

5 **Summary**

[0004] ~~An aspect of the disclosure includes~~ The present disclosure refers to subject matter as defined in the attached set of claims. In one example, there is disclosed a method of producing hepatocyte lineage cells from an extended nodal agonist treated induced endodermal cell population, the method comprising:

(a) specifying the extended nodal agonist treated induced endodermal cell population to obtain a cell population comprising hepatocyte progenitors by contacting the extended nodal agonist treated induced endodermal cell population with specification media comprising a FGF agonist and a BMP4 agonist and/or active conjugates and/or fragments thereof;

(b) inducing maturation, optionally further lineage specification and/or expansion of the hepatocyte progenitors of the cell population to obtain a population comprising hepatocyte lineage cells such as hepatoblasts, hepatocytes and/or cholangiocytes, the inducing maturation step comprising generating aggregates of the cell population.

[0005] In an ~~embodiment~~example, the hepatocyte and/or cholangiocyte lineage cells are hepatoblasts. In an ~~embodiment~~example, the method produces an expanded population of hepatoblasts. In another ~~embodiment~~example the hepatocyte lineage cells are mature hepatocytes or the cholangiocyte lineage cells are mature cholangiocytes.

[0006] In some ~~embodiment~~examples, the extended nodal agonist treated induced endodermal cell population is induced from pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). The pluripotent stem cells are optionally human ESCs (hESCs) or human iPSCs (hiPSCs).

[0007] The extended nodal agonist treated induced endoderm population is, in an ~~embodiment~~example, obtained by inducing endoderm cells in embryoid bodies (EBs). In another ~~embodiment~~example, the extended nodal agonist treated induced endodermal population is obtaining by inducing endoderm cells that are in a monolayer. In each case, the induced endodermal population is cultured in the presence of a nodal agonist, for example activin, for an extended period to produce an extended nodal agonist treated induced endodermal population.

[0008] In an ~~embodiment~~example, the extended nodal agonist treated induced endodermal population comprises at least, 80%, 85%, 90%, 95 CXCR4 + and cKIT + positive cells and/or at least 70%, 75%, 80% SOX17+ cells.

[0009] In an ~~embodiment~~example, the specifying step comprises contacting an extended nodal agonist treated (e.g. activin treated) induced endodermal population with specification media comprising a FGF and BMP4. The FGF can for example be bFGF, FGF10, FGF2 or FGF4 or combinations thereof. The combinations can for example be added sequentially.

[0010] In an ~~embodiment~~example, the specifying step comprises first contacting an extended nodal agonist treated induced endodermal population with specification media comprising FGF10 and BMP4 for approximately 40 to 60 hours, optionally approximately 40, 42, 44, 46, 48, 50, 52, 54, 56, 58 or 60 hours and then contacting the extended nodal agonist treated induced endodermal population

5 with specification media comprising bFGF and BMP4 for about 4 to 7 days, optionally about 4, 5, 6 or 7 days.

[0011] In another embodimentexample, the aggregates are generated from a cell population comprising at least 70%, 80%, 85%, or 90% albumin positive cells. In another embodimentexample, the aggregates are generated after 24, 25, 26, 27, or 28 days in culture.

10 [0012] In some embodimentexamples, aggregates are generated from a monolayer of the cell population comprising hepatocyte and/or cholangiocyte progenitors by enzymatic treatment and/or manual dissociation.

[0013] Inducing maturation, and optionally further lineage specification and/or expansion can comprise one or more additional steps. In a further embodimentexample, the cell population
15 comprising hepatocyte and/or cholangiocyte progenitors and/or the aggregates are cultured in the presence of hepatocyte growth factor (HGF), dexamethasone (DEX) and/or Oncostatin M (OSM) and/or active conjugates and/or fragments thereof.

[0014] In one embodimentexample, inducing maturation, and optionally further lineage specification and/or expansion further comprises activating the cAMP pathway within the cells of the
20 aggregates to induce the maturation of the hepatocyte and cholangiocyte progenitors into hepatocytes and/or cholangiocytes. In another embodimentexample, activating the cAMP pathway comprises contacting the aggregates with cAMP and/or a cAMP analog (e.g. such as 8-bromoadenosine-3'5'-cyclic monophosphate, dibutyryl-cAMP, Adenosine- 3', 5'-cyclic monophosphorothioate, Sp- isomer (Sp-cAMPS) and/or 8-Bromoadenosine-3', 5'-cyclic
25 monophosphorothioate, Sp-isomer (Sp-8-Br-cAMPS)) and/or any other cAMP agonist.

[0015] For example, in an embodimentexample, a maturation media comprising a cAMP agonist and DEX and optionally HGF is added to the aggregates subsequent to culturing the pre-aggregate population in a maturation media comprising HGF, DEX and OSM, for example for about 10, 11, 12, 13 or 14 days.

30 [0016] In an embodimentexample, the population of hepatocytes produced is a population comprising functional hepatocytes.

[0017] In embodimentexamples, the hepatocytes, optionally functional hepatocytes, comprise increased expression of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more genes or protein selected from the group consisting of ALB, CPS1, G6P, TDO, CYP2C9, CYP2D6, CYP7A1, CYP3A7, CYP1A2,
35 CYP3A4, CYP2B6, NAT2 and UGT1A1 compared to a cell population comprising hepatocyte and/or cholangiocyte progenitors, and/or hepatocytes produced from a non-extended nodal agonist treated induced endodermal cell population (e.g. from an induced endodermal population that was not treated with a nodal agonist such as activin for an extended period of time), produced without aggregation and/or cAMP signaling induction. In other embodimentexamples, at least 40%, 50%, 60%, 70%, 80%
40 or 90% of the hepatocytes, optionally functional hepatocytes, are ASGPR-1+ cells.

5 [0018] In an ~~embodiment~~example, cholangiocyte fate is specified by treating aggregates of the cell population with a notch agonist.

[0019] In an ~~embodiment~~example, the population of cholangiocytes produced is a population of functional cholangiocytes. The functional cholangiocytes comprise for example increased expression of at least 1, at least 2 or 3 genes or proteins selected from Sox9, CK19 and CFTR (Cystic fibrosis transmembrane conductance regulator) compared to the cells of the cell population comprising hepatocyte and cholangiocyte progenitors and/or compared to a population cells produced from aggregates not treated with a notch agonist. In other ~~embodiment~~examples, at least 40%, 50%, 60%, 70%, 80% or 90% of the population of cholangiocytes are CK19+ cholangiocytes. In other ~~embodiment~~examples, at least 40%, 50%, 60%, 70%, 80% or 90% of the functional cholangiocytes are CFTR+ cholangiocytes.

[0020] As mentioned, the method can be applied to an endodermal cell population grown in a monolayer.

[0021] Accordingly a further ~~aspect~~example includes a method of producing hepatocytes and/or cholangiocytes from a pluripotent stem cell population, the method comprising:

a) contacting the pluripotent stem cells cultured as a monolayer, with an induction media comprising nodal agonist such as ActA and optionally a wnt/beta-catenin agonist such as i) Wnt3a and/or ii) a GSK-3 selective inhibitor such as CHIR-99021, to provide an induced endodermal cell population;

b) contacting the induced endodermal cell population with a nodal agonist to provide an extended nodal agonist treated induced endodermal cell population;

c) specifying the extended nodal agonist treated induced endodermal cell population by contacting the extended nodal agonist treated induced endodermal cell population with a specification media comprising a FGF agonist and a BMP4 agonist and/or active conjugates and/or fragments thereof to obtain a cell population comprising hepatocyte and/or cholangiocyte progenitors;

d) optionally contacting the cell population comprising hepatocyte and/or cholangiocyte progenitors with a maturation media comprising HGF, dexamethasone and/or Oncostatin M and/or active conjugates and/or fragments thereof; and

e) inducing maturation, optionally further lineage specification and/or expansion of hepatocyte and cholangiocyte progenitors of the cell population into expanded hepatoblasts, hepatocytes and/or cholangiocytes, the inducing maturation comprising generating aggregates of the cell population.

[0022] Further, the endodermal population can also be comprised in embryoid bodies.

[0023] Accordingly, a further ~~aspect~~example of the disclosure ~~provides~~describes a method of producing hepatocytes and/or cholangiocytes from a pluripotent stem cell population, the method comprising:

- 5 a) forming embryoid bodies (EBs) of the pluripotent stem cells, optionally by contacting the pluripotent stem cells with a BMP4 agonist;
- b) contacting the EBs with an induction media comprising a nodal agonist such as ActA and optionally a wnt/beta-catenin agonist such as i) Wnt3a and/or ii) a GSK-3 selective inhibitor such as CHIR-99021, to provide an induced endodermal cell population;
- 10 c) dissociating the induced endodermal cell population to provide a dissociated induced endodermal cell population;
- d) contacting the dissociated induced endodermal cell population with a nodal agonist to provide an extended nodal agonist treated induced endodermal cell population;
- 15 e) specifying the extended nodal agonist treated induced endodermal cell population by contacting the extended nodal agonist treated induced endodermal cell population with a specification media comprising a FGF agonist and a BMP4 agonist and/or active conjugates and/or fragments thereof to obtain a cell population comprising hepatocyte and/or cholangiocyte progenitors,
- 20 f) optionally contacting the cell population comprising hepatocyte and/or cholangiocyte progenitors with a maturation media comprising HGF, dexamethasone and/or Oncostatin M and/or active conjugates and/or fragments thereof; and
- g) inducing maturation, further lineage specification and/or expansion of hepatocyte and cholangiocyte progenitors of the cell population into hepatocytes and/or cholangiocytes, the inducing maturation, further lineage specification and/or expansion comprising generating
- 25 aggregates of the cell population.

[0024] In some ~~embodiment~~examples, the inducing maturation, further lineage specification and/or expansion step further comprises activating the cAMP pathway within the aggregates to induce the maturation of hepatocyte and/or cholangiocyte progenitors of the cell population into a population comprising hepatocytes and/or cholangiocytes. In an ~~embodiment~~example, the method comprises

30 contacting the aggregates with a cAMP analog and/or cAMP agonist.

[0025] In an ~~embodiment~~example, the monolayer or EBs are contacted with a nodal agonist in induction media for at least about 1 day, 2 days, 3 days or about 4 days.

[0026] In an ~~embodiment~~example, in a step prior to dissociation of the endodermal population (e.g. embryoid bodies (EB) stage), the EBs are cultured with a nodal agonist for at least 36, 38, 42,

35 44, 46, 48, 50, 52, 56, 58 or 60 hours or for at least about 1 day, 2 days, 3 days or about 4 days.

[0027] Accordingly in another ~~aspect~~example of the disclosure ~~relates to~~describes a method of producing hepatocytes and/or cholangiocytes from pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), the method comprising:

- 40 a) contacting the pluripotent stem cells cultured as a monolayer or formed into embryoid bodies, with an induction media comprising nodal agonist such as ActA and

optionally a wnt/beta-catenin agonist such as i) Wnt3a and/or ii) a GSK-3 selective inhibitor such as CHIR-99021 to provide an induced endodermal cell population;

b) contacting the induced endodermal cell population with a nodal agonist to provide an extended nodal agonist treated induced endodermal cell population; and

c) specifying the extended nodal agonist treated induced endodermal cell population by contacting the extended nodal agonist treated induced endodermal cell population with a specification media comprising at least one FGF agonist and one BMP4 agonist and/or active conjugates and/or fragments thereof to obtain a cell population comprising hepatocyte and/or cholangiocyte progenitors, and

d) inducing maturation, further lineage specification and/or expansion of hepatocyte and/or cholangiocyte progenitors into hepatocytes and/or cholangiocytes, the inducing maturation, further lineage specification and/or expansion comprising:

(i) culturing the cell population comprising hepatocyte and/or cholangiocyte progenitors with a maturation media comprising HGF, OSM and DEX;

(ii) generating aggregates of the cell population, optionally when the cell population comprises at least 70%, 80%, 85%, or 90% albumin positive cells or after about 20 to about 40 days of culture for example after about 24 to about 28 days of culture;

(iii) culturing the aggregated cells in an aggregated cell maturation media: and

(iv) activating the cAMP pathway in the aggregated cells, optionally within about 1 to about 10 days of aggregation, for example within 6 days of aggregation, optionally after about 27 to about 36 days of culture,.

[0028] In an embodimentexample, the aggregated cell maturation media can comprise factors which promote hepatocyte maturation or factors which promote cholangiocyte development or both.

[0029] In another embodimentexample, aggregated cells are upon aggregation treated with a wnt agonist such as CHIR 99021, optionally in the presence of a TGFbeta antagonist such as SB431542. As demonstrated herein, activation of the Wnt pathway and SMAD pathway at for example day 26 (or optionally one or two days later e.g. day 27, in embodimentexamples using EBs), promotes expansion of an albumin+/HNF4+ progenitor population. It is demonstrated for example that up to a 10 fold expansion of said population can be obtained when a wnt agonist is added.

[0030] In an embodimentexample, the aggregated cells are treated with a wnt agonist and optionally a TGFbeta antagonist (such as SB431542) for about 6 to about 12 days, preferably about 8 to about 10 days, optionally for about 9 days.

[0031] In yet a further embodimentexample, a Wnt antagonist such as XAV939 (also referred to as XAV for short) and/or a Mek/Erk antagonist, for example PD0325901 (also referred to as PD for short) is added during the cAMP activation step. Addition of a Wnt antagonist and/or a MEK/Erk antagonist during activation of cAMP signaling enhances expression of CYP enzymes, for example up to levels

5 or greater than levels seen in adult liver cells. For example, an inhibitor of MEK/Erk added in the presence of cAMP, for example, added to about day 28 to about day 32 cultures, results in hepatocytes with increased levels of CYP3A4. Addition of a MEK/Erk antagonist in combination with a Wnt antagonist is shown to also increase levels of CYP1A2. In an embodimentexample, the Wnt antagonist is XAV939. In another embodimentexample, the MEK/Erk antagonist is PD0325901.

10 **[0032]** In an embodimentexample, approximately 1 to about 4 days after aggregation, the cells are treated with a notch agonist. Addition of a notch agonist at such stages promotes cholangiocyte maturation. In some embodimentexamples, for example where cholangiocyte maturation is preferred, inducing cAMP signaling is omitted.

15 **[0033]** Further inhibiting Notch signaling for example with a Notch antagonist such as gamma-secretase inhibitor (GSI) L695,458 is demonstrated herein to inhibit cholangiocyte development and cells produced retain the characteristics of hepatocytes. In an embodimentexample, the method comprises approximately 1 to about 4 days after aggregation, treating the cells with a notch antagonist, for example in embodimentexamples where hepatocyte differentiation is desired.

20 **[0034]** In another embodimentexample, the method of producing hepatocytes and/or cholangiocytes from pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), comprises:

25 a) contacting the pluripotent stem cells cultured as a monolayer or formed into embryoid bodies, with an induction media comprising a nodal agonist such as ActA and optionally a wnt/beta-catenin agonist such as i) Wnt3a and/or ii) a GSK-3 selective inhibitor such as CHIR-99021, optionally for about 4 to about 8 days, to provide an induced endodermal cell population;

b) contacting the induced endodermal cell population with a nodal agonist, optionally for about 1, 2, 3, or about 4 days, to provide an extended nodal agonist treated induced endodermal cell population;

30 c) specifying the extended nodal agonist treated induced endodermal cell population by contacting the extended nodal agonist treated induced endodermal cell population with a specification media comprising at least one FGF agonist and at least one BMP4 agonist and/or active conjugates and/or fragments thereof, optionally for about 4 to about 10 days, to obtain a cell population comprising hepatocyte and/or cholangiocyte progenitors, and

35 d) inducing maturation, further lineage specification and/or expansion of the hepatocyte or cholangiocyte progenitors into hepatocytes and/or cholangiocytes, the inducing maturation, further lineage specification and/or expansion comprising:

40 (i) culturing the cell population comprising hepatocyte and/or cholangiocyte progenitors with a maturation media comprising comprising HGF, Dex and/or OSM, optionally for about 10 to 14 days;

- 5 (ii) generating aggregates of the cell population, optionally when the cell population comprises at least 70%, 80% 85%, or 90% albumin positive cells or after about 20 to about 40 days for example after about 24 to about 28 days of culture;
- 10 (iii) culturing the aggregates in maturation medium comprising Dex for about 1 to 10 days;
- 15 iv) a) culturing aggregates in a maturation medium comprising Dex and a cAMP analog and/or cAMP agonist for about 6 days to about 10 days, optionally adding the cAMP analog and/or cAMP agonist within about 1 to about 10 days of the generating aggregates step, for example within 6 days of the generating aggregates step, optionally after about 27 to about 36 days of culture; or
- 20 b) culturing the aggregates in a maturation medium comprising a notch agonist and optionally a cAMP agonist, HGF, and/or EGF for about 6 days to about 20 days, optionally adding the notch agonist within about 1 to about 10 days of the generating aggregates step, for example within 6 days of the generating aggregates step, optionally after about 20 to 40 days of culture.

[0035] In an ~~embodiment~~example, the method comprises aggregating for example after about 20 days of culture and/or before 40 days of culture

25 **[0036]** The disclosure also provides a method of inducing maturation, further lineage specification and/or expansion of cholangiocyte progenitors into cholangiocytes, the inducing maturation, further lineage specification and/or expansion comprising:

- 30 (i) culturing a cell population comprising cholangiocyte progenitors with a Notch agonist to induce the maturation of at least one cholangiocyte progenitor into a cholangiocyte, optionally a functional cholangiocyte.

[0037] The notch agonist can for example be any notch ligand bound to a surface such as a cell, plastic, ECM or bead. In one ~~embodiment~~example, the notch ligand is notch ligand delta. In one ~~embodiment~~example, inducing maturation, further lineage specification and/or expansion comprises contacting a cell population comprising cholangiocyte progenitors with a notch signaling donor (e.g. notch agonist) such as OP9, OP9delta, and/or OP9 Jagged1 cells and optionally in the presence of EGF, TGFbeta1, HGF and EGF, and/or HGF, TGFbeta1 and EGF for at least or about 5 to about 90 days, to induce the maturation of cholangiocyte progenitors into functional cholangiocytes.

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[0038] Optionally, contacting a cell population comprising cholangiocyte progenitors with a notch agonist (e.g. a notch signaling donor) comprises co-culturing the cell population comprising cholangiocyte progenitors with a notch signaling donor such as OP9, OP9delta, and/or OP9 Jagged1 cells and optionally in maturatoin media comprising EGF, TGFbeta1, HGF and EGF, and/or HGF,

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5 TGFbeta1 and EGF, for at least or about 5 to at least or about 90 days, optionally for at least or about 5 to at least or about 60 days, at least or about 30 days, at least or about 25 days, 2 at least or about 1 days and/or at least or about 14 days to induce the maturation of cholangiocyte progenitors into cholangiocytes, optionally functional cholangiocytes, optionally wherein the functional cholangiocytes form branched, cyst, tubular or sphere type structures.

10 [0039] In another ~~embodiment~~example, the application provides a method comprising:

(a) producing a population of cells comprising hepatocytes and/or cholangiocytes according to any of the methods described herein; and

(b) introducing the population of cells, or optionally a hepatocyte and/or a cholangiocyte enriched or isolated population, into a subject.

15 [0040] In some ~~embodiment~~examples, the method further comprises enriching or isolating a hepatocyte and/or cholangiocyte population of cells. Optionally, the hepatocyte and/or cholangiocyte population of cells comprises at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or up to about 95% hepatocytes and/or cholangiocytes (e.g. optionally functional hepatocytes and/or cholangiocytes).

20 [0041] The disclosure also provides the use of the population of hepatocytes and/or cholangiocytes for drug discovery, drug metabolism analysis, development of bioartificial liver devices and/or as cell replacement therapy for the treatment of liver conditions and disease.

[0042] Other features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the
25 specific examples while indicating preferred embodiments of the disclosure are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

Brief description of the drawings

30 [0043] An embodiment of the present disclosure will now be described in relation to the drawings in which:

[0044] **Figure 1** shows endoderm induction in hESC-derived embryoid bodies. Figure 1(a) is a schematic representation of the differentiation protocol. EBs are trypsinized at day six and plated as a monolayer in the presence of activin for two days to generate appropriate-staged definitive endoderm.
35 The hepatic lineage is specified from this endoderm population by culture in the presence of BMP4 and FGF. Hepatic maturation is induced through a step wise process, first by the addition of HGF, Dexamethasone (Dex) and Oncostatin M (OSM) for 12 days followed by the generation of 3D aggregates that are cultured for eight days in hepatocyte medium supplemented with Dex and subsequently in this medium with the addition of the cAMP analog, 8-br-cAMP for another 12 days
40 (day 32-44). Figure 1(b) shows flow cytometric analyses showing the proportion of CXCR4+, CKIT+ (CD117) and EPCAM+ cells in day six activin- and activin/Wnt3a-induced populations. Figure 1(c)

Claims:

1. ~~A method of producing hepatocyte and/or cholangiocyte lineage cells from an extended nodal agonist treated induced endodermal cell population, the method comprising:
 - ~~—— (a) specifying the extended nodal agonist treated induced endodermal cell population to obtain a cell population comprising hepatocyte and/or cholangiocyte progenitors by contacting the extended nodal agonist treated induced endodermal cell population with specification media comprising a FGF agonist and a BMP4 agonist and/or active conjugates and/or fragments thereof;~~
 - ~~—— (b) inducing maturation, and optionally further lineage specification and/or expansion of the hepatocyte and/or cholangiocyte progenitors of the cell population to obtain a population comprising hepatocyte lineage cells such as hepatoblasts, hepatocytes and/or cholangiocytes, the inducing maturation step comprising generating aggregates of the cell population.~~~~
2. ~~The method of claim 1, wherein the extended nodal agonist treated induced endoderm population is obtained by inducing endoderm cells in embryoid bodies (EBs) or by inducing endoderm cells that are in a monolayer, and wherein the induced endodermal population is cultured in the presence of a nodal agonist, for example activin, for an extended period to produce an extended nodal agonist treated induced endodermal population.~~
3. ~~The method of any claim 1 or 2, wherein maturation, further lineage specification and/or expansion can comprise one or more additional steps, optionally wherein the cell population comprising hepatocyte and/or cholangiocyte progenitors and/or the aggregates are cultured in the presence of hepatocyte growth factor (HGF), dexamethasone (DEX) and/or Oncostatin M (OSM) and/or active conjugates and/or fragments thereof.~~
4. ~~A method of producing hepatocytes and/or cholangiocytes from a pluripotent stem cell population, the method comprising:
 - ~~—— a) contacting the pluripotent stem cells cultured as a monolayer, with an induction media comprising nodal agonist such as ActA and optionally a wnt/beta-catenin agonist such as i) Wnt3a and/or ii) a GSK-3 selective inhibitor such as CHIR-99021, to provide an induced endodermal cell population;~~
 - ~~—— b) contacting the induced endodermal cell population with a nodal agonist to provide an extended nodal agonist treated induced endodermal cell population;~~
 - ~~—— c) specifying the extended nodal agonist treated induced endodermal cell population according to claim 1 step a);~~~~

- ~~— d) optionally contacting the cell population comprising hepatocyte and/or cholangiocyte progenitors with a maturation media comprising HGF, dexamethasone and/or Oncostatin M and/or active conjugates and/or fragments thereof; and~~
 - ~~— e) inducing maturation, and optionally further lineage specification and/or expansion of hepatocyte and cholangiocyte progenitors of the cell population into hepatocytes and/or cholangiocytes, the inducing maturation step comprising generating aggregates of the cell population.~~
5. ~~A method of producing hepatocytes and/or cholangiocytes from a pluripotent stem cell population, the method comprising:~~
- ~~— a) forming embryoid bodies (EBs) of the pluripotent stem cells, optionally by contacting the pluripotent stem cells with a BMP4 agonist;~~
 - ~~— b) contacting the EBs with an induction media comprising a nodal agonist such as ActA and optionally a wnt/beta-catenin agonist such as i) Wnt3a and/or ii) a GSK-3 selective inhibitor such as CHIR-99021, to provide an induced endodermal cell population;~~
 - ~~— c) dissociating the induced endodermal cell population to provide a dissociated induced endodermal cell population;~~
 - ~~— d) contacting the dissociated induced endodermal cell population with a nodal agonist to provide an extended nodal agonist treated induced endodermal cell population;~~
 - ~~— e) specifying the extended nodal agonist treated induced endodermal cell population according to claim 1 step a);~~
 - ~~— f) optionally contacting the cell population comprising hepatocyte and/or cholangiocyte progenitors with a maturation media comprising HGF, dexamethasone and/or Oncostatin M and/or active conjugates and/or fragments thereof;~~
 - ~~— g) inducing maturation, and optionally further lineage specification and/or expansion of hepatocyte and cholangiocyte progenitors of the cell population into an expanded hepatoblast population and/or hepatocytes and/or a cholangiocytes, the inducing maturation, comprising generating aggregates of the cell population.~~
6. ~~The method of any one of claims 1 to 5, wherein the extended nodal agonist treated induced endodermal population comprises at least, 80%, 85%, 90%, 95 CXCR4 + and eKIT + positive cells and/or at least 70%, 75%, 80% SOX17+ cells.~~
7. ~~The method of any one of claims 1 to 6, wherein the specifying step comprises contacting an extended nodal agonist treated (e.g. activin treated) induced endodermal population with specification media comprising a FGF and BMP4, optionally wherein the FGF is bFGF, FGF10, FGF2 or FGF4 or combinations thereof.~~
8. ~~The method of any one of claims 1 to 7, wherein the aggregates are generated from a cell population comprising at least 70%, 80%, 85%, or 90% albumin positive cells.~~

9. ~~The method of any one of claims 1 to 8, wherein inducing maturation, and optionally further lineage specification and/or expansion further comprises activating the cAMP pathway within the cells of the aggregates to induce the maturation of the hepatocyte and cholangiocyte progenitors into hepatocytes and/or cholangiocytes, optionally wherein, activating the cAMP pathway comprises contacting the aggregates with a cAMP analog such as 8-bromoadenosine 3'5'-cyclic monophosphate.~~

10.1. A method of producing hepatocytes and/or cholangiocytes lineage cells from human pluripotent embryonic stem cells (PSCs) such as embryonic stem cells (ESCs) or induced human pluripotent stem cells (iPSCs), the method comprising:

a) a) generating an induced endodermal cell population from by either:

i. contacting culturing the PSCs or iPSCs pluripotent stem cells cultured as in a monolayer in a medium comprising Activin A and a Wnt/beta-catenin agonist for 3 days, and then 2 days in a medium comprising bFGF and Activin A; or

ii. ~~or~~ culturing the PSCs or iPSCs in medium comprising BMP4 for 1 day to promote formation ~~offormed into~~ embryoid bodies (EBs), and then culturing the EBs inwith an induction media-medium comprising bFGF, Activin A, a Wnt/beta-catenin agonist, and BMP4 for 6 dayscomprising nodal agonist such as ActA and optionally a wnt/beta-catenin agonist such as i) Wnt3a and/or ii) a GSK-3 selective inhibitor such as CHIR-99021 to provide an induced endodermal cell population;

b) ~~contacting culturing~~ the induced endodermal cell population with a ~~nodal agonist~~Activin A for 1 to 4 days to provide an extended nodal agonist-treated induced endodermal cell population; and

c) specifying the extended nodal agonist-treated induced endodermal cell population according to claim 1 step a)to obtain a cell population comprising hepatoblasts by contacting the extended nodal agonist-treated induced endodermal cell population with specification media comprising:

i. a FGF agonist, selected from FGF-2, FGF-4, FGF-10, and active conjugates thereof of fragments thereof; and

ii. a BMP4 agonist selected from BMP4, BMP2, and BMP7 and/or active conjugates and/or fragments thereof; and

d) inducing maturation, and optionally further lineage specification and/or expansion of the hepatocyte and/or cholangiocyte progenitors into hepatoblasts, hepatocytes and/or cholangiocytes, the inducing maturation, and optionally further lineage specification and/or expansion comprising:

~~(i) culturing the cell population comprising hepatocyte and/or cholangiocyte progenitors with a maturation media comprising HGF, OSM and DEXdissociating the progenitor cell population;~~

~~(ii) generating aggregates of the dissociated cell population, optionally when the cell population comprises at least 70%, 80%, 85%, or 90% albumin positive cells or after about 20 to about 40 days of culture for example after about 24 to about 28 days of culture; and~~

~~(iii) culturing the aggregated cells in an aggregated cell maturation medium, comprising at least one of: hepatocyte growth factor (HGF); dexamethasone (DEX); and Oncostatin M (OSM), for 1 to 40 days to produce hepatocyte and/or cholangiocyte lineage cells; and~~

~~(iv) optionally activating the cAMP pathway in the aggregated cells, optionally within about 1 to about 10 days of aggregation, for example within 6 days of aggregation, optionally after about 27 to about 36 days of culture.~~

2. The method of claim 1, wherein the Wnt/beta-catenin agonist is a GSK-3 selective inhibitor.

3. The method of claim 2, wherein the GSK-3 inhibitor is CHIR-99021.

4. The method of claim 1, wherein the Wnt/beta-catenin agonist is Wnt3a.

5. The method of any one of claims 1 to 4, wherein the extended nodal agonist treated induced endodermal population comprises at least, 80%, 85%, 90%, or 95% of the cells that are CXCR4⁺ and cKIT⁺ and/or at least 70%, 75%, or 80% SOX17⁺ as determined by flow cytometric analysis.

6. The method of any one of claims 1 to 5, wherein a cAMP signaling pathway activator is added to the maturation medium within, and including, 1 to 10 days of generating cell aggregates.

~~11. The method of claim 10, wherein the aggregated cell maturation media comprises one or more factors which promote maturation, further lineage specification and/or expansion, optionally:~~

~~a. a wnt agonist such as CHIR 99021, optionally in combination with a TGFbeta antagonist such as SB431542 which promotes expansion of an albumin⁺/HNF4⁺ progenitor population; or~~

~~b. a Wnt antagonist such as XAV939 and/or a Mek/Erk antagonist, for example PD0325901 which is added during the cAMP activation step, which enhances expression of CYP enzymes and promotes maturation of hepatocyte precursors;~~

- ~~12. The method of claim 10 wherein the aggregated cell maturation media comprises one or more factors which promote maturation, further lineage specification and/or expansion, optionally:~~
- ~~a. a Notch agonist which promotes cholangiocyte lineage specification;~~
 - ~~b. a Notch antagonist such as gamma secretase inhibitor (GSI) L695,458 which promotes hepatocyte lineage specification.~~
- ~~13. The method of claim 11 wherein the aggregated cells are treated with a wnt agonist and optionally a TGFbeta antagonist (such as SB431542) for about 6 to about 12 days, preferably about 8 to about 10 days, optionally for about 9 days.~~
- ~~14. The method of claim 12, wherein the maturation media and/or the aggregate specification media comprises a notch agonist, cAMP activation is omitted.~~
- ~~15. A method of producing hepatocytes and/or cholangiocytes from pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), comprises:~~
- ~~a) contacting the pluripotent stem cells cultured as a monolayer or formed into embryoid bodies, with an induction media comprising a nodal agonist such as ActA and optionally a wnt/beta-catenin agonist such as i) Wnt3a and/or ii) a GSK-3 selective inhibitor such as CHIR-99021, optionally for about 4 to about 8 days, to provide an induced endodermal cell population;~~
 - ~~b) contacting the induced endodermal cell population with a nodal agonist, optionally for about 1, 2, 3, or about 4 days, to provide an extended nodal agonist treated induced endodermal cell population;~~
 - ~~c) specifying the extended nodal agonist treated induced endodermal cell population according to claim 1 step a), optionally for about 4 to about 10 days, to obtain a cell population comprising hepatocyte and/or cholangiocyte progenitors, and~~
 - ~~d) inducing maturation, and optionally further lineage specification and/or expansion of the hepatocyte or cholangiocyte progenitors into hepatocytes and/or cholangiocytes, the inducing maturation, and optionally further lineage specification and/or expansion comprising:~~
 - ~~(i) culturing the cell population comprising hepatocyte and/or cholangiocyte progenitors with a maturation media comprising comprising HGF, Dex and/or OSM, optionally for about 10 to 14 days;~~
 - ~~(ii) generating aggregates of the cell population, optionally when the cell population comprises at least 70%, 80% 85%, or 90% albumin positive cells or after about 20 to about 40 days for example after about 24 to about 28 days of culture;~~

~~(iii) culturing the aggregates in maturation medium comprising Dex for about 1 to 10 days;~~

~~iv)~~

~~a) culturing aggregates in a hepatocyte maturation medium comprising Dex and optionally a cAMP agonist, optionally a cAMP analog, for about 6 days to about 10 days, optionally adding the cAMP agonist within about 1 to about 10 days of the generating aggregates step, for example within 6 days of the generating aggregates step, optionally after about 27 to about 36 days of culture; or~~

~~b) culturing the aggregates in a cholangiocyte maturation/specification medium comprising a notch agonist, optionally a notch signal donor such as OP-9 cells, and optionally a cAMP agonist, optionally EGF, TGF β 1, HGF and EGF, and/or EGF, TGF β 1 and HGF for about 5 days to about 90 days, optionally adding the notch agonist within about 1 to about 10 days of the generating aggregates step, for example within 6 days of the generating aggregates step, optionally after about 20 to 40 days of culture and/or coculturing the aggregates when the notch agonist is a notch signaling donor cell.~~

7. The method of claim 6, wherein the maturation medium further comprises a Wnt antagonist or a Mek/Erk antagonist, or both.

8. The method of claim 6, wherein the maturation medium further comprises a Notch antagonist to promote hepatocyte lineage specification.

9. The method of claim 7, wherein the maturation medium further comprises a Wnt agonist or a TGF β antagonist, or both, for 6 to 12 days, 8 to 10 days, or 9 days after cell aggregation.

10. The method of claim 8, wherein the aggregates are cultured in maturation medium comprising (EGF and TGF β 1), (HGF and EGF) or (EGF, TGF β 1 and HGF) for 1 to 10 days after generating cell aggregates, and then Dex for 1 to 10 days, and wherein the maturation medium further comprises a Notch agonist for 5 days to 90 days, wherein the Notch agonist is added within 1 to 10 days of generating aggregates.

~~16,11.~~ The method of ~~any one of claims 1 to 13 and 15~~ claim 8, wherein the hepatocytes produced are functional hepatocytes.

~~17,12.~~ The method of claim ~~16~~11, wherein the functional hepatocytes comprises increased expression of: (a) and/or activity of at least one gene or protein selected from the group consisting of ALB, CPS1, G6P, TDO, CYP7A1, CYP3A7, CYP1A2, CYP3A4, CYP2B6, CYP2C9, CYP2D6, NAT2 and UGT1A1; (b) at least one protein selected from the group consisting of
consisting of ALB, CPS1, G6P, TDO, CYP7A1, CYP3A7, CYP1A2,

- CYP3A4, CYP2B6, CYP2C9, CYP2D6, NAT2 and UGT1A1, or (c) both (a) and (b), compared to the cells of the cell population comprising hepatocyte and/or cholangiocyte progenitors a hepatoblast population.
- ~~18.13.~~ The method of any one of claims 1 to 13 and 15 to 17claim 11, wherein at least 40, 50, 60, 70, 80 or 90% of the functional hepatocytes, optionally functional hepatocytes, are ASGPR-1⁺ cells.
- ~~19.14.~~ The method of any one of claims 1 to 15, wherein the cholangiocyte fate is specified by including a Notch agonist in the maturation medium~~treating aggregates of the cell population during the inducing maturation, further lineage specification and/or expansion step with a notch agonist.~~
15. The method of claim 14, wherein the hepatoblasts are cultured with the Notch agonist for at least 5, 8, 9, 10, 11, 12, 13, or 14 days, to induce further lineage specification, or both, maturation of the hepatocytes into cholangiocytes or functional cholangiocytes.
20. The method of claim 19 wherein the notch agonist comprises a notch ligand bound to a surface such as a cell, plastic, ECM or bead.
21. The method of claim 20, wherein the notch agonist comprises a notch signaling donor such as OP9, OP9delta and/or OP9Jagged1 cells.
22. The method of claim 19 or 20 wherein, the notch agonist comprises a notch ligand selected from notch ligand delta, Jagged-1 Jagged1 peptide, or Pref-1/DLK-1/FA1.
23. The method of any one of claims 19 to 22 wherein the method comprises contacting a cell population comprising cholangiocyte progenitors with a notch agonist such as a notch signaling donor such as OP9, OP9delta and/or OP9Jagged1 cells, optionally in the presence of EGF, TGFbeta1, HGF and EGF, and/or HGF, TGFbeta1 and EGF for at least or about 5 to about 14 days, to induce further lineage specification and/or maturation of the cholangiocyte progenitors into cholangiocytes, optionally functional cholangiocytes.
- ~~24.16.~~ The method of any one of claims 1 to 23claim 15, wherein the population of cholangiocytes produced is a population of functional cholangiocytes comprise optionally wherein the functional cholangiocytes comprise for example increased expression of (a) at least 1, at least 2 or 3 genes or proteinsgene selected from SOX9, CK19, and CFTR (Cystic fibrosis transmembrane conductance regulator); (b) at least 1 protein selected from SOX9, CK19, and CFTR; or (c) both (a) and (b), Sox9, CK19 and CFTR (Cystic fibrosis transmembrane conductance regulator) compared to the cells of the cell population comprising hepatocyte and cholangiocyte progenitors and/or compared to a population cells produced from aggregates a hepatoblast population not treated with a notchNotch agonist, optionally wherein at least 40%, 50%, 60%, 70%, 80% or 90% of the population of cholangiocytes are CK19⁺ cholangiocytes and/or CFTR⁺ cholangiocytes

~~25. The method of any one of claims 1 to 24, wherein the extended nodal agonist treated induced endodermal cell population is induced from pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). The pluripotent stem cells are optionally human ESCs (hESCs) or human iPSCs (hiPSCs).~~

~~26. The method of any one of claims 19 to 25, wherein inducing maturation, and optionally further lineage specification and/or expansion comprises co-culturing the cell population comprising cholangiocyte progenitors with a notch signalling donor such as OP9, OP9delta and/or OP9Jagged1 cells and optionally in the presence of EGF, TGFbeta1, HGF and EGF, and/or HGF, TGFbeta1 and EGF, for at least 5, 8, 9, 10, 11, 12, 13 or 14 days, to induce the maturation of at least one hepatic progenitor into a functional cholangiocyte.~~

~~27.17. A method for generating a functional hepatocyte and/or cholangiocyte comprising:~~

~~a. producing a population of cells comprising hepatoblasts, optionally day 25 stage hepatoblasts; hepatocytes and/or cholangiocytes, according to the method of any one of claims 1 to 26; and~~

~~b. co-culturing the population of hepatoblasts for at least 4, 6, 8, 10, 12, 15, 20, 30, 40, 50, 60, or 90 days with:~~

~~i. co-culturing the population of cells, optionally wherein the population of cells comprises hepatoblasts, with:~~

~~1. a culture of aggregates of CD34+ endothelial cells, optionally CD34+ endothelial cells, optionally embryonic CD34+ endothelial cells, optionally cultured in chimeric aggregates optionally wherein the aggregates are embedded in a gel/matrix to form a co-culture of chimeric aggregates to produce a population of cells comprising functional hepatocytes; or~~

~~2. a culture of Notch signaling donor cells to form a co-culture of notch agonist, optionally notch signaling donor cells, optionally OP9 cells, OP9-Jagged 1 cells and/or OP9delta1 cells, optionally in chimeric aggregates, optionally wherein the aggregates are embedded in a gel/matrix and wherein the aggregates are cultured with (EGF, TGFβ1, EGF and HGF), or EGF, TGFβ1 and HGF to produce a population of cells comprising functional cholangiocytes; and/or~~

~~ii. introducing the population of cells from a) or b i), or a functional hepatocyte and/or cholangiocyte enriched or isolated population therefrom into a subject.~~

~~28. The method of claim 27 wherein the population of cells is co-cultured with: i) endothelial cells for at least about 4 days, about 6 days, about 8 days, about 10 days, about 12 days or more days, up to for example 15 days, 20 days, 30 days, 40 days, 50 days, 60 days or~~

~~90 days, or ii) a notch agonist, optionally OP9 cells, OP9-Jagged 1 cells and/or OP9delta1 cells for at least about about 4 days, about 6 days, about 8 days, about 10 days, about 12 days or more days, up to for example about 90 days or about 60 days.~~

~~29.18. The method of any one of claims 1 to 28-17 wherein the aggregates are embedded in a gel/matrix and 3D cultured.~~

~~19. A method of screening a candidate drug for use in treatment of a liver disease, comprising contacting the candidate drug with a population of cells produced according to a method comprising the method of any one of claims 1-5, and assessing the effect of the candidate drug on hepatocyte or cholangiocyte maturation or function.~~

~~20. A bioartificial liver device comprising a population of cells produced according to a method of any one of claims 1-17.~~

~~21. The method of claim 9, wherein the TGF β antagonist is SB431542.~~

~~22. The method of claim 7 or 9, wherein the Wnt antagonist is XAV939, and the Mek/Erk antagonist is PD0325901.~~

~~23. The method of any one of claims 6 to 13, wherein the cAMP signaling pathway activator is 8-bromoadenosine-3'5"-cyclic monophosphate.~~

~~24. The method of claim 10, wherein the Notch agonist is a Notch signaling donor, comprising a Notch ligand bound to a cell surface, a plastic surface, an extracellular matrix (ECM) or the surface of a bead.~~

~~25. The method of claim 26, wherein the Notch signaling donor is a Notch ligand-expressing OP9 cell, OP9delta cell, and/or an OP9Jagged1 cell, and wherein the Notch ligand is Notch ligand delta, Jagged-1, Jagged1 peptide, or Pref 1/DLK 1/FA1.~~

~~30. The method of any one of claims 1 to 29 further comprising enriching or isolating a hepatocyte and/or cholangiocyte lineage population of cells, optionally a functional hepatocyte population of cells.~~

~~31. The method of claim 30 comprising at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or up to about 95% hepatocytes and/or cholangiocytes, optionally functional hepatocytes.~~

~~32. A population of cells produced according to any one of claims 1 to 31.~~

~~33. Use of the population of cells produced according to the method of any one of claims 1 to 29 or the population of cells of claim 32 for drug discovery, drug metabolism analysis, the development of bioartificial liver devices and/or as cell replacement therapy for the treatment of liver and/or biliary disease.~~

~~34. A kit comprising one or more of the agonists, antagonists, maturation factors etc e.g. described above, one or more medias, vessels for growing cells and the like, which can~~

be used in a method described herein and/or cells expanded and/or prepared according to a method described herein or for use in a method described herein, and optionally instructions for use according to a method herein.