammals, such as cats and dogs. The term diluent, excipient, and/or "carrier" can refer to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Such pharmaceutical diluent, excipient, and/or carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin. Water, saline solutions and aqueous dextrose and glycerol solutions can be employed as liquid diluents, excipients, and/or carriers, particularly for injectable solutions. Suitable pharmaceutical diluents and/or excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. A non-limiting example of a physiologically acceptable carrier is an aqueous pH buffered solution. The physiologically acceptable carrier may also comprise one or more of the following: antioxidants, such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, such as serum albumin, gelatin, immunoglobulins, hydrophilic polymers such as polyvinylpyrrolidone, amino acids, carbohydrates such as glucose, mannose, or dextrins, chelating agents such as EDTA, sugar alcohols such as mannitol or sorbitol, salt-forming counterions such as sodium, and nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS®. The composition, if desired, can also contain minor amounts of wetting, bulking, emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, sustained release formulations and the like. The formulation typically suits the mode of administration.

[0305] Cryoprotectants are cell composition additives to improve efficiency and yield of low temperature cryopreservation by preventing formation of large ice crystals. Cryoprotectants include but are not limited to DMSO, ethylene glycol, glycerol, propylene glycol, trehalose, formamide, methyl-formamide, dimethyl-formamide, glycerol 3-phosphate, proline, sorbitol, diethyl glycol, sucrose, triethylene glycol, polyvinyl alcohol, polyethylene glycol, or hydroxyethyl starch. Cryoprotectants can be used as part of a cryopreservation medium, which

include other components such as nutrients (e.g. albumin, serum, bovine serum, fetal calf serum [FCS]) to enhance post-thawing survivability of the cells. In these cryopreservation media, at least one cryoprotectant may be found at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, or any percentage within a range defined by any two of the aforementioned numbers.

[0306] Additional excipients with desirable properties include but are not limited to preservatives, adjuvants, stabilizers, solvents, buffers, diluents, solubilizing agents, detergents, surfactants, chelating agents, antioxidants, alcohols, ketones, aldehydes, ethylenediaminetetraacetic acid (EDTA), citric acid, salts, sodium chloride, sodium bicarbonate, sodium phosphate, sodium borate, sodium citrate, potassium chloride, potassium phosphate, magnesium sulfate sugars, dextrose, fructose, mannose, lactose, galactose, sucrose, sorbitol, cellulose, serum, amino acids, polysorbate 20, polysorbate 80, sodium deoxycholate, sodium taurodeoxycholate, magnesium stearate, octylphenol ethoxylate, benzethonium chloride, thimerosal, gelatin, esters, ethers, 2-phenoxyethanol, urea, or vitamins, or any combination thereof. Some excipients may be in residual amounts or contaminants from the process of manufacturing, including but not limited to serum, albumin, ovalbumin, antibiotics, inactivating agents, formaldehyde, glutaraldehyde, β-propiolactone, gelatin, cell debris, nucleic acids, peptides, amino acids, or growth medium components or any combination thereof. The amount of the excipient may be found in composition at a percentage that is, is about, is at least, is at least about, is not more than, or is not more than about, 0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100% w/w or any percentage by weight in a range defined by any two of the aforementioned numbers.

[0307] The term "pharmaceutically acceptable salts" has its plain and ordinary meaning as understood in light of the specification and includes relatively non-toxic, inorganic and organic acid, or base addition salts of compositions or excipients, including without limitation, analgesic agents, therapeutic agents, other materials, and the like. Examples of pharmaceutically acceptable salts include those derived from mineral acids, such as hydrochloric acid and sulfuric acid, and those derived from organic acids, such as ethanesulfonic acid, benzenesulfonic acid, p-

toluenesulfonic acid, and the like. Examples of suitable inorganic bases for the formation of salts include the hydroxides, carbonates, and bicarbonates of ammonia, sodium, lithium, potassium, calcium, magnesium, aluminum, zinc, and the like. Salts may also be formed with suitable organic bases, including those that are non-toxic and strong enough to form such salts. For example, the class of such organic bases may include but are not limited to mono-, di-, and trialkylamines, including methylamine, dimethylamine, and triethylamine; mono-, di-, or trihydroxyalkylamines including mono-, di-, and triethanolamine; amino acids, including glycine, arginine and lysine; guanidine; N-methylglucosamine; N-methylglucamine; L-glutamine; N-methylpiperazine; morpholine; ethylenediamine; N-benzylphenethylamine; trihydroxymethyl aminoethane.

[0308] Proper formulation is dependent upon the route of administration chosen. Techniques for formulation and administration of the compounds described herein are known to those skilled in the art. Multiple techniques of administering a compound exist in the art including, but not limited to, enteral, oral, rectal, topical, sublingual, buccal, intraaural, epidural, epicutaneous, aerosol, parenteral delivery, including intramuscular, subcutaneous, intra-arterial, intravenous, intraportal, intra-articular, intradermal, peritoneal, intramedullary injections, intrathecal, direct intraventricular, intraperitoneal, intranasal or intraocular injections. Pharmaceutical compositions will generally be tailored to the specific intended route of administration.

[0309] As used herein, a "carrier" has its plain and ordinary meaning as understood in light of the specification and refers to a compound, particle, solid, semi-solid, liquid, or diluent that facilitates the passage, delivery and/or incorporation of a compound to cells, tissues and/or bodily organs.

[0310] As used herein, a "diluent" has its plain and ordinary meaning as understood in light of the specification and refers to an ingredient in a pharmaceutical composition that lacks pharmacological activity but may be pharmaceutically necessary or desirable. For example, a diluent may be used to increase the bulk of a potent drug whose mass is too small for manufacture and/or administration. It may also be a liquid for the dissolution of a drug to be administered by injection, ingestion or inhalation. A common form of diluent in the art is a buffered aqueous solution such as, without limitation, phosphate buffered saline that mimics the composition of human blood.

[0311] The term "basement membrane matrix" or "extracellular matrix" as used herein has its plain and ordinary meaning in light of the specification and refers to any biological or synthetic compound, substance, or composition that enhances cell attachment and/or growth. Any extracellular matrix, as well as any mimetic or derivative thereof, known in the art can be used for the methods disclosed herein. Some examples of extracellular matrices, or mimetics or derivative thereof, include but are not limited to cell-based feeder layers, polymers, proteins, polypeptides, nucleic acids, sugars, lipids, poly-lysine, poly-ornithine, collagen, collagen IV, gelatin, fibronectin, vitronectin, laminin, laminin-511 elastin, tenascin, heparan sulfate, entactin, nidogen, osteopontin, perlecan, fibrin, basement membrane, Matrigel, hydrogel, PEI, WGA, or hyaluronic acid, or any combination thereof. A common basement membrane matrix that is used in laboratories are those isolated from murine Engelbreth-Holm-Swarm (EHS) sarcoma cells. However, these basement membrane matrices are derived from non-human animals and therefore contain xenogeneic components that prevent its use towards humans. They are also not defined, which can lead to variability in manufacturing, as well as potentially harbor pathogens. Accordingly, in some embodiments, the methods for culturing cells may involve the use of synthetic and/or defined alternatives to these xenogeneic basement membrane matrices. The use of non-xenogeneic basement membrane matrices or mimetics or derivatives thereof enables manufacturing of biological products better suited for human use.

[0312] The terms "passage" and "passaging" as used herein have their plain and ordinary meaning as understood in light of the specification, and refer to the conventional approaches performed in biological cell culture methods to maintain a viable population of cells for prolonged periods of time. As cells are generally proliferative in cell culture, they undergo multiple cycles of mitosis until occupying the available space, which is typically a surface of a cell culture container (e.g., a plate, dish, or flask) submerged under culture medium. For example, the cells may grow out as a monolayer on a cell culture container surface or aggregate in a culture medium. If the growing cells occupy the entire available space of surface or form too large an aggregate, they cannot proliferate further and may exhibit senescent behavior. In order to continue growth of the cells, which may be performed to maintain the viability and proliferative nature of the cells and/or to expand the number of cells for downstream purposes, the cells may be passaged by taking a fraction of the cells and seeding this fraction onto a fresh surface or into fresh culture medium following dissociation of the aggregates. This fraction of

the cells will continue to proliferate and multiply until they occupy the available space of the new surface or again form aggregates, upon which this passaging can be repeated successively.

[0313] The term "three-dimensional" as used in "three-dimensional pluripotent stem cell (PSC) aggregate(s)," "three-dimensional suspension," "suspension culture," "three-dimensional culture," three-dimensional culturing," "three-dimensional expansion," or "three-dimensional aggregates" refers to the ability of cells, PSCs, PSC aggregates, DE, spheroids, and/or organoids, to be able to grow, develop, reproduce, expand, and interact with their surrounding framework in three dimensions. Such growth, development, reproduction, expansion, and/or interaction may be facilitated by a suspension of said cells, PSCs, PSC aggregates, DE, spheroids, and/or organoids in the framework. In some aspects, such growth, development, reproduction, expansion, and/or interaction may be facilitated by a suspension of beads or bead-like structures holding or otherwise providing a place for said cells, PSCs, PSC aggregates, DE, spheroids, and/or organoids in the framework. The usage of "three-dimensional" in the above referenced terminology and/or phrases may be contrasted with "two-dimensional" as used in "two-dimensional PSC(s)" "two-dimensional culture," "two-dimensional culturing," or "two-dimensional monolayer" where cells, PSCs, PSC aggregates, DE, spheroids, and/or organoids to be able to grow, develop, reproduce, expand, and interact with their surrounding framework in two dimensions (e.g., along a monolayer of a plate).

[0314] The term "% w/w" or "% wt/wt" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a percentage expressed in terms of the weight of the ingredient or agent over the total weight of the composition multiplied by 100. The term "% v/v" or "% vol/vol" as used herein has its plain and ordinary meaning as understood in the light of the specification and refers to a percentage expressed in terms of the liquid volume of the compound, substance, ingredient, or agent over the total liquid volume of the composition multiplied by 100.

Stem Cells

[0315] The term "totipotent stem cells" (also known as omnipotent stem cells) as used herein has its plain and ordinary meaning as understood in light of the specification and are stem cells that can differentiate into embryonic and extra-embryonic cell types. Such cells can

construct a complete, viable organism. These cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent.

[0316] The term "embryonic stem cells (ESCs)," also commonly abbreviated as ES cells, as used herein has its plain and ordinary meaning as understood in light of the specification and refers to cells that are pluripotent and derived from the inner cell mass of the blastocyst, an early-stage embryo.

[0317] The term "pluripotent stem cells (PSCs)" as used herein has its plain and ordinary meaning as understood in light of the specification and encompasses any cells that can differentiate into nearly all cell types of the body, i.e., cells derived from any of the three germ layers (germinal epithelium), including endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), and ectoderm (epidermal tissues and nervous system). PSCs can be the descendants of inner cell mass cells of the preimplantation blastocyst or obtained through induction of a non-pluripotent cell, such as an adult somatic cell, by forcing the expression of certain genes. Pluripotent stem cells can be derived from any suitable source. Examples of sources of pluripotent stem cells include mammalian sources, including human, rodent, porcine, and bovine.

[0318] The term "induced pluripotent stem cells (iPSCs)," also commonly abbreviated as iPS cells, as used herein has its plain and ordinary meaning as understood in light of the specification and refers to a type of pluripotent stem cells artificially derived from a normally non-pluripotent cell, such as an adult somatic cell, by inducing a "forced" expression of certain genes. hiPSC refers to human iPSCs. In some methods known in the art, iPSCs may be derived by transfection of certain stem cell-associated genes into non-pluripotent cells, such as adult fibroblasts. Transfection may be achieved through viral transduction using viruses such as retroviruses or lentiviruses. Transfected genes may include the master transcriptional regulators *Oct-3/4 (POU5F1)* and *Sox2*, although other genes may enhance the efficiency of induction. After 3-4 weeks, small numbers of transfected cells begin to become morphologically and biochemically similar to pluripotent stem cells, and are typically isolated through morphological selection, doubling time, or through a reporter gene and antibiotic selection. As used herein, iPSCs include first generation iPSCs, second generation iPSCs in mice, and human induced pluripotent stem cells. In some methods, a retroviral system is used to transform human fibroblasts into pluripotent stem cells using four pivotal genes: *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*.

In other methods, a lentiviral system is used to transform somatic cells with *OCT4*, *SOX2*, *NANOG*, and *LIN28*. Genes whose expression are induced in iPSCs include but are not limited to *Oct-3/4* (*POU5F1*); certain members of the *Sox* gene family (e.g., *Soxl*, *Sox2*, *Sox3*, and *Sox15*); certain members of the *Klf* family (e.g., *Klfl*, *Klf2*, *Klf4*, and *Klf5*), certain members of the *Myc* family (e.g., *C-myc*, *L-myc*, and *N-myc*), *Nanog*, *LIN28*, *Tert*, *Fbx15*, *ERas*, *ECAT15-1*, *ECAT15-2*, *Tcl1*, β-Catenin, *ECAT1*, *Esg1*, *Dnmt3L*, *ECAT8*, *Gdf3*, *Fth117*, *Sal14*, *Rex1*, *UTF1*, *Stella*, *Stat3*, *Grb2*, *Prdm14*, *Nr5a1*, *Nr5a2*, or E-cadherin, or any combination thereof.

[0319] The term "precursor cell" as used herein has its plain and ordinary meaning as understood in light of the specification and encompasses any cells that can be used in methods described herein, through which one or more precursor cells acquire the ability to renew itself or differentiate into one or more specialized cell types. In some embodiments, a precursor cell is pluripotent or has the capacity to becoming pluripotent. In some embodiments, the precursor cells are subjected to the treatment of external factors (e.g., growth factors) to acquire pluripotency. In some embodiments, a precursor cell can be a totipotent (or omnipotent) stem cell; a pluripotent stem cell (induced or non-induced); a multipotent stem cell; an oligopotent stem cells and a unipotent stem cell. In some embodiments, a precursor cell can be from an embryo, an infant, a child, or an adult. In some embodiments, a precursor cell can be a somatic cell subject to treatment such that pluripotency is conferred via genetic manipulation or protein/peptide treatment. Precursor cells include embryonic stem cells (ESC), embryonic carcinoma cells (ECs), and epiblast stem cells (EpiSC).

[0320] In some embodiments, one step is to obtain stem cells that are pluripotent or can be induced to become pluripotent. In some embodiments, pluripotent stem cells are derived from embryonic stem cells, which are in turn derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation in vitro. Embryonic stem cells are pluripotent stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo. Methods for deriving embryonic stem cells from blastocytes are well known in the art. It would be understood by one of skill in the art that the methods and systems described herein are applicable to any stem cells.

[0321] Additional stem cells that can be used in embodiments in accordance with the present disclosure include but are not limited to those provided by or described in the database hosted by the National Stem Cell Bank (NSCB), Human Embryonic Stem Cell Research Center

at the University of California, San Francisco (UCSF); WISC cell Bank at the Wi Cell Research Institute; the University of Wisconsin Stem Cell and Regenerative Medicine Center (UW-SCRMC); Novocell, Inc. (San Diego, Calif.); Cellartis AB (Goteborg, Sweden); ES Cell International Pte Ltd (Singapore); Technion at the Israel Institute of Technology (Haifa, Israel); and the Stem Cell Database hosted by Princeton University and the University of Pennsylvania. Exemplary embryonic stem cells that can be used in embodiments in accordance with the present disclosure include but are not limited to SA01 (SA001); SA02 (SA002); ES01 (HES-1); ES02 (HES-2); ES03 (HES-3); ES04 (HES-4); ES05 (HES-5); ES06 (HES-6); BG01 (BGN-01); BG02 (BGN-02); BG03 (BGN-03); TE03 (13); TE04 (14); TE06 (16); UCOl (HSF1); UC06 (HSF6); WA01 (HI); WA07 (H7); WA09 (H9); WA13 (H13); WA14 (H14). Exemplary human pluripotent cell lines include but are not limited to TkDA3-4, 1231A3, 317-D6, 317-A4, CDH1, 5-T-3, 3-34-1, NAFLD27, NAFLD77, NAFLD150, WD90, WD91, WD92, L20012, C213, 1383D6, FF, or 317-12 cells.

[0322] In developmental biology, cellular differentiation is the process by which a less specialized cell becomes a more specialized cell type. As used herein, the term "directed differentiation" describes a process through which a less specialized cell becomes a particular specialized target cell type. The particularity of the specialized target cell type can be determined by any applicable methods that can be used to define or alter the destiny of the initial cell. Exemplary methods include but are not limited to genetic manipulation, chemical treatment, protein treatment, and nucleic acid treatment.

[0323] In some embodiments, an adenovirus can be used to transport the requisite four genes, resulting in iPSCs substantially identical to embryonic stem cells. Since the adenovirus does not combine any of its own genes with the targeted host, the danger of creating tumors is eliminated. In some embodiments, non-viral based technologies are employed to generate iPSCs. In some embodiments, reprogramming can be accomplished via plasmid without any virus transfection system at all, although at very low efficiencies. In other embodiments, direct delivery of proteins is used to generate iPSCs, thus eliminating the need for viruses or genetic modification. In some embodiment, generation of mouse iPSCs is possible using a similar methodology: a repeated treatment of the cells with certain proteins channeled into the cells via poly-arginine anchors was sufficient to induce pluripotency. In some embodiments, the

expression of pluripotency induction genes can also be increased by treating somatic cells with FGF2 under low oxygen conditions.

[0324] The term "definitive endoderm" or "DE" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to the developmental cell type that gives rise to the gut tube and resultant gastrointestinal organs, including the esophagus, stomach, small intestine, colon, liver, and pancreas. The anterior DE forms the foregut and its associated organs, including the liver and pancreas, and the posterior DE forms the midgut and hindgut, which forms the small and large intestines and parts of the genitourinary system. Markers of DE include SOX17 and FOXA2. During development, the Wnt and FGF signaling pathways establish regionalization between anterior and posterior patterning of the DE.

[0325] Pluripotent stem cells can be differentiated into definitive endoderm by known methods in the art. In some embodiments, definitive endoderm cells can be differentiated from pluripotent cells by contacting the definitive endoderm with the Nodal, Activin (e.g., Activin A or Activin B), and/or BMP subgroups of the TGFβ superfamily of growth factors. In some embodiments, the pluripotent stem cells are contacted with Nodal, Activin A, Activin B, a BMP signaling pathway activator, or any combination thereof, to differentiate the pluripotent stem cells to definitive endoderm. In some embodiments, the pluripotent stem cells are contacted with Activin A to differentiate the pluripotent stem cells to definitive endoderm. In some embodiments, the one or more growth factors are selected from the group consisting of Nodal, Activin A, Activin B, a BMP signaling pathway activator, or combinations of any of these growth factors. In some embodiments, the stem cells are contacted with Activin A and a BMP signaling pathway activator. In some embodiments, PSCs are treated with 100 ng/ml of Activin A for 3 days as previously described.

[0326] The term "feeder cell" as used herein has its plain and ordinary meaning as understood in light of the specification and refers to cells that support the growth of pluripotent stem cells, such as by secreting growth factors into the medium or displaying on the cell surface. Feeder cells are generally adherent cells and may be growth arrested. For example, feeder cells are growth-arrested by irradiation (e.g. gamma rays), mitomycin-C treatment, electric pulses, or mild chemical fixation (e.g. with formaldehyde or glutaraldehyde). However, feeder cells do not necessarily have to be growth arrested. Feeder cells may serve purposes such as secreting growth factors, displaying growth factors on the cell surface, detoxifying the culture medium, or

synthesizing extracellular matrix proteins. In some embodiments, the feeder cells are allogeneic or xenogeneic to the supported target stem cell, which may have implications in downstream applications. In some embodiments, the feeder cells are mouse cells. In some embodiments, the feeder cells are human cells. In some embodiments, the feeder cells are mouse fibroblasts, mouse embryonic fibroblasts, mouse STO cells, mouse 3T3 cells, mouse SNL 76/7 cells, human fibroblasts, human foreskin fibroblasts, human dermal fibroblasts, human adipose mesenchymal cells, human bone marrow mesenchymal cells, human amniotic mesenchymal cells, human amniotic epithelial cells, human umbilical cord mesenchymal cells, human fetal muscle cells, human fetal fibroblasts, or human adult fallopian tube epithelial cells. In some embodiments, conditioned medium prepared from feeder cells is used in lieu of feeder cell co-culture or in combination with feeder cell co-culture. In some embodiments, feeder cells are not used during the proliferation of the target stem cells.

[0327] The term "three-dimensional" as used in "three-dimensional pluripotent stem cell (PSC) aggregate(s)," "three-dimensional suspension," "three-dimensional culture," two-dimensional culturing," "three-dimensional expansion," or "three-dimensional aggregates" refers to the ability of cells, PSCs, PSC aggregates, DE, spheroids, and/or organoids to be able to grow, develop, reproduce, expand, and interact with their surrounding framework in three dimensions. Such growth, development, reproduction, expansion, and/or interaction may be facilitated by a suspension of said cells, PSCs, PSC aggregates, DE, spheroids, and/or organoids in the framework. The usage of "three-dimensional" in the above referenced terminology and/or phrases may be contrasted with "two-dimensional" as used in "two-dimensional PSC(s)" "two-dimensional culture," "two-dimensional culturing," or "two-dimensional monolayer" where cells, PSCs, PSC aggregates, DE, spheroids, and/or organoids to be able to grow, develop, reproduce, expand, and interact with their surrounding framework in two dimensions (e.g., along a monolayer of a plate).

[0328] Known methods for making downstream cell types, such as definitive endoderm, foregut endoderm, ventral foregut endoderm, and hepatic lineages from pluripotent cells (e.g., iPSCs or ESCs) are applicable to the methods described herein. In some embodiments, iPSCs are used to produce definitive endoderm or other downstream cell types such as foregut endoderm, ventral foregut endoderm, and hepatic lineages. In some embodiments, human iPSCs

(hiPSCs) are used to produce definitive endoderm or other downstream cell types such as foregut endoderm, ventral foregut endoderm, and hepatic lineages.

[0329] In some embodiments, PSCs, such as ESCs and iPSCs, undergo directed differentiation into embryonic germ layer cells, organ tissue progenitor cells, and then into tissue such as liver tissue or any other biological tissue. In some embodiments, the directed differentiation is done in a stepwise manner to obtain each of the differentiated cell types where molecules (e.g. growth factors, ligands, agonists, antagonists) are added sequentially as differentiation progresses. In some embodiments, the directed differentiation is done in a non-stepwise manner where molecules (e.g. growth factors, ligands, agonists, antagonists) are added at the same time. In some embodiments, directed differentiation is achieved by selectively activating certain signaling pathways in the PSCs or any downstream cells.

[0330] In some embodiments, the embryonic stem cells or iPSCs are treated with one or more small molecule compounds, activators, inhibitors, or growth factors for a time that is, is about, is at least, is at least about, is not more than, or is not more than about, 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, 96 hours, 120 hours, 150 hours, 180 hours, 240 hours, 300 hours or any time within a range defined by any two of the aforementioned times, for example 6 hours to 300 hours, 24 hours to 120 hours, 48 hours to 96 hours, 6 hours to 72 hours, or 24 hours to 300 hours. In some embodiments, more than one small molecule compounds, activators, inhibitors, or growth factors are added. In these cases, the more than one small molecule compounds, activators, inhibitors, or growth factors can be added simultaneously or separately.

[0331] In some embodiments, the embryonic stem cells or iPSCs are treated with one or more small molecule compounds, activators, inhibitors, or growth factors at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 10 ng/mL, 20 ng/mL, 50 ng/mL, 75 ng/mL, 100 ng/mL, 120 ng/mL, 150 ng/mL, 200 ng/mL, 500 ng/mL, 1000 ng/mL, 1200 ng/mL, 1500 ng/mL, 2000 ng/mL, 5000 ng/mL, 7000 ng/mL, 10000 ng/mL, or 15000 ng/mL, or any concentration that is within a range defined by any two of the aforementioned concentrations, for example, 10 ng/mL to 15000 ng/mL, 100 ng/mL to 5000 ng/mL, 500 ng/mL to 2000 ng/mL, 10 ng/mL to 2000 ng/mL, or 1000 ng/mL to 15000 ng/mL. In some embodiments, concentration of the one or more small molecule compounds, activators, inhibitors, or growth factors is maintained at a constant level throughout the treatment. In some

embodiments, concentration of the one or more small molecule compounds, activators, inhibitors, or growth factors is varied during the course of the treatment. In some embodiments, more than one small molecule compounds, activators, inhibitors, or growth factors are added. In these cases, the more than one small molecule compounds, activators, inhibitors, or growth factors can differ in concentrations.

[0332] In some embodiments, the ESCs or iPSCs are cultured in growth media that supports the growth of stem cells. In some embodiments, the ESCs or iPSCs are cultured in stem cell growth media. In some embodiments, the stem cell growth media is RPMI 1640, DMEM, DMEM/F12, or Advanced DMEM/F12. In some embodiments, the stem cell growth media comprises fetal bovine serum (FBS). In some embodiments, the stem cell growth media comprises FBS at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20%, or any percentage within a range defined by any two of the aforementioned concentrations, for example 0% to 20%, 0.2% to 10%, 2% to 5%, 0% to 5%, or 2% to 20%. In some embodiments, the stem cell growth media does not contain xenogeneic components. In some embodiments, the growth media comprises one or more small molecule compounds, activators, inhibitors, or growth factors.

[0333] In some embodiments, pluripotent stem cells are prepared from somatic cells. In some embodiments, pluripotent stem cells are prepared from biological tissue obtained from a biopsy. In some embodiments, the pluripotent stem cells are cryopreserved. In some embodiments, the somatic cells are cryopreserved. In some embodiments, pluripotent stem cells are prepared from PBMCs. In some embodiments, human PSCs are prepared from human PBMCs. In some embodiments, pluripotent stem cells are prepared from cryopreserved PBMCs. In some embodiments, PBMCs are grown on a feeder cell substrate. In some embodiments, PBMCs are grown on a mouse embryonic fibroblast (MEF) feeder cell substrate. In some embodiments, PBMCs are grown on an irradiated MEF feeder cell substrate.

[0334] In some embodiments, definitive endoderm (DE) can further undergo anterior endoderm pattering, foregut specification and morphogenesis, dependent on FGF, Wnt, BMP, or retinoic acid, or any combination thereof. In some embodiments, human PSCs are efficiently directed to differentiate *in vitro* into liver epithelium and mesenchyme. It will be understood that

molecules such as growth factors can be added to any stage of the development to promote a particular type of hepatic tissue formation. In some embodiments, siRNA and/or shRNA targeting cellular constituents associated with the FGF, Wnt, BMP, or retinoic acid signaling pathways are used to inhibit or activate these pathways.

Intestinal and Colonic Organoids and Methods of Making

[0335] The intestinal and colonic organoids disclosed herein are produced by a differentiation process from pluripotent stem cells (such as embryonic stem cells or induced pluripotent stem cells) or an intermediate thereof (such as definitive endoderm), and comprise epithelial cell types and mesenchymal cell types, along with intestinal or colonic specialized cell types. Exemplary methods for making intestinal and colonic organoids can be found in U.S. Patents 9,719,068 and 10,781,425, U.S. Patent Application Publication US 2020/190478, and PCT Publication WO 2011/140441, WO 2016/061464, WO 2018/106628, WO 2018/200481, WO 2019/126626, WO 2020/160371, WO 2020/056158, WO 2020/243633, and WO 2021/030373, each of which are incorporated herein by reference in their entirety.

[0336] In some embodiments, intestinal and colonic organoids are differentiated through the culture of definitive endoderm cells. These definitive endoderm cells can be differentiated from pluripotent cells by contacting the definitive endoderm with the Nodal, Activin, and/or BMP subgroups of the TGFβ superfamily of growth factors. In some embodiments, the pluripotent stem cells are contacted with Nodal, Activin A, Activin B, a BMP signaling pathway activator, or any combination thereof, to differentiate the pluripotent stem cells to definitive endoderm. In some embodiments, the pluripotent stem cells are contacted with Activin A to differentiate the pluripotent stem cells to definitive endoderm.

[0337] Definitive endoderm can further be subjected to FGF/Wnt-induced posterior endoderm patterning to direct hindgut specification.

[0338] In some embodiments, to produce intestinal and colonic organoids, definitive endoderm is first contacted with a Wnt signaling pathway activator and an FGF signaling pathway activator to posteriorize the definitive endoderm to hindgut endoderm. During this culture process, hindgut endoderm grows as monolayer but also spontaneously buds off as clumps of cells called hindgut spheroids in suspension. In some embodiments, the Wnt signaling pathway activator comprises Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6,

Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, or Wnt16, or any combination thereof. In some embodiments, the Wnt signaling pathway activator is Wnt3a. In some embodiments, the Wnt signaling pathway activator comprises a glycogen synthase kinase-3 (GSK3) inhibitor, which acts as a Wnt signaling pathway activator. In some embodiments, the GSK3 inhibitor is CHIR99021. In some embodiments, the FGF signaling pathway activator comprises FGF1, FGF2, FGF3, FGF4, FGF4, FGF5, FGF6, FGF7, FGF8, FGF8, FGF9, FGF10, FGF11, FGF12, FGF13, FGF14, FGF15 (FGF19, FGF15/FGF19), FGF16, FGF17, FGF18, FGF20, FGF21, FGF22, FGF23, or any combination thereof. In some embodiments, the FGF signaling pathway activator is FGF4. The hindgut endoderm and hindgut spheroids produced comprise CDX2+ polarized epithelium surrounded by CDX2+ mesenchyme, and lack Alb and Pdx1, which denote foregut endoderm.

[0339] Following formation of hindgut endoderm, or hindgut spheroids, the BMP signaling pathway regulates formation of distinct regional types of intestine. Inhibition of BMP signaling after the hindgut stage promotes a proximal intestinal fate (duodenum/jejunum). Activation of BMP signaling after the hindgut stage promotes a more distal intestinal cell fate (cecum/colon). In some embodiments, the hindgut endoderm is contacted with a BMP signaling pathway activator to differentiate the hindgut endoderm into an intestinal organoid. In some embodiments, the hindgut endoderm is contacted with a BMP signaling pathway inhibitor to differentiate the hindgut endoderm into a colonic organoid. In some embodiments, the BMP signaling pathway activator comprises BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10, BMP11, BMP15, IDE1, or IDE2, or any combination thereof. In some embodiments, the BMP signaling pathway activator comprises BMP2. In some embodiments, the BMP signaling pathway inhibitor comprises Noggin, RepSox, LY364947, LDN193189, or SB431542, or any combination thereof. In some embodiments, the BMP signaling pathway inhibitor comprises Noggin.

Dissociation Methods

[0340] In some embodiments, the methods disclosed herein include dissociation of cellular aggregates (e.g., PSC aggregates) and/or spheroids (e.g., hindgut spheroids), and/or organoids (e.g., intestinal and/or colonic organoids). In some embodiments, the dissociated cell population are prepared by chemical, enzymatic dissociation and/or mechanical dissociation of the aggregate, spheroid and/or organoid. In some embodiments, the dissociation is chemical,

e.g., EDTA. In some embodiments, enzymatic dissociation comprises dissociating the aggregate, spheroid and/or organoid with trypsin, chymotrypsin, collagenase, papain, hyaluronidase, elastase, thermolysin, neutral protease, or any combination thereof. In some embodiments, the enzymatic dissociation comprises incubating the aggregate, spheroid and/or organoid with a proteolytic and/or a collagenolytic enzyme. In some embodiments, the enzymatic dissociation utilizes Accutase. In some embodiments, mechanical dissociation comprises passing the aggregate, spheroid and/or organoid through successively narrower bore channels.

[0341] In some embodiments, the concentration of the dissociated cell population in the cell suspension is, is about, is at least, is at least about, is not more than, or is not more than about, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} cells/mL, or any concentration of cells within a range defined by any two of the aforementioned concentrations, for example, 10^5-10^{11}, 10^5-10^8, 10^9-10^{11} or 10^6-10^{10} cells/mL. In some embodiments, the concentration of cells in the dissociated cell population that are mesenchymal cell types is, is about, is at least, is at least about, is not more than, or is not more than about, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} cells/mL, or any concentration of cells within a range defined by any two of the aforementioned concentrations, for example, 10^5-10^{11}, 10^5-10^8, 10^9-10^{11} or 10^6-10^{10} cells/mL. In some embodiments, the concentration of cells in the dissociated cell population that are epithelial cell types is, is about, is at least, is at least about, is not more than, or is not more than about, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} cells/mL, or any concentration of cells within a range defined by any two of the aforementioned concentrations, for example, 10^5-10^{11}, 10^5-10^8, 10^9-10^{11} or 10^6-10^{10} cells/mL.

[0342] In some embodiments, the dissociated cell population is made up of multi-cellular fragments at a percentage that is, is about, is at least, is at least about, is not more than, or is not more than about, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% of the total cells in the dissociated cell population, or any percentage within a range defined by any two of the aforementioned percentages, for example, 30-100%, 50-100%, 75-100%, 90-100%, 30-75%, or 50-95%. In some embodiments, the dissociated cell population is in the form of 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% multi-cellular fragments. In some embodiments, the dissociated cell population is in the form of at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% multi-cellular fragments.

Culture and Expansion of Foregut Endoderm Cells and Downstream Cell Types

[0343] Methods of making liver organoids have been explored previously in, for example, Ouchi et al. "Modeling Steatohepatitis in Humans with Pluripotent Stem Cell-Derived Organoids" Cell Metabolism (2019) 30(2):374-384; Shinozawa et al. "High-Fidelity Drug-Induced Liver Injury Screen Using Human Pluripotent Stem Cell-Derived Organoids" Gastroenterology (2021) 160(3):831-846; PCT Publications WO 2018/085615, WO 2018/191673, WO 2018/226267, WO 2019/126626, WO 2020/023245, WO 2020/069285, and WO 2021/262676, each of which is hereby expressly incorporated by references in its entirety. Disclosure of liver organoid compositions and methods of making thereof are applicable to the human liver organoids (HLOs) described herein.

[0344] In some embodiments, pluripotent stem cells, definitive endoderm, foregut endoderm, ventral foregut endoderm, or downstream liver cell types are contacted with a TGF-β pathway inhibitor. In some embodiments, the TGF-β pathway inhibitor comprises one or more of A83-01, RepSox, LY365947, and SB431542. In some embodiments, the cells are not treated with a TGF-β pathway inhibitor. The TGF-β pathway inhibitor provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0345] In some embodiments, pluripotent stem cells, definitive endoderm, foregut endoderm, ventral foregut endoderm, or downstream liver cell types are contacted with an FGF pathway activator. In some embodiments, the FGF pathway activator comprises an FGF protein. In some embodiments, the FGF protein comprises a recombinant FGF protein. In some embodiments, the FGF pathway activator comprises one or more of FGF1, FGF2, FGF3, FGF4, FGF4, FGF5, FGF6, FGF7, FGF8, FGF8, FGF9, FGF10, FGF11, FGF12, FGF13, FGF14, FGF15 (FGF19, FGF15/FGF19), FGF16, FGF17, FGF18, FGF20, FGF21, FGF22, or FGF23. In some embodiments, the cells are not treated with an FGF pathway activator. The FGF pathway activator provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0346] In some embodiments, pluripotent stem cells, definitive endoderm, foregut endoderm, ventral foregut endoderm, or downstream liver cell types are contacted with a Wnt pathway activator. In some embodiments, the Wnt pathway activator comprises a Wnt protein.

In some embodiments, the Wnt protein comprises a recombinant Wnt protein. In some embodiments, the Wnt pathway activator comprises Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, Wnt16, BML 284, IQ-1, WAY 262611, or any combination thereof. In some embodiments, the Wnt pathway activator comprises a GSK3 signaling pathway inhibitor. In some embodiments, the Wnt pathway activator comprises CHIR99021, CHIR 98014, AZD2858, BIO, AR-A014418, SB 216763, SB 415286, aloisine, indirubin, alsterpaullone, kenpaullone, lithium chloride, TDZD 8, or TWS119, or any combination thereof. In some embodiments, the Wnt pathway activator is CHIR99021. In some embodiments, the cells are not treated with a Wnt pathway activator. The Wnt pathway activator provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0347] In some embodiments, pluripotent stem cells, definitive endoderm, foregut endoderm, ventral foregut endoderm, or downstream liver cell types are contacted with a VEGF pathway activator. In some embodiments, the VEGF pathway activator comprises one or more of VEGF or GS4012. In some embodiments, the cells are not treated with a VEGF pathway activator. The VEGF pathway activator provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0348] In some embodiments, pluripotent stem cells, definitive endoderm, foregut endoderm, ventral foregut endoderm, or downstream liver cell types are contacted with an EGF pathway activator. In some embodiments, the EGF pathway activator comprises EGF. In some embodiments, the cells are not treated with an EGF pathway activator. The EGF pathway activator provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0349] In some embodiments, pluripotent stem cells, definitive endoderm, foregut endoderm, ventral foregut endoderm, or downstream liver cell types are contacted with ascorbic acid. In some embodiments, the cells are not treated with ascorbic acid. Ascorbic acid as provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0350] In some embodiments, pluripotent stem cells, definitive endoderm, foregut endoderm, ventral foregut endoderm, or downstream liver cell types are contacted with a BMP pathway activator or BMP pathway inhibitor. In some embodiments, the BMP pathway activator

comprises a BMP protein. In some embodiments, the BMP protein is a recombinant BMP protein. In some embodiments, the BMP pathway activator comprises BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10, BMP11, BMP15, IDE1, or IDE2, or any combination thereof. In some embodiments, the BMP pathway inhibitor comprises Noggin, RepSox, LY364947, LDN-193189, SB431542, or any combination thereof. In some embodiments, the cells are not treated with a BMP pathway activator or BMP pathway inhibitor. The BMP pathway activator or BMP pathway inhibitor provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0351] In some embodiments, pluripotent stem cells, definitive endoderm, foregut endoderm, ventral foregut endoderm, or downstream liver cell types are contacted with a retinoic acid pathway activator. In some embodiments, the retinoic acid pathway activator comprises retinoic acid, all-trans retinoic acid, 9-cis retinoic acid, CD437, EC23, BS 493, TTNPB, or AM580, or any combination thereof. In some embodiments, the cells are not treated with a retinoic acid pathway activator. The retinoic acid pathway activator provided herein may be used in combination with any of the other growth factors, pathway activators, or pathway inhibitors provided herein.

[0352] In some embodiments, pluripotent stem cells are converted into liver cell types via a "one step" process. For example, one or more molecules that can differentiate pluripotent stem cells into DE culture (e.g., Activin A) are combined with additional molecules that can promote directed differentiation of DE culture (e.g., FGF4, CHIR99021, RA) to directly treat pluripotent stem cells.

[0353] In some embodiments, pluripotent stem cells (e.g., ESCs or iPSCs) are expanded in suspension culture as described above and elsewhere herein. In some embodiments, the pluripotent stem cells are expanded in cell culture comprising a ROCK inhibitor (e.g. Y-27632). In some embodiments, the iPSCs are differentiated into definitive endoderm cells. In some embodiments, the pluripotent stem cells are differentiated into definitive endoderm cells by contacting the pluripotent stem cells with Activin A, a BMP activator, or both. In some embodiments, the pluripotent stem cells are contacted with a concentration of Activin A that is, is about, is at least, is at least about, is not more than, or is not more than about, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 ng/mL, or any

concentration of Activin A within a range defined by any two of the aforementioned concentrations, for example, 10 to 200 ng/mL, 10 to 100 ng/mL, 100 to 200 ng/mL, or 50 to 150 ng/mL. In some embodiments, the pluripotent stem cells are contacted with Activin A at a concentration of 100 ng/mL or about 100 ng/mL. In some embodiments, the pluripotent stem cells are contacted with a concentration of a BMP signaling pathway activator that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 ng/mL, or any concentration of a BMP signaling pathway activator within a range defined by any two of the aforementioned concentrations, for example, 1 to 200 ng/mL, 1 to 100 ng/mL, 25 to 200 ng/mL, 1 to 80 ng/mL, or 25 to 100 ng/mL. In some embodiments, the pluripotent stem cells are contacted with a BMP signaling pathway activator at a concentration of 50 ng/mL or about 50 ng/mL.

[0354] In some embodiments of the methods provided herein, the TGF-b pathway inhibitor is selected from the group consisting of A83-01, RepSox, LY365947, and SB431542. In some embodiments, the TGF-b pathway inhibitor is A83-01. In some embodiments, the TGF-b pathway inhibitor is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nM, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 100-1000 nM, 100-500 nM, 500-1000 nM, or 300-700 nM. In some embodiments, the TGF-b pathway inhibitor is provided at a concentration of, or of about, 500 nM.

[0355] In some embodiments of the methods provided herein, the FGF pathway activator is selected from the group consisting of FGF1, FGF2, FGF3, FGF4, FGF4, FGF5, FGF6, FGF7, FGF8, FGF8, FGF9, FGF10, FGF11, FGF12, FGF13, FGF14, FGF15, FGF16, FGF17, FGF18, FGF19, FGF20, FGF21, FGF22, and FGF23. In some embodiments, the FGF pathway activator is FGF2. In some embodiments, the FGF pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 ng/mL, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 1-10 ng/mL, 1-5 ng/mL, 5-10 ng/mL, or 3-7 ng/mL. In some embodiments, the FGF pathway activator is provided at a concentration of, or of about, 5 ng/mL.

[0356] In some embodiments of the methods provided herein, the Wnt pathway activator is selected from the group consisting of Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, Wnt16, BML 284, IQ-1, WAY 262611, CHIR99021, CHIR 98014, AZD2858, BIO, AR-A014418, SB 216763, SB 415286, aloisine, indirubin, alsterpaullone, kenpaullone, lithium chloride, TDZD 8, and TWS119. In some embodiments, the Wnt pathway activator is CHIR99021. In some embodiments, the Wnt pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, or 8 μM, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 1-8 μM, 1-3 μM, 3-8 μM, or 2-4 μM. In some embodiments, the Wnt pathway activator is provided at a concentration of, or of about, 3 μM.

[0357] In some embodiments of the methods provided herein, the VEGF pathway activator is selected from the group consisting of VEGF or GS4012. In some embodiments, the VEGF pathway activator is VEGF. In some embodiments, the VEGF pathway activator is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 ng/mL, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 1-20 ng/mL, 1-10 ng/mL, 10-20 ng/mL, or 5-15 ng/mL. In some embodiments, the VEGF pathway activator is provided at a concentration of, or of about, 10 ng/mL.

[0358] In some embodiments of the methods provided herein, the foregut endoderm cells of step c) are cultured in a media that further comprises EGF. In some embodiments, the EGF is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 ng/mL, or any concentration within a range defined by any two of the aforementioned concentrations, for example, 10-30 ng/mL, 10-20 ng/mL, 20-30 ng/mL, or 15-25 ng/mL. In some embodiments, the EGF is provided at a concentration of, or of about, 20 ng/mL. In some embodiments, the foregut endoderm cells of step c) are cultured in a media that does not comprise EGF.

[0359] In some embodiments of the methods provided herein, the foregut endoderm cells of step c) are cultured in a media that further comprises ascorbic acid. In some embodiments, the ascorbic acid is provided at a concentration that is, is about, is at least, is at least about, is not

more than, or is not more than about, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 μg/mL or any concentration within a range defined by any two of the aforementioned concentrations, for example, 10-100 μg/mL, 10-50 μg/mL, 50-100 μg/mL, or 30-70 μg/mL. In some embodiments, the ascorbic acid is provided at a concentration of, or of about, 50 μg/mL. In some embodiments, the foregut endoderm cells of step c) are cultured in a media that does not comprise ascorbic acid.

[0360] In some embodiments of the methods provided herein, the foregut endoderm cells of step c) are cultured in a media that further comprises a ROCK inhibitor (ROCKi). In some embodiments, the ROCK inhibitor is provided at a concentration that is, is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 μM or any concentration within a range defined by any two of the aforementioned concentrations, for example, 1-20 μM, 1-10 μM, 10-20 μM, or 5-15 μM. In some embodiments, the ROCK inhibitor is provided at a concentration of, or of about, 10 μg/mL. In some embodiments, the ROCK inhibitor is Y-27632 In some embodiments, the foregut endoderm cells of step c) are cultured in a media that does not comprise the ROCK inhibitor.

Apoptotic Agents and Anti-Adhesion Agents in Culture Media

[0361] In some embodiments of the methods provided herein, the culture media used in any one or more of the aforementioned processes (e.g., the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, the liquid hindgut differentiation culture media, the liquid IO maturation culture media, the liquid differentiation culture media, and/or the liquid organoid maturation culture media) may include an anti-apoptotic agent. Examples of anti-apoptotic agent may include but are not limited to CEPT or ROCKi. As discussed herein, the use of the anti-apoptotic agent, such as CEPT, may result in higher cell (e.g., PSC) recovery during passaging. In some embodiments, CEPT may be present in the culture medium at a concentration that is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 μM or any concentration within a range defined by any two of the aforementioned concentrations, for example, 1-20 μM, 1-10 μM, 10-20 μM, or 5-15 μM. In some embodiments, the CEPT is provided at a concentration of, or of about, 10 μg/mL.

[0362] In some embodiments of the methods provided herein, the culture media used in any one or more of the aforementioned processes (e.g., the liquid culture media, the second liquid

culture media, the liquid definitive endoderm differentiation culture media, the liquid hindgut differentiation culture media, the liquid IO maturation culture media, the liquid differentiation culture media, and/or the liquid organoid maturation culture media) may include an anti-adhesion agent. In some embodiments, the anti-adhesion agent may include one or more of dextran sulphate sodium (DSS), xantham gum, A-205804, I-CAM1, carboxymethyl cellulose, and/or Neural Organoid Basal Medium 2 (NOBM). In some embodiments, the anti-adhesion agent may comprise DSS. In some embodiments, the anti-adhesion agent (e.g., DSS) may be present in the culture media at a concentration that is about, is at least, is at least about, is not more than, or is not more than about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 75, 100, 150, 200, 300, 400, 500, 750, or 1000 μg/ml or any concentration within a range defined by any two of the aforementioned concentrations, for example, 1-1000 μg/ml, 1-500 μg/ml, 5-100 μg/ml, or 5-50 μg/ml. In some embodiments, anti-adhesion agent (e.g., DSS)is provided at a concentration of, or of about, 10 μg/mL of the culture media (e.g., the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, the liquid hindgut differentiation culture media, the liquid IO maturation culture media, the liquid differentiation culture media, and/or the liquid organoid maturation culture media). As will be discussed herein, DSS is found to moderate (e.g., reduce) the average size of 3D PSC aggregates.

Compositions

EXAMPLES

[0363] Some aspects of the embodiments discussed herein are disclosed in further detail in the following examples, which are not in any way intended to limit the scope of the present disclosure. Those in the art will appreciate that many other embodiments also fall within the scope of the disclosure, as it is described herein and in the claims.

Example 1. Determination of Conditions for Matrix-free Suspension Culture of PSC

[0364] A series of experiments were conducted to determine conditions to successfully expand and maintain PSCs in a suspension culture. FIG. 1A depicts an embodiment of an experimental protocol to explore the impact of various culture conditions on the maintenance

and expansion of PSCs. For example, cell culture media (e.g., TeSR AOF) was obtained (see column 102) for inoculation in a bioreactor (column 104). A low-shear/shear-free bioreactor (Clinostar, CellVivo) was utilized in the following experiments. FIG. 1B illustrates the operation of a suspension culture where the chamber is rotated around its longitudinal axis when the longitudinal axis is oriented parallel to the ground. The depicted rotating vessel bioreactor (cell culture systems), rotates continuously to keep the cells, PSC aggregates, spheroids and/or organoids suspended by counterbalancing the gravitation forces, thereby ideally keeping them in statical orbit. As a result, cells grown in the rotating vessel bioreactor experience very low shear forces. The bioreactor allows for suspension of cells in the liquid culture media. In various embodiments, the composition comprising the suspended cell culture undergo serial passaging (column 106). Furthermore, an analysis of the composition may be performed during or after one or more passages (column 108). The analysis may include but is not limited to determination of aggregate size distribution, flow cytometry, immunohistochemistry, PCR, and/or tri-lineage differentiation.

Inoculation Density

[0365] Experiments were conducted to examine the impact of inoculation density on the resulting PSC expansion in a suspension culture. FIG. 2 depicts the results of an embodiment of a study examining the impact of the inoculation density of PSC in the suspension culture media on the formation of PSC aggregates and cell death at various time points following culture inoculation. The suspension culture media (10 ml) was inoculated with 2, 5 or 10 million PSCs. The resulting PSC aggregates were examined after 1, 2, 3 or 4 days. While all of the seeding densities resulted in the formation of PSC aggregates, the 5M and 10M conditions resulted in significantly more cell death (not aggregating single cells), as well as more significantly larger aggregates (see markers 202 and 204 in FIG. 2) that could result in formation of the hypoxic core (see markers 206 and 208 in FIG. 2). As a result, the 2M cell density (200K cells/ml culture media) was therefore used as the inoculation density in subsequent experiments discussed below.

Dissociation Reagent

[0366] Experiments were conducted to compare various dissociation reagents to create single cell culture. TrypLE, GCDR and Accutase were utilized to create single cell PSC

inoculants which were cultured in a suspension culture, and the resulting PSC aggregates were examined 24 and 96 hours after inoculation. FIG. 3 depicts the results of the experiment. TrypLE resulted in the formation of large cellular clumps 24h post-inoculation. GCDR gave suboptimal results as it was much harder to generate single cell suspension with the use of this reagent and its use resulted in creation of more heterogenous aggregates (size and shape). Accutase dissociation showed to be the most optimal method of those tested for obtaining single cell suspension for the purpose of inoculation, passaging and downstream cell analysis. Accutase was therefore used in subsequent experiments discussed below.

Growth Stage of PSCs Used for Inoculation

[0367] Experiments were conducted to compare how the stage of growth of PSC impacts the yield of aggregate formation upon their inoculation to a suspension culture. PSCs grown in adherent cultures were harvested on day 0 (d0), which refers to the day at which the PSC line would usually be passaged (80-90% confluency), or on day -1 (d-1), which refers to one day before the adherent PSC culture reached "passage ready" confluency, about 40-50% confluency. FIG. 4A depicts an iPSC growth curve and the two timepoints the PSCs were harvested. Equal amounts of these d0 or d-1 PSCs were used to inoculate a suspension culture, and the number and size of the resulting PSC aggregates were examined on day 4 (d4). FIG. 4B depicts an embodiment of photographs of a starting two-dimensional PSC adherent culture at d-1 and d0, and the resulting PSC aggregates at day 4 (d4) of suspension culture. FIG. 4C depicts an embodiment of a graph of the PSC aggregate size distribution at d4 of the suspension culture using either d-1 or d0 PSC inoculates. The results show that d-1 PSC inoculants resulted in significantly more PSC aggregates on d4 as compared to d0 PSC inoculates.

Passaging Timepoint for PSC Suspension Cultures

[0368] Experiments were conducted to compare how the size of PSC aggregates at the time of their passaging impacts the yield of aggregates formed after re-inoculation. PSC aggregates of a suspension culture were passaged on day 3 or day 4 by dissociating the PSC aggregates and using the resulting single cells to re-inoculate a suspension culture. The resulting passaged cultures were examined after 3 days of suspension culturing. FIG. 5 depicts the results of the experiment, showing the culture at the time of passaging (day 3 or day 4), and the resulting

passaged culture on day 3 after passaging. The results show that passaging of PSC aggregates at Day 3, when the diameter of the majority of aggregates is <400 μm, results in a successful propagation of the culture. In contrast, passaging of PSC aggregates at Day 4, when the diameter of a majority of the PSC aggregates exceeds 400 μm results in a much lower yield of aggregates. It was concluded that passaging of PSC aggregates of the suspension culture should be performed before their diameter exceeds 350 μm to improve the maintenance of PSC in suspension culture.

Bioreactor Rotation Speed

[0369] Experiments were conducted comparing how the bioreactor rotation speed impacts the yield of PSC aggregates formed (see FIGs. 6A-6C). The Clinostar bioreactor utilizes a chamber holding 10 ml of culture media which is a cylindric section and which is rotated around its longitudinal axis The speed of rotation of the chamber can be varied. FIG. 6A depicts an embodiment of PSC aggregates on day 3 (d3) of suspension culture at various bioreactor chamber rotation speeds. FIG. 6B depicts an embodiment of a graph of the PSC aggregate size distribution at d3 of the suspension culture at various bioreactor chamber rotation speeds, and FIG. 6C depicts an embodiment of a chart comparing the total cellular yield and fold cell expansion of PSC suspension cultures at the first (P1), second (P2) and third (P3) passaging at various bioreactor chamber rotation speeds. While almost all bioreactor chamber rotation speeds (except for 80 rpm) resulted in successful formation of PSC aggregates, 5 and 40 rpm showed maintenance of consistent cellular expansion across 3 passages.

Apoptotic Agent

[0370] An experiment was conducted comparing the effects of using various anti-apoptic agents on the cell recovery of PSCs across passages (see FIG. 7). In particular, the anti-apoptotic agents ROCKi and CEPT were compared across two passages and the cumulative cell count was measured. As shown in FIG. 7, the use of anti-apoptotic agents increased cell (e.g., PSCs) recovery with each passage. Moroever, the anti-apoptotic agent CEPT was found to provide higher cell recovery than the anti-apoptotic agent ROCKi.

Culture Media

[0371] An experiment was conducted to compare how culture media, mTeSR 1 (research media) and mTeSR AOF (animal product free media), impacts PSC expansion and maintenance, as well as expression of stem cell markers. FIG. 8 depicts the results of PSC suspension culturing

using mTeSR 1 (research media) or mTeSR AOF (animal product free media) at the first (P1), second (P2) and third (P3) passaging of a suspension culture. Both media tested supported successful cellular expansion and maintenance of PSC aggregates, with similar levels of PSC expansion and aggregate formation efficiency across multiple passages. FIGs. 9A and 9B depict the results of the impact of culture media, mTeSR 1 (research media; FIG. 9A) and mTeSR AOF (animal product free media; FIG. 9B) on expression of stem cell markers: Oct4, SSEA4 and TRA 1-60. Both media tested, mTeSR 1 and mTeSR AOF supported successful maintenance of stemness (>90% cells expressing stem cell markers Oct4, SSEA4 and TRA 1-60) by PSC aggregates grown in suspension culture.

PSC Cell Line

[0372] FIG. 10 depicts the results of a study comparing how cell line (research-grade, PSC cell line 72.3; clinical-grade PSC cell line FF3 produced under GMP) impact the production of PSC aggregates at the first (P1), second (P2) and third (P3) passaging of a suspension culture. Successful cellular expansion and maintenance of expression of stem cell markers (Oct4, SSEA4 and TRA 1-60) was observed in both cell lines grown in mTeSR AOF culture media across 3 passages. FIGs. 11A and 11B depict the results of a study comparing the impact of two-dimensional (2D) culturing (FIG. 11A) to suspension culturing (3D) (FIG. 11B) on the expression of stem cell markers Oct4, SSEA4 and TRA 1-60 in research-grade PSC cell line 72.3. The results show that >95% of 72.3 cells maintained expression of stem cell markers in the mTeSR AOF suspension culture of PSC aggregates. Expression of Oct4, SSEA4 and TRA 1-60 is comparable (if not higher) as compared to standard adherent 2D culture as static monolayers. FIGs. 12A and 12B depict the results of a study comparing the impact of two-dimensional (2D) culturing (FIG. 12A) to suspension culturing (3D) (FIG. 12B) on the expression of stem cell markers Oct4, SSEA4 and TRA 1-60 in clinical-grade PSC cell line FF3 produced under GMP. The results show that >95% of FF3 cells maintained expression of stem cell markers in the mTeSR AOF suspension culture of PSC aggregates. Expression of Oct4, SSEA4 and TRA 1-60 is comparable (if not higher) as compared to standard adherent 2D culture as static monolayers.

Pluripotency

[0373] Experiments were also conducted assessing pluripotency in three-dimensional cultures. For example, FIGs. 13A-13B depict the result of an embodiment based on a study investigating the formation of three-dimensional PSC aggregates from PSCs, in which the three-dimensional PSC aggregates were found to retain their pluripotency. As shown in the images of FIG. 13A, PSCs are able to form three-dimensional PSC aggregates progressing from Day 1 to Day 4. As shown in FIG. 13B, number of such aggregates increased from 600 in Day 1 to ~1400 in Day 4. In addition, markers of pluripotency, such as OCT4 and SSEA4 were evident in the three-dimensional PSC aggregates as shown in the confocal imaging. FIGs. 14A-14B depict the results of an evaluation of pluripotent 3D PSC aggregates across multiple lines and passages. As shown in FIG. 14A, there was a marked formation of three-dimensional PSC aggregates across passages for iPSC line 72.3 and ESC line H1. FIG. 14B shows an increase in cell count as well as an increase in diameter of the PSC aggregates across the passages for both lines. Furthermore, FIG. 14B shows that an expression of pluripotency markers (e.g., OCT4 and SSEA-4) remained at least 90% across all the passages and lines tested for three-dimensional PSC aggregates, similar to the expression in conventional, two-dimensionally grown PSC monolayers. Furthermore, FIG. 15 depicts the results an evaluation comparing the pluripotency of PSC lines grown in three-dimensional suspension culture according to methods described herein, compared to PSC lines grown in conventional 2D monolayers. As shown in FIG. 15, based on the higher gene expression of the pluripotency markers OCT4, SOX2, and KLF4, there is an increased pluripotency of PSC lines (H1 and 72.3) grown in three-dimensional suspension culture in comparison to those lines grown in 2D monolayers.

Example 2. Development of HIOs in Suspension Culture

[0374] FIG. 16 depicts an embodiment of an experimental protocol for the matrix-free suspension culture production of HIOs from hiPSCs. As shown in FIG. 16, the protocol may involve formation of a 3D iPSC culture (e.g., using a bioreactor), including the formation of 3D PSC aggregates. The acclimation to the 3D PSC culture may involve single cell dissociation. The protocol may further involve a differentiation of the 3D iPSC to 3D definitive endoderm (DE) (e.g., via use of Activin A in the culture media for DE induction). The protocol may further involve a differentiation of the 3D DE culture to hindgut spheroids (e.g., via use of CHIR99021

and/or FGF4 in the culture media). The protocol may further involve the development of HIO (e.g., via single cell dissociation and the use of EGF).

PSC Acclimatization to Suspension Culture

[0375] FIG. 17 depicts the results of a study examining the impact of acclimatization of PSCs to suspension culturing on the production of HIOs at various time points. Comparison between cells which were differentiated toward HIOs either: a) right after their inoculation from 2D monolayers into 3D suspension culture (Not acclimatized); or b) after being subjected to 1 passage in 3D suspension culture (Acclimatized). Inoculation methods and culture conditions are provided in more detail below. The results show that lack of cell acclimatization to 3D suspension culture (by passaging and re-introduction into the 3D bioreactor culture) resulted in significant cellular death and failure in HIOs development. One passage in suspension culture was demonstrated to be sufficient to acclimatize cells to 3D suspension culture. Therefore, PSC acclimatization to suspension culture is important for successful formation of human intestinal organoids.

Definitive Endoderm Induction in Suspension Culture

[0376] FIGs. 18A and 18B depict the results of a study comparing the efficiency of DE induction in two-dimensional (2D) aggregate monolayer culturing (FIG. 18A) and suspension culturing (3D) (FIG. 18B) by examining expression of definitive endoderm markers Sox 17 and FoxA2. DE was induced using the methods described in more detail below. Successful DE induction in 3D suspension culture was confirmed by immunofluorescent staining and flow cytometry analysis. The results show that comparable efficiency of DE induction (~50% +/+ Sox17/FoxA2 cells) between 2D and 3D culture. Experiments also showed Activin A to contribute to DE differentiation efficiency when applied at specific time points. FIG. 19 depicts the results of an embodiment demonstrating the effect of exposure to Activin A at different time points (e.g., at 24 hours, at 48 hours, or at 72 hours post-passaging) to DE induction efficiency in 3D suspension culture. As shown in FIG. 19, 3D PSC aggregates exposed to Activin A at 48 hour post-passaging showed the highest DE differentiation efficiency, based on the expression of FoxA2 and Sox17.

Effect of 3D PSC Aggregate Size on Intestinal Tissue Differentiation

[0377] Experiments were also conducted comparing the effects of the size of 3D PSC aggregates on intestinal tissue differentiation across various stages of the differentiation process. For example, FIG. 20 shows the results of an embodiment demonstrating the effect of 3D PSC aggregate size on intestinal tissue differentiation at the DE stage. As shown in FIG. 20, the size of the 3D PSC aggregates at the time of the exposure to Activin determines DE induction efficiency. Furthermore, FIG. 20 shows that smaller size of the 3D PSC aggregates (e.g., less than about 400 μm (e.g., less than about 300 μm) in average diameter of the aggregates) ensures better DE induction. FIG. 21 depicts the results of an embodiment demonstrating the effect of 3D PSC aggregate size on intestinal tissue differentiation at the HGS stage. The effects were demonstrated by the expression of CDX2, which is a marker for intestinal tissue differentiation. As shown in FIG. 21, there is a strong expression of CDX2 at the hindgut stage of differentiation of 3D PSC aggregates with diameters smaller than about 300 μm at the time of DE induction. However, there is a weaker and more sparse expression of CDX2 for 3D PSC aggregates having diameters greater than about 300 μm at the time of DE induction. The results further confirm the importance of initial size of the 3D PSC aggregates on differentiation efficiency. FIG. 22 depicts the results of an embodiment demonstrating the effect of 3D PSC aggregate size on intestinal tissue differentiation at the HIO stage. The effects were demonstrated by the expression of CDX2, a marker for intestinal tissue differentiation. As shown in FIG. 22, differentiation of 3D PSC aggregates having diameters no more than about 300 μm resulted in apical-in HIO formation and substantially homogenous expression of CDX2 across generated HIOs. However, differentiation of 3D PSC aggregates having diameters at least 300 μm resulted in mixed apical-out and apical-out structures and epithelial structures with weak or no CDX2 expression.

Effect of Anti-adhesion Agent On The Size Of 3D PSC Aggregates

[0378] Experiments were also conducted investigating the effect of an anti-adhesion agent on 3D PSC aggregates, as other experiments discussed herein noted the beneficial effects of having a smaller average size of 3D PSC aggregates. For example, as previously discussed, 3D PSC aggregates having a smaller size (e.g., diameters of less than about 400 μm (e.g., less than about 300 μm)) is favorable for intestinal tissue differentiation.

[0379] In particular, FIGs. 23-27 depicts the results of an embodiment based on a study investigating the effect of dextran sulfate sodium (DSS), an anti-adhesion agent, mediating the

size of 3D PSC aggregates. However, it is contemplated that other anti-adhesion agents, such as xantham gum, may deliver similar effects on the size of 3D PSC aggregates. The images of FIG. 23 shows the effects of varied concentrations of DSS on the size of 3D PSC aggregates, with a concentration of 10 µg/ml having the highest effect of reducing the size of the 3D PSC aggregates. FIG. 24 further shows that the effects of DSS on reducing aggregate size were noted across different PSC lines (72.3, FF3, H1, and H1 GFP). FIG. 25 further shows that a DSS concentration of 10 µg/ml had the highest reduction in the average diameter across the lines, while also increasing the yield of aggregates being formed. FIGS. 26 and 27 further explore the effect of 10 µg/ml DSS on average size of PSC aggregates across different lines. In particular, FIG. 26 shows that, 10 µg/ml of DSS is sufficient to induce smaller diameter of PSC aggregates, with an average of ~200 µm decrease in mean diameter of PSCs upon DSS treatment in comparison to untreated controls. FIG. 27 shows that this effect is consistent across different iPSC and ESC lines and leads to the shift in the frequency of PSC aggregate size distribution.

Effect of Anti-adhesion Agent On The Pluripotency Of 3D PSC Aggregates

[0380] Experiments also demonstrated that anti-adhesion agents do not have any negative effects on pluripotency, thus making anti-adhesion agents a useful means for moderating 3D PSC aggregate size. In particular, FIG. 28 depicts the results of an embodiment based on a study investigating the effect of DSS on the pluripotency of 3D PSC aggregates, as measured by pluripotency markers SOX2 and OCT4. As shown in the confocal images of FIG. 28, DSS had no negative effect on the pluripotency of the 3D PSC aggregates any of the concentrations tested. The study also investigated the effect of DSS on the viability of 3D PSC aggregates, as measured by the release of a viability marker lactate dehydrogenase (LDH). The study found that DSS at concentrations at or below 1000 µg/ml has no negative effect on the viability of PSC aggregates.

Comparison of Treatment Regimes for Applying Anti-adhesion Agent

[0381] FIG. 29 depicts the results of an embodiment based on a study investigating the effects of various treatment regimes for applying DSS on the average size, numbers, and pluripotency of 3D PSC aggregates. As shown in FIG. 29, the treatment regimes that were tested included control (i.e., no treatment regime), at inoculation, and throughout. The results show that Treatment with 10 ug/ml of DSS at the time of inoculation is sufficient to maintain PSC aggregates <400 um, while having no negative effect on the PSC aggregate numbers or

expression of pluripotency genes. However, prolonged treatment (Throughout) with DSS lead to a decreased OCT4 expression.

Effect of Anti-adhesion Agent In Controlling Size Of 3D PSC Aggregate Maintained Across Passages

[0382] An experiment also demonstrated that the aforementioned beneficial effects of anti-adhesion agents in controlling size (e.g., reducing the average diameter) of 3D PSC aggregates is maintained across passages. FIG. 30 depicts the results of an embodiment based on a study investigating the effects of DSS on the average size of 3D PSC aggregates across passages. As shown in FIG. 30, the effect of DSS treatment on PSC aggregate size (a decrease in diameter) was maintained across multiple passages.

Effect of Anti-adhesion Agent On Propensity Of 3D PSC Aggregates To Differentiate

[0383] FIG. 31 depicts the results of an embodiment based on a study investigating the effects of DSS on the propensity of 3D PSC aggregates to differentiate. The propensity is measured based on differentiation efficiency, which is marked by an expression of markers FOXA2 and SOX17. As shown in FIG. 31, similar differentiation efficiency of 3D PSC aggregates toward differentiation to DE was found, as noted by the expression of Sox17 and FoxA2 in the presence and absence of DSS, demonstrating that there is no negative effect of DSS treatment on cells propensity to differentiation.

Development of HIOs in Suspension Culture

[0384] FIG. 32 depicts the results of an experiment demonstrating the development of well-patterned HIOs in suspension culture having either an apical-out epithelial cell polarity or an apical-in epithelial polarity (see below for additional details). Induction of HIOs from DE was performed using the methods described in more detail below. Successful development of correctly patterned HIOs in suspension culture confirmed by immunofluorescent staining for CDx2, E-cadherin. FIG. 33 depicts the results of an embodiment based on the development of well-patterned HIOs in 3D suspension culture. The successful development of correctly patterned HIOs in the suspension culture is confirmed by immunofluorescent staining. The markers CDX2, ZO-1, and Vim1 signify the differentiation into HIOs. FIGs. 34A and 34B depict the results of an experiment demonstrating the *in vivo* maturation of HIOs developed in suspension culture following transplantation under the kidney capsule of a mouse. FIG. 34A is an embodiment of a photograph of an HIO 9 weeks post-transplant under the kidney capsule.

FIG. 34B is an embodiment of H & E staining of an HIO 9 weeks post-transplant under the kidney capsule. HIOs developed using the described suspension culture methods showed efficient engraftment and appropriate maturation upon transplantation under the kidney capsule.

Modification of HIO Epithelial Cell Polarity in Suspension Culture

[0385] FIG. 35 depicts the results of an experiment demonstrating that the polarity of epithelial cells in HIOs developed in suspension culture can be modified. The methods used for development of the HIOs are described in more detail below. Dissociation of hindgut spheroids at day 7 (+ dissociation) and their reaggregation in suspension culture leads to the apical surface on the inside of the HIO (apical-in). If the DE is not dissociated at day 7 (- dissociation), the result is the apical surface on the outside of the HIO (apical-out). These results show that dissociation of hindgut spheroids at Day 7 and their re-aggregation in suspension culture leads to reversal of epithelial polarity from Apical-Out to Apical-In.

Materials and Methods

[0386] The following are exemplary procedures used in Experiment 2.

Bioreactor Inoculation for iPSC Maintenance

[0387] In some embodiments, bioreactor inoculation for iPSC maintenance may begin after one or more passages (e.g., at day 3 of a 5 day passage, at day 4 of the 5 day passage, at day 5 of the 5 day passage). In some embodiments, cells may be maintained in a 2D (e.g., monolayer) culture until they are deemed appropriate for inoculation.

[0388] In some embodiments, the inoculation may begin with aspiration of media and the addition of a dissociation reagent (e.g., TrypLE, GCDR and/or Accutase) to each well of a multi-well plate containing the samples to be inoculated. The contents of the multi-well plate may be incubated until enough cells have sloughed off of the plate.

[0389] In some embodiments, the inoculation may further include collecting cells and adding to a conical tube. Media may be added to the tube and may be gently titrated to break up colonies further into a single cell suspension.

[0390] In some embodiments, the culture may be spun down, supernatants may discarded, and culture media may be readded. The cells may be gently resuspended in the media to create single cell suspension.

[0391] In some embodiments, a anti-adhesion agent, such as 10uM (1:1000 dilution) of CEPT or ROCKi, may be added to the suspension to prevent cell death. Cells may then be counted to determine the amount needed to obtain an appropriate number or range of cells. In some embodiments, that appropriate number may be about 0.5x10^6 million cells, 1x10^6 million cells, 1.5x10^6 million cells, 2x10^6 million cells, 2.5x10^6 million cells, 3x10^6 million cells, 3.5x10^6 million cells, 4x10^6 million cells, 4.5x10^6 million cells, or 5x10^6 million cells. In some embodiments, the appropriate range of cells may be a range formed by any two of the aforementioned number of cells.

[0392] The composition comprising the culture media, the cells, and any of the aforementioned ingredients (anti-adhesion agents, dissociation agents, etc.) may then be added to a bioreactor (e.g., Clinostar) to fill the inner chamber. In some embodiments, an amount of anti-adhesion agent, such as 10uM CEPT or ROCKi, may be added to the system, taking into account the amount of ROCKi added with the cells. The bioreactor may be rotated (e.g., at about 5RPM) to allow the cells to aggregate (e.g., for about 24hr).

Media Changes for any Application

[0393] In some embodiments, changes in the composition within the bioreactor may be visualized to ensure that the sample is free from any abnormalities (e.g., large clumps, contamination, etc.). In some embodiments, the bioreactor may be placed upright to allow cell aggregates to settle to the bottom of the bioreactor. In some embodiments, culture media may be slowly added to the bioreactor, while ensuring that bubbles are removed from the system, and excess media outside the bioreactor system is aspirated off. In some embodiments, the bioreactor may be rotated horizontally to disperse cells throughout inner chamber. The bioreactor speed may be adjusted to reduce or eliminate the possibility of aggregates hitting the walls of the inner chamber.

Passaging of iPSC Aggregates in ClinoStar System

[0394] In some embodiments, a dissociation agent (e.g., TrypLE, GCDR and/or Accutase) and the culture media may be pre-warmed (e.g., to about 37 C). The contents of the bioreactor may be visualized to evaluate quality and size of aggregates.

[0395] In some embodiments, the aggregates may be split before they reach a given size (e.g., split before the aggregates reach about 500μm, 450μm, 400μm, 350μm, or 300μm).

[0396] The aggregates may continue to be cultured based on the aforementioned steps until they are deemed ready for passaging.

[0397] In some embodiments, the passaging may begin by allowing the aggregates to settle (e.g., by placing the bioreactor vertically), and then exposing the inside of the bioreactor chamber (e.g., by turning the bioreactor onto its side and lifting the lid).

[0398] In some embodiments, the cell aggregates may be gently transferred to one or more wells of a multi-well plate for size quantification.

[0399] In some embodiments, the cell aggregates may be imaged for BF image analysis (described herein).

[0400] In some embodiments, the aggregates may be transferred to a conical tube to remove and discard excess media.

[0401] In some embodiments, a dissociation agent (e.g., 1ml Accutase) may be added to the aggregates. In some embodiments, the aggregates may be incubated in a warm bead bath for some time, while ensuring that the aggregates are mixed. The aggregates may be periodically visualized to evaluate progress of dissociation. After aggregates are dissociated, an amount of media (e.g., about 5 mL) may be added (e.g., via a conical tube). The culture may be spun down, and supernatants may be removed. The cells may be resuspended in culture media and an amount of anti-adhesion agent (e.g., 10uM CEPT or ROCKi) may be added. The sample may be gently titrated to achieve single cell suspension. Cells and fold expansion may be counted and recorded.

[0402] In some embodiments, the cells may be inoculated in a new bioreactor using the methods described herein.

iPSC Culture & Maintenance (e.g., Using ClinoStar System)

[0403] In some embodiments, the process may begin by preparing the bioreactor and adding cells to the bioreactor as previously discussed. A culture schedule may be following as shown in Table 1 below. If aggregates are larger than a specified size (e.g., 500μm 450μm 400μm, 350μm, or 300μm), passage may be performed prior to reaching d4.

Table 1: TeSR AOF 2D Media Change Schedule

Day	TeSR AOF 2D
0	Inoculation, 5RPM
1	
2	90% Media Change
3	Split

Definitive Endoderm (DE) Generation

[0404] In some embodiments, the process may begin with inoculating the bioreactor with an appropriate number of cells to allow for growth over one or more passages. The sample may be split on d3 and reinoculated back into the bioreactor to allow for 3D adjustment.

[0405] In some embodiments, the process may further include creating media based on Table 2 as shown below. DE Induction may begin about 48 hr post second inoculation.

[0406] At, D0, D0 media may be added following media changing protocols. The starting material may be imaged. At D1, the D1 media may be added following media changing protocols. The material may be imaged to ensure minor changes.

[0407] At D2, the D2 media may be added following media changing protocols. The material may be imaged to ensure minor changes.

[0408] At D3, take a small subset of the material may be obtained. In some embodiments, the subset may be fixed in PFA for some time (e.g., about 30min-1hr). In some embodiments, the subset may change its fixing from PFA to PBS +/+ and be left to cool. The material may be imaged to ensure expression of Sox17+/FoxA2+ aggregates.

[0409] In some embodiments, a subset of cells may be obtained to perform DE FACS. The subset may be tested to ensure 80% Sox17+/FoxA2+ of Oct4- population.

[0410] In some embodiments, with remaining material, the process may proceed with Mid-Hindgut Generation.

Table 2: Exemplary Definitive Endoderm Differentiation Media

Day	D0	D1	D2
Total ml	10	10	10
RPMI 1640 +	10	10	9.8ml

NEAA 1X (ml)			
a BMP signaling pathway activator (e.g., BMP4) (ng/ml)	15	0	0
Activin A (ng/ml)	100	100	100
FBS (%)	0	0.20	2.0

Mid-Hindgut Generation

[0411] In some embodiments, the process for mid-hindgut generation may involve making d3-d6 media according to Table 3. The media may be changed daily and imaged.

Table 3: Exemplary Hindgut Differentiation Media

Day	D3-D6
Total ml	10
RPMI 1640 + NEAA 1X (ml)	9.7
CHIR99021 (uM)	3
FGF4 (ng/ml)	500
FBS (%)	2
CEPT	10uM

HiO Maturation

[0412] In some embodiments, the process for HiO maturation may begin with mid-hindgut dissociation. On D7, aggregates at the bottom of the bioreactor may be collected (e.g., using a cut tip). The aggregates may be moved to a conical tube.

[0413] In some embodiments, the remaining media may be aspirated once the aggregates have settled again. In some embodiments, a pre-warmed dissociation agent (e.g., TrypLE, GCDR and/or Accutase) may be added, and the tube holding the sample may be placed in a warm bead bath. The aggregates may be periodically resuspended until visually broken down.

[0414] In some embodiments, media containing serum may be added to the mixture and gently pipetted. The sample may then be spun. Liquid may be aspirated. The aggregates may be

resuspended aggregates in HiO Media as shown below in Table 4. The process may further involve counting and recording the amount generated.

[0415] In some embodiments, an appropriate amount of cells (e.g., about ~4x10^6 cells) may be added into a new bioreactor in HiO Media with an anti-adhesion agent (e.g., 1:1000 of CEPT or ROCKi). The bioreactor may be rotated (e.g., at about 5RPM) overnight for aggregate formation.

[0416] In some embodiments, the HiO Maturation process may involve changing media on a periodic bases (e.g., every other day or when yellow) with the media shown in Table 4.

[0417] In some embodiments, aggregates may be moved into separate bioreactors if the sample aggregates become too dense. Indicators of too dense cultures may include but are not limited to: media becoming acidic quickly, cells falling off due to shear stress, visually more than a large number of HiOs.

[0418] The sample may be monitored daily to ensure continued growth until about d34.

Table 4: HIO Maturation Media

Day	D7-D37
Total ml	50
Adv. DMEM/F12 (ml)	47.25
HEPES (M)	0.01
L-Glutamine (mM)	2
B-27 (ul)	1000
N-2 (ul)	500
EGF (ng/ml)	100

Aggregate Size BF Analysis

[0419] In some embodiments, the process for aggregate size BF analysis may begin by allowing aggregates to settle (e.g., by placing the bioreactor vertically). After aggregates settle, the process may involve exposing the inside of the bioreactor (e.g., by turning the bioreactor onto its side laying flat and remove the front panel). The process may further include gently transfer cell aggregates (e.g., using a pipet tip) to one or more wells of a multi-well plate for size quantification. The samples may be imaged, and the images may be saved (e.g., on Leica Software) and opened (e.g., on FIJI).

[0420] In some embodiments, the image formats may be converted, and color imbalances may be removed or adjusted via thresholding. In some embodiments, masks may be applied or adjusted.

[0421] In some embodiments, the process may further involve opening a software (e.g., MICROSOFT EXCEL) for size calculation, and calculating the diameter based on area measurement. Buckets may be created for each aggregate size range in increments (e.g., of about 100um). Appropriate visualizations (e.g., bar graphs) may be created.

[0422] In at least some of the previously described embodiments, one or more elements used in an embodiment can interchangeably be used in another embodiment unless such a replacement is not technically feasible. It will be appreciated by those skilled in the art that various other omissions, additions and modifications may be made to the methods and structures described herein without departing from the scope of the claimed subject matter. All such modifications and changes are intended to fall within the scope of the subject matter, as defined by the appended claims.

[0423] With respect to the use of substantially any plural and/or singular terms herein, those having skill in the art can translate from the plural to the singular and/or from the singular to the plural as is appropriate to the context and/or application. The various singular/plural permutations may be expressly set forth herein for sake of clarity.

[0424] It will be understood by those within the art that, in general, terms used herein, and especially in the appended claims (e.g., bodies of the appended claims) are generally intended as "open" terms (e.g., the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.). It will be further understood by those within the art that if a specific number of an introduced claim recitation is intended, such an intent will be explicitly recited in the claim, and in the absence of such recitation no such intent is present. For example, as an aid to understanding, the following appended claims may contain usage of the introductory phrases "at least one" and "one or more" to introduce claim recitations. However, the use of such phrases should not be construed to imply that the introduction of a claim recitation by the indefinite articles "a" or "an" limits any particular claim containing such introduced claim recitation to embodiments containing only one such recitation,

even when the same claim includes the introductory phrases "one or more" or "at least one" and indefinite articles such as "a" or "an" (e.g., "a" and/or "an" should be interpreted to mean "at least one" or "one or more"); the same holds true for the use of definite articles used to introduce claim recitations. In addition, even if a specific number of an introduced claim recitation is explicitly recited, those skilled in the art will recognize that such recitation should be interpreted to mean at least the recited number (e.g., the bare recitation of "two recitations," without other modifiers, means at least two recitations, or two or more recitations). Furthermore, in those instances where a convention analogous to "at least one of A, B, and C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (e.g., "a system having at least one of A, B, and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). In those instances where a convention analogous to "at least one of A, B, or C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (e.g., " a system having at least one of A, B, or C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or "B" or "A and B."

[0425] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0426] As will be understood by one skilled in the art, for any and all purposes, such as in terms of providing a written description, all ranges disclosed herein also encompass any and all possible sub-ranges and combinations of sub-ranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as "up to," "at least,"

"greater than," "less than," and the like include the number recited and refer to ranges which can be subsequently broken down into sub-ranges as discussed herein. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 articles refers to groups having 1, 2, or 3 articles. Similarly, a group having 1-5 articles refers to groups having 1, 2, 3, 4, or 5 articles, and so forth.

[0427] While various aspects and embodiments have been disclosed herein, other aspects and embodiments will be apparent to those skilled in the art. The various aspects and embodiments disclosed herein are for purposes of illustration and are not intended to be limiting, with the true scope and spirit being indicated by the following claims.

[0428] All references cited herein, including but not limited to published and unpublished applications, patents, and literature references, are incorporated herein by reference in their entirety and are hereby made a part of this specification. To the extent publications and patents or patent applications incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

WHAT IS CLAIMED IS:

1. A method comprising:

(a) inoculating a liquid culture media with PSCs;

(b) culturing, in a bioreactor, the liquid culture media that is inoculated with the PSCs such that three-dimensional PSC aggregates form in the liquid culture media, wherein the culturing in the bioreactor comprises suspending the PSCs in the liquid culture media;

(c) passaging the PSCs by:

(i) dissociating at least a portion of the three-dimensional PSC aggregates into single cells; and

(ii) inoculating a second liquid culture media with the dissociated three-dimensional PSC aggregates of (i) with PSCs.

2. The method of claim 1, wherein the liquid culture media and the second liquid culture media are free of materials of animal or human origin; optionally wherein the liquid culture media and the second liquid culture media are free of any extracellular matrix and/or basement membrane matrix.

3. The method of any one of the preceding claims, wherein the PSCs are passaged when the diameter of a majority of the three-dimensional PSC aggregates formed is not more than 500 μm.

4. The method of any one of the preceding claims, wherein the PSCs are passaged when the diameter of a majority of the three-dimensional PSC aggregates formed is not more than 400 μm.

5. The method of any one of the preceding claims, wherein the PSCs are passaged when the diameter of a majority of the three-dimensional PSC aggregates formed is not more than 300 μm.

6. The method of any one of the preceding claims, wherein at least 80% of the three-dimensional PSC aggregates are dissociated into single cells.

7. The method of any one of the preceding claims, optionally wherein at least 90% of PSC aggregates are dissociated into single cells.

8. The method of claim 1, where the method comprises passaging the PSCs two or more times by culturing the PSCs of inoculated second liquid culture media until additional three-dimensional PSC aggregates form.

9. The method of any one of the preceding claims, wherein the liquid culture media of (a) and/or the second liquid culture media of (c)(ii) is inoculated at a density of about 100,000-220,000 PSCs/ml.

10. The method of any one of the preceding claims, wherein the liquid culture media of (a) and/or the second liquid culture media of (c)(ii) is inoculated at a density of about 180,000-220,000 PSCs/ml.

11. The method of any one of the preceding claims, wherein the passaging occurs after a period of time following the inoculation in (a) that is between about 40-168 hours.

12. The method of any one of the preceding claims, wherein the passaging occurs after a period of time following the inoculation in (a) that is between about 40-84 hours.

13. The method of any one of the preceding claims, wherein the passaging occurs after a period of time following the inoculation in (a) that is between about 66-78 hours.

14. The method of any one of the preceding claims, wherein the method further comprises exchanging a portion of the culture media in the bioreactor of (a) after a period of time following the inoculation in (a) and/or (c)(ii) that is between about 36-60 hours.

15. The method of any one of the preceding claims, wherein the method further comprises exchanging a portion of the culture media in the bioreactor of (a) after a period of time following the inoculation in (a) and/or (c)(ii) that is between about 42-54 hours.

16. The method of any one of the preceding claims, wherein the method further comprises exchanging a portion of the liquid culture media in the bioreactor of (a) after a period of time following the inoculation in (a) and/or (c)(ii), wherein the portion of the liquid culture media exchanged is at least 50% of the liquid culture media in the bioreactor of (a).

17. The method of any one of the preceding claims, the method further comprising, prior to the inoculation in (a):

culturing the PSCs on the surface of a substrate; and

collecting the PSCs from the surface of the substrate for use in the inoculation of (a) when the PSCs are in a logarithmic growth phase and/or at 35-55% confluency, optionally wherein said collecting comprises dissociating the PSCs prior to the inoculation of (a).

18. The method of claim 17, wherein the PSCs are collected from the surface of the substrate for use in the inoculation of (a) when the PSCs are in a logarithmic growth phase and/or at 40-50% confluency.

19. The method of any one of the preceding claims, wherein the dissociation is chemical, enzymatic and/or mechanical dissociation.

20. The method of any one of the preceding claims, wherein the dissociation is enzymatic, optionally wherein the enzyme comprises a proteolytic and/or a collagenolytic enzyme, optionally wherein the enzyme is Accutase.

21. The method of any one of the preceding claims, wherein the bioreactor of (a) and/or (b) comprises a rotating chamber comprising the liquid culture media, wherein the rotation speed of the rotating chamber is selected such that the number of PSCs in the liquid culture media at (c) is at least 2 or 2.5-fold the number of PSCs used to inoculate the liquid culture media, optionally wherein the number of PSCs in the liquid culture media at (c) is at least 2 or 2.5-fold the number of PSCs used to inoculate the liquid culture media for at least two passages of the PSCs.

22. The method of any one of the preceding claims, wherein the liquid culture media is a serum-free media; optionally, wherein the media comprises recombinant human basic fibroblast growth factor (rh bFGF) and/or recombinant human transforming growth factor β (rh TGFβ).

23. The method of any one of the preceding claims, wherein, following one or more passages, the portion of PSCs expressing Oct4, SSEA1 and TRA 1-60 at levels at least as high as the average expression level of the PSCs used in the inoculation in (a) is at least 85%.

24. The method of claim 23, wherein, following the one or more passages, the portion of PSCs expressing Oct4, SSEA1 and TRA 1-60 at levels at least as high as the average expression level of the PSCs used in the inoculation in (a) is at least 95%.

25. The method of any one of the preceding claims, wherein the PSCs express SOX2 and KLF4.

26. A method for differentiating PSCs into definitive endoderm (DE) in a three dimensional suspension culture, the method comprising:

(d) culturing, in a bioreactor, a liquid culture media inoculated with PSCs;

wherein the culturing of the liquid culture media inoculated with PSCs of (d) comprises suspending the PSCs in the liquid culture media; and

(e) culturing, in a bioreactor, the PSCs of (d) in liquid definitive endoderm differentiation culture media for a period of time sufficient to differentiate the PSCs into DE, wherein the culturing of the PSCs of (d) in the liquid definitive endoderm differentiation culture media comprises suspending the PSCs in the liquid definitive endoderm differentiation culture media.

27. The method of claim 26, wherein the liquid culture media or the liquid definitive endoderm differentiation culture is free of materials of animal or human origin; optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

28. The method of claim 26 or 27, wherein the culturing in (d) is for a period of time between about 18-54 hours.

29. The method of any one of claims 26-28, wherein the culturing in (d) is for a period of time between about 24-48 hours.

30. The method of any one of claims 26-29, wherein the liquid culture media inoculated with PSCs cultured in (d) is the PSC inoculated culture media of (c)(ii) of any one of claims 1-24.

31. The method of any one of claims 26-30, wherein the period of time sufficient to differentiate the PSCs into DE is a period of time that is between about 48-96 hours.

32. The method of any one of claims 26-31, wherein the period of time sufficient to differentiate the PSCs into DE is a period of time that is between about 60-84 hours.

33. The method of any one of claims 26-32, wherein the period of time sufficient to differentiate the PSCs into DE is a period of time that is between about 66-78 hours.

34. The method of any one of claims 26-33, wherein culturing the PSCs in the liquid definitive endoderm differentiation culture media for a period of time sufficient to differentiate the PSCs into DE comprises culturing the PSCs in a culture media comprising a nodal signaling pathway activator and/or a Wnt signaling pathway activator for a first period, then culturing the PSCs in a culture media comprising the nodal signaling pathway activator and/or the Wnt signaling pathway activator and a serum or serum replacement for a second period, and then culturing the PSCs in a culture media comprising the nodal signaling pathway activator and/or the Wnt signaling pathway activator and the serum or serum replacement for a third period.

35. The method of claim 34, wherein the culture media in which the PSCs are cultured for the first period further comprises a BMP activator.

36. The method of claim 34 or 35, wherein the culture media in which the PSCs are cultured for the second period, and the culture media in which the PSCs are cultured for the third period, comprise:

the nodal signaling pathway activator and/or the Wnt signaling pathway activator and a serum; optionally wherein the serum is FBS.

37. The method of claim 34 or 35, wherein the culture media in which the PSCs are cultured for the second period, and the culture media in which the PSCs are cultured for the third period, comprise:

the nodal signaling pathway activator and/or the Wnt signaling pathway activator and a serum replacement; optionally wherein the serum replacement is knockout serum replacement (KSR).

38. The method of any one of claims 34-37, wherein each of the first, second and third period of time is between about 18-30 hours.

39. The method of any one of claims 34-38, wherein each of the first, second and third period of time is between about 20-28 hours.

40. The method of any one of claims 26-39, wherein the efficiency of DE induction is at least about 35%.

41. The method of any one of claims 26-40, wherein the efficiency of DE induction is at least about 45-55%.

42. The method of any one of claims 26-41, wherein the DE expresses Sox17 and FoxA2.

43. A method for differentiating definitive endoderm (DE) into hindgut spheroids (HGS) in a three dimensional suspension culture, the method comprising:

(f) culturing, in a bioreactor, DE in liquid hindgut differentiation culture media for a period of time sufficient to differentiate the DE into HGS, wherein the culturing of the DE comprises suspending the DE in the liquid hindgut differentiation culture media.

44. The method of claim 43, wherein the liquid hindgut differentiation culture media is free of materials of animal or human origin; optionally wherein the liquid hindgut differentiation culture media is free of any extracellular matrix and/or basement membrane matrix.

45. The method of claim 43, wherein the DE cultured in (f) is the DE of any one of claims 25-39.

46. The method of any one of claims 43-45, wherein the period of time sufficient to differentiate the DE into HGS is a period of time that is between about 60-120 hours.

47. The method of any one of claims 43-46, wherein the period of time sufficient to differentiate the DE into HGS is a period of time that is between about 84-108 hours.

48. The method of any one of claims 43-47, wherein the period of time sufficient to differentiate the DE into HGS is a period of time that is between about 90-102 hours.

49. The method of any one of claims 43-48, wherein the liquid hindgut differentiation culture media is changed after a period of time that is between about 20-28 hours.

50. The method of any one of claims 43-49, wherein the liquid hindgut differentiation culture media is changed after a period of time that is between about 22-26 hours.

51. The method of any one of claims 43-50, wherein the liquid hindgut differentiation culture media comprises a Wnt signaling pathway activator, an FGF signaling pathway activator, and optionally FBS.

52. The method of claim 51, wherein the Wnt signaling pathway activator comprises CHIR99021.

53. The method of claim 51 or 52, wherein the FGF signaling pathway activator comprises FGF4.

54. The method of any one of claims 51-53, wherein the FGF signaling pathway activator is FGF4 at a concentration between about 50-750 ng/ml.

55. The method of any one of any one of claims 51-54, wherein the Wnt pathway activator is CHIRON 99021 at a concentration between about 0.5 – 6 μM.

56. A method for differentiating hindgut spheroid (HGS) into intestinal organoids (IO) in a three dimensional suspension culture, the method comprising:
(g) culturing, in a bioreactor, HGS in liquid IO maturation culture media for a period of time sufficient to differentiate the HGS into IO, wherein the culturing of the HGS comprises suspending the HGS in the liquid IO maturation culture media.

57. The method of claim 56, wherein the liquid IO maturation culture media is free of materials of animal or human origin; optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

58. The method of claim 56 or 57, wherein the HGS cultured in (g) is the HGS of any one of claims 40-52.

59. The method of any one of claims 56-58, wherein the period of time sufficient to differentiate the HGS into IO is a period of time that is between about 12-30 days.

60. The method of any one of claims 56-59, wherein the period of time sufficient to differentiate the HGS into IO is a period of time that is between about 15-28 days.

61. The method of any one of claims 56-60, wherein the IO maturation culture media is changed after a period of time that is between about 24-54 hours.

62. The method of any one of claims 56-61, wherein the IO maturation culture media is changed after a period of time that is between about 46-50 hours.

63. The method of any one of claims 56-62, wherein the IO maturation culture media comprises one or more of EGF, R-spondin, Noggin, Gremlin 1, and/or Epiregulin (EREG).

64. The method of claim 63, wherein the concentration of EGF, R-sponding, Noggin, Gremlin 1, and/or EREG is between about 25-150 ng/ml.

65. The method of claim 63 or 64, wherein the concentration of EGF R-sponding, Noggin, Gremlin 1, and/or EREG is between about 50-100 ng/ml.

66. The method of any one of claims 56-65, wherein the HGS are not dissociated prior to culturing in the IO maturation culture media, wherein epithelial cells of the IO formed have a polarity wherein the apical surface is oriented to the outside of the IO.

67. A method for differentiating hindgut spheroids (HGS) into intestinal organoids (IO) having an apical-in polarity in a three dimensional suspension culture, the method comprising the method of any one of claims 56-66;

wherein the method further comprises dissociating at least a portion of the HGS into HGS single cells prior to incubation in the IO maturation culture media;

wherein the culturing of the HGS comprises suspending the dissociated HGS single cells and any non-dissociated HGS in the liquid IO maturation culture media;

wherein epithelial cells of the IO formed from the dissociated HGS single cells have a polarity wherein the apical surface is oriented to the inside of the IO.

68. The method of claim 67, wherein the liquid IO maturation culture media is free of materials of animal or human origin; optionally wherein the liquid IO maturation culture media is free of any extracellular matrix and/or basement membrane matrix.

69. The method of claim 67 or 68, wherein at least 80% of the HGS are dissociated into single cells.

70. The method of any one of claims 67-69, wherein at least 90% of HGS are dissociated into single cells.

71. The method of any one of claims 67-70, wherein a concentration of dissociated HGS single cells are in the IO maturation culture media, wherein the concentration is between about $0.05x10^5$ - $80x10^5$ dissociated HGS single cells/ml of IO maturation culture media.

72. The method of any one of claims 67-71, wherein a concentration of dissociated HGS single cells are in the IO maturation culture media, wherein the concentration is between about $10x10^5$-$80x10^5$ dissociated HGS single cells/ml of IO maturation culture media.

73. The method of any one of claims 67-72, wherein a concentration of dissociated HGS single cells are in the IO maturation culture media, wherein the concentration is between about $20x10^5$-$60x10^5$ dissociated HGS single cells/ml of IO maturation culture media.

74. The method of any one of claims 67-73, wherein the dissociation is chemical, enzymatic and/or mechanical dissociation.

75. The method of any one of claims 67-74, wherein the dissociation is enzymatic, optionally wherein the enzyme comprises a proteolytic and/or a collagenolytic enzyme, optionally wherein the enzyme is Accutase.

76. The method of any one of claims 67-75, wherein the method further comprises transplanting the IO into a subject.

77. The method of claim 76, wherein transplanting the IO into the subject comprises transplanting the IO under the kidney capsule of a non-human animal, optionally for a period of time that is between about 6-20 weeks.

78. The method of claim 77, wherein the IO is transplanted under the kidney capsule of a non-human animal for a period of time that is between about 12-20 weeks.

79. The method of any one of claims 77-78, wherein the IO is transplanted under the kidney capsule of a non-human animal for a period of time that is between about 16-20 weeks.

80. The method of claim 76, wherein transplanting the IO into the subject comprises transplanting the IO into an intestinal lumen of the subject for treatment of an intestine of the subject.

81. The method of any one of claims 76-80, wherein the IO is matured *in vitro* for a period of time prior to transplantation, optionally wherein the period of time is between about 7-28 days.

82. The method of any one of claims 76-81, wherein the IO is matured *in vitro* for a period of time prior to transplantation that is between about 14-28 days.

83. The method of any one of claims 76-82, wherein the IO is matured *in vitro* for a period of time prior to transplantation that is between about 21-28 days.

84. The method of any one of claims 26-42, the method further comprising differentiating the DE into a spheroid, optionally wherein the differentiating comprises:
(h) culturing, in a bioreactor, the DE in liquid differentiation culture media for a period of time sufficient to differentiate the DE into a spheroid, wherein the culturing of the DE comprises suspending the DE in the liquid differentiation culture media; optionally wherein the spheroid is a foregut or a hindgut spheroid.

85. The method of claim 84, wherein the liquid differentiation culture media is free of materials of animal or human origin; optionally wherein the liquid differentiation culture media is free of any extracellular matrix and/or basement membrane matrix.

86. The method of claim 84 or 85, the method further comprising:

(i) culturing, in a bioreactor, the spheroids in liquid organoid maturation culture media for a period of time sufficient to differentiate the spheroids into an organoid, wherein the culturing of the spheroid comprises suspending the spheroid in the liquid organoid maturation culture media;

optionally wherein the organoid is selected from the group consisting of a liver, pancreatic, gastric, antral gastric, fundal gastric, intestinal, lung, or colonic organoid.

87. The method of any one of claims 84-86, wherein the liquid organoid maturation culture media is free of materials of animal or human origin; optionally wherein the liquid organoid maturation culture media is free of any extracellular matrix and/or basement membrane matrix.

88. The method of any one of the preceding claims, wherein the PSC is an induced PSC (iPSC) or an embryonic stem cells (ESC).

89. The method of any one of the preceding claims, wherein the PSC is a human PSC, optionally a human iPSC (hiPSC).

90. A PSC or three-dimensional PSC aggregate made by the method of any one of claims 1-25 or 88-89.

91. DE made by the method of any one of claims 26-42 or 88-89.

92. An HGS made by the method of any one of claims 43-552, or 88-89.

93. An IO made by the method of any one of claims 56-89.

94. An IO having an apical-in polarity, wherein epithelial cells of the IO have a polarity wherein the apical surface is oriented to the inside of the IO, optionally wherein the IO is a human IO (hIO).

95. The IO having an apical-in polarity made by the method of any one of claims 67-89.

96. A spheroid made by the method of any one of claims 84, 85, or -89.

97. An organoid made by the method of any one of claims 86-89.

98. A method of treatment comprising transplanting the IO of any one of claims 93-95, or cells derived therefrom, into an animal, optionally wherein the animal is suffering from a GI disease state; optionally wherein the animal is human.

99. A method of screening a compound for activity comprising contacting the IO of any one of claims 93-95, or cells derived therefrom, with the compound and measuring a response of the IO to the compound.

100. A method of screening a compound for activity comprising contacting the organoid of claim 97, or cells derived therefrom, with the compound and measuring a response of the organoid to the compound.

101. The method of any one of the preceding claims, wherein the method is free of any xenogenic material, optionally wherein the organoids are clinical grade and suitable for transplantation in a human.

102. The method of any one of the preceding claims,

wherein the bioreactor of (a), (b), (d), (e), (f), (g), (h), and/or (i) comprises a rotating chamber comprising the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media;

wherein the rotating chamber is a cylindric section which is rotated around its longitudinal axis, thereby suspending the PSCs and/or three-dimensional PSC aggregates in the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation

culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media;

optionally wherein the chamber is oriented such that its longitudinal axis is parallel to the ground.

103. The method of any one of the preceding claims, wherein the bioreactor of (a), (b), (d), (e), (f), (g), (h), and/or (i) comprises a rotating chamber comprising the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media in a volume that is between about 5ml to about 50 L.

104. The method of any one of the preceding claims,

wherein the bioreactor of (a), (b), (d), (e), (f), (g), (h), and/or (i) comprises a rotating chamber comprising the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media;

wherein the rotation of the chamber is between about 3-7 rpm; and

optionally wherein the rotational speed is a speed selected to keep the PSCs, the three-dimensional PSC aggregates, the spheroids, and/or the organoids in statical orbit.

105. The method of any one of the preceding claims, wherein the bioreactor of (a), (b), (d), (e), (f), (g), (h), and/or (i) comprises a rotating chamber comprising the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, liquid hindgut differentiation culture media, liquid IO maturation culture media, liquid differentiation culture media, and/or liquid organoid maturation culture media;

wherein the average shear stress on the PSCs, the three-dimensional PSC aggregates, the spheroids, and/or the organoids is less than about 5.0 dynes/cm^2.

106. The method of any one of the preceding claims, wherein the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, the liquid hindgut differentiation culture media, the liquid IO maturation culture media, the liquid differentiation culture media, and/or the liquid organoid maturation culture media comprises an anti-apoptotic agent.

107. The method of any one of the preceding claims, wherein the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, the liquid hindgut differentiation culture media, the liquid IO maturation culture media, the liquid differentiation culture media, and/or the liquid organoid maturation culture media comprises an anti-adhesion agent.

108. The method of claim of any one of the preceding claims, wherein the liquid culture media comprises an anti-adhesion agent.

109. The method of claim 108, wherein the anti-adhesion agent is DSS, xantham gum, A-205804, I-CAM1, carboxymethyl cellulose, and/or Neural Organoid Basal Medium 2 (NOBM).

110. The method of any one of claims 108-109, wherein the anti-adhesion agent is DSS at a concentration that is between about 1 μg/ml – 1000 μg/ml of the liquid culture media, the second liquid culture media, the liquid definitive endoderm differentiation culture media, the liquid hindgut differentiation culture media, the liquid IO maturation culture media, the liquid differentiation culture media, and/or the liquid organoid maturation culture media.

111. A composition for a three-dimensional expansion and maintenance of pluripotent stem cell (PSC) cultures, the composition comprising:

a liquid culture media comprising recombinant human basic fibroblast growth factor (rh bFGF), and/or a recombinant human transforming growth factor β (rh TGFβ); and

PSCs suspended in the culture media.

112. The composition of claim 111, wherein the liquid culture media is a serum-free media,

wherein the liquid culture media is free of materials of animal or human origin;

optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

113. The composition of claim 111 or 112, further comprising:

an anti-apoptotic agent.

114. The composition of any one of claims 111-113, wherein the PSCs express Oct4, SSEA1, TRA 1-60, Sox 2, and/or TRA-1-81.

115. The composition of any one of claims 111-114, wherein the PSCs express Oct4, SSEA1, TRA 1-60, Sox 2, and TRA-1-81.

116. The composition of any one of claims 111-115, further comprising:

an anti-adhesion agent.

117. The composition of claim 116, wherein the anti-adhesion agent is one or both of DSS or xantham gum.

118. The composition of any one of claims 111-117, wherein the PSCs are suspended in the liquid culture media at a density that is about 50,000 – 1,000,000 PSCs/ml of culture media.

119. The composition of any one of claims 111-117, wherein the PSCs are suspended in the liquid culture media at a density that is about 100,000 – 300,000 PSCs/ml of culture media

120. The composition of any one of claims 111-119, wherein the PSCs are suspended in the culture media at a density that is about 180,000 - 220,000 PSCs/ml of culture media.

121. A composition for differentiating PSCs into definitive endoderm (DE) in a three dimensional suspension culture, the composition comprising:

a liquid DE differentiation culture media; and

PSCs suspended in the liquid DE differentiation culture media.

122. The composition of claim 121, wherein the liquid DE differentiation culture media is a serum-free media, wherein the liquid DE differentiation culture media is free of materials of animal or human origin; optionally wherein the liquid DE differentiation culture media is free of any extracellular matrix and/or basement membrane matrix.

123. The composition of claim 121 or 122, wherein the PSCs have an average diameter of less than about 500μm.

124. The composition of any one of claims 121-123, wherein the PSCs have an average diameter of less than about 400μm.

125. The composition of any one of claims 121-124, wherein the PSCs have an average diameter of less than about 300μm.

126. The composition of any one of claims 121-125, wherein the liquid DE differentiation culture media comprises a nodal signaling pathway activator and/or a Wnt signaling pathway activator. at a concentration of about 10 to 200 ng/mL of liquid DE differentiation culture media.

127. The composition of claim 126, wherein the nodal signaling pathway activator or the Wnt signaling pathway activator is at a concentration of about 10 to 200 ng/mL of liquid DE differentiation culture media

128. The composition of any one of claims 126-127, wherein the nodal signaling pathway activator or the Wnt signaling pathway activator is at a concentration of about 50 to 150 ng/mL ng/mL of liquid DE differentiation culture media.

129. The composition of any one of claims 126-128, wherein the nodal signaling pathway activator or the Wnt signaling pathway activator at a concentration of about 100 to 200 ng/mL ng/mL of liquid DE differentiation culture media.

130. The composition of any one of claims 126-129, wherein the liquid DE differentiation culture media further comprises serum or a serum replacement at a concentration of about 0% to 20%.

131. The composition of any one of claims 126-130, wherein the liquid DE differentiation culture media further comprises serum or serum replacement at a concentration of about 2% to 5%.

132. The composition of any one of claims 111-131, further comprising:
DE differentiated from the PSCs.

133. The composition of claim 132, wherein the DE differentiated from the PSCs expresses Sox17 and/or FoxA2.

134. The composition of claim 132 or 133, wherein the DE differentiated from the PSCs expresses Sox17 and FoxA2.

135. A composition for differentiating DE into hindgut spheroids (HGS) in a three dimensional suspension culture, the composition comprising:
a liquid hindgut differentiation culture media comprising a Wnt signaling pathway activator, an FGF signaling pathway activator, and optionally FBS; and
DE suspended in the liquid hindgut differentiation culture media.

136. The composition of claim 135, wherein the liquid hindgut differentiation culture media is free of materials of animal or human origin; optionally wherein the culture media is free of any extracellular matrix and/or basement membrane matrix.

137. The composition of claim 135 or 136, wherein the Wnt signaling pathway activator comprises CHIR99021, and wherein the FGF signaling pathway activator comprises FGF4.

138. The composition of any one of claims 135-137, wherein the FGF signaling pathway activator is at a concentration that is at least about 50 ng/ml of the liquid hindgut differentiation culture media.

139. The composition of any one of claims 135-138, wherein the FGF signaling pathway activator is at a concentration that is at least about 500 ng/ml of the liquid hindgut differentiation culture media.

140. The composition of any one of claims 135-139, wherein the Wnt pathway activator is at a concentration that is at least about 0.5 μM of the liquid hindgut differentiation culture media

141. A composition for differentiating HGS into intestinal organoids (IO) in a three dimensional suspension culture, the composition comprising:

a liquid IO maturation culture media comprising EGF; and

HGS suspended in the liquid IO maturation culture media.

142. The composition of claim 141, wherein the liquid IO maturation culture media is free of materials of animal or human origin; optionally wherein the liquid IO maturation culture media is free of any extracellular matrix and/or basement membrane matrix.

143. The composition of claim 141 or 142, wherein lumen of the HGS suspended in the liquid IO maturation culture media is exterior facing relative to the liquid IO maturation culture media.

144. The composition of any one of claims 141-143, wherein the concentration of EGF is at least about 25 ng/ml.

145. The composition of any one of claims 141-144, wherein the concentration of EGF is at least about 100 ng/ml.

146. The composition of any one of claims 141-145, wherein at least a portion of the HGS suspended in the liquid IO maturation culture media comprises dissociated HGS single cells.

147. The composition of claim 146, wherein at least 80% of the HGS are dissociated HGS single cells; optionally wherein at least 90% of HGS are dissociated single cells.

148. The composition of any one of claims 146 or 147, wherein a concentration of the dissociated HGS single cells in the liquid IO maturation culture media is in a range that about $0.1x10^5$-$80x10^5$ dissociated HGS single cells/ml of liquid IO maturation culture media.

149. The composition of any one of claims 146-148, wherein a concentration of the dissociated HGS single cells in the liquid IO maturation culture media is in a range that is about $20x10^5$-$60x10^5$ dissociated HGS single cells/ml of liquid IO maturation culture media.

150. The composition of any one of claims 141-149, further comprising:
IO differentiated from the HGS.

151. The composition of claim 150, wherein epithelial cells of the IO formed from the dissociated HGS single cells have a polarity such that an apical surface is oriented to the inside of the IO.

152. The composition of any one of claims 141-151, wherein the HGS express CdX2.

153. The composition of any one of claims 141-152, wherein the HGS expresses FOX-F1 but does not express SOX2.

154. The composition of claims 141-153, wherein the liquid IO maturation culture media further comprises noggin.

155. A composition comprising:
a liquid culture media; and
three-dimensional PSC aggregates suspended in the liquid culture media.

156. The composition of claim 155, wherein the liquid culture media is free of materials of animal or human origin; optionally wherein the liquid culture media is free of any extracellular matrix and/or basement membrane matrix.

157. The composition of claim 155 or 156, wherein at least a portion of the three-dimensional PSC aggregates are dissociated as single cells.

158. The composition of any one of claims 155-157, wherein an average size of diameters of the three-dimensional PSC aggregates is less than 400 μm

159. The composition of any one of claims 155-158, wherein an average size of diameters of the three-dimensional PSC aggregates is less than 350 μm.

160. The composition of any one of claims 155-159, wherein an average size of diameters of the three-dimensional PSC aggregates is less than 300 μm.

161. The composition of any one of claims 155-160, further comprising:
an anti-adhesion agent.

162. The composition of claim 161, wherein the anti-adhesion agent is DSS, xantham gum, A-205804, I-CAM1, carboxymethyl cellulose, and/or Neural Organoid Basal Medium 2 (NOBM).

163. The composition of claim 161 or 162, wherein the anti-adhesion agent is at a concentration that is between about 1 μg/ml – 1000 μg/ml of the liquid culture media.

102
104
106
108
2D Cell Culture
Bioreactor Inoculation
Serial Passaging
Analysis
6 well Matrigel-coated
tissue culture plate
Culture Media: TeSR AOF
Culture Media: TeSR AOF
Spin Speed: 6 RPM
Inoculation Density: 2e6 Cells
Culture Media: TeSR AOF
Spin Speed: Changed Daily
Media Change: Alternate Days
Daily Measurements
• Nutrients
• Aggregate Size Distribution
Final Readouts
• Aggregate Size Distribution
• Flow Cytometry
• Immunohistochemistry
• qRT-PCR
• Tri-Lineage Differentiation

FIG. 1A

FORCE BY UPLIFT MOVEMENT
FORCE BY GRAVITY
1
2
3

FIG. 1B

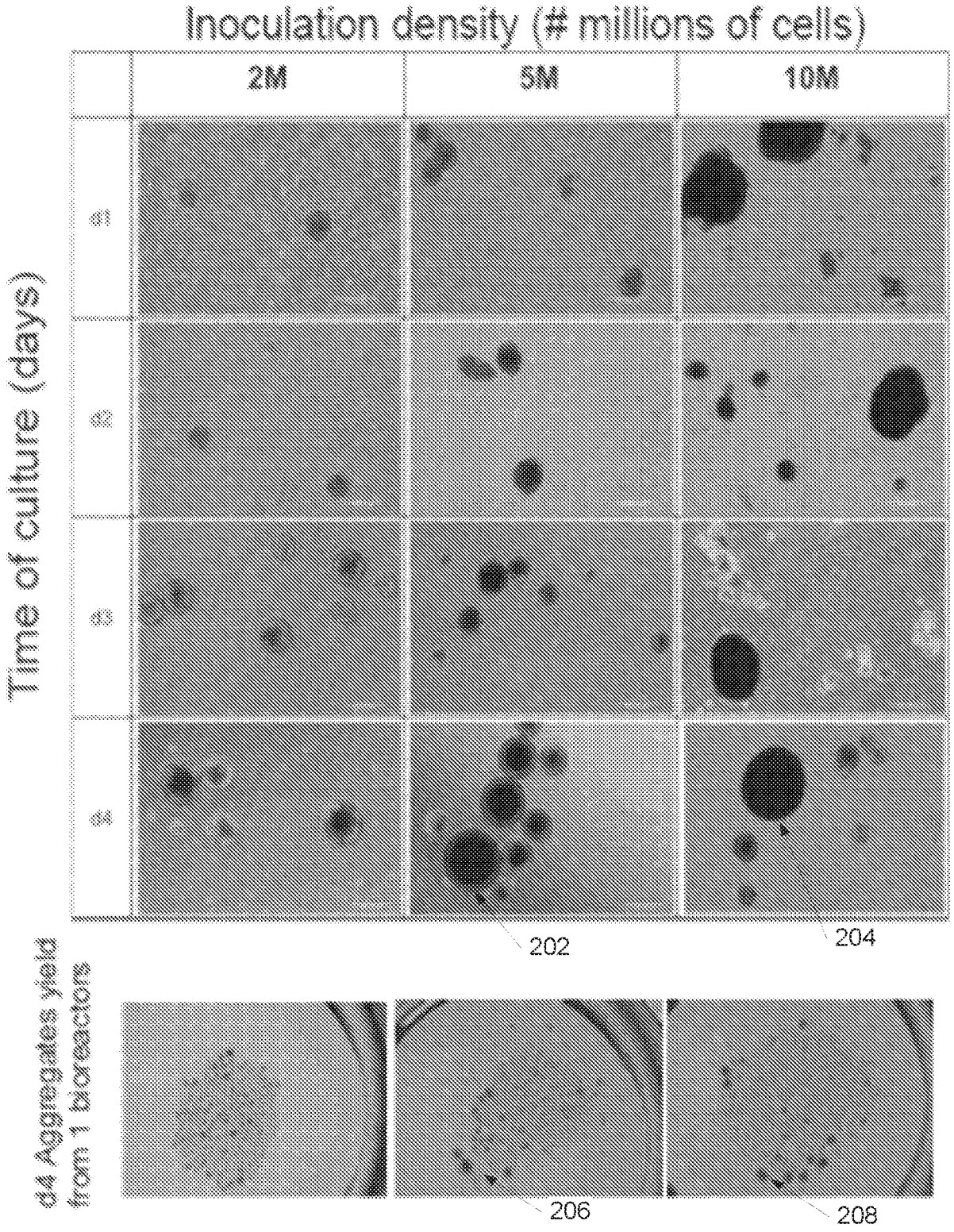

Inoculation density (# millions of cells)
2M
5M
10M
Time of culture (days)
d1
d2
d3
d4
202
204
d4 Aggregates yield from 1 bioreactors
206
208

FIG. 2

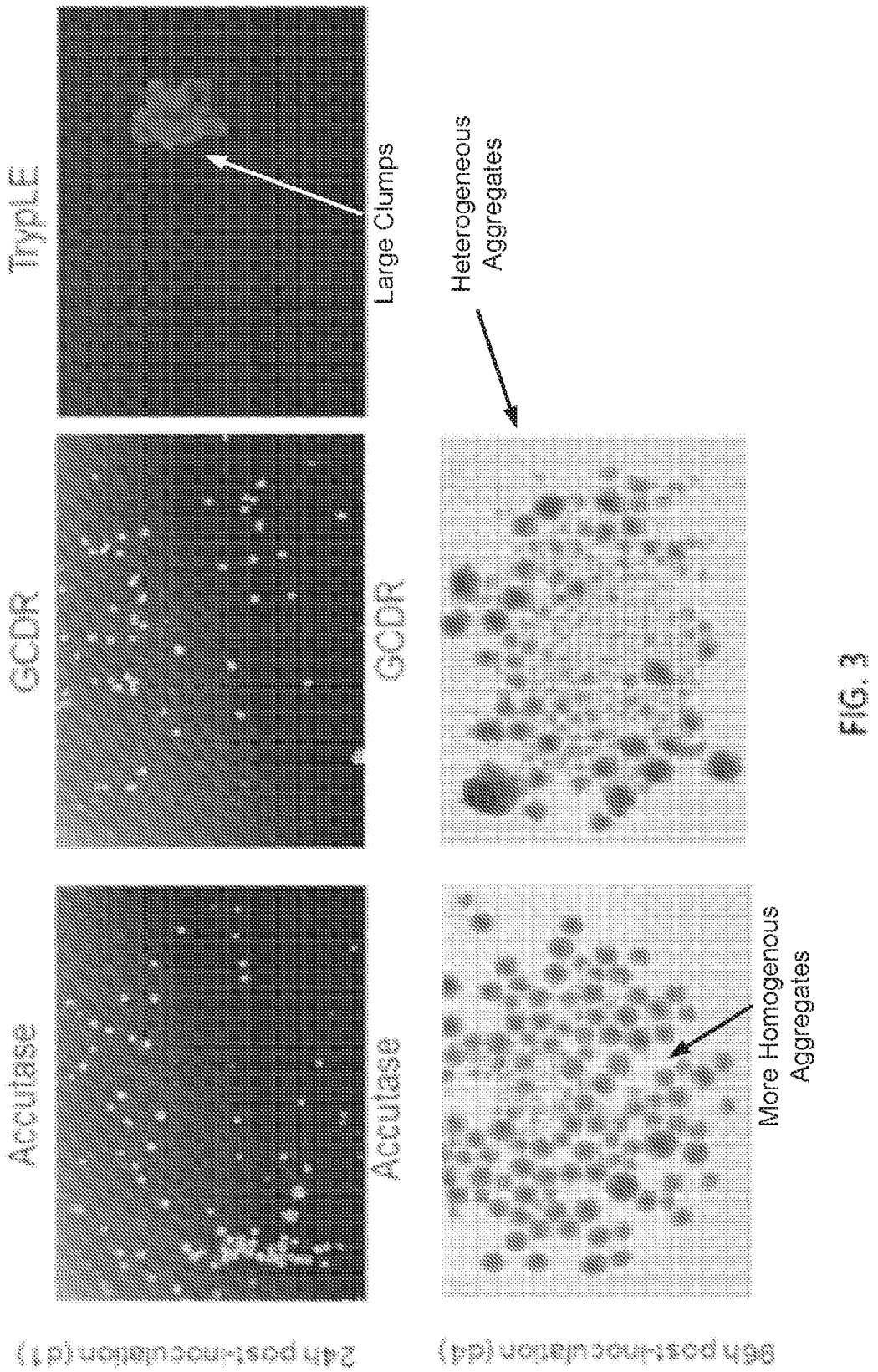

TrypLE
Large Clumps
GCDR
GCDR
Heterogeneous Aggregates
Accutase
Accutase
More Homogenous Aggregates
2-h post-inoculation (d1)
6-h post-inoculation (d4)

FIG. 3

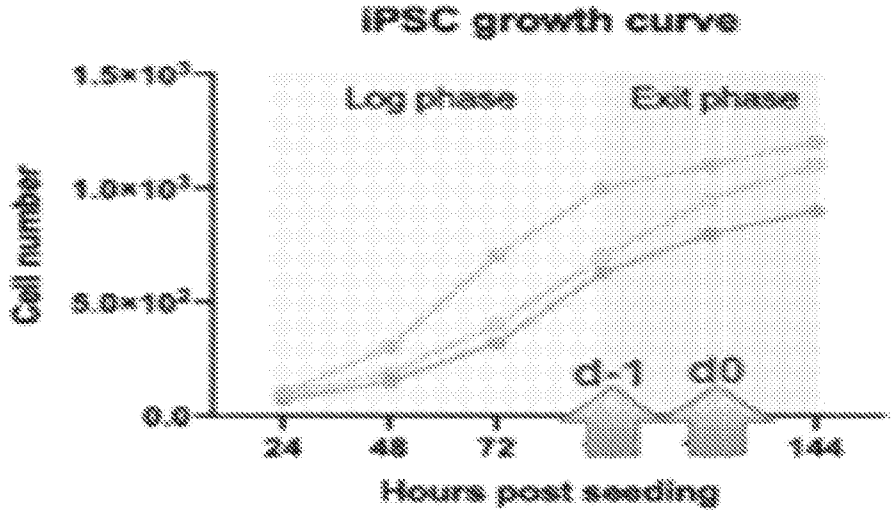


FIG. 4A

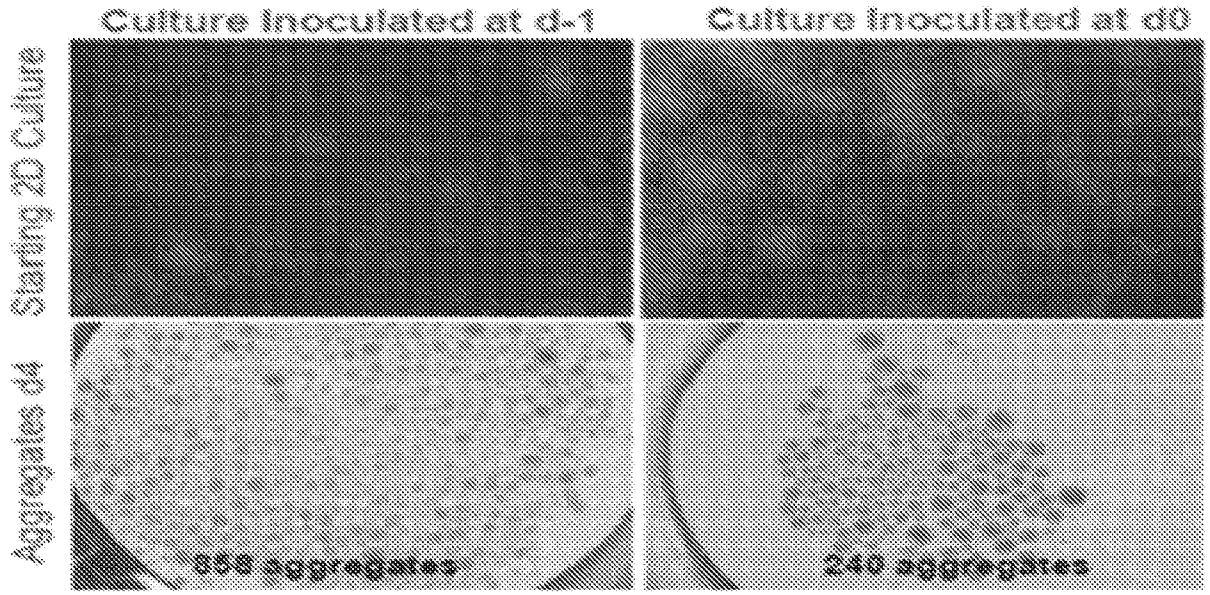


FIG. 4B

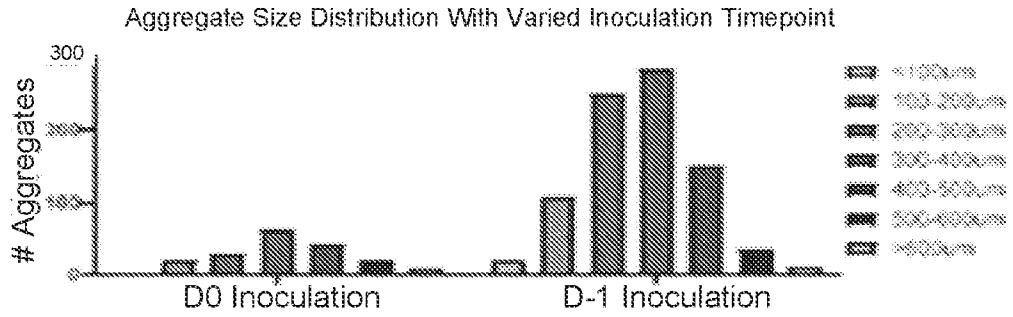


FIG. 4C

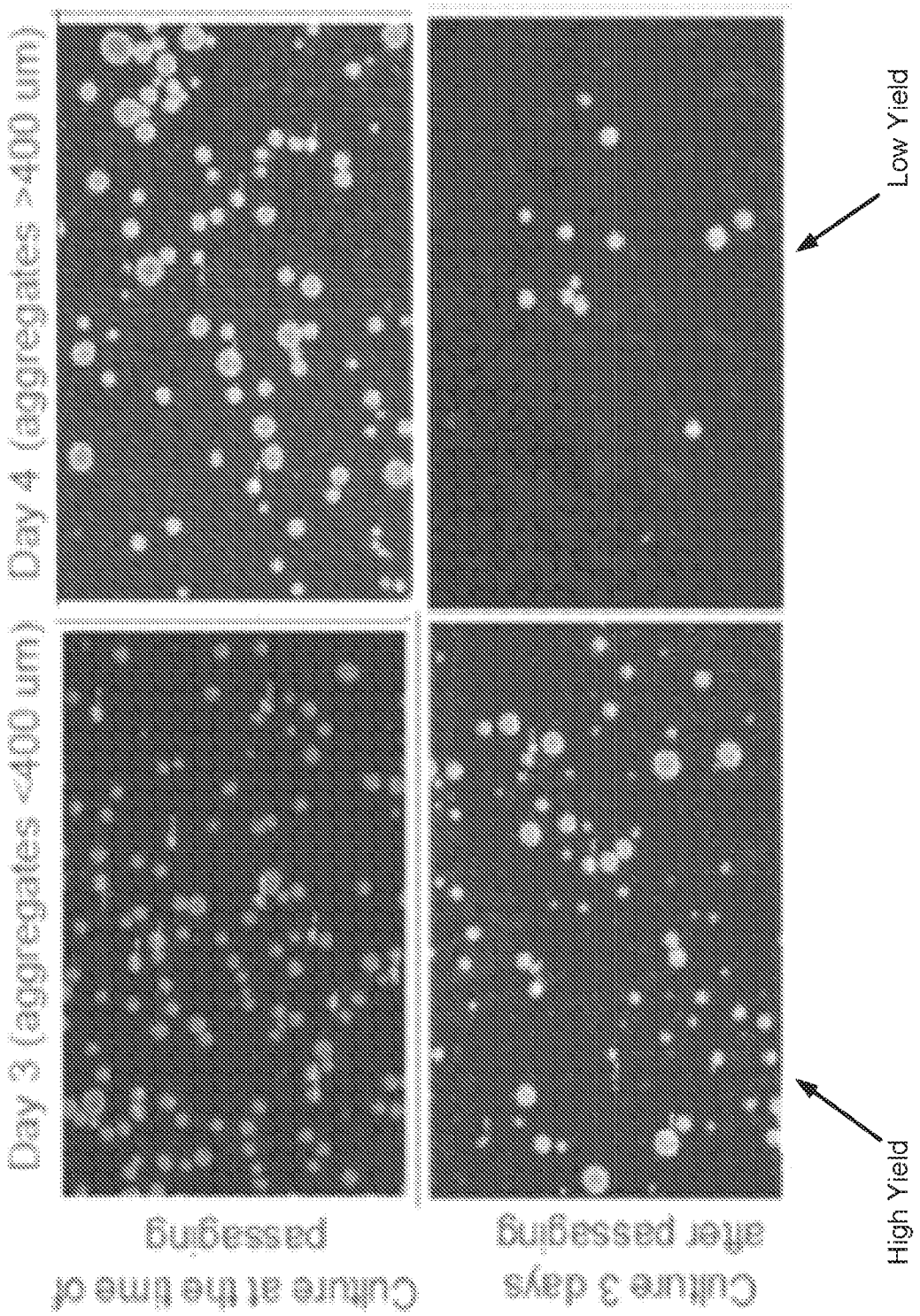

Culture 3 days after passaging
Culture at the time of passaging
Day 3 (aggregates <400 um)
Day 4 (aggregates >400 um)
High Yield
Low Yield

FIG. 5

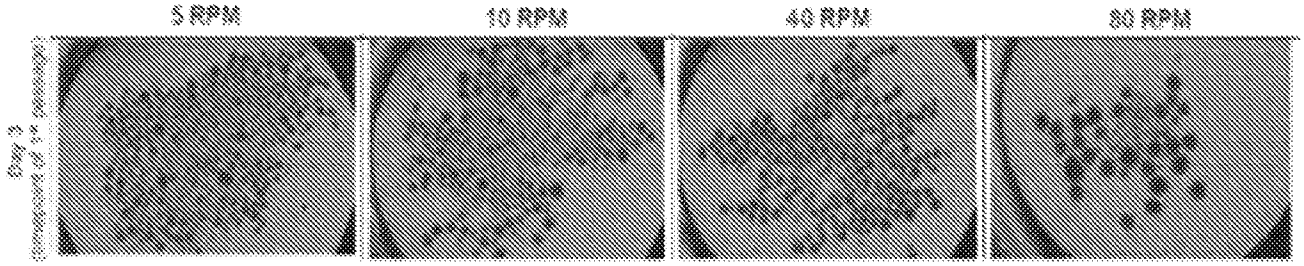


FIG. 6A

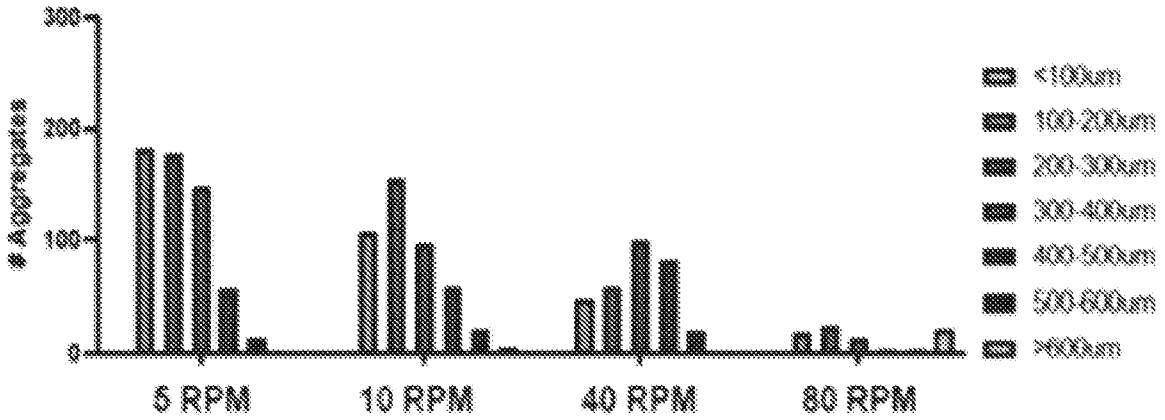
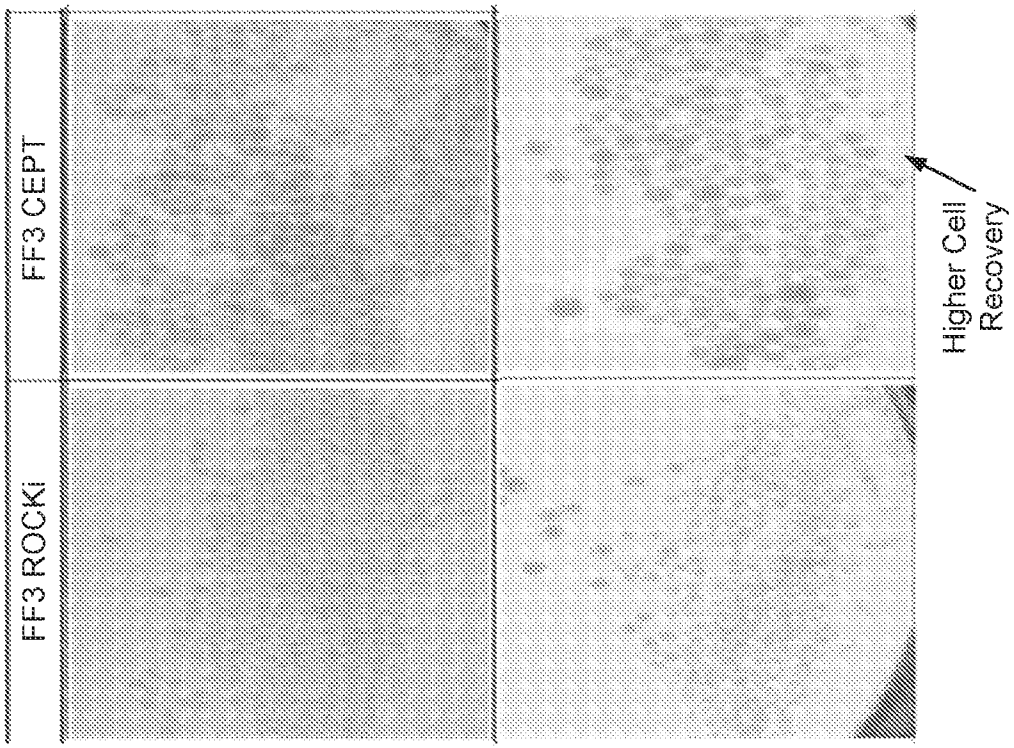


FIG. 6B

Consistent cellular expansion

	5RPM	10RPM	40RPM	80RPM
Inoculum (# million of cells)	2	2	2	2
Output at p1 (# millions of cell) Fold expansion at p1 (output/inoculum)	**4.48** 2.5X	**5.7** 3.0X	**5.6** 3.0X	**1.4** 0.75X
Output at p2 (# millions of cell) Fold expansion at p1(output/inoculum)	**5.24** 2.6X	**1.9** 0.95X	**6.12** 3.0X	-
Output at p3 (# millions of cell) Fold expansion at p1(output/inoculum)	**7.36** 3.68X	-	**6.88** 3.44X	-

FIG. 6C


FF3 ROCKi
FF3 CEPT
Higher Cell Recovery

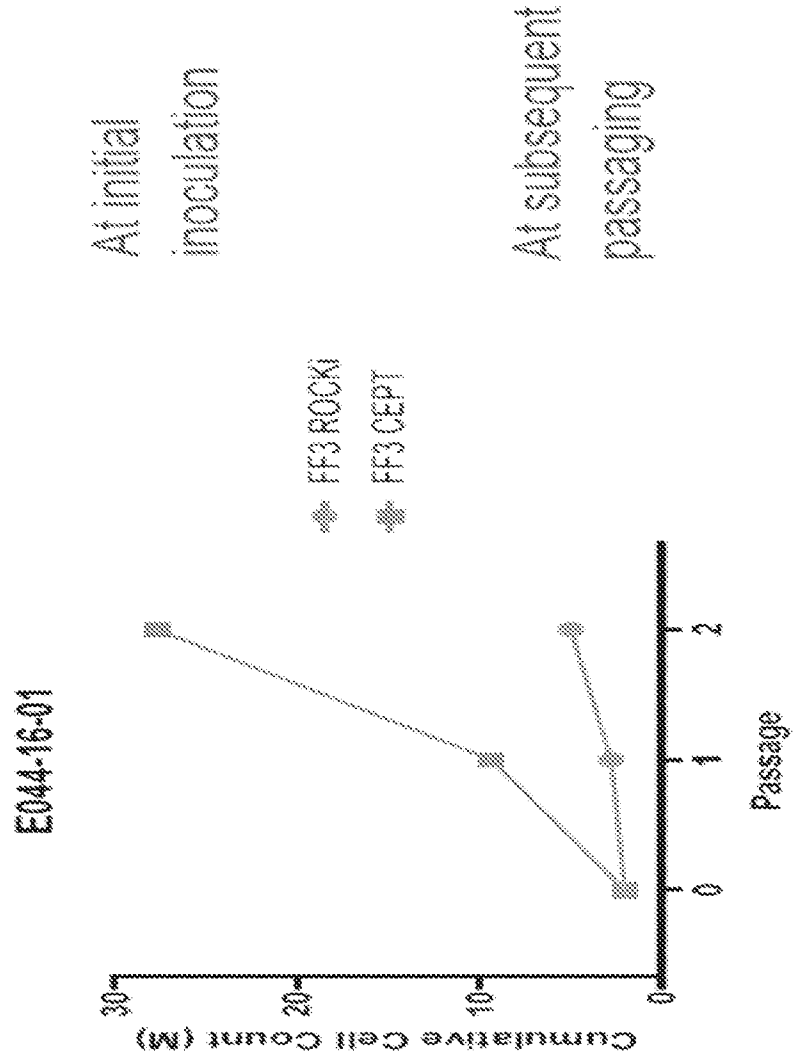

At initial inoculation
At subsequent passaging
FF3 ROCKi
FF3 CEPT
E044-16-01
Cumulative Cell Count (M)
30
20
10
0
0
1
2
Passage

FIG. 7

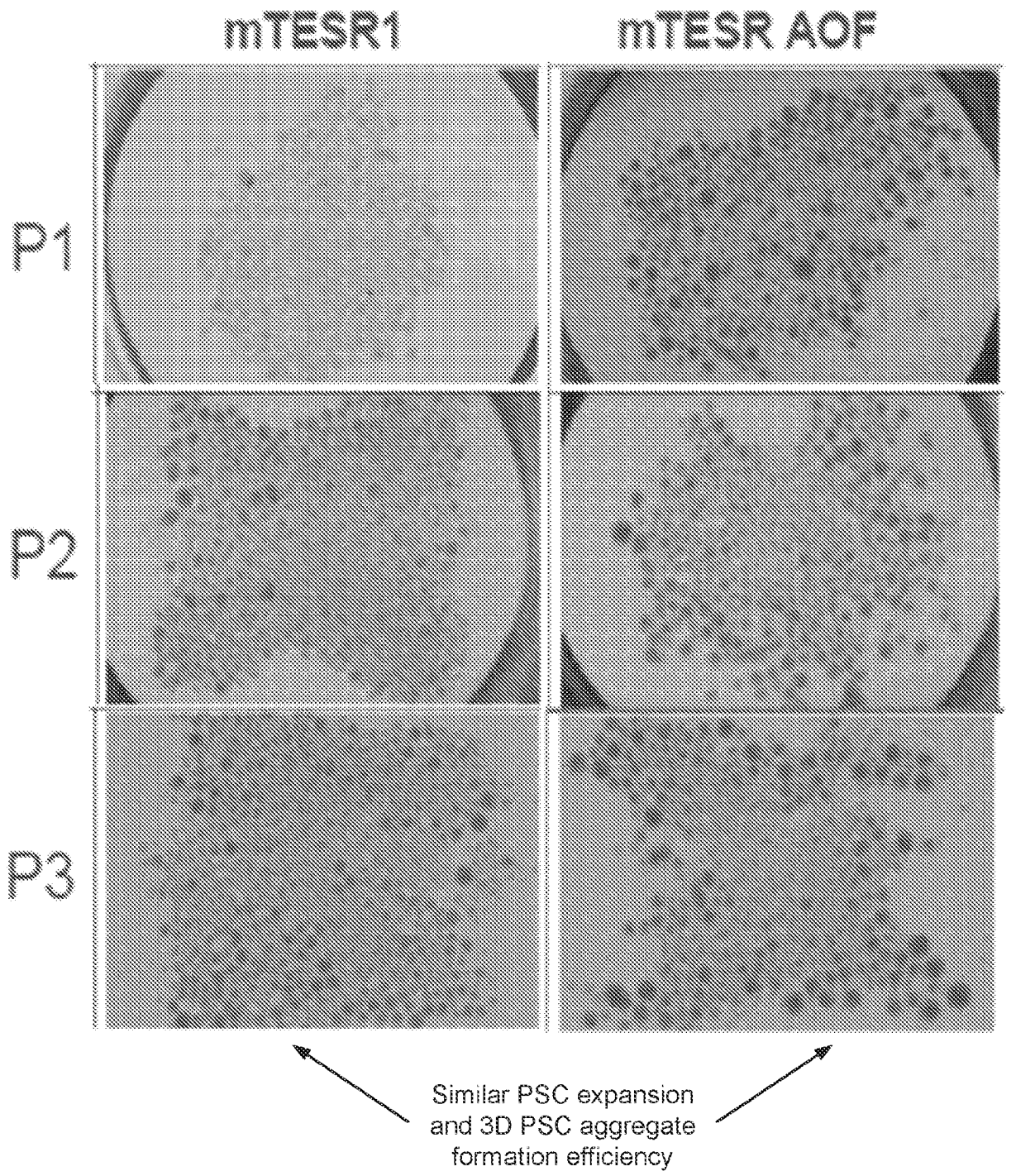

mTESR1
mTESR AOF
P1
P2
P3
Similar PSC expansion and 3D PSC aggregate formation efficiency

FIG. 8

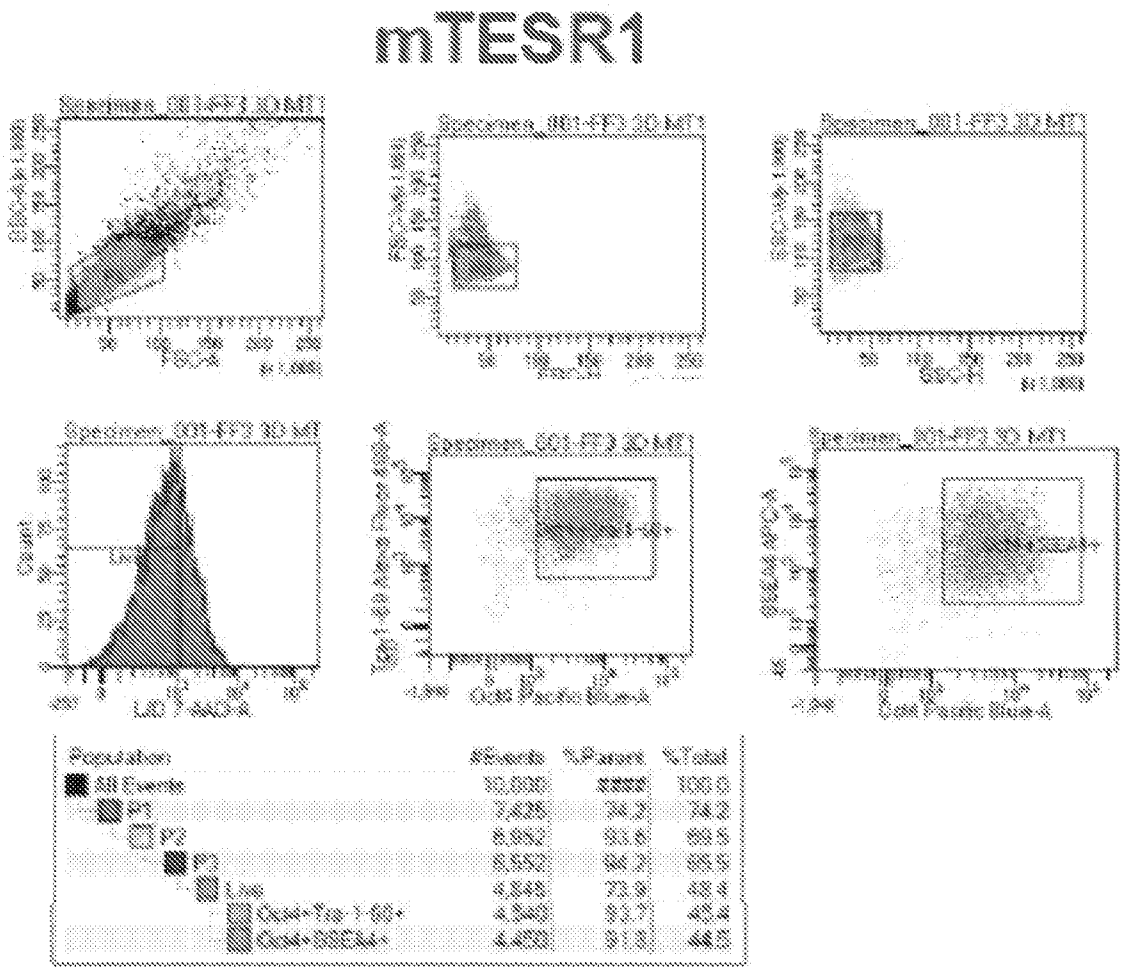


FIG. 9A

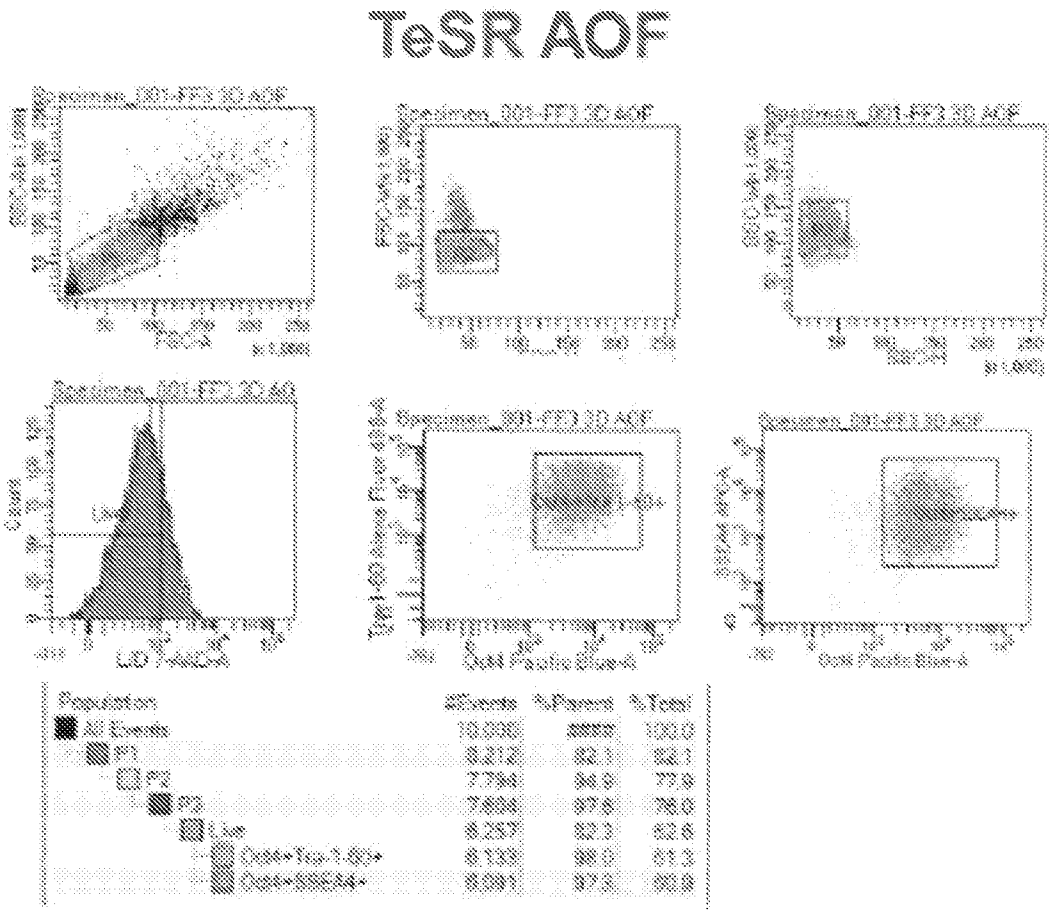


FIG. 9B

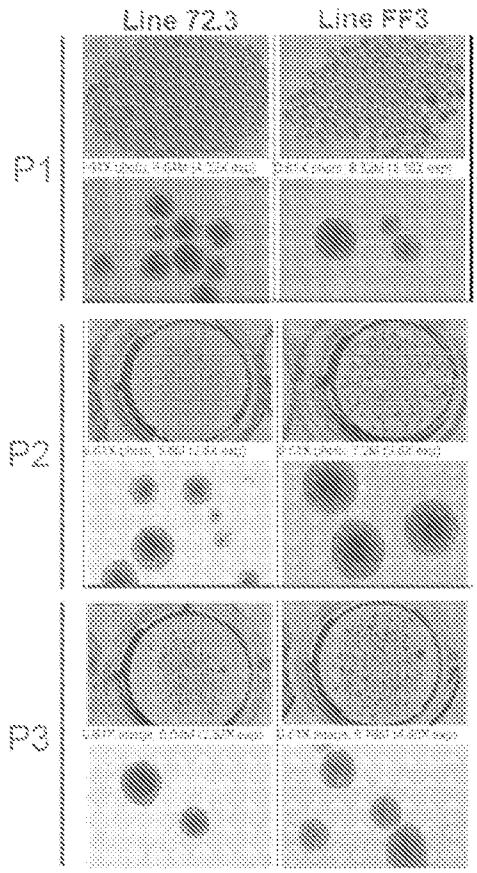


FIG. 10

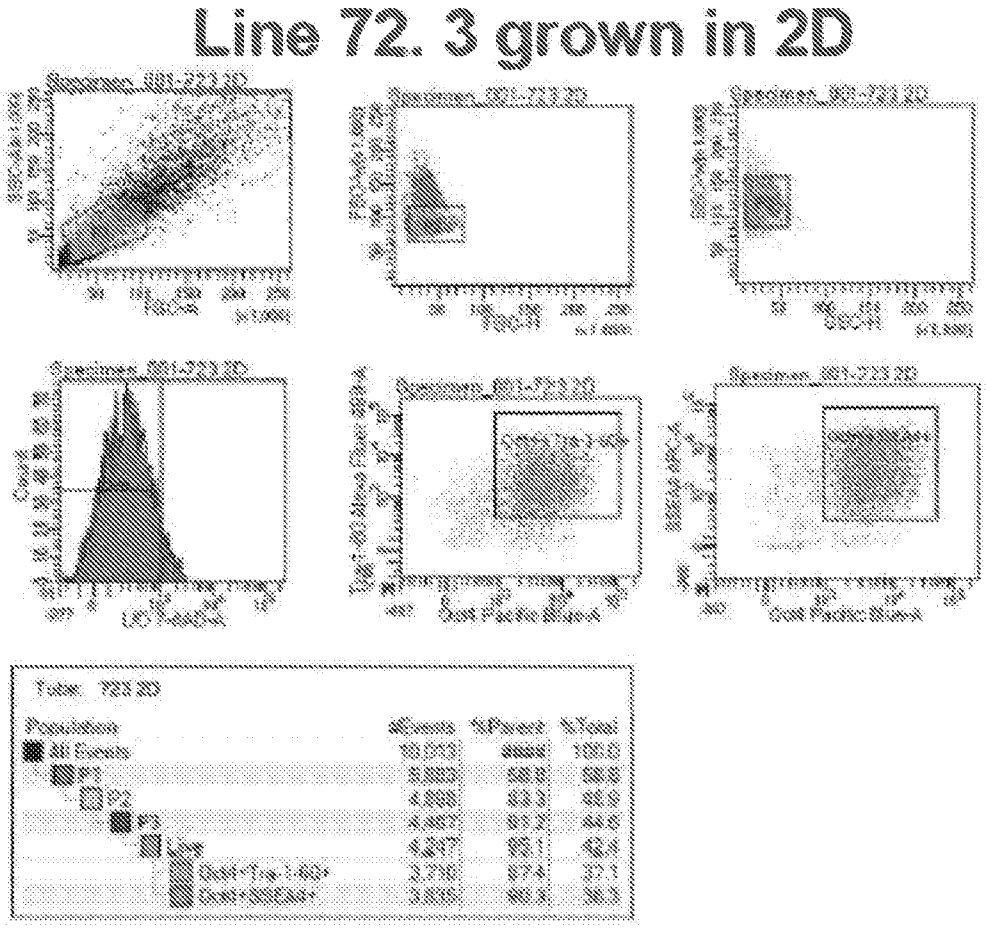


FIG. 11A

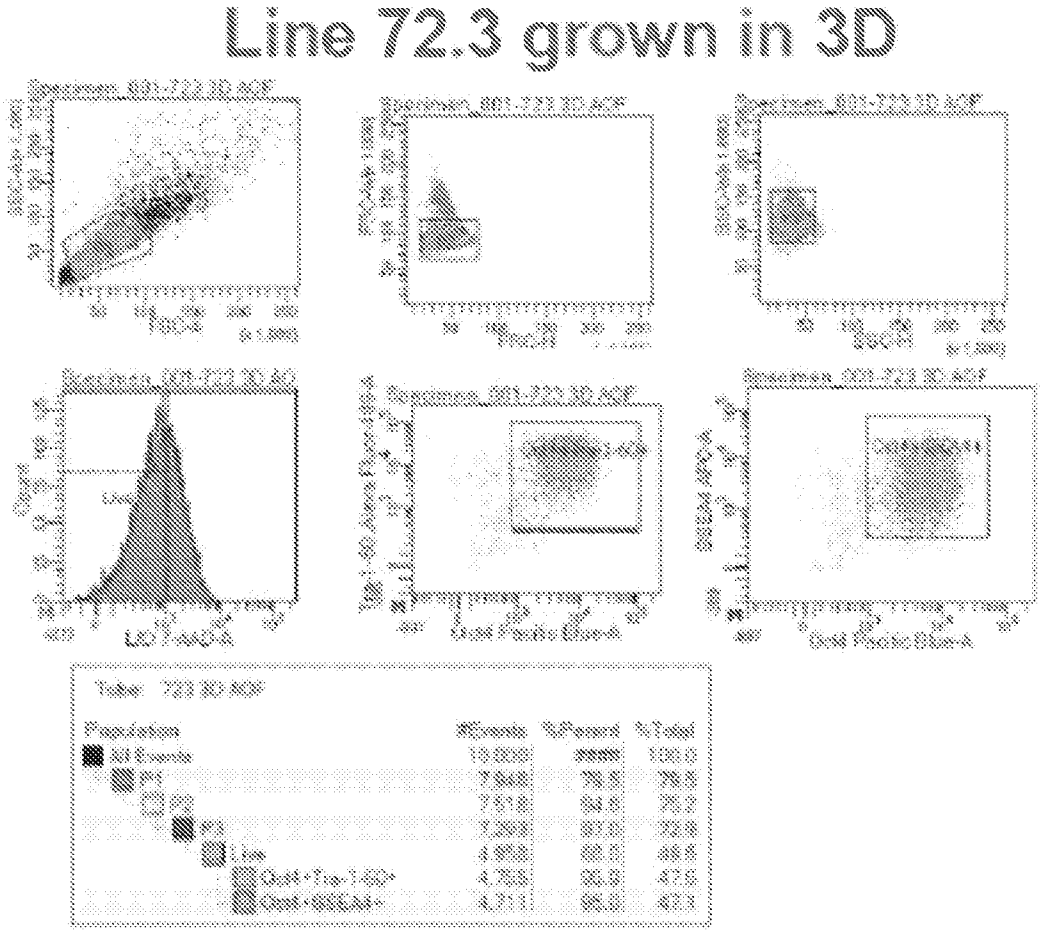


FIG. 11B

Line FF3 grown in 2D

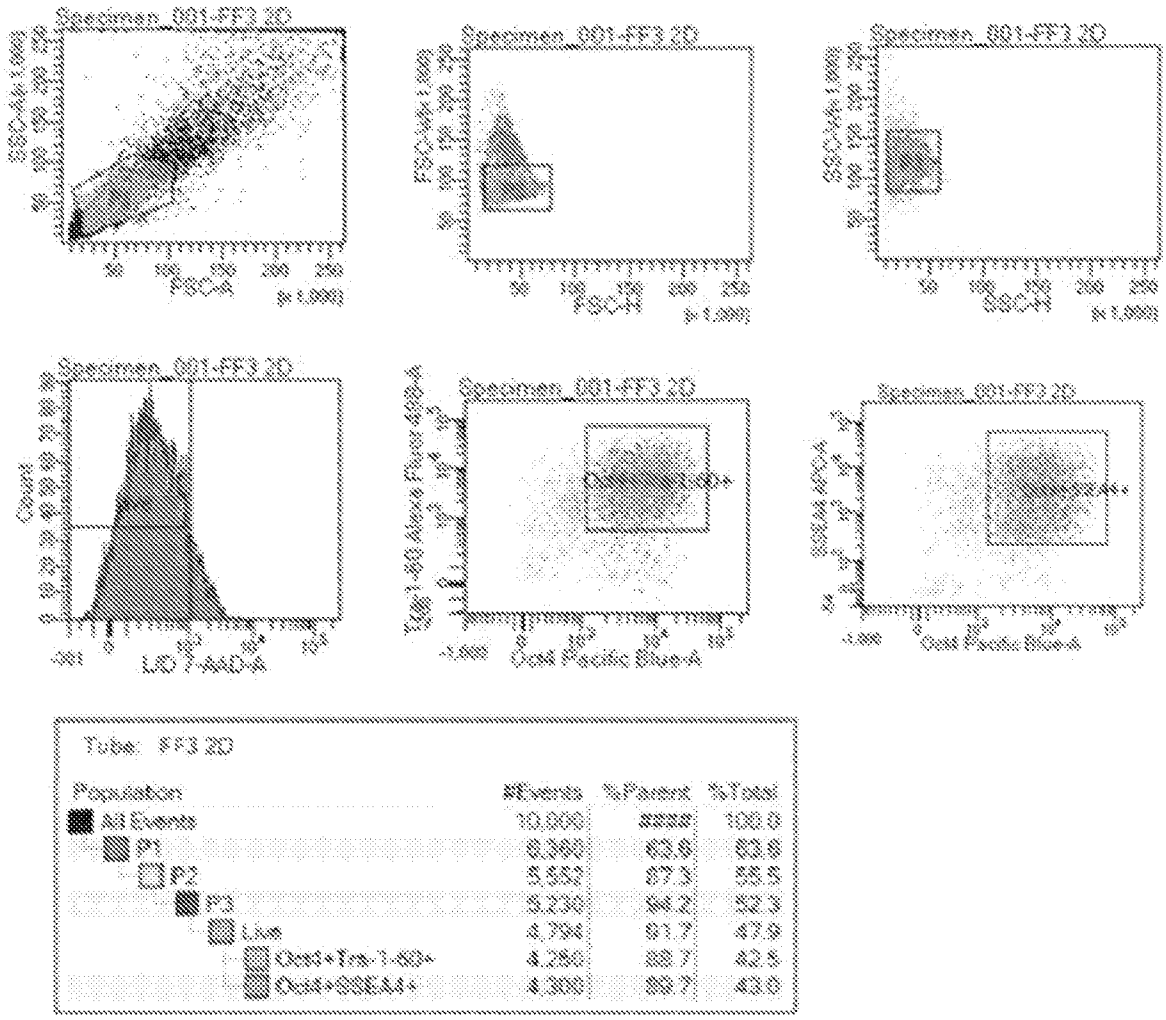


FIG. 12A

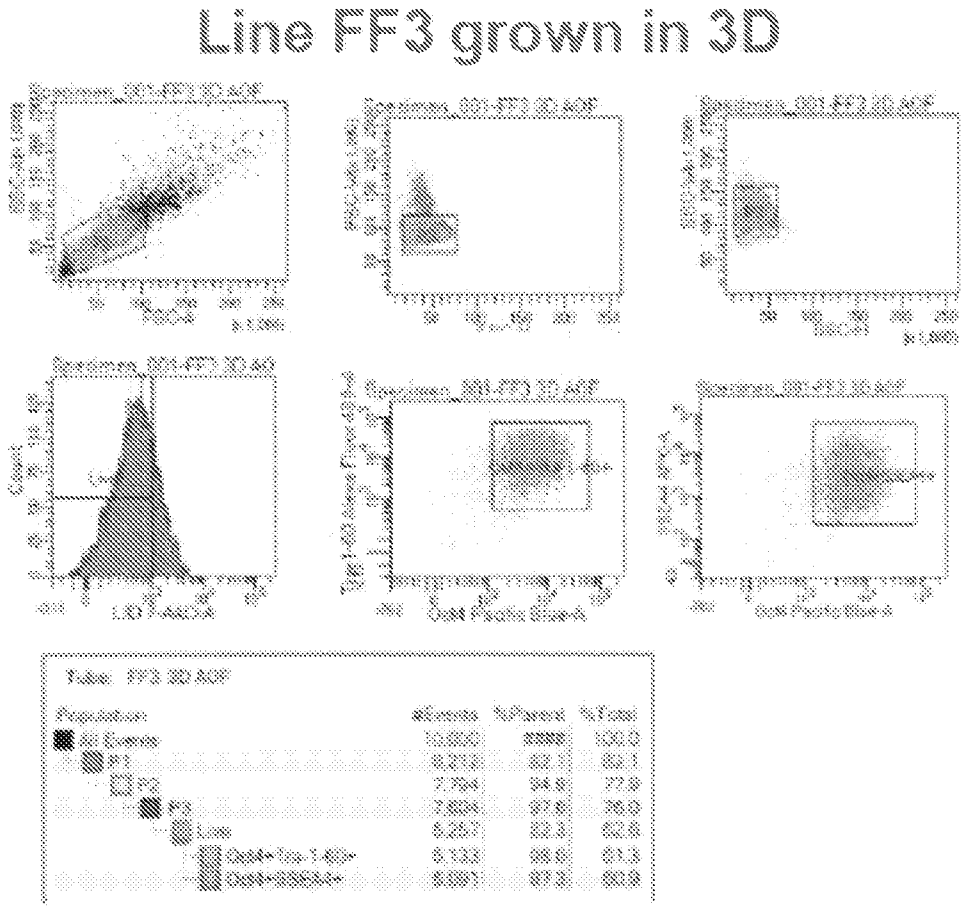


FIG. 12B

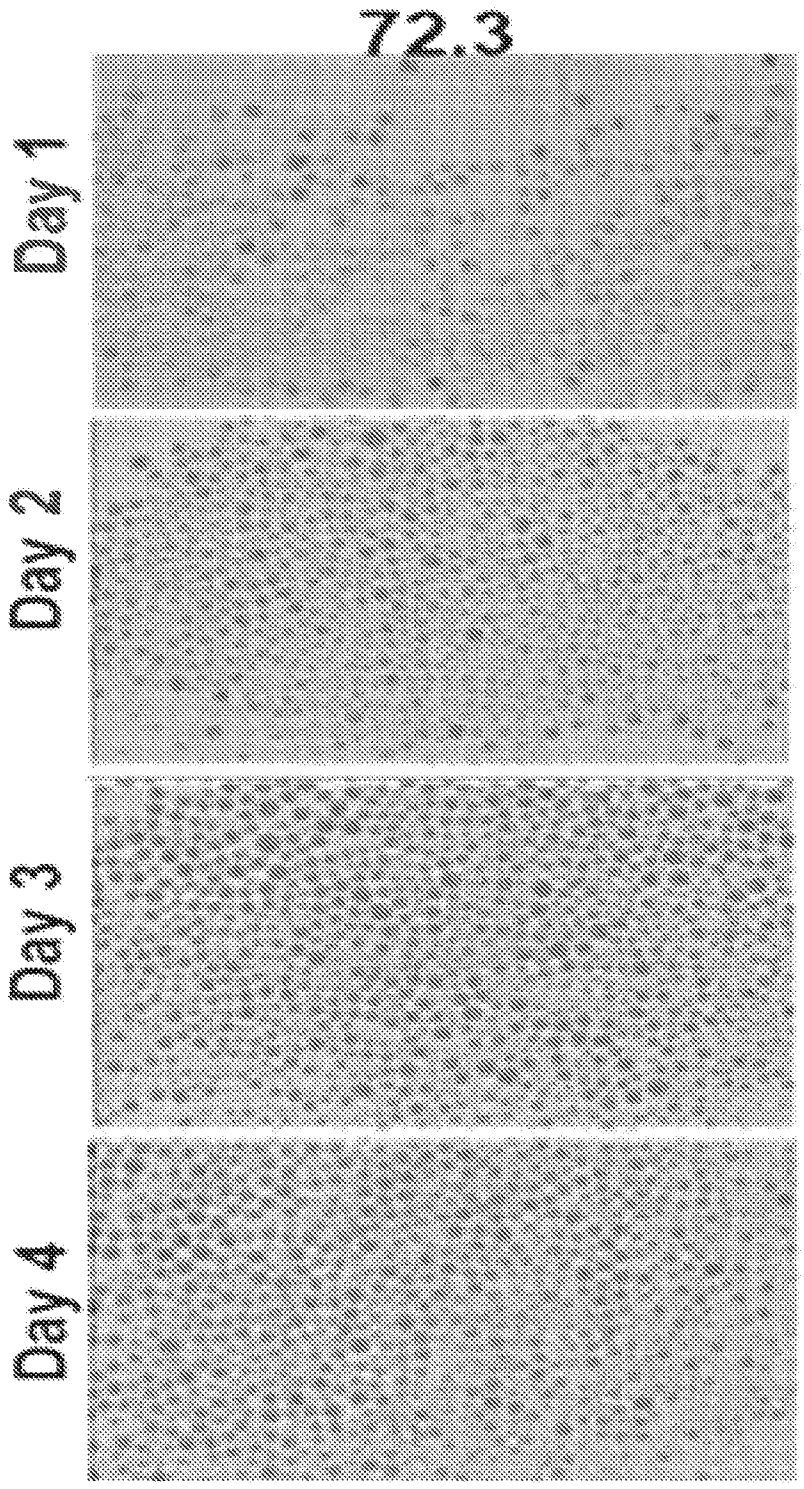


FIG. 13A

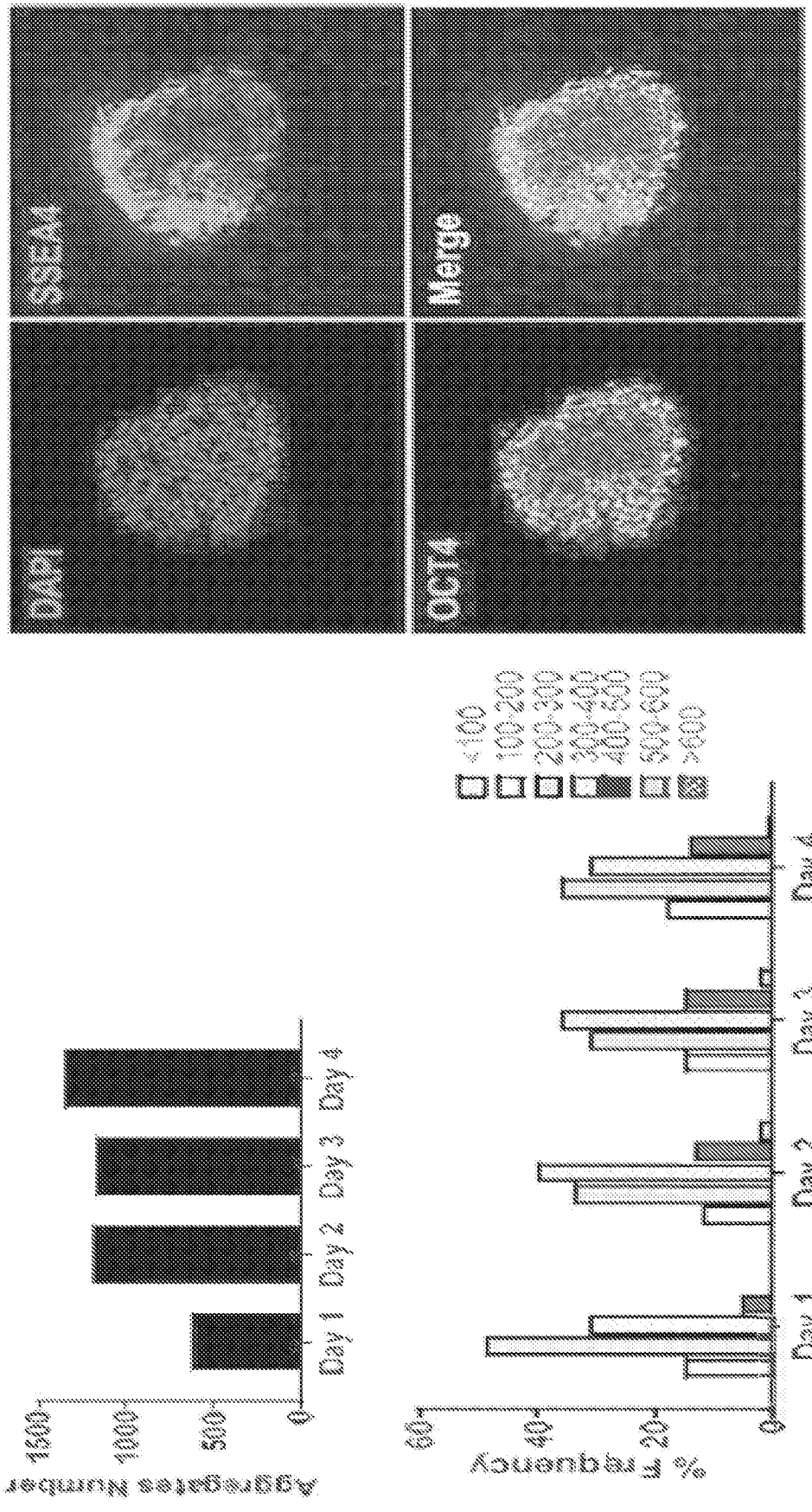

SSEA4
Merge
OCT4
DAPI
% Frequency
0
20
40
60
Day 1
Day 2
Day 3
Day 4
<100
100-200
200-300
300-400
400-500
500-600
>600
Aggregates Number
0
500
1000
1500
Day 1
Day 2
Day 3
Day 4

FIG. 13B

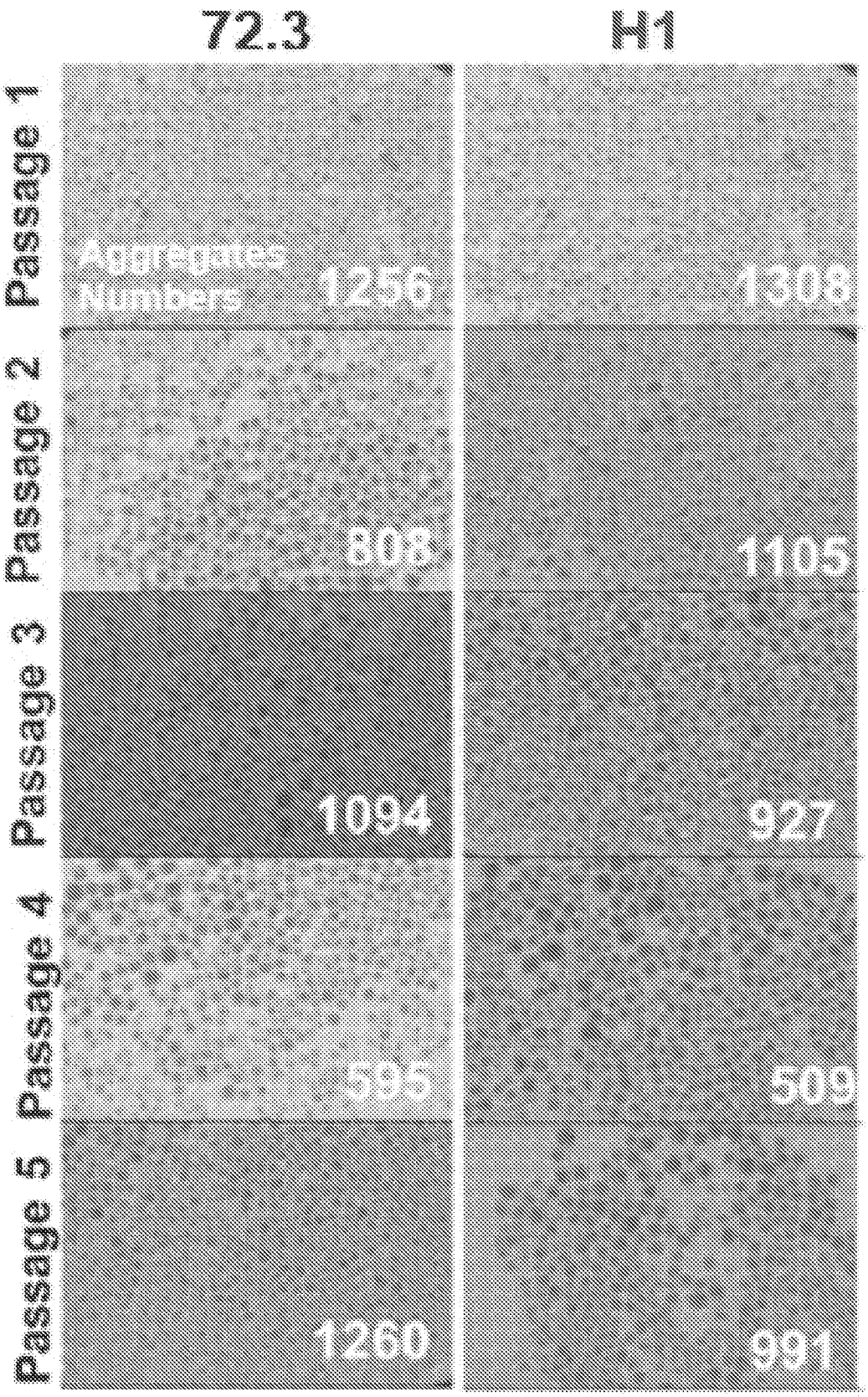

72.3
H1
Passage 1
Passage 2
Passage 3
Passage 4
Passage 5
Aggregates Numbers
1256
1308
808
1105
1094
927
595
509
1260
991

FIG. 14A

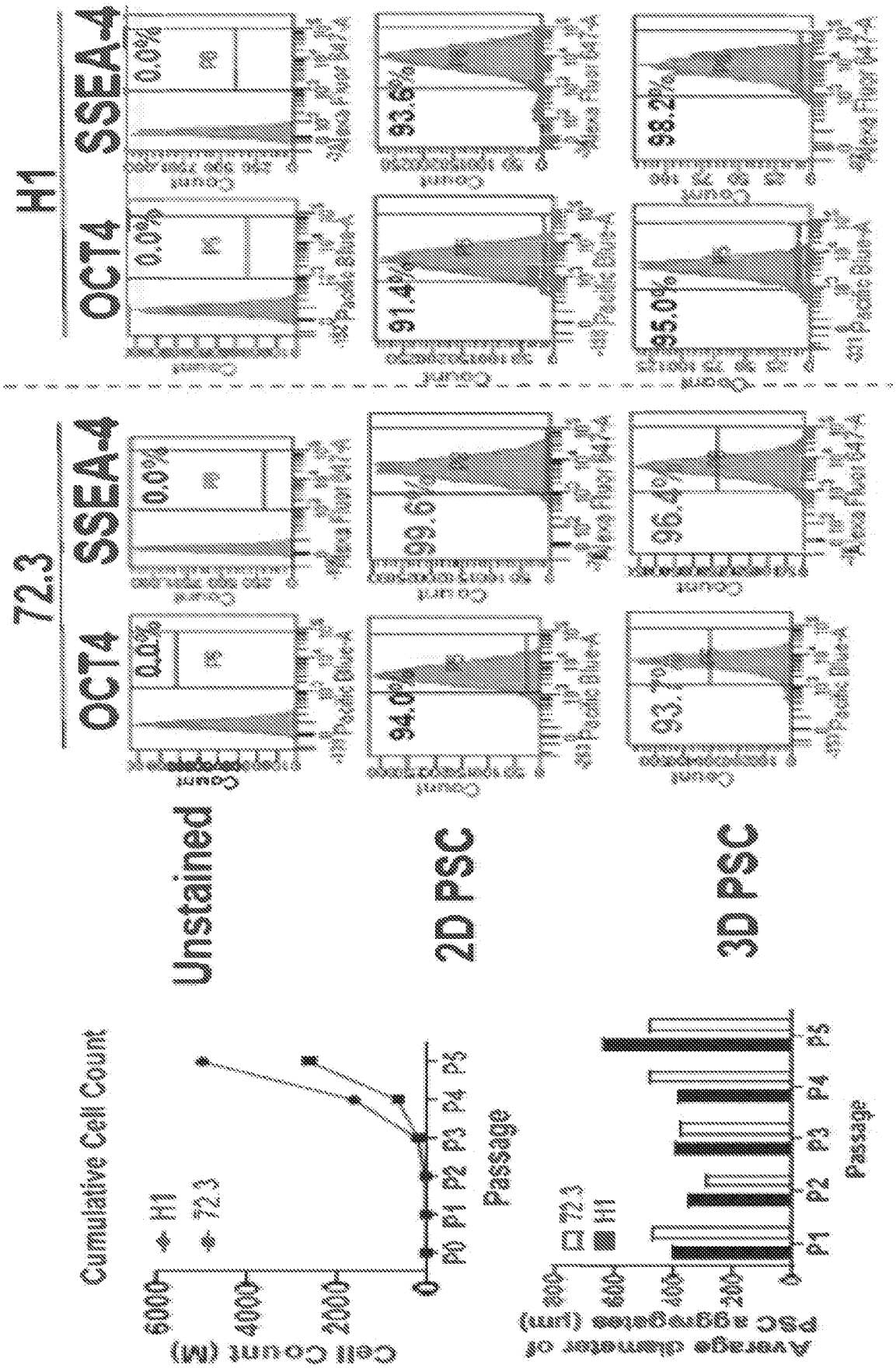

H1
OCT4
SSEA-4
72.3
Unstained
2D PSC
3D PSC
0.0%
91.4%
93.6%
95.0%
98.2%
94.0%
96.4%
Cumulative Cell Count
Cell Count (M)
6000
4000
2000
0
H1
72.3
P0 P1 P2 P3 P4 P5
Passage
Average diameter of PSC aggregates (μm)
400
600
400
200
0
72.3
H1
P1 P2 P3 P4 P5
Passage

FIG. 14B

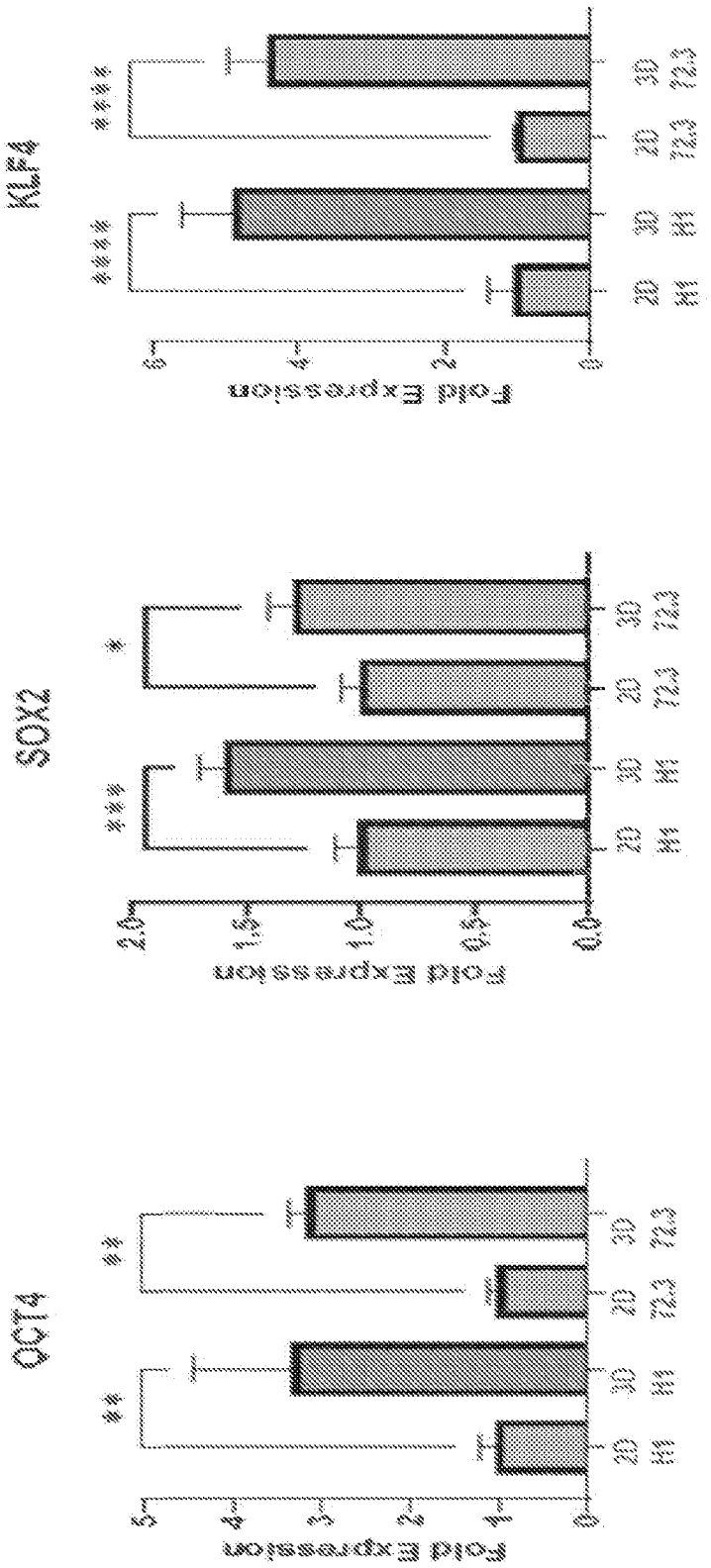

KLF4
SOX2
OCT4
Fold Expression
20 H1
30 H1
20 72.3
30 72.3
T
*
**
***
****
0.0
0.5
1.0
1.5
2.0
0
2
4
6

FIG. 15

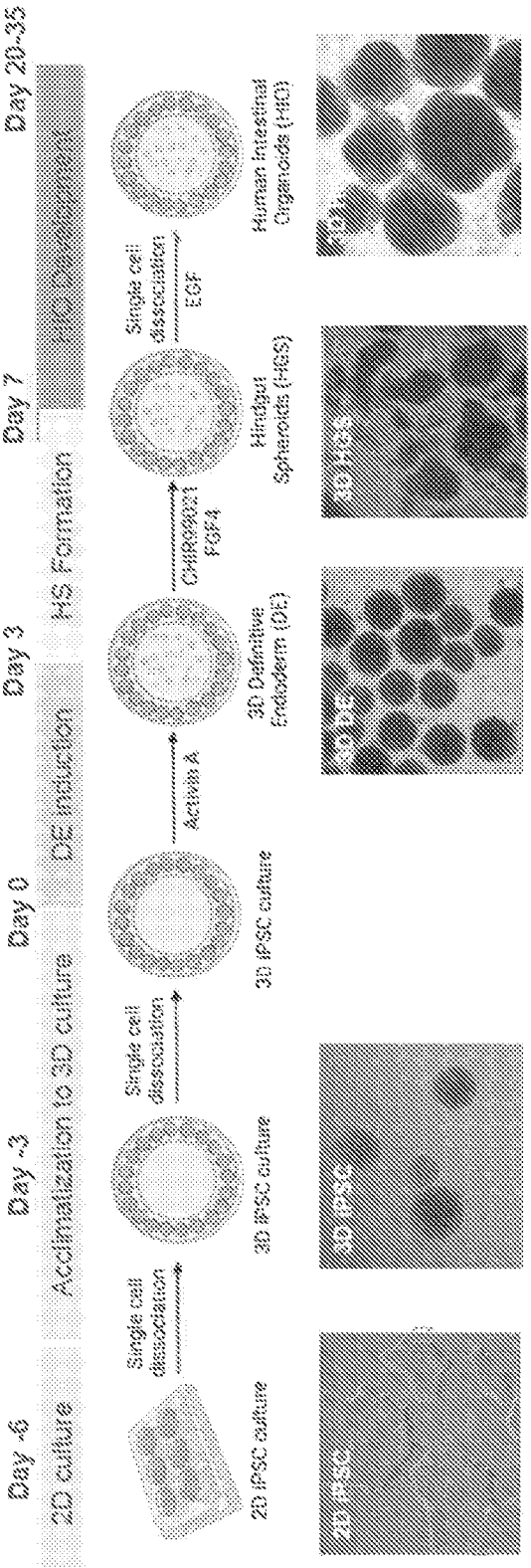

Day -6
Day -3
Day 0
Day 3
Day 7
Day 20-35
2D culture
Acclimatization to 3D culture
DE induction
HS Formation
Single cell dissociation
Single cell dissociation
Single cell dissociation
2D iPSC culture
3D iPSC culture
3D iPSC culture
Activin A
CHIR99021
FGF4
EGF
3D Definitive Endoderm (DE)
Hindgut Spheroids (HBS)
Human Intestinal Organoids (HIO)
2D iPSC
3D iPSC
3D DE
3D HBS
HIO

FIG. 16

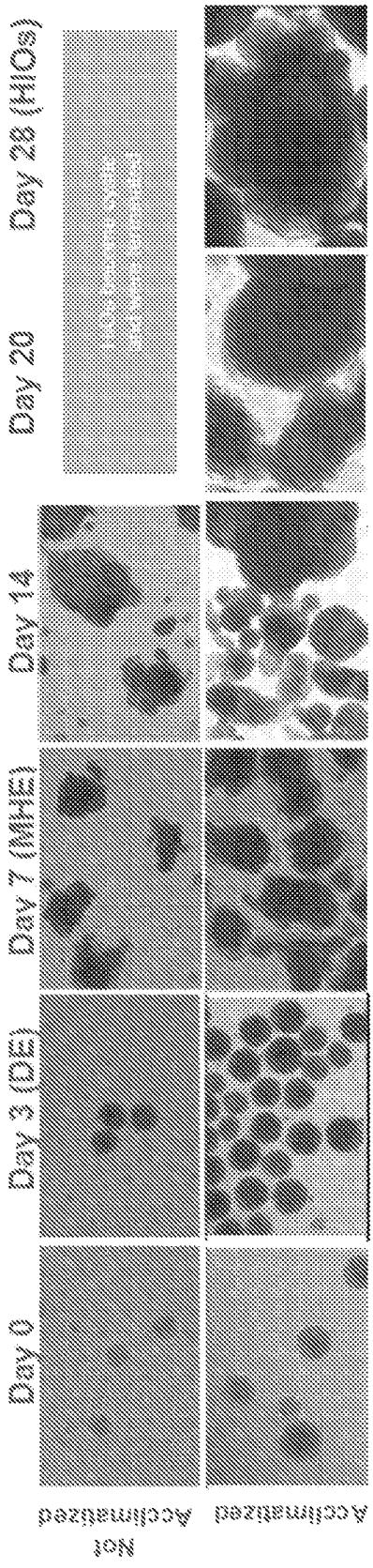

Day 0
Day 3 (DE)
Day 7 (MHE)
Day 14
Day 20
Day 28 (HIOs)
Not Acclimatized
Acclimatized

FIG 17

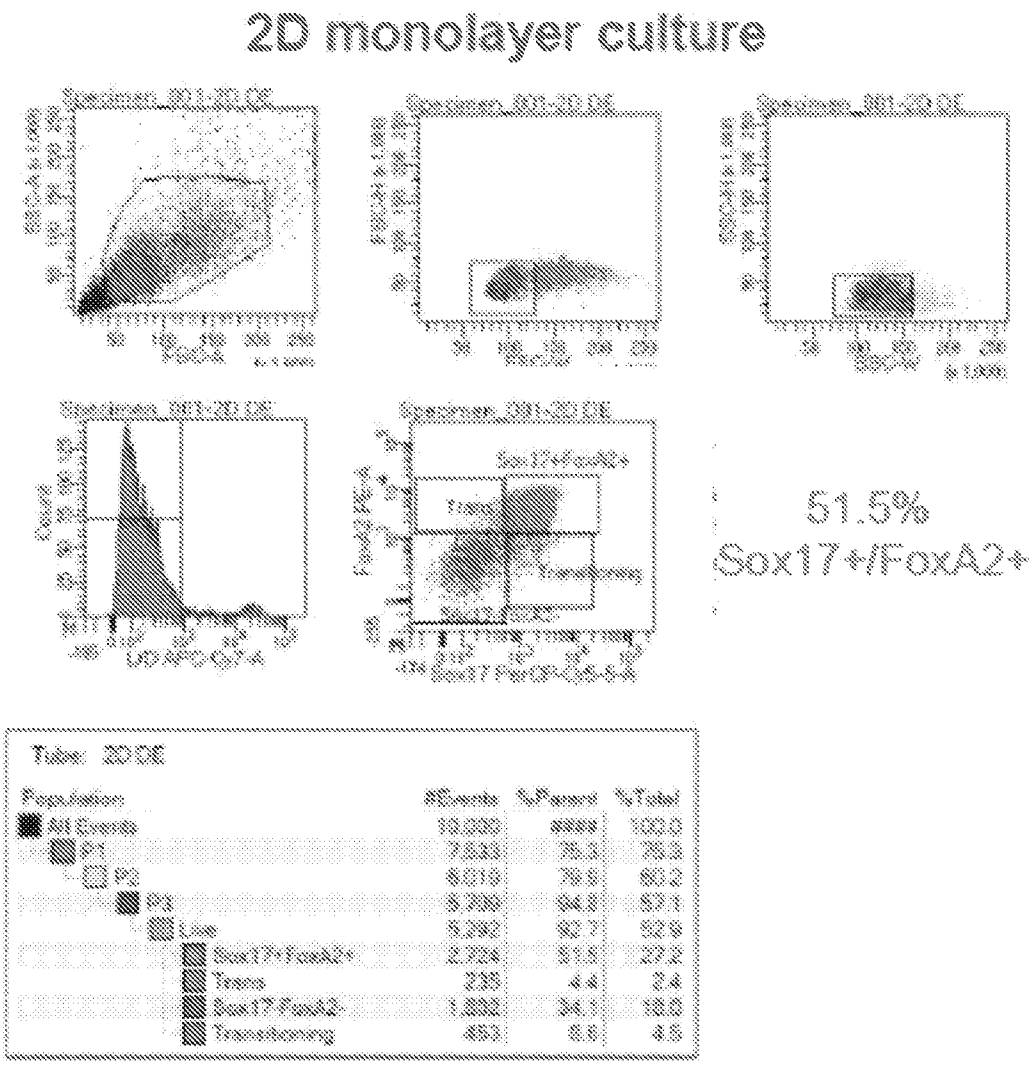


FIG. 18A

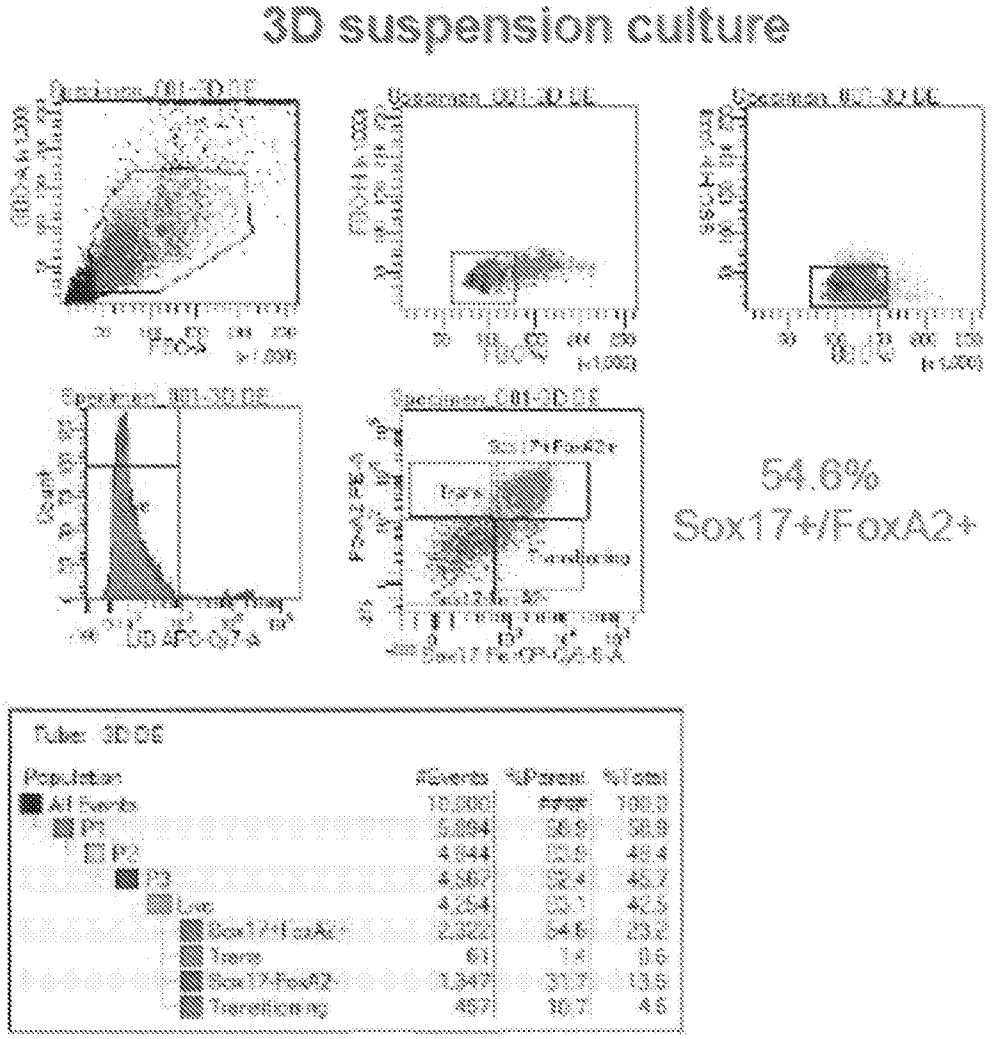


FIG. 18B

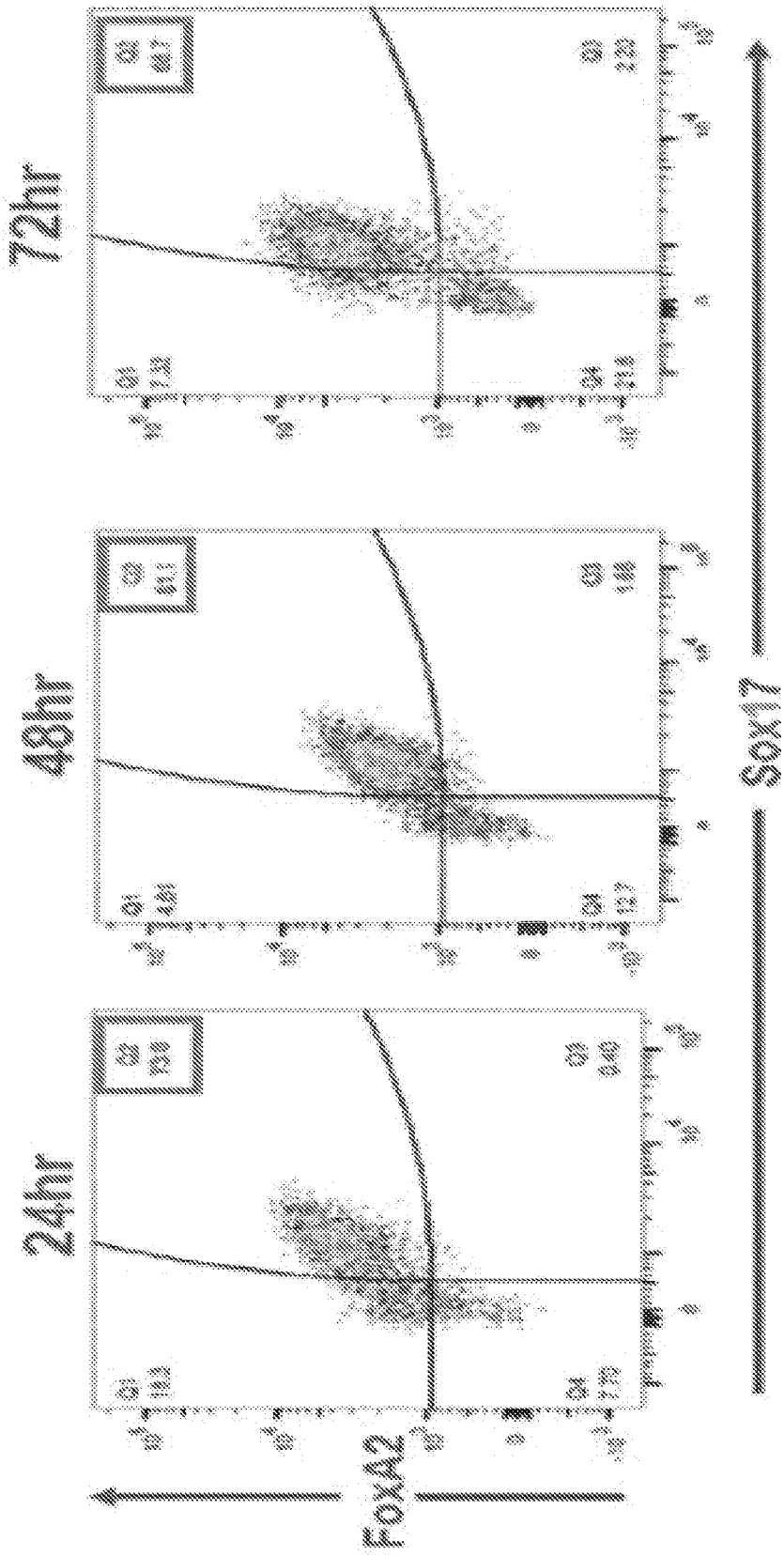


FIG. 19

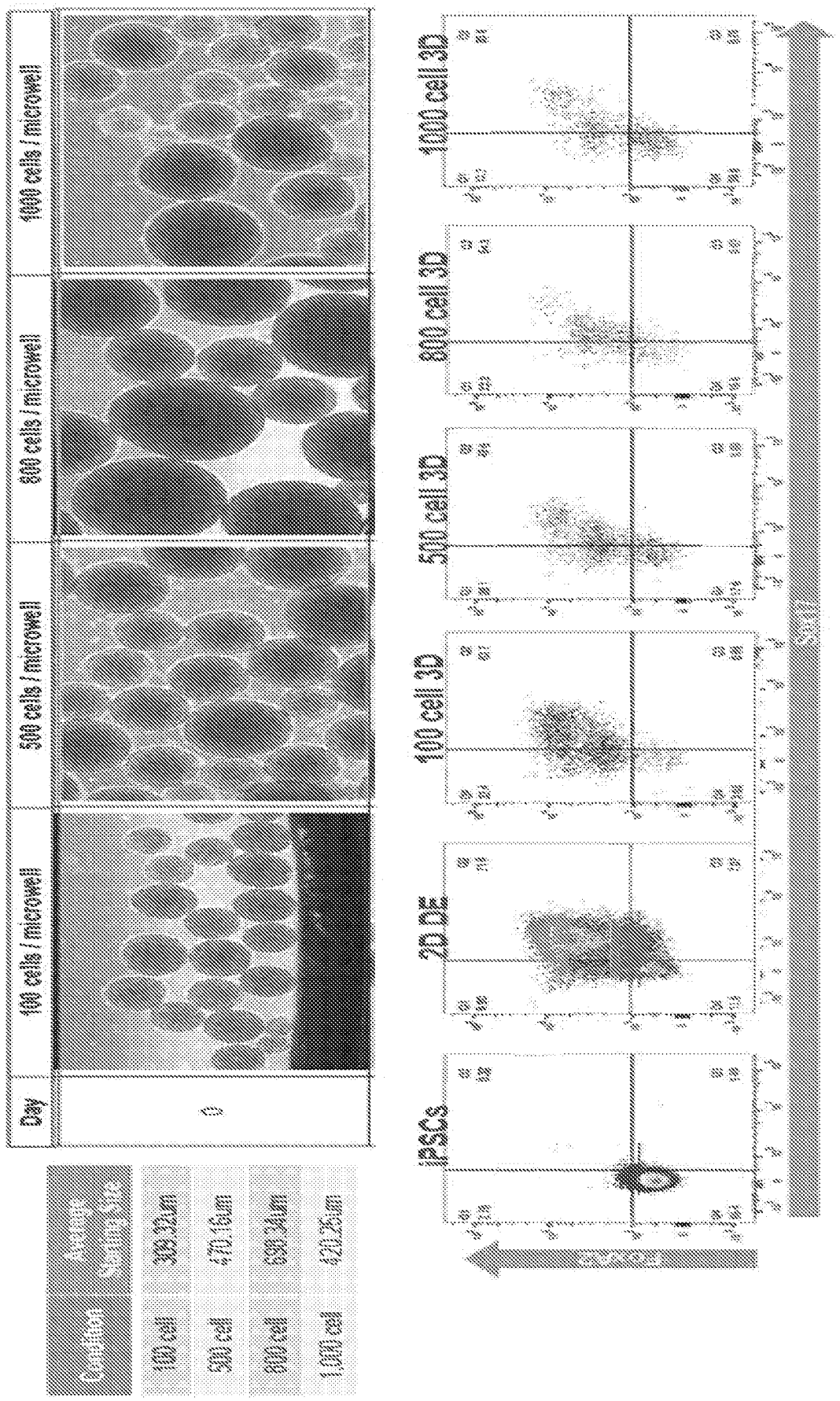

Day
100 cells / microwell
500 cells / microwell
800 cells / microwell
1000 cells / microwell
0
Condition
Average Diameter
100 cell
309.32um
500 cell
470.16um
800 cell
698.34um
1,000 cell
420.25um
iPSCs
2D DE
100 cell 3D
500 cell 3D
800 cell 3D
1000 cell 3D
SSEA4
CXCR4
CD117
CD56

FIG. 20

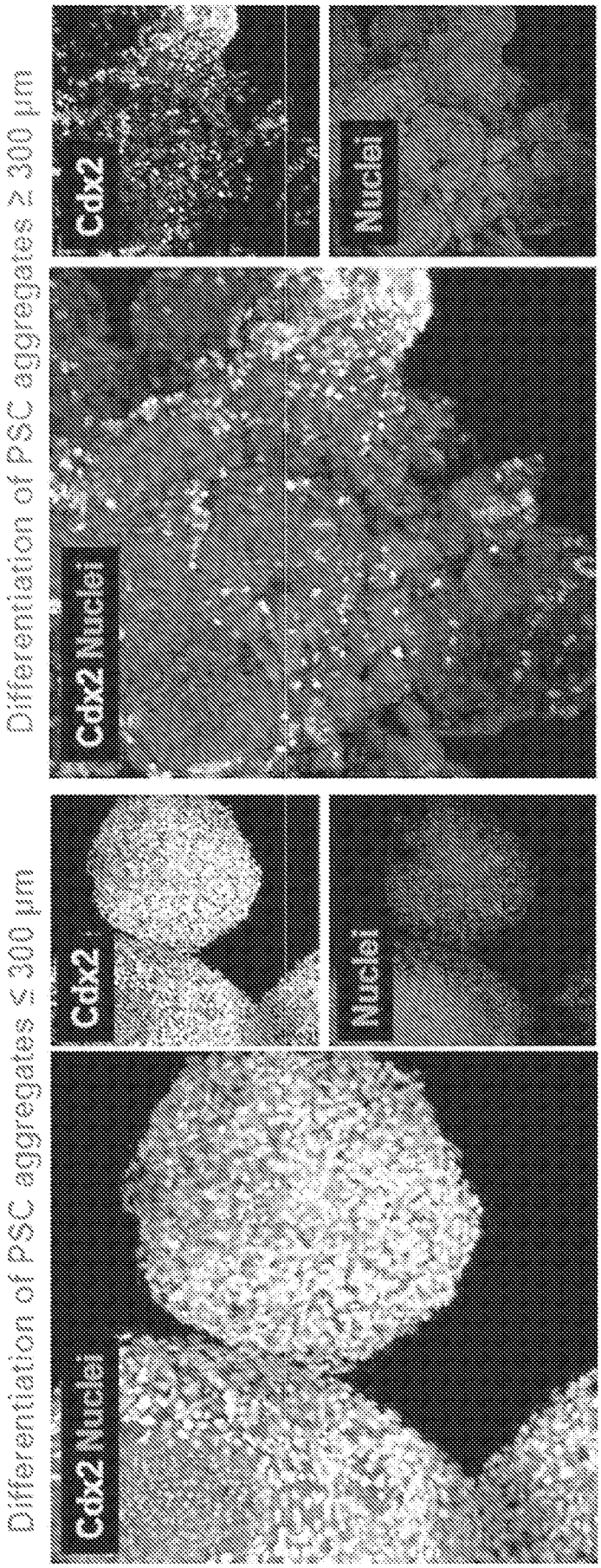

Differentiation of PSC aggregates ≤ 300 µm
Cdx2 Nuclei
Cdx2
Nuclei
Differentiation of PSC aggregates ≥ 300 µm
Cdx2 Nuclei
Cdx2
Nuclei

FIG. 21

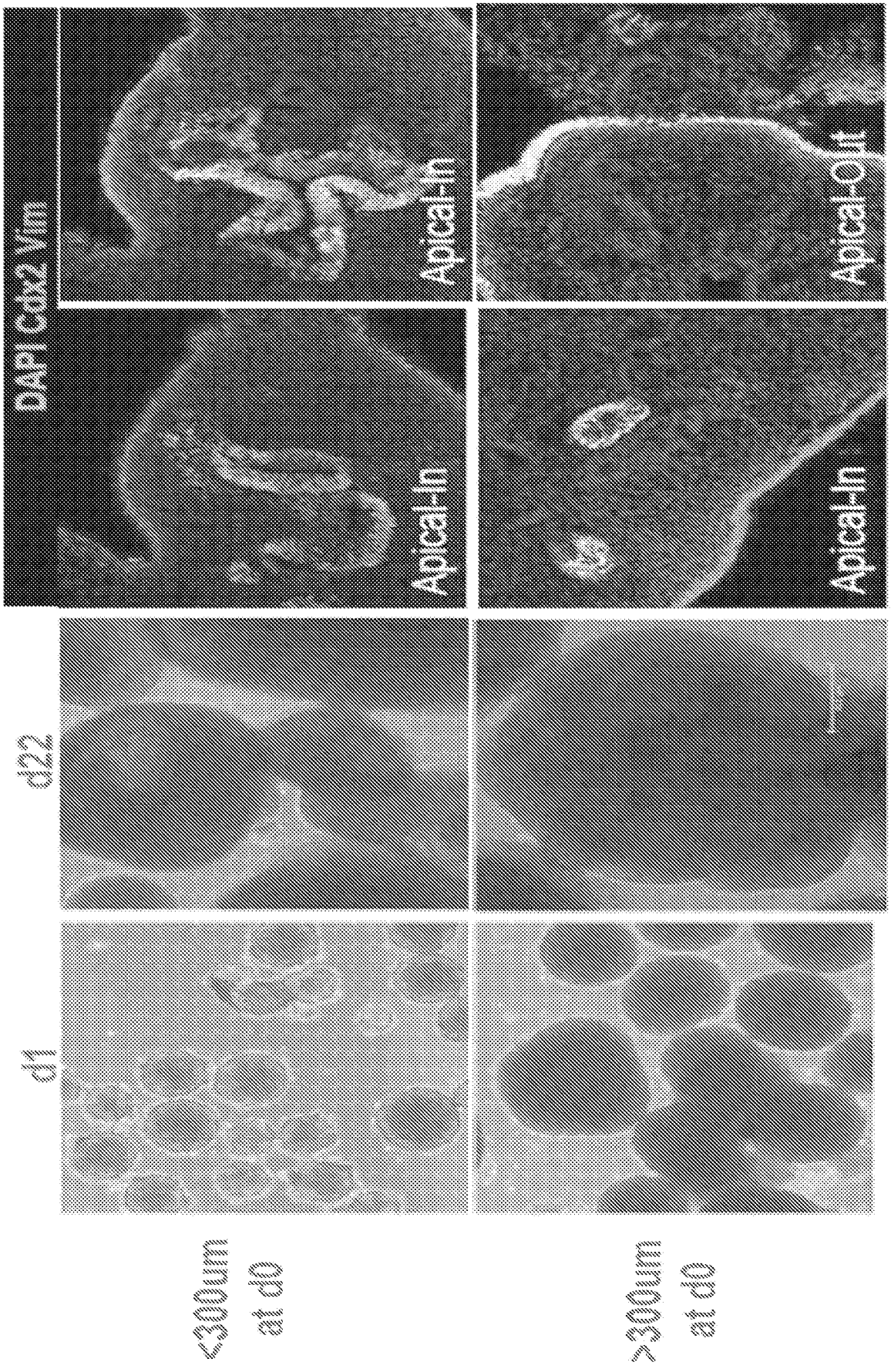

DAPI Cdx2 Vim
Apical-In
Apical-Out
Apical-In
Apical-In
d1
d22
<300um at d0
>300um at d0

FIG. 22

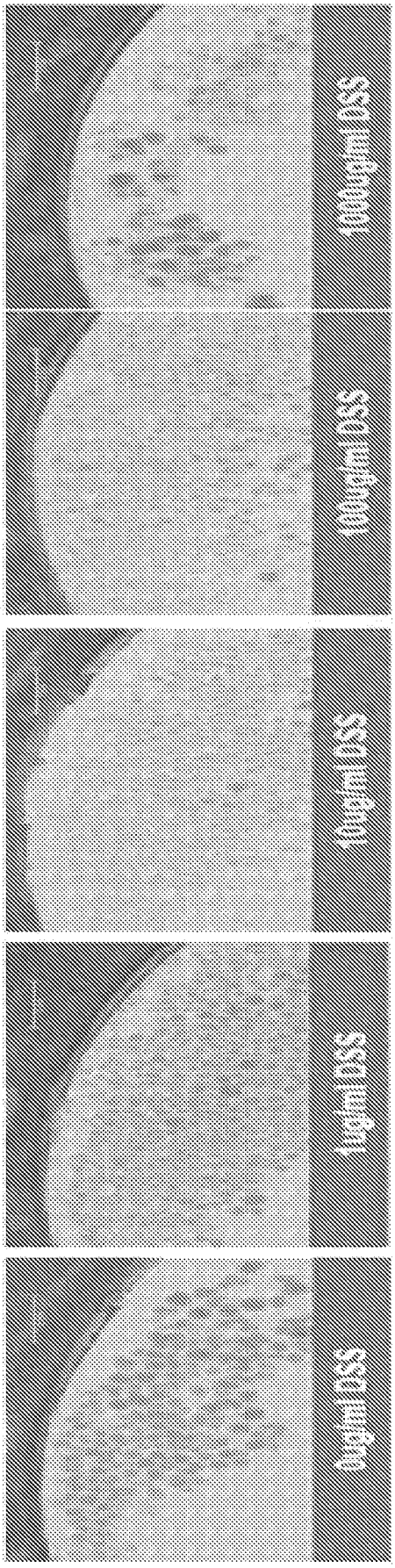


FIG. 23

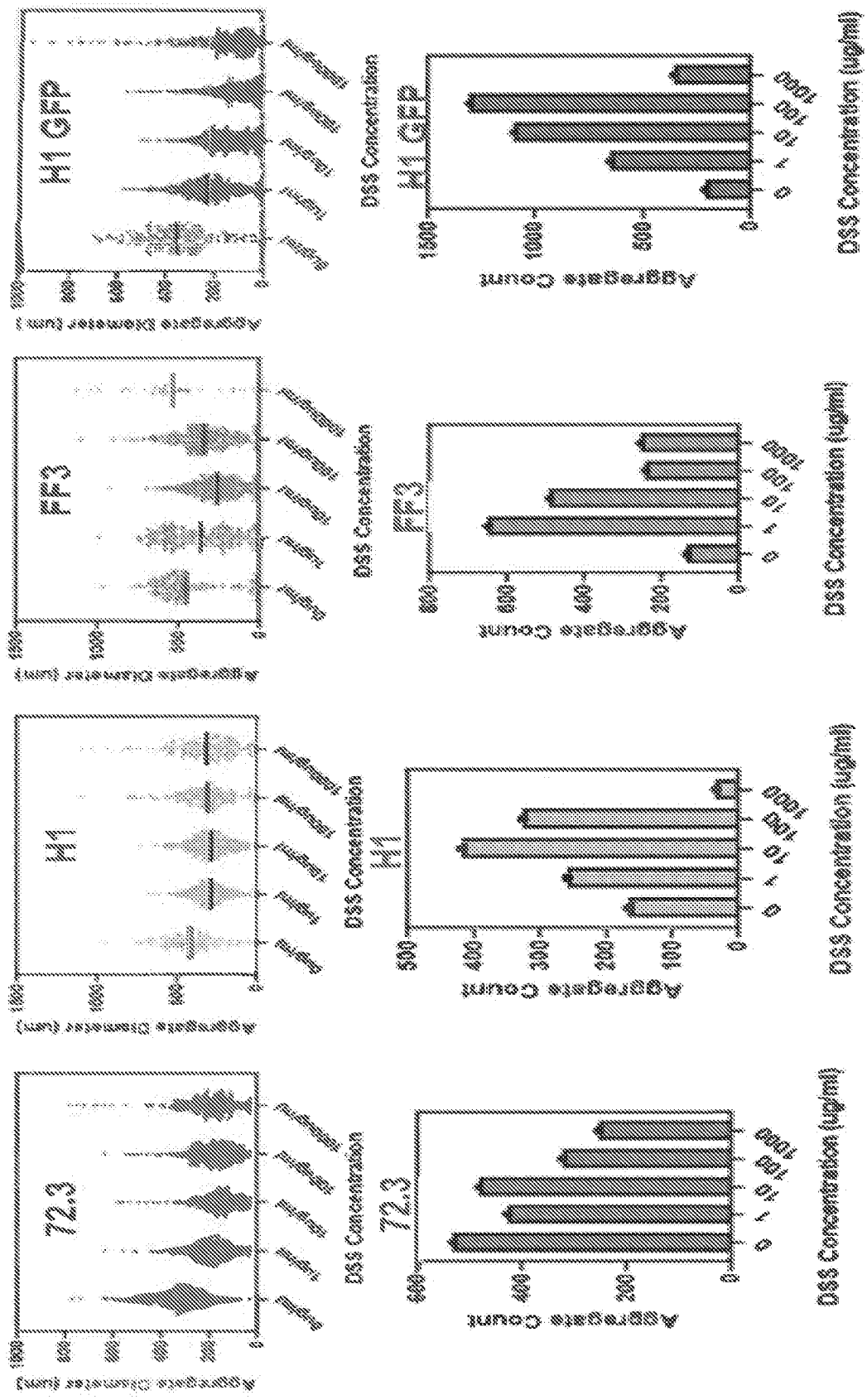

72.3
H1
FF3
H1 GFP
Aggregate Diameter (um)
Aggregate Count
DSS Concentration
DSS Concentration (ug/ml)
0
100
200
400
600
800
1000
1200
1600

FIG. 24

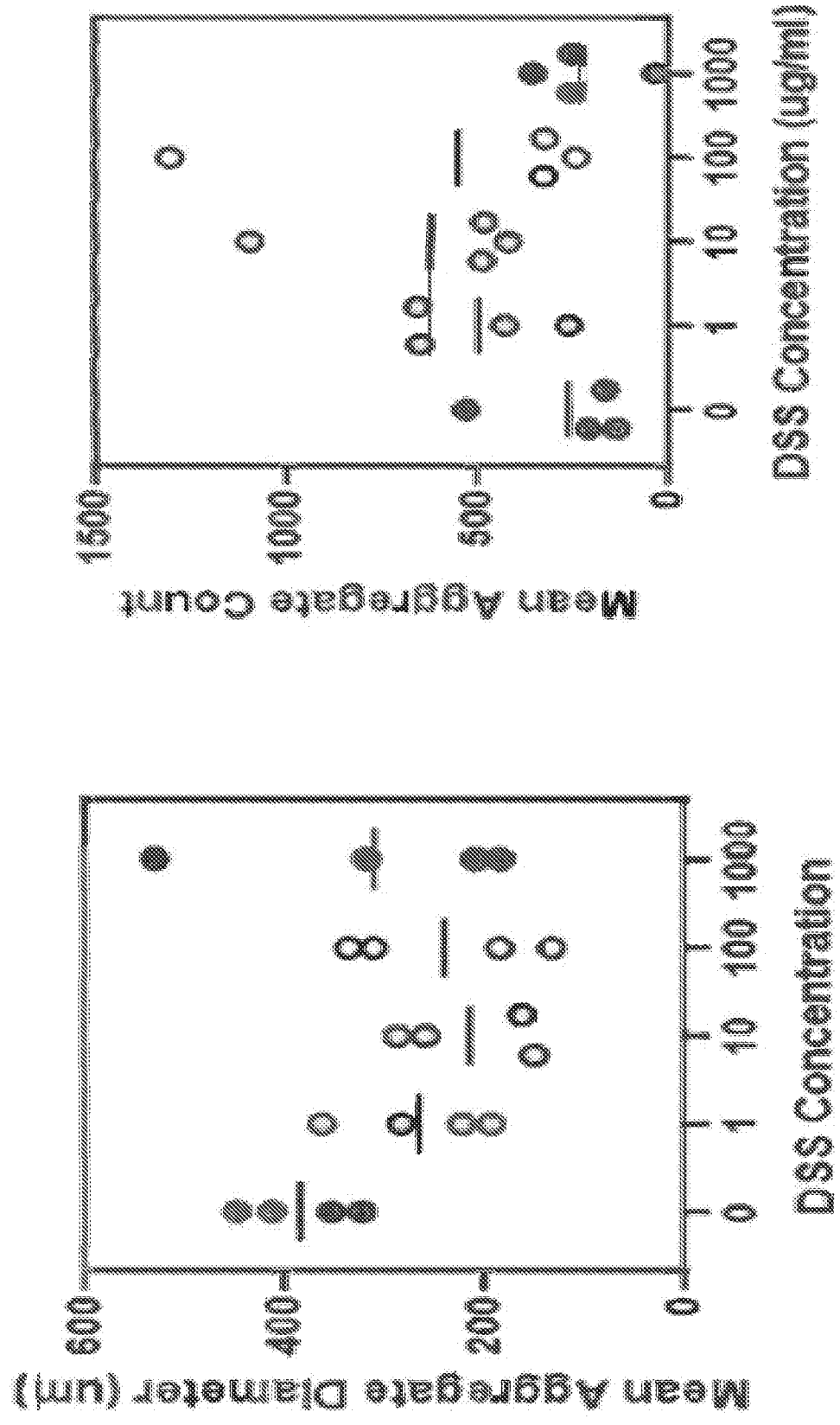

Mean Aggregate Count
1500
1000
500
0
0
1
10
100
1000
DSS Concentration (ug/ml)
Mean Aggregate Diameter (um)
600
400
200
0
0
1
10
100
1000
DSS Concentration

FIG. 25

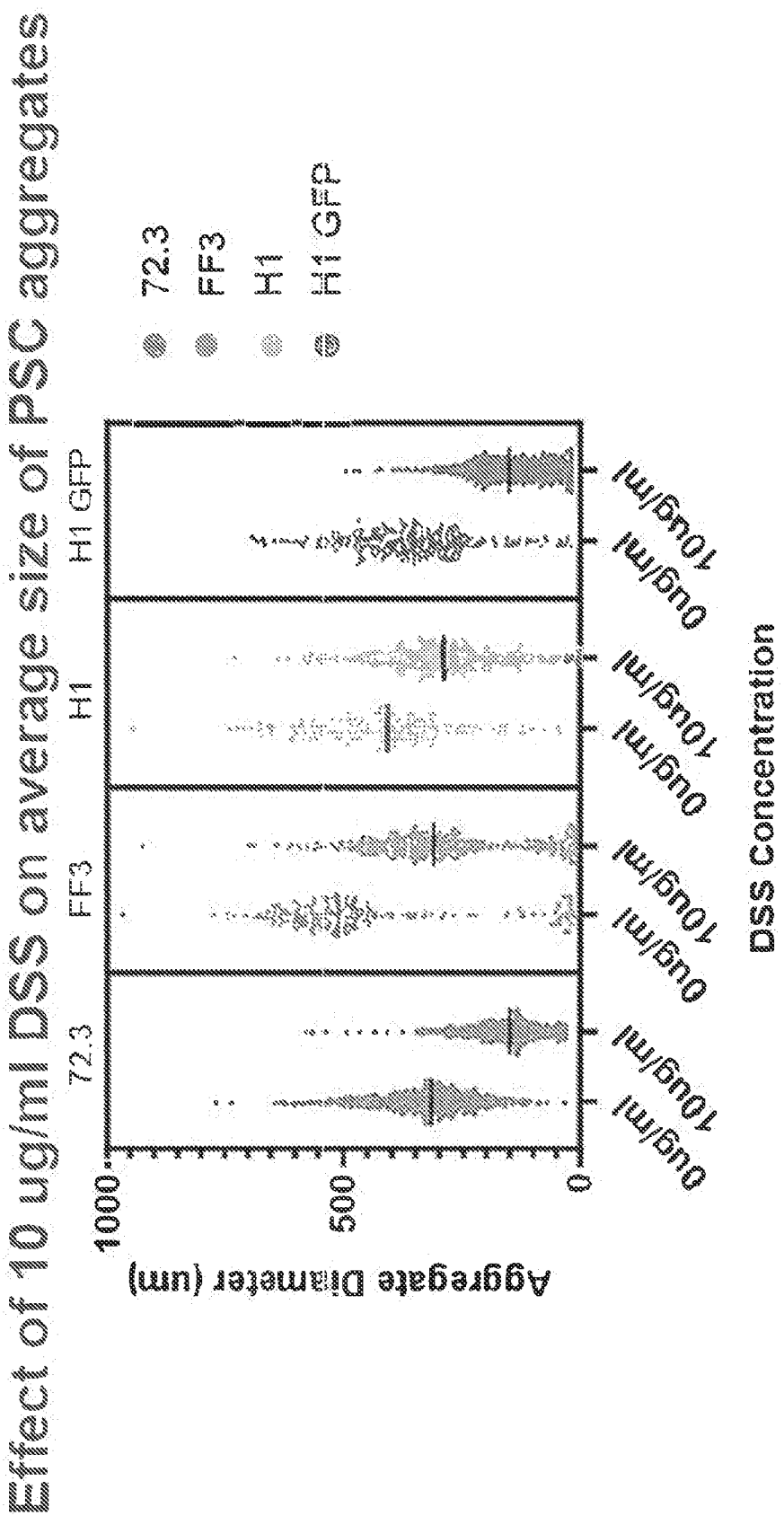

Effect of 10 ug/ml DSS on average size of PSC aggregates
72.3
FF3
H1
H1 GFP
Aggregate Diameter (um)
1000
500
0
72.3
FF3
H1
H1 GFP
10ug/ml
0ug/ml
DSS Concentration

FIG. 26

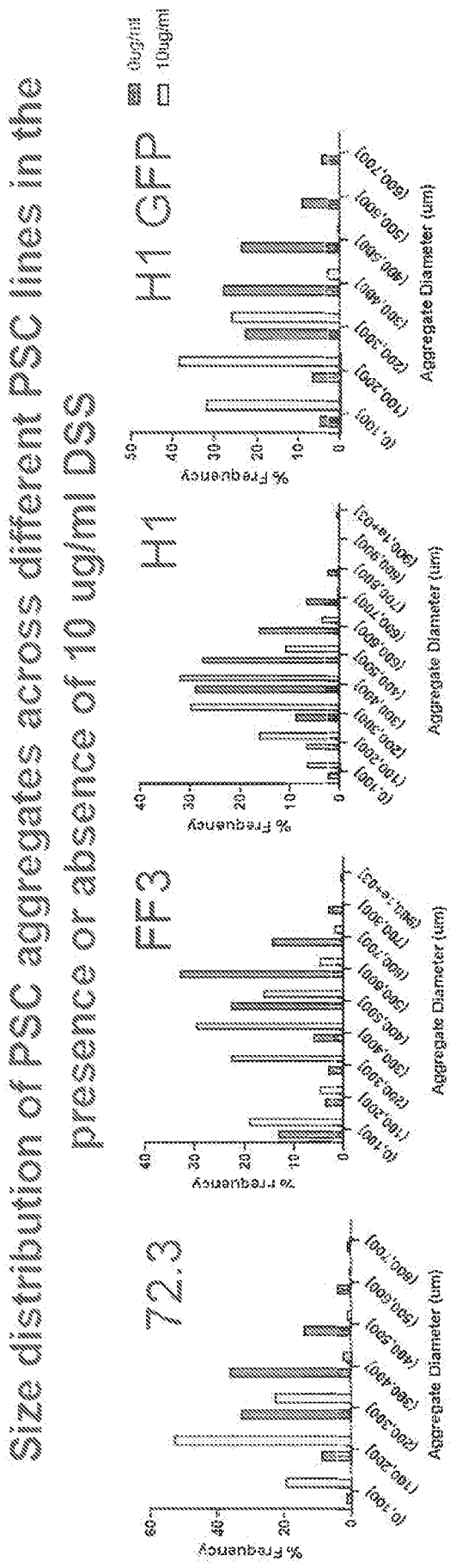

Size distribution of PSC aggregates across different PSC lines in the presence or absence of 10 ug/ml DSS
0ug/ml
10ug/ml
H1 GFP
H1
FF3
72.3
% Frequency
Aggregate Diameter (um)

FIG. 27

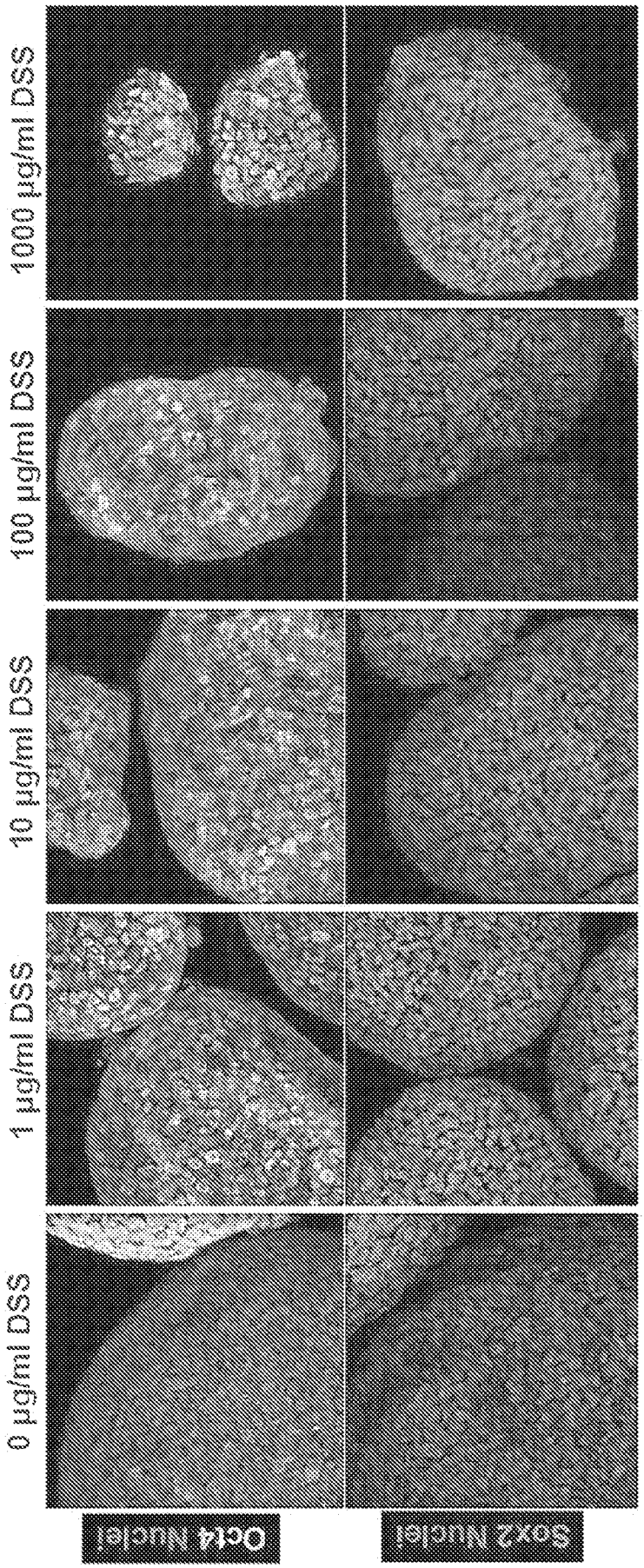

0 µg/ml DSS
1 µg/ml DSS
10 µg/ml DSS
100 µg/ml DSS
1000 µg/ml DSS
Oct4 Nuclei
Sox2 Nuclei

FIG. 28

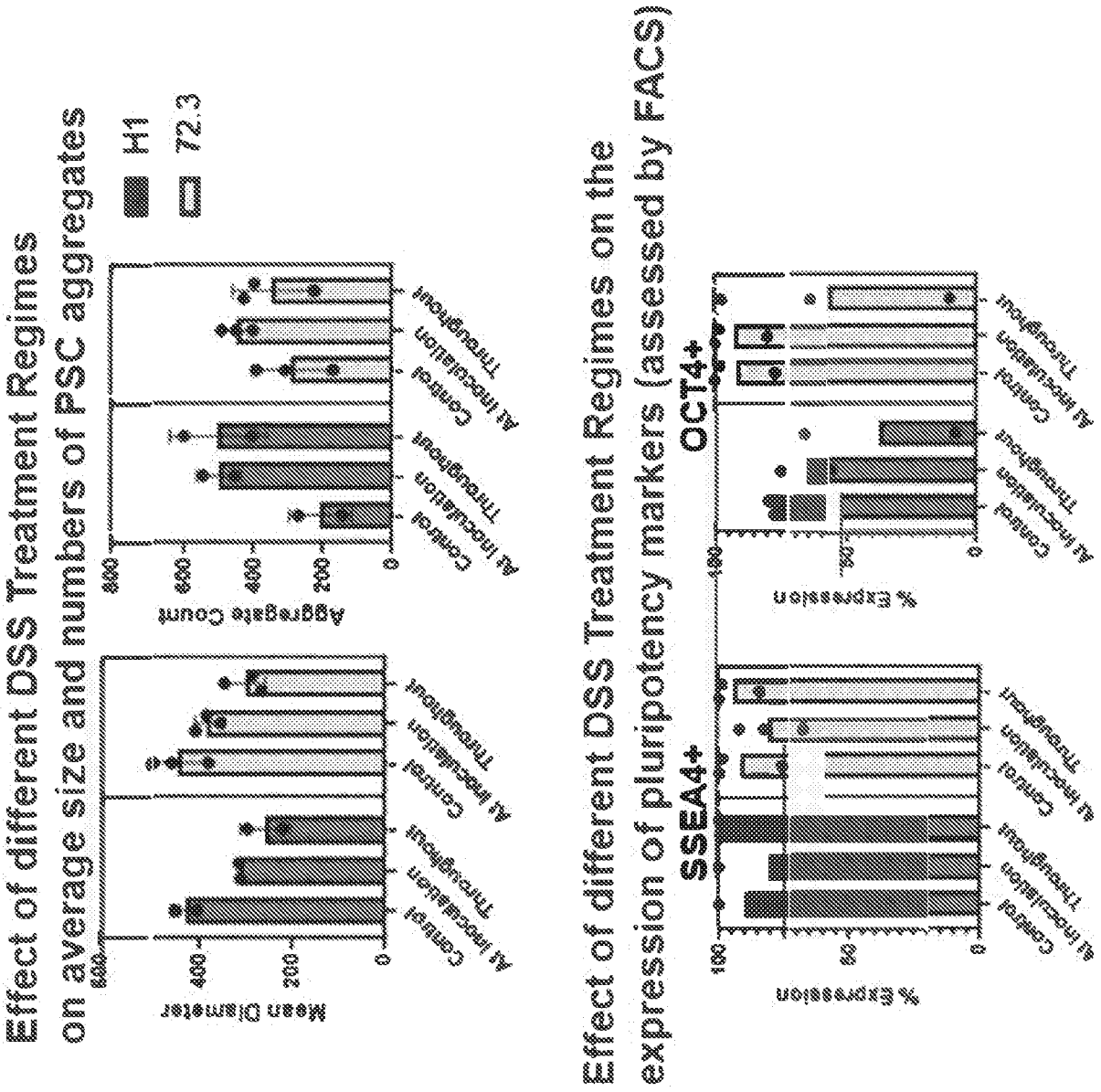

Effect of different DSS Treatment Regimes
on average size and numbers of PSC aggregates
H1
72.3
Mean Diameter
Aggregate Count
Control
At Inoculation
Throughout
Effect of different DSS Treatment Regimes on the
expression of pluripotency markers (assessed by FACS)
SSEA4+
OCT4+
% Expression
Control
At Inoculation
Throughout
Representative brightfield images of 72.3
Control
At Inoculation
Throughout

FIG. 29

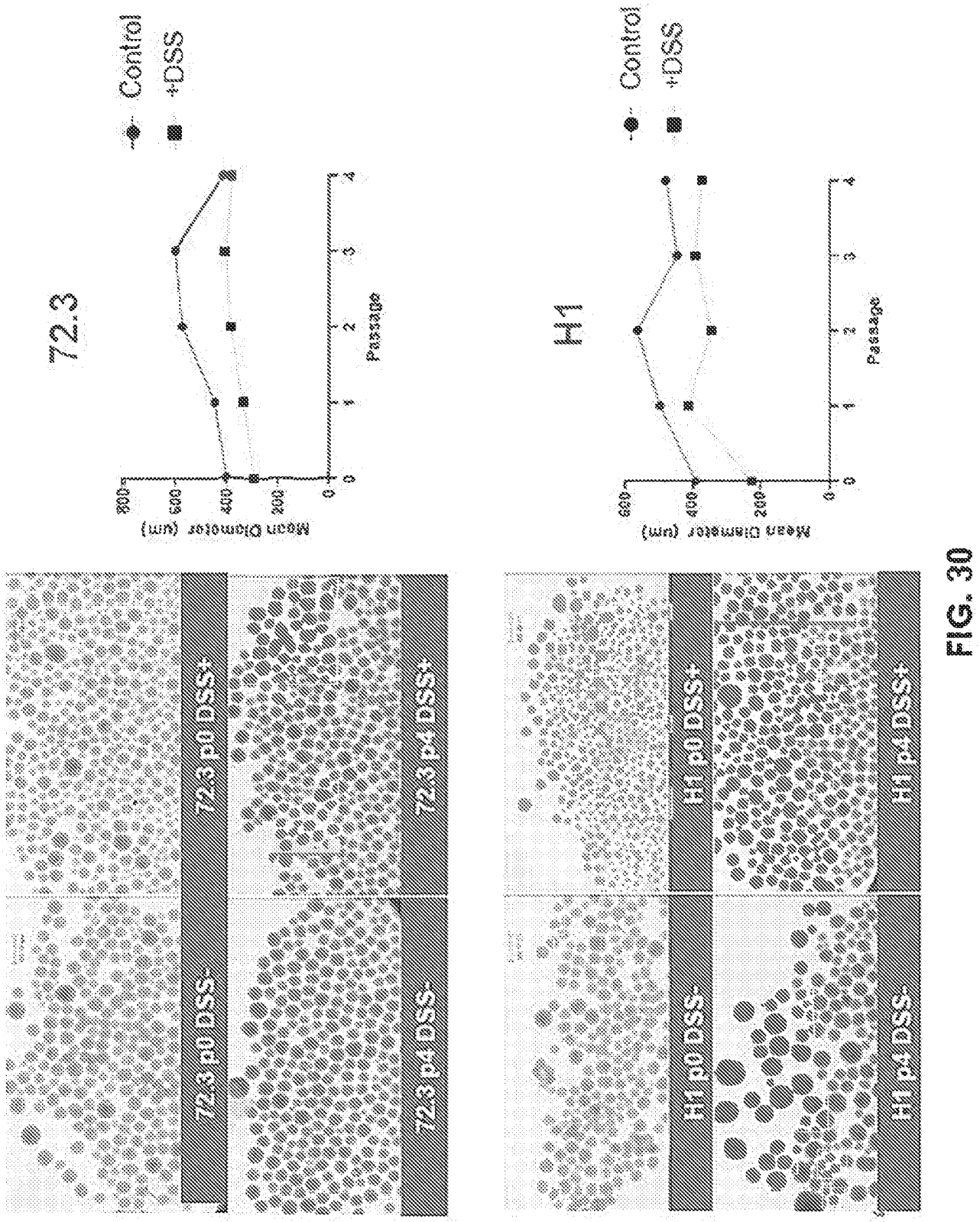

72.3
Control
+DSS
Mean Diameter (um)
Passage
H1
72.3 P0 DSS-
72.3 P0 DSS+
72.3 P4 DSS-
72.3 P4 DSS+
H1 P0 DSS-
H1 P0 DSS+
H1 P4 DSS-
H1 P4 DSS+

FIG. 30

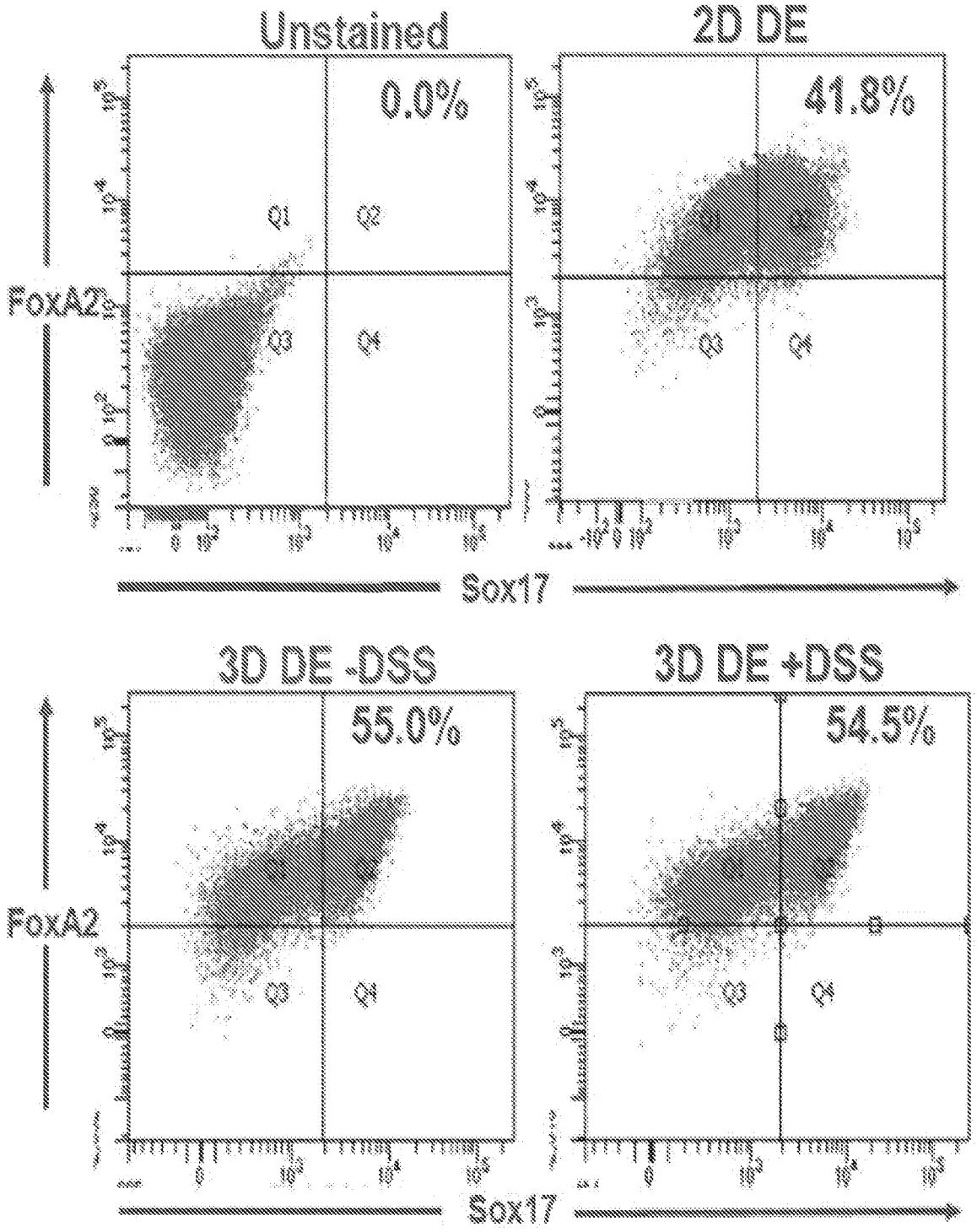

Unstained
0.0%
2D DE
41.8%
3D DE -DSS
55.0%
3D DE +DSS
54.5%
FoxA2
Sox17
Q1
Q2
Q3
Q4

FIG. 31

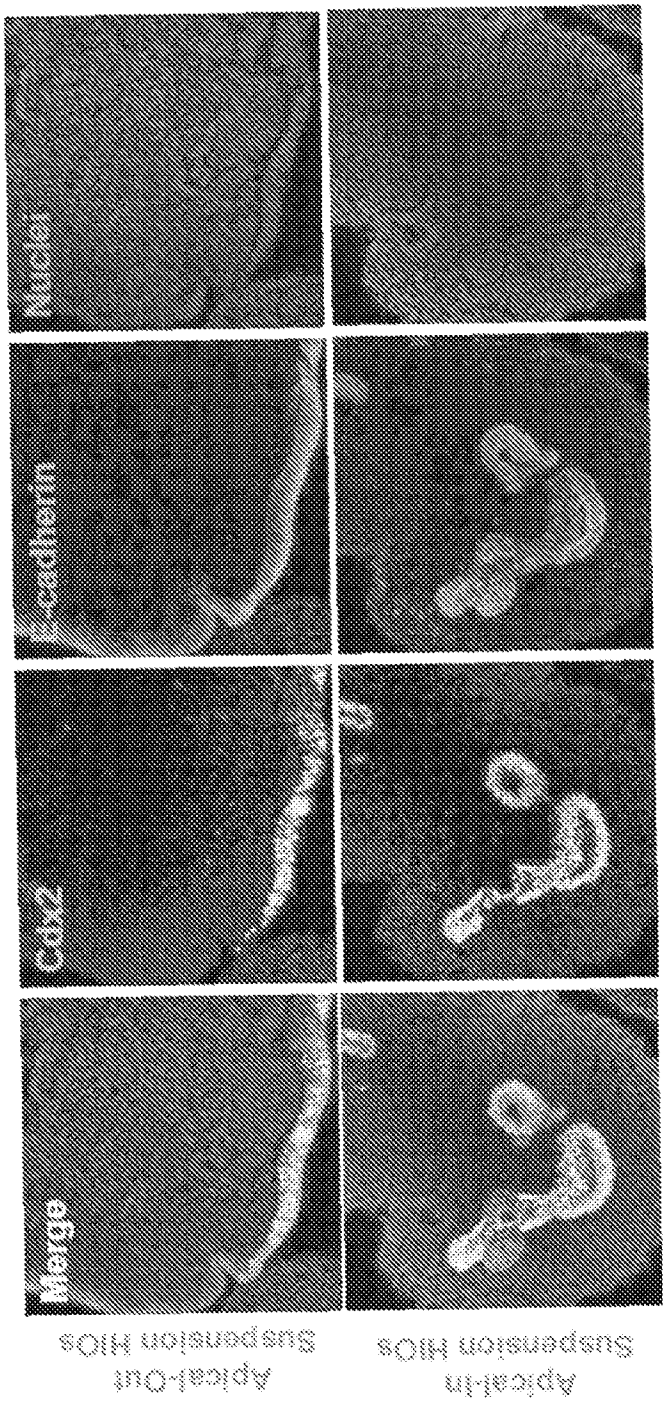

Nuclei
E-cadherin
Cdx2
Merge
Apical-In
Suspension hIOs
Apical-Out
Suspension hIOs

FIG. 32

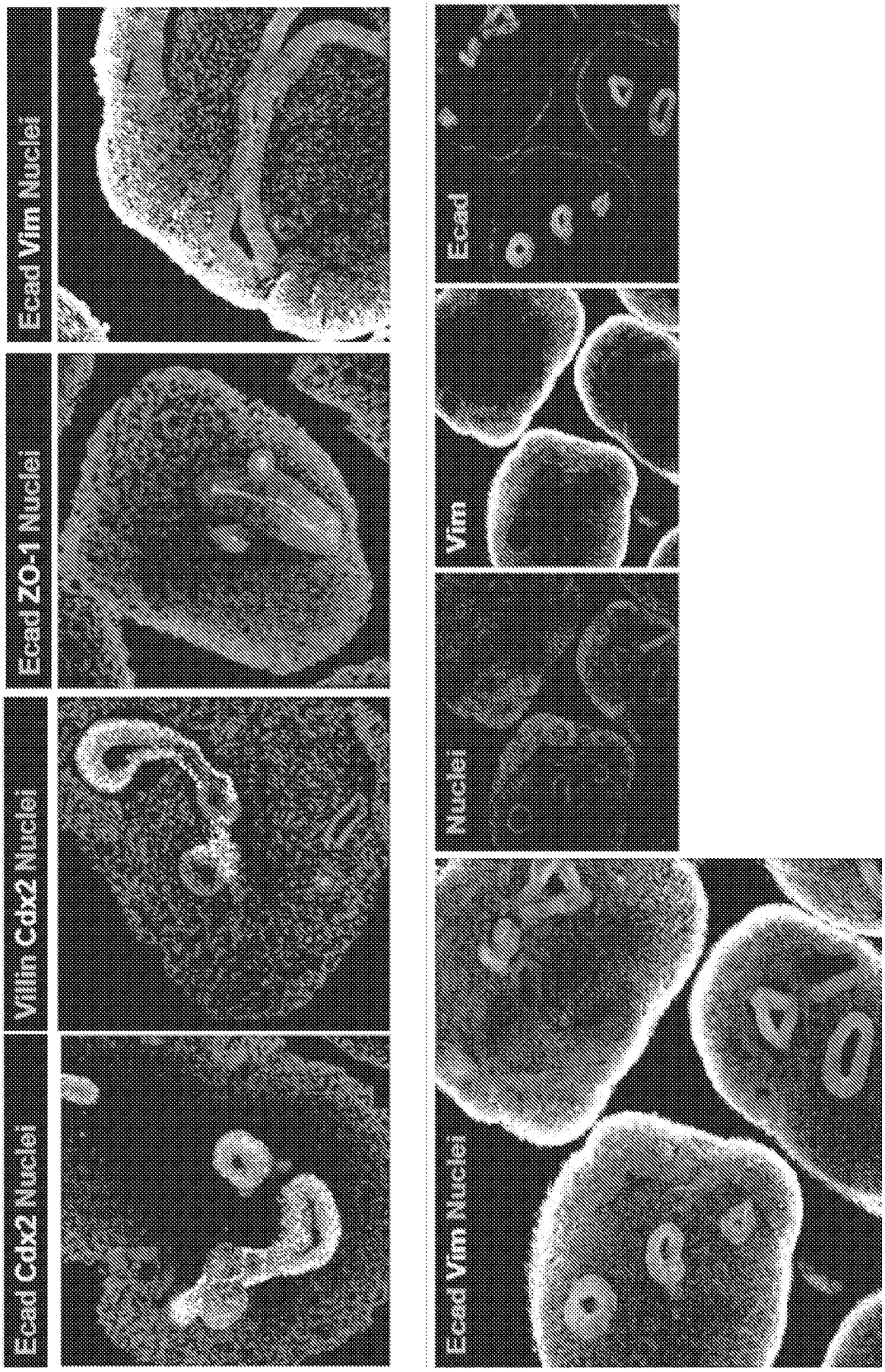

Ecad Cdx2 Nuclei
Villin Cdx2 Nuclei
Ecad ZO-1 Nuclei
Ecad Vim Nuclei
Ecad Vim Nuclei
Nuclei
Vim
Ecad

FIG. 33

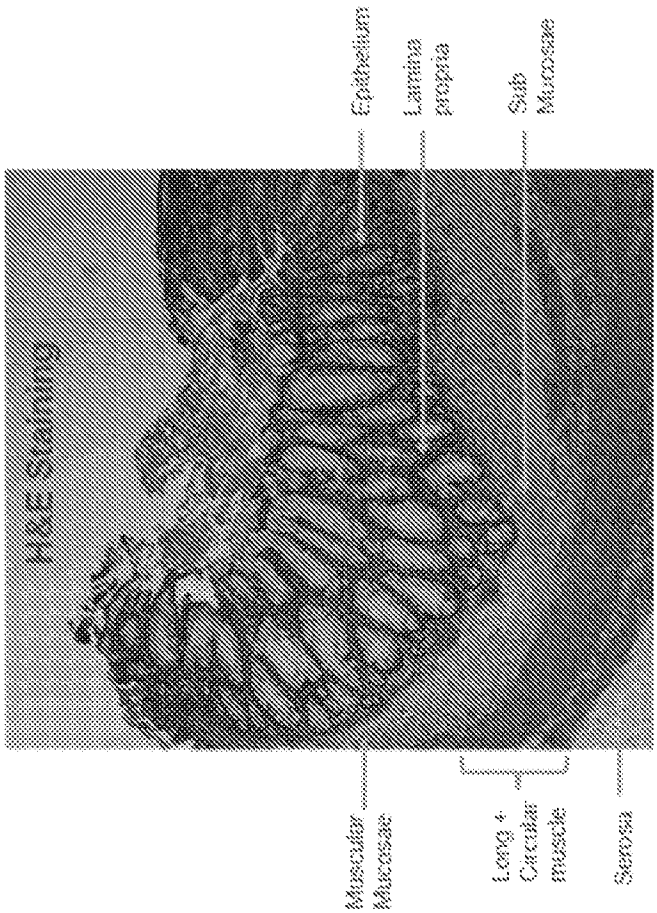


FIG. 34B

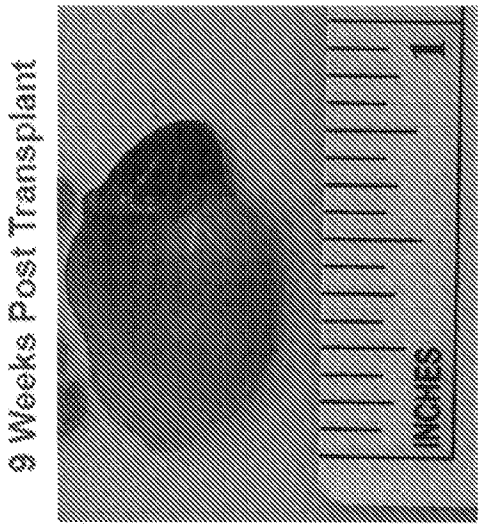


FIG. 34A

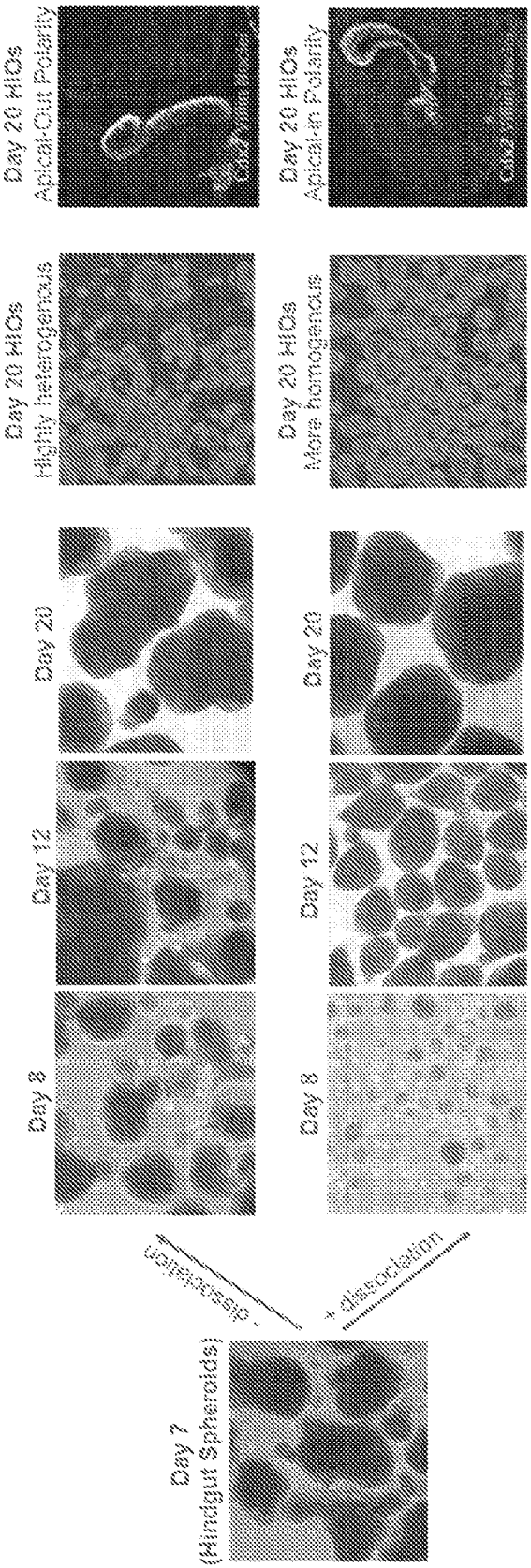

Day 20 HIOs
Apical-Out Polarity
Day 20 HIOs
Apical-In Polarity
Day 20 HIOs
Highly Heterogeneous
Day 20 HIOs
More homogeneous
Day 20
Day 12
Day 8
Day 20
Day 12
Day 8
collagenase
+ dissociation
Day 7
(Hindgut Spheroids)

FIG. 35