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(54) Title: HEPATOCYTE EXPANSION METHODS

(57) Abstract: This invention relates to culture methods and media for *in vitro* expansion of hepatocytes, particularly primary hepatocytes; hepatocyte cultures and organoids obtainable and obtained by said methods;and uses of said hepatocyte cultures and organoids.

HEPATOCYTE EXPANSION METHODS

TECHNICAL FIELD

This invention provides, *inter-alia*, culture methods and media for *in vitro* expansion of hepatocytes, hepatocyte cultures and organoids obtainable and obtained by said methods, and uses of said hepatocyte cultures and organoids.

BACKGROUND TO THE INVENTION

The mammalian liver possesses a remarkable regenerative ability. Two modes of damage response have been described: 1) The 'oval cell' response emanates from the biliary tree when all hepatocytes are affected by chronic liver disease. 2) A massive, proliferative response of mature hepatocytes occurs upon acute liver damage such as partial hepatectomy (PHx). While the oval cell response has been captured *in vitro* by growing organoids from cholangiocytes, the hepatocyte proliferative response has not been recapitulated in culture.

The liver displays a remarkable regenerative capacity in terms of recovery of mass and function upon surgical removal of up to 2/3 of its size partial hepatectomy (PHx), or after liver-wide chemical or infectious injury (Stanger, 2015). The response to partial removal of the liver is particularly impressive as it involves massive cell cycle entry of remaining mature hepatocytes without an apparent de-differentiation into a progenitor/stem cell-like state (Miyajima et al., 2014). The liver will thus grow back to its original size within two weeks after damage (Michalopoulos, 2010). There is good evidence for direct, slow hepatocyte expansion under steady-state conditions *in vivo* (Wang et al., 2015, Font-Burgada et al., 2015). Proliferation of adult, functional hepatocytes represents the principle mechanism for replacement of lost liver tissue (Stanger, 2015).

An alternative mechanism of liver repair comes into play when noxious agents (toxins, viruses) chronically affect all hepatocytes. Under these circumstances, small cells near the bile duct tree become proliferative ('oval cells'). The prevailing school of thought states that the cholangiocyte-like oval cells represent activated liver stem cells, capable of regenerating hepatocytes as well as new cholangiocytes (Evarts et al., 1987). A long-term 3D liver expansion system of mouse and human adult biliary epithelial-derived progenitor cells that mirrors the *in vivo* oval cell response has been described (Huch et al., 2013; Huch et al., 2015). Under these defined 3D organoid culture conditions, up to a third of mature Epcam+ biliary cells are capable to undergo rapid dedifferentiation into bipotent progenitor cells that expand as cystic structures and can be passaged for over 6 months (Huch et al., 2015; Li et al., 2017; WO2012/014076; WO2015/173425).

Adult mature hepatocytes can also be reprogrammed into proliferative bipotent progenitor cells in response to chronic liver injury (Tanimizu et al., 2014; Tarlow et al., 2014; Yimlamai et al., 2014; Yanger et al., 2013). Indeed, mature hepatocytes have been demonstrated to display plasticity *in vivo*

by transdifferentiating into cholangiocytes/biliary epithelial cells (Sekiya and Suzuki, 2014; Tanimizu et al., 2014; Yanger et al., 2014; Yanger et al., 2013). Lineage tracing has underscored the existence of hepatocyte-derived progenitor cells (hepPD), yet attempts to culture these in 3D culture initially failed (Malato et al., 2011; Tarlow et al., 2014). A recent study has described a cocktail of three small molecules that can convert rat and mouse hepatocytes *in vitro* into small, proliferative bipotent cells, termed 'chemically induced liver progenitors' (CLiPs) in 2D. In long-term culture, CLiPs do not resemble hepatocytes morphologically, yet they retain their proliferative capacity and their hepatic differentiation ability, and can repopulate a chronically injured liver (Katsuda et al., 2017).

However, it has generally remained challenging to maintain functional, mature hepatocytes in culture for > 1-2 weeks, let alone to induce such hepatocytes to enter the cell cycle and to undergo long term expansion *in vitro*. Co-culture systems or expression of HPV genes have been described to support limited hepatocyte expansion (Khetani and Bhatia, 2008; Levy et al., 2015). Recent efforts have focused on producing hepatocytes *in vitro* by differentiation from pluripotent stem cells (embryonic stem cells or induced pluripotent (iPSCs) (Li et al., 2010a; Liang and Zhang, 2013; Lund et al., 2012). Alternatively, it has been proven feasible to induce trans-differentiation of fibroblasts by transfection with reprogramming genes (Huang et al., 2011; Huang et al., 2014; Swenson, 2012; Zhu et al., 2014). While encouraging results are being reported for these approaches, *in vitro*-produced hepatocyte-like cells do not yet resemble freshly isolated, primary hepatocytes in terms of maturation (Si-Tayeb et al., 2010).

There therefore remains a need for methods which enable expanding hepatocytes in culture for a prolonged period of time, while the expanded cells maintain the morphological, functional and biochemical characteristics of hepatocytes.

SUMMARY OF THE INVENTION

The present invention relates to novel methods for expanding hepatocytes *in vitro*. According to some embodiments, primary hepatocytes from fetal or adult liver may be expanded using the methods of the invention, optionally wherein the primary hepatocytes to be expanded are extracted from fetal or adult liver of a subject to be treated by cells expanded using the method.

According to some embodiments, hepatocytes expanded using the methods of the invention form hepatocyte organoids (referred to herein also as "Hep-orgs", "the hepatocyte organoids" or "the hepatocyte organoids of the invention"). The hepatocyte organoids may be expanded in culture for a prolonged period of time while maintaining key morphological, functional and gene expression features of primary hepatocytes. According to some embodiments, the hepatocyte organoids and/or cells derived therefrom (optionally single cells derived therefrom) are able to recapitulate the hepatocyte proliferative response (such as that seen following partial hepatectamy) both *in vitro* and upon transplantation into the liver of a subject. The hepatocyte organoids of the invention are distinct from organoids which originate from expansion of cholangiocytes (also referred to herein as "Chol-

orgs") in their morphology, gene-expression, functionality and/or their ability to be engrafted and proliferate in the liver of a subject. Chol-orgs have been previously described, for example in Huch et al., 2013 and Huch et al., 2015.

According to one aspect, provided herein is a method for expanding hepatocytes *in vitro* (also referred to herein as "the method of the invention"), wherein the method comprises culturing hepatocytes in the hepatocyte culture medium of the invention. The methods of the invention are based in part on the discovery of the hepatocyte culture medium of the invention which enables optimally expanding primary hepatocytes while maintaining their key morphological, functional and gene expression properties. As exemplified herein below (for example in Fig. 12B), removing key components of the hepatocyte culture medium of the invention reduces its ability to enable expansion of primary hepatocytes. According to some embodiments, the method of the invention is a method for generating the hepatocyte organoids of the invention.

According to some embodiments, culturing hepatocytes in the hepatocyte culture medium of the invention is performed while primary hepatocytes, and/or hepatocytes and/or Hep-orgs expanded from them, are in contact with a three dimensional extracellular matrix (ECM), optionally wherein the primary hepatocytes and/or Hep-orgs are grown on and/or embedded in the three dimensional extracellular matrix. Thus, according to some embodiments, the method of the invention comprises culturing primary hepatocytes on and/or within a three dimensional extracellular matrix in the presence of the hepatocyte culture medium of the invention, thus expanding primary hepatocytes, optionally wherein the cells and/or Hep-orgs expanded from the primary hepatocytes are similarly cultured on and/or within a three dimensional extracellular matrix in the presence of the hepatocyte culture medium of the invention.

According to some embodiments, provided herein is the hepatocyte culture medium of the invention. According to some embodiments, the hepatocyte culture medium of the invention comprises:

i. 50 to 500 ng/ml of a Fibroblast Growth Factor (FGF), optionally selected from the group consisting of FGF7, FGF10 and a combination thereof;

ii. a Wnt agonist comprising an R-spondin and at least one glycogen synthase kinase 3 (GSK3) inhibitor;

iii. 5 to 100 ng/ml of epidermal growth factor (EGF);

iv. 5 to 100 ng/ml of hepatocyte growth factor (HGF); and

v. a transforming growth factor beta (TGF-β) inhibitor, comprising an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7.

Thus, according to some embodiments there is provided herein a method for expanding hepatocytes *in vitro*, wherein the method comprises culturing hepatocytes in a hepatocyte culture medium, wherein the hepatocyte culture medium comprises:

i. 50 to 500 ng/ml of a Fibroblast Growth Factor (FGF), optionally selected from the group consisting of FGF7, FGF10 and a combination thereof;

ii. a Wnt agonist comprising an R-spondin and at least one glycogen synthase kinase 3 (GSK3) inhibitor;

iii. 5 to 100 ng/ml of epidermal growth factor (EGF);

iv. 5 to 100 ng/ml of hepatocyte growth factor (HGF); and

v. a transforming growth factor beta (TGF-β) inhibitor, comprising an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7.

The culture medium of the invention is particularly useful for expanding primary hepatocytes. Therefore, according to some embodiments there is provided herein a method for expanding primary hepatocytes *in vitro*, wherein the method comprises culturing primary hepatocytes in a hepatocyte culture medium, wherein the hepatocyte culture medium comprises:

i. 50 to 500 ng/ml of a Fibroblast Growth Factor (FGF), optionally selected from the group consisting of FGF7, FGF10 and a combination thereof;

ii. a Wnt agonist comprising an R-spondin and at least one glycogen synthase kinase 3 (GSK3) inhibitor;

iii. 5 to 100 ng/ml of epidermal growth factor (EGF);

iv. 5 to 100 ng/ml of hepatocyte growth factor (HGF); and

v. a transforming growth factor beta (TGF-β) inhibitor, comprising an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7.

In addition, the culture medium of the invention may be used for expanding hepatocytes obtained by the expansion methods of the invention, including hepatocyte organoids or cells derived therefrom. Therefore, according to some embodiments there is provided herein a method for expanding a hepatocyte organoid or hepatocyte cell derived therefrom *in vitro*, wherein the method comprises culturing the hepatocyte organoid or hepatocyte cell derived therefrom in a hepatocyte culture medium, wherein the hepatocyte culture medium comprises:

i. 50 to 500 ng/ml of a Fibroblast Growth Factor (FGF), optionally selected from the group consisting of FGF7, FGF10 and a combination thereof;

ii. a Wnt agonist comprising an R-spondin and at least one glycogen synthase kinase 3 (GSK3) inhibitor;

iii. 5 to 100 ng/ml of epidermal growth factor (EGF);

iv. 5 to 100 ng/ml of hepatocyte growth factor (HGF); and

v. a transforming growth factor beta (TGF-β) inhibitor, comprising an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7.

In WO 2015/173425 the inventors demonstrated that adding an activator of the cAMP pathway (also referred to as "a cAMP pathway activator") to a culture medium allows human epithelial stem cells to be cultured for an increased number of passages compared to when the cAMP pathway activator is absent from the medium. However, according to preferred embodiments of the present invention, the hepatocyte culture medium of the invention does not comprise a cAMP pathway activator.

According to some embodiments, the R-spondin is selected from the group consisting of: R-spondin 1 (RSPO1), R-spondin 2 (RSPO2), R-spondin 3 (RSPO3), R-spondin 4 (RSPO4), an active fragment or variant thereof, and a combination thereof. According to some embodiments, the R-spondin in the R-spondin conditioned medium is R-spondin 1 (RSPO1).

According to some embodiments, the R-spondin is provided as conditioned medium and is at a concentration of 5%-50%, 5%-40%, 5%-35%, 10%-30%, 10%-20%, 12%-17%, or preferably about 15% (vol/vol) within the hepatocyte culture medium. According to some embodiments, the R-spondin conditioned medium is at a concentration of about 15% (vol/vol) within the hepatocyte culture medium. According to some embodiments, the Wnt agonist in the hepatocyte culture medium of the invention comprises of about 15% (vol/vol) of R-spondin.

According to some embodiments, the GSK3 inhibitor in the hepatocyte culture medium of the invention is at a concentration of 1 to 10 μM, 0.5 to 5 μM, 1 to 5 μM, 2 to 4 μM, or preferably about 3 μM. According to some embodiments, the GSK3 inhibitor in the hepatocyte culture medium of the invention is at a concentration of about 3 μM.

According to some embodiments, the GSK3 inhibitor in the hepatocyte culture medium of the invention is selected from the group consisting of: CHIR99021, SB216763, TWS119, 5-bromoindole, SB415286, BIO, AZD2858, AZD1080, AR-A014418, TDZD-8, LY2090314, BIO-acetoxime, 1-Azakenpaullone and a combination thereof. According to some embodiments, the GSK3 inhibitor in the hepatocyte culture medium of the invention is CHIR99021. According to some embodiments, the GSK3 inhibitor in the hepatocyte culture medium of the invention is CHIR99021 in a concentration of about 3 μM.

According to some embodiments, the hepatocyte culture medium of the invention comprises FGF7. According to some embodiments, the hepatocyte culture medium of the invention comprises FGF7 at a concentration of 50 to 400 ng/ml, 50 to 300 ng/ml, 75 to 200 ng/ml, 80 to 150 ng/ml or preferably about 100 ng/ml. According to some embodiments, the hepatocyte culture medium of the invention comprises FGF7 at a concentration of about 100 ng/ml.

According to some embodiments, the hepatocyte culture medium of the invention comprises FGF10. According to some embodiments, the hepatocyte culture medium of the invention comprises FGF10

at a concentration of 50 to 400 ng/ml, 50 to 300 ng/ml, 75 to 200 ng/ml, 80 to 150 ng/ml or preferably about 100 ng/ml. According to some embodiments, the hepatocyte culture medium of the invention comprises FGF10 at a concentration of about 100 ng/ml.

According to some embodiments, the hepatocyte culture medium of the invention comprises FGF7 and FGF10. According to some embodiments, the hepatocyte culture medium of the invention comprises FGF7 and FGF10, each at a concentration of about 100 ng/ml.

According to some embodiments, the hepatocyte culture medium of the invention comprises EGF at a concentration of 10 ng/ml to 100 ng/ml, 20 ng/ml to 100 ng/ml, 20 ng/ml to 80 ng/ml, 40 ng/ml to 80 ng/ml, or preferably about 50 ng/ml. According to some embodiments, the hepatocyte culture medium of the invention comprises EGF at a concentration of about 50 ng/ml.

According to some embodiments, the hepatocyte culture medium of the invention comprises HGF at a concentration of 10 ng/ml to 100 ng/ml, 20 ng/ml to 100 ng/ml, 20 ng/ml to 80 ng/ml, 40 ng/ml to 80 ng/ml, or preferably about 50 ng/ml. According to some embodiments, the hepatocyte culture medium of the invention comprises HGF at a concentration of about 50 ng/ml.

According to some embodiments, the hepatocyte culture medium of the invention comprises TGF-β inhibitor at a concentration of 1 to 10 μM, 0.5 to 5 μM, 1 to 4 μM, 1 to 3 μM, or preferably about 2 μM. According to some embodiments, the hepatocyte culture medium of the invention comprises a TGF-β inhibitor at a concentration of about 2 μM. According to some embodiments, the TGF-β inhibitor is an inhibitor of the activin receptor-like kinase ALK4, ALK5 and/or ALK7 signalling pathway. According to some embodiments, the TGF-β inhibitor is an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7. According to some embodiments, the TGF-β inhibitor is an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7. According to some embodiments, the TGF-β inhibitor is an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7 selected from the group consisting of: A83-01, SB-431542, SB-505124, EW-7197, LY-2157299, GW6604 and a combination thereof. According to some embodiments, the TGF-β inhibitor is A83-01, an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7. According to some embodiments, the TGF-β inhibitor of the hepatocyte culture medium of the invention is A83-01 at a concentration of about 2 μM.

According to some embodiments, the hepatocyte culture medium of the invention comprises nicotinamide. According to some embodiments, the hepatocyte culture medium of the invention comprises nicotinamide at a concentration of 1 to 100 mM, 1 to 50 mM, 1 to 30 mM, 1 to 20 mM, 5 to 20 mM, 5 to 15 mM, or preferably about 10 mM. According to some embodiments, the hepatocyte culture medium of the invention comprises nicotinamide at a concentration of about 10 mM.

According to some embodiments, the hepatocyte culture medium of the invention comprises gastrin. According to some embodiments, the hepatocyte culture medium of the invention comprises gastrin at

a concentration of 1 to 100 nM, 1 to 50 nM, 1 to 30 nM, 1 to 20 nM, 5 to 20 nM, 5 to 15 nM, or preferably about 10 nM. According to some embodiments, the hepatocyte culture medium of the invention comprises gastrin at a concentration of about 10 nM.

According to some embodiments, the hepatocyte culture medium of the invention comprises further comprises transforming growth factor alpha (TGF-α) (also referred to herein as TGFa). According to some embodiments, the hepatocyte culture medium of the invention comprises TGF-α at a concentration of 1 to 50 ng/ml, 5 to 35 ng/ml, 10 to 30 ng/ml, 15 to 25 ng/ml, or preferably about 20 ng/ml. According to some embodiments, the hepatocyte culture medium of the invention comprises TGF-α at a concentration of about 20 ng/ml.

According to some embodiments, the hepatocyte culture medium of the invention comprises further comprises a Rho-associated protein kinase (ROCK) inhibitor. According to some embodiments, the ROCK inhibitor is selected from the group consisting of: Y-27632, GSK429286A, Fasudil, Thiazovivin, Rho Kinase Inhibitor IV and a combination thereof. According to some embodiments, the ROCK inhibitor is Y-27632. According to some embodiments, the hepatocyte culture medium of the invention comprises ROCK inhibitor at a concentration of 1 to 50 μM, 1 to 30 μM, 1 to 20 μM, 5 to 20 μM, 5 to 15 μM, or preferably about 10 μM. According to some embodiments, the hepatocyte culture medium of the invention comprises ROCK inhibitor is at a concentration of about 10 μM. According to some embodiments, the hepatocyte culture medium of the invention comprises Y-27632 at a concentration of about 10 μM.

According to some embodiments, the hepatocyte culture medium of the invention comprises further comprises N-acetylcysteine. According to some embodiments, the hepatocyte culture medium of the invention comprises further comprises N-acetylcysteine at a concentration of 0.5 to 5 mM, 0.5 to 4 mM, 1 to 4 mM, 1 to 2 mM, 1 to 1.5 mM, or preferably about 1.25mM. According to some embodiments, the hepatocyte culture medium of the invention comprises N-acetylcysteine at a concentration of about 1.25mM.

According to some embodiments, the hepatocyte culture medium of the invention further comprises the B27 supplement. According to some embodiments, the hepatocyte culture medium of the invention comprises further comprises the B27 supplement not containing vitamin A. As used herein the B27 supplement not containing vitamin A refers to 'B27 Supplement minus Vitamin A' (available from Invitrogen, Carlsbad, CA; www.invitrogen.com; currently catalog no. 12587010; and from PAA Laboratories GmbH, Pasching, Austria; www.paa.com; catalog no. F01-002; Brewer et al., J Neurosci Res., 35(5):567-76, 1993).

According to some embodiments, the hepatocyte culture medium of the invention further comprises a cell growth medium. According to some embodiments, the cell growth medium comprises antibiotics, optionally penicillin and streptomycin. According to some embodiments, the cell growth medium is

AdDMEM/F12 (by Invitrogen), optionally comprising Hepes (e.g. 10mM) and/or the Glutamax supplement (Gibco).

According to some embodiments, hepatocytes expanded according to the method of the present invention are cultured in contact with an extracellular matrix (ECM) in the presence of the hepatocyte culture medium of the invention. The extracellular matrix is preferably an exogenous extracellular matrix (meaning that it is not secreted by the cells that are being cultured). In some embodiments the extracellular matrix is a three dimensional extracellular matrix. In other embodiments the extracellular matrix is in suspension. According to some embodiments, the method of the present invention further comprises contacting the primary hepatocytes with a three dimensional extracellular matrix (ECM). According to some embodiments, contacting cells with a three dimensional extracellular matrix (ECM) comprising seeding cells on and/or within the three dimensional extracellular matrix. According to some embodiments, contacting cells with a three dimensional extracellular matrix refers to mixing the cells with a non-solid three dimensional matrix and then allowing the matrix to solidify, thus embedding the cells in and/or on the three dimensional matrix. According to some embodiments, the method of the present invention further comprises contacting the primary hepatocytes with a three dimensional extracellular matrix (ECM) prior to culturing the primary hepatocytes with the hepatocyte culture medium of the invention. According to some embodiments, the method of the invention comprises a step of embedding the primary hepatocytes in a three dimensional extracellular matrix (ECM), followed by culturing the embedded cells and three dimensional matrix in the hepatocyte culture medium of the invention. According to some embodiments, the three dimensional extracellular matrix comprises and/or is prepared using the hepatocyte culture medium of the invention. According to some embodiments, the three dimensional extracellular matrix is selected from the group consisting of: a synthetic ECM, a natural ECM and a combination thereof. According to some embodiments, the three dimensional ECM comprises Matrigel™. According to some embodiments, the three dimensional extracellular matrix comprises the hepatocyte culture medium of the invention at a medium:matrix ratio of 1:1, 1:2, or preferably 1:3. According to some embodiments, the three dimensional ECM comprises Matrigel™ and the hepatocyte culture medium of the invention, optionally in a medium:matrix ratio of 1:3.

According to some embodiments, the primary hepatocytes expanded by the method of the invention are isolated from liver tissue. According to some embodiments, the primary hepatocytes expanded by the method of the invention are isolated from liver tissue, wherein the liver tissue is an explant or a liver sample, optionally from a subject to be administered with the expanded hepatocytes. According to some embodiments, the method of the invention further comprises isolating the primary hepatocytes to be expanded from liver tissue. According to some embodiments, isolating primary hepatocytes comprises collagenase digestion.

According to some embodiments, the primary hepatocytes which are expanded by the method of the invention are mammalian hepatocytes, such as murine or human hepatocytes. Without wishing to be

bound by any theory or mechanism, the expanded murine hepatocytes and/or Hep-orgs may be used in hepatocyte or liver research, or to test the potential effect of certain substances on the liver (e.g. therapeutics or toxins). Expanded human hepatocytes and/or Hep-orgs may be similarly used in research but may also be transplanted into a subject in need of liver regeneration (e.g. following partial hepatectamy) or a subject afflicted with a disease or condition which results in or is associated with liver damage.

According to some embodiments, the hepatocytes which are expanded by the method of the invention may be fetal hepatocytes, adult hepatocytes and a combination thereof. According to some embodiments, the primary hepatocytes which are expanded by the method of the invention may be fetal primary hepatocytes, adult primary hepatocytes (also referred to herein as "adult primary human hepatocytes") and a combination thereof, preferably wherein the primary hepatocytes are human ("PHHs"). According to some embodiments, the primary hepatocytes which are expanded by the method of the invention are albumin-expressing hepatocytes. According to some embodiments, the primary hepatocytes which are expanded by the method of the invention are diploid or tetraploid. According to some embodiments, the primary hepatocytes which are expanded by the method of the invention are not octaploid.

According to some embodiments, expanding hepatocytes using the method of the invention results in formation of one or more hepatocyte organoids (also referred to herein as "the hepatocyte organoids of the invention" or "Hep-orgs"). According to some embodiments, expanding hepatocytes using the method of the invention results in hepatocyte organoids and/or in hepatocytes which are not part of hepatocyte organoids. According to some embodiments, expanding fetal hepatocytes using the method of the invention results in hepatocyte organoids also referred to herein as "Fetal-organoids" or "Fetal-Orgs". According to some embodiments, expanding adult primary human hepatocytes using the method of the invention results in hepatocyte organoids also referred to herein as "PHH-orgs". According to some embodiments, PHH-orgs re-express alpha-fetoglobulin (Afp).

According to some embodiments, the hepatocyte organoids and/or the hepatocytes expanded by the method of the invention are capable of being further expanded in culture for at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45 or 50 passages. According to some embodiments, the hepatocyte organoids and/or the hepatocytes expanded by the method of the invention are capable of being further expanded in culture for at least 3, 4, 5, 6, 10, 12, 15, 18, 20 or 24 months. According to some embodiments, PHH-orgs expanded by the method of the invention are capable of being further expanded in culture for at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 or 5 months. According to some embodiments, the Fetal-orgs expanded by the method of the invention are capable of being further expanded in culture for at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45 or 50 passages.

According to some embodiments, the hepatocyte organoids generated by the method of the invention expand exponentially, optionally wherein the expansion is at a ratio of about 1:3 every 7-10 days.

According to some embodiments, the hepatocyte organoids generated by the method of the invention have a plating efficiency of at least 0.5%, 0.6%, 0.7%, 0.8%, 0.9% or 1%.

According to another aspect, provided herein are hepatocyte organoids generated by the method of the invention. According to some embodiments, provided herein is a culture of hepatocytes, comprising hepatocyte organoids generated by the method of the invention. According to some embodiments, provided herein are single cells and/or a culture of single cells prepared from the hepatocyte organoids generated by the method of the invention.

According to some embodiments, the hepatocyte organoids generated by the method of the invention comprise a lumen. According to some embodiments, the hepatocyte organoids generated by the method of the invention comprise a small lumen and large cells, as opposed to large lumen and flat cells typical for organoids generated from cholangiocytes.

According to some embodiments, the hepatocyte organoids generated by the method of the invention comprise characteristics of whole liver and primary hepatocyte morphology and function selected from the group consisting of: glycogen accumulation, functional uptake of Low Density Lipoprotein (LDL), nuclei comprising prominent nucleoli having a fibrilar centre and de-condensed chromatin, large numbers of mitochondria with few and short cristae, bile canaliculi, tight junctions, peroxisomes, lysosomes, multi-vesicular bodies, autophagic vacuoles and a combination thereof.

According to some embodiments, the hepatocyte organoids generated by the method of the invention secrete at least one of albumin, alpha-1 antitrypsin (A1AT), alpha-fetoglobulin (Afp) and a combination thereof.

According to some embodiments, the hepatocyte organoids generated by the method of the invention demonstrate gene expression comparable to that of the primary hepatocytes for genes selected from the group consisting of: albumin, Apolipoprotein A2 (APOA2), Serpin family member 1 (SERPINA1) and a combination thereof.

According to some embodiments, the hepatocyte organoids generated by the method of the invention do not express marker genes typically expressed in cholangiocytes. According to some embodiments, the hepatocyte organoids generated by the method of the invention express genes which are typically not expressed in cholangiocytes. According to some embodiments, the expression of functional hepatocyte genes, such as, but not limited to, genes related to cytochrome P450 activity, glycogen/lipid metabolism and/or urea cycle, is comparable in Hep-orgs from fetal or adult origin and fetal hepatocytes or adult primary human hepatocytes, respectively. According to some embodiments, the hepatocyte organoids generated by the method of the invention demonstrate lower expression of progenitor/cholangiocyte marker genes than that seen organoids originating in cholangiocytes. Non-limiting examples of such progenitor/cholangiocyte marker genes are EPCAM, SOX9, KRT8/18 and KRT7/19.

According to another aspect, provided herein are the hepatocyte organoids generated by the method of the invention and/or single cells derived therefrom for use in medicine or diagnostics.

According to some embodiments, provided herein are the hepatocyte organoids generated by the method of the invention for use in screening the potential effect of a substance on hepatocytes *in-vivo*, optionally for use in screening for a therapeutic or a toxic effect. Without wishing to be bound by theory or mechanism, the hepatocyte organoids generated by the method of the invention may provide a suitable *in vitro* model for accurately screening and predicting the effect of substances on liver tissue *in-vivo* due to its high morphological and functional similarity to liver tissue

According to some embodiments, provided herein are the hepatocyte organoids generated by the method of the invention for use in treating a disease or condition which is caused by and/or associated with damaged hepatocytes in the liver of a subject. Without wishing to be bound by theory or mechanism, the hepatocyte organoids generated by the method of the invention are able to become engrafted and proliferate in the liver of a subject, thus may be able to replace damaged liver tissue and treat diseases and conditions in which liver tissue is damages (and in particular hepatocytes). According to some embodiments, a disease or condition which is caused by and/or associated with damaged hepatocytes in the liver of a subject is selected from the group consisting of: Hepatocellular Carcinoma, Alagille Syndrome, Alpha- 1- Antitrypsin Deficiency, Autoimmune Hepatitis, Biliary Atresia, Chronic Hepatitis, Cancer of the Liver, Cirrhosis, Liver Cysts, Fatty Liver Disease, Galactosemia Gilbert's Syndrome, Primary Biliary Cirrhosis, Hepatitis A, Hepatitis B, Hepatitis C, Primary Sclerosing Cholangitis, Reye's Syndrome, Sarcoidosis, Tyrosinemia, Type I Glycogen Storage Disease, Wilson's Disease, Neonatal Hepatitis, Non-alchoholic SteatoHepatitis, Porphyria, Hemochromatosis, Progressive familial intrahepatic cholestasis, Glycogen storage disease type III, Tyrosinemia, Deoxyguanosine kinase deficiency, Pyruvate carboxylase deficiency, Congenital dyserythropoietic anemia, Polycystic Liver Disease Polycystic Kidney Disease, Alpha-1 antitrypsine deficiency, Ureum cycle defects, Organic acidemiea, lysosomal storage diseases, Fatty Acid Oxydation Disorders, and a combination thereof.

According to some embodiments, provided herein are the hepatocyte organoids generated by the method of the invention for use in a method of repairing a liver which underwent damage and/or underwent at least partial hepatectamy (HPx), the method comprising transplanting the hepatocyte organoids and/or the single cells derived therefrom into the liver. According to some embodiments, provided herein are the hepatocyte organoids generated by the method of the invention for use in a method for regenerating liver tissue in a subject in need thereof, the method comprising transplanting the hepatocyte organoids and/or the single cells derived therefrom into the liver. According to some embodiments, transplanting the hepatocyte organoids and/or the single cells derived therefrom is via splenic injection. According to some embodiments, methods of treatment, transplantation and/or regeneration as disclosed herein are performed using single cells derived from the hepatocyte organoids of the invention. The skilled person will be able to select an appropriate method and route

of administration depending on the material that is being transplanted (i.e. population of cells, single cells in cell suspension, organoids or fragments of organoids).

Without wishing to be bound by theory or mechanism, the hepatocyte organoids of the invention can engraft onto the liver of the transplanted subject and recapitulate the hepatocyte proliferative response following damage, thus enabling regeneration of damaged and/or removed liver tissue.

According to some embodiments, methods comprising transplanting Fetal-orgs or cells (e.g. single cells) derived therefrom into the liver of a subject further comprise a step of differentiating the cells. According to some embodiments, methods comprising treatment, diagnosis and/or screening using Fetal-orgs or single cells derived therefrom into the liver of a subject further comprise a step of differentiating the cells. According to preferred embodiments, differentiating the cells of Fetal-orgs or cells derived therefrom is performed prior to transplanting or the cells into a subject, using the cells to treat a subject or using the cells *in vitro* for screening and/or diagnosis. Without wishing to be bound by theory or mechanism, differentiating organoids derived from fetal hepatocytes prior to transplanting them into a subject may improve transplantability and engraftment of the cells into the liver of the subject, thus potentially accelerating and/or improving liver regeneration/treatment. According to some embodiments, fetal-orgs and/or single cells derived therefrom that may benefit from differentiation prior to transplantation into a subject are derived from primary hepatocytes isolated from a human fetus, optionally wherein the fetus is at about week 5-30 of gestation, optionally at about week 10-20 of gestation.

Thus, according to some embodiments, provided herein is a method for repairing a liver which underwent damage and/or underwent at least partial hepatectamy (HPx), the method comprising transplanting the hepatocyte organoid and/or the single cells derived therefrom into the liver, wherein the hepatocyte organoid was expanded from fetal primary hepatocytes and wherein the method for repairing a liver further comprises differentiating the hepatocyte organoid and/or the single cells derived therefrom, optionally wherein differentiating is prior to transplanting the hepatocyte organoid and/or the single cells derived therefrom.

According to some embodiments, differentiating the cells of Fetal-orgs and/or single cells derived therefrom is performed by culturing the cells in a differentiation medium (also referred to herein as "DM"). Thus the invention also provides a differentiation medium. According to some embodiments, the differentiation medium increases maturation of the fetal-orgs and/or cells derived therefrom. According to some embodiments, the differentiation medium comprises at least one of dexamethasone and oncostatin M. According to some embodiments, the differentiation medium comprises dexamethasone and oncostatin M. According to some embodiments, the differentiation medium comprises the hepatocyte culture medium of the invention and at least one of dexamethasone and oncostatin M. According to some embodiments, the differentiation medium comprises the hepatocyte culture medium of the invention supplemented by dexamethasone and oncostatin M. According to some embodiments, the dexamethasone is at a concentration of about 1 μM. According to some

embodiments, the oncostatin M is at a concentration of about 10 ng/ml. According to some embodiments, the dexamethasone is at a concentration of about 1 μM and the oncostatin M is at a concentration of about 10 ng/ml. According to some embodiments, the dexamethasone is at a concentration of 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.2, 1.3, 1.4, 1.5, 2, 3, 4 or 5 μM. According to some embodiments, the oncostatin M is at a concentration of about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20 ng/ml.

According to some embodiments, provided herein is a differentiation medium for maturation of fetal hepatocytes, wherein the differentiation medium comprises the hepatocyte medium of the invention and further comprises dexamethasone and oncostatin M, optionally at concentrations of about 1 μM and about 10 ng/ml, respectively. According to some embodiments, the differentiation medium of the fetal hepatocyte organoids of the invention comprises all elements indicated in Table 2 under "Human Fetal-Orgs differentiation medium (DM)". According to some embodiments, provided herein is a differentiation medium for maturation of fetal hepatocytes, wherein the differentiation medium comprises all elements indicated in Table 2 under "Human Fetal-Orgs differentiation medium (DM)".

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 - Establishment of 3D culture system of murine hepatocyte organoids.

(A) Schematic depicting the isolation and seeding of primary hepatocytes, and the expansion and passage of Hep-Orgs.

(B) Differential interference contrast (DIC) images of Hep-Orgs at Passage 0 (P0) Day 20 cultured from primary hepatocytes of wildtype C57BL/6 mice. Lower magnification (Left, Black Scale Bar=50μm), Higher magnification (Right, Black Scale Bar=12.5 μm).

(C) Numbers of organoids formed per 10,000 hepatocytes. Experiments were performed in triplicate and on independent C57BL/6 mice. Data are represented as mean ± SEM.

(D) Differential interference contrast (DIC) images of Chol-Orgs at Passage 2 Day 3 (Left, Black Scale Bar =50μm); Higher magnification (Right, Black Scale Bar =12.5 μm).

(E) Confocal image of paraffin section of a Hep-Org co-stained for proliferation marker mKi67 (Red), the adhesion junction marker *β*-catenin (Yellow) and DAPI (Blue). Scale Bar=25μm.

(F) Transmission EM (TEM) of Hep-Orgs shows typical hepatocyte structures. (I) Overview of cell morphology; (II)-(V) Typical hepatocyte structures (Indicated by arrows). N=Nucleus;

Mv=Microvilli; Tj=Tight junction; Bc=Bile canaliculi-like structures; Cv= Coated vesicles; Ld=Lipid droplets, Si=Sinusoid. Scale Bar=2 μm.

(G) A clonal Hep-Org grown from a single primary hepatocyte (tdTomato^{+}) from a tamoxifen- induced *Albumin-CreERT2*; *Rosa26-LSL-tdTomato* mouse. Images were taken on different days of primary culture, as indicated. Scale Bar=25 μm.

Figure 2 - Characterization of mouse hepatocyte organoids.

(A) Confocal z-stack (left) and single plane (right) images of Hep-Orgs. Albumin (Green), E- cadherin (Red, right panel), and DAPI (Blue). Scale Bar=20μm.

(B) Confocal z-stack (left) and single plane (right) images of mouse Chol-Orgs. Krt7 (Blue), Krt19 (Red), and DAPI (White). Scale Bar=20μm.

(C) + (D) qRT-PCR analysis of gene expression of hepatocyte markers (C) and cholangiocyte/progenitor markers (D) in Hep-Orgs and Chol-Orgs relative to primary hepatocytes. Graph presents mean results from 4 replicates from three independent mice. Data are represented as mean ±SEM. ** indicates $p<0.01$, *** indicates $p<0.001$.

(E) Glycogen accumulation evaluated by Periodic-Acid Schiff (PAS) staining (Dark Pink) in Hep- Orgs. Nuclei were stained with hematoxylin (Blue). Scale Bar=20μm.

(F) Low density lipoprotein (LDL) uptake was analyzed by Dil-ac-LDL fluorescent staining (Red) in cultured Hep-Orgs. Nuclei were stained with DAPI (Blue). Scale Bar=20μm.

(G) Albumin secretion measured after 24h culturing of primary hepatocytes, Hep-Orgs of Passage 0 (P0) Day15 and Passage 3 (P3) and Chol-Orgs in expansion medium (EM) or differentiation medium (DM). Results are indicated as picograms of albumin per cell. Data are represented as mean ±SEM.

(H) Measurement of cytochrome activity (Cyp1a2) in cultured primary hepatocytes, Hep-Orgs of p0 Day15 and p3. Relative light units (RLU) per ml per million cells is indicated. Data are represented as mean ±SEM.

(I) Heatmap of liver gene expression determined by mRNA sequencing comparing three independent Hep-Orgs, (p1) with primary hepatocytes (n=1), and three independent Chol-Orgs (p8-p12) in expansion medium (EM) (Full gene list in Figure 9K).

Figure 3 - Hep-Orgs recapitulate hepatocyte proliferation upon partial hepatectomy.

(A) PCA plot showing the clustering of Hep-Orgs at different passages and their clear distinction from Chol-Orgs (Passage 1, 3, and 7 is labelled as P1, P3 and P7). Note the very small variance (5%) on PC2.

(B) Heatmap of major hepatic markers in primary hepatocytes, Hep-Orgs and Chol-Orgs (from M1 and

M2, two separate mouse donors, Passage 1, 3, and 7 is labelled as P1, P3 and P7).

(C) Correlation plot showing Pearson's correlation coefficient between biological replicates of Chol-Orgs, Hep-Orgs at Passage 1, 3, and 7, PHx and undamaged (Hep) hepatocytes. Color intensities and the size of the circles are proportional to the correlation coefficients. Samples are ordered based on hierarchical clustering and rectangles in the graph are based on the results of the hierarchical clustering. Correlation is based on the Top 1000-highest expressed genes.

(D) GSEA enrichment analysis of Hep-Orgs (biological replicates at Passage 1, 3, 7) vs Primary Hepatocytes as compared to the list of differentially expressed genes between mouse liver three days post-PHx compared with non-damaged liver obtained by mRNA sequencing (top) or microarray (bottom). Enrichment of up-regulated genes after 3 days of partial hepatectomy: Left panel); enrichment of down-regulated genes after 3 days of partial hepatectomy: Right panel.

Figure 4 - Single cell transcriptome analysis of Hep-Orgs

(A) Overview of single cell sequencing experiment of Hep-Orgs and Chol-Orgs and hepatocytes isolated from *Albumin-CreERT2*; *Rosa26-LSL-tdTomato* mice ("undamaged control") or 3 days after 2/3 partial hepatectomy ("regeneration").

(B) t-SNE maps indicating origin of individual cells: Hep-Org cells (Green), Chol-Org cells (Blue).

(C) + (D) t-SNE plot showing the expression of Alb (C) and Krt7 (D) in single cells derived from Hep-Orgs and Chol-Orgs. Expression is given as normalized log2 value.

(E) t-SNE map of all cell clusters from Hep-Orgs obtained by RaceID2 algorithm.

(F) – (H) t-SNE plot showing the expression levels of Alb (F), Pcna (G), and Krt7 (H) in Hep-Orgs. Expression is given as normalized log2 value.

(I) GSEA of genes in Hep-Orgs vs primary hepatocytes. Expression enrichment was compared to a gene set of differentially expressed genes generated by comparing mouse liver three days after partial hepatectomy compared with control non-damaged liver. Enrichment of up-regulated genes at 3 days post-PHx: upper panel; enrichment of down-regulated genes at 3 days of post- PHx (lower panel).

(J) Violin plot comparing the expression of markers in undamaged hepatocytes, post-PHx hepatocytes isolated and Hep-Orgs. Expression of cell cycle-/growth-related genes is given in the top row. Markers were selected for their specific expression in regenerating liver, but unrelated to cell cycling (bottom-three rows). Transcript counts are provided on log10 scale.

Figure 5 -Establishment of Hep-Orgs from human hepatocytes.

(A) DIC images of Hep-Orgs from fetal liver on p0 Day21 (Fetal-Orgs, left panel), Black Scale Bar=40μm or from primary human hepatocytes (PHHs) on p0 Day19 (PHH-Orgs, right panel), Black Scale Bar=20μm.

(B) Numbers of Hep-Orgs formed per 10,000 Fetal-Heps or PHHs. Experiments were performed in triplicate. Data are represented as mean ±SEM.

(C) DIC image showing p5 Chol-Orgs from human adult liver, Black Scale Bar=200μm

(D) Transmission EM of Fetal-Orgs (D) I Overview of cell morphology, Scale Bar=2 μm; II typical hepatocyte structures, Scale Bar=1 μm; III typical hepatocyte structures, Scale Bar=2 μm. N=Nuclear; Nu=Nucleoli, Gly=Glycogen, Mit=Mitochondria, Mv=Microvilli, Tj=Tight junction, GJ= Gap junction, RER=Rough endoplasmic reticulum, Bc=Bile canaliculi-like structures (black arrow), Av=Autophagic vacuoles (white arrow).

(E) Transmission EM of PHH-Orgs and Chol-Orgs (right panel). PHH-Orgs show typical hepatocyte structures. Mvb=Multivesicular bodies, Po=Peroxisomes.

(F) Confocal images of (z-stack projection) of human Fetal-Orgs. ALB (Cyan), HNF4A (Green), F-ACTIN (Red), and DAPI (White), Scale bale=20μm.

(G) Confocal image of whole mount staining of MRP2 and F-ACTIN (3D reconstruction) of human Fetal-Orgs (P26).

(H) Low density lipoprotein (LDL) uptake was analyzed by Dil-ac-LDL fluorescent staining (Red) in cultured human Fetal-Orgs. Nuclei were stained with DAPI (Blue). Scale bale=20μm.

(I) Glycogen accumulation evaluated by PAS staining (Dark Pink) in human Fetal-Orgs. Nuclei were stained with hematoxylin (Blue). Scale bale=20μm.

(J) Albumin secretion by PHHs (White), PHH-Orgs derived from the same PHH batch (White striped), fetal hepatocytes (Fetal-Heps, Grey), six independent Fetal Hep-Org cultures (Grey striped) and differentiated Chol-Orgs (Black). "Late" indicates P22 cultures from one well. Results are indicated as micrograms of albumin per day per million cells. Data are represented as mean ±SEM.

(K) Measurement of cytochrome activity CYP3A4 in PHH-Orgs (Striped) compared with PHHs from which they were derived (White) and with four independent Fetal-Org cultures (same numbering as in Figure 5J). Relative light units (RLU) per ml per million cells is given. Data are represented as mean ±SEM.

Figure 6 - Transcriptional characterization of human Hep-Orgs

(A) Heatmap of top expressed hepatic genes in two independent PHH batches, PHH-Orgs, Fetal-Heps, Fetal-Orgs and two differentiated Chol-Orgs. Hep-Org lines labelled as in Figure 5.

(B) t-SNE map combining Fetal-Org cells (Purple) with Chol-Org cells (Grey).

(C) t-SNE map showing the clustering results of the combined Fetal-Org/Chol-Org dataset. Six main clusters were assigned: Hepatocytes (i), Hepatic mesenchymal-like cells (ii), Progenitors (iii-iv), and Cholangiocytes (v-iv).

(D) – (G) t-SNE plot emphasizing the expression levels of hepatocyte markers ALB, SERPINA1 (D), ASGR1 and ASGR2 (E), cholangiocyte markers EPCAM and KRT7 (F) and ribosome gene RPS10 (G). Dataset as in Figure 6B. Expression is given as normalized log2 value.

Figure 7 - Engraftment of Human Hep-Orgs in FNRG mouse liver

(A) Human ALB and MKI67 immunofluorescent (top) and human NuMA immunohistochemical (bottom) staining of a representative liver section of an FNRG mouse transplanted with human Fetal-Org cells 30 days after transplantation.

(B) Human ALB, MKI67 and KRT19 immunofluorescent staining (top) and human NuMA immunohistochemical staining (bottom) of a representative liver section of a human Fetal-Org transplanted FNRG mouse 90 days after transplantation.

(C) Human ALB, MRP2, CYP2E1, MKI67 and AFP immunofluorescence staining of a representative liver section of a human Fetal Hep-Org transplanted FNRG mouse 90 days after transplantation.

(D) Left panel compares serum hALB time courses of transplanted PHHs (Primary Human Hepatocytes), transplanted PHH-Orgs (Primary Human Hepatocyte Organoids) and transplanted Fetal Hep-Orgs, with each dot value representing the average of the transplanted group. Right panel: data from the 45 day time point for all mice in each group, where each plot represents one mouse.

Figure 8 - Hepatocyte organoids originate mainly from central vein hepatocytes

(A) Numbers of Hep-Orgs per well in medium of indicated composition. Organoid numbers were counted on day 10 after seeding. Data are represented as mean SEM in triplicate wells.

(B) DIC images of Hep-Orgs growing in Hep-Medium at Passage 0 (p0) on Day 8 and Day 13 (the same field). Lipid droplet in organoids indicated with black arrow (Black Scale Bar=100 µm).

(C) Growth curve of Hep-Orgs (derived from primary mouse hepatocytes) followed from Day 0 to Day 100 after seeding. Please note leveling off during months 2-3. Data are represented as mean SEM for three adult male mice of the same age.

(D) DIC images of Hep-Orgs (derived from primary mouse hepatocytes of BALB/c mice or C57BL/6 x BALB/c F1 progeny) cultured in Hep-Medium analyzed at Day 14 (Black Scale Bar=25µm).

(E) Immunohistochemical staining for tdTomato on mouse liver tissue section of Albumin-CreERT2; Rosa26-LSL-tdTomato mouse five days after a single dose of tamoxifen induction (Black Scale Bar=50µm).

(F) Numbers of organoids formed per 5,000 cells FACS-sorted as Axin2 positive (Axin2+) or Axin2 negative (Axin2-). Experiments were performed in triplicate. Data are represented as mean ±SEM.

(G) Numbers of organoids formed per 5,000 Epcam+ cells. Experiments were performed in triplicate. Data are represented as mean ±SEM.

(H)Numbers of organoids formed per 10,000 cells (diploid, tetraploid, octaploid hepatocytes, FACS-sorted using Hoechst34580 and non-sorted hepatocytes). Data are represented as mean ±SEM.

Figure 9 - Comparison of murine Hep-Orgs and Chol-Orgs

(A) Immunohistochemical staining of biliary epithelial cell markers of Krt7 and Krt19 in Hep- Orgs and Chol-Orgs. Scale bale=20μm.

(B) Paraffin sections of Hep-Orgs and Chol-Orgs HE-stained (top) and stained for β-catenin (bottom). Arrows indicated binucleated cells in the Hep-Orgs (Upper panel, Scale Bar=20μm. Bottom panel, Scale Bar=25 μm).

(C) Confocal images of Chol-Orgs (z-stack, top; single confocal section, bottom). F-actin (Blue), Epcam (Green), and DAPI (White). Scale bale=20μm.

(D)– (J) Heatmaps comparing Hep-Orgs (n=3) with primary hepatocytes (n=1) and Chol-Orgs (n=3). Cytochrome activity (D), glycogen metabolism (E), glucose metabolism (F), lipid metabolism (G), complement activation (H), urea cycle (I), and steroid metabolism (J). Gene set was basically from figure S2 (Katsuda et al., 2017). Samples are the same as in Figure 2I.

(K) Heatmap of expression profile of liver genes comparing Hep-Orgs (n=3), with Primary-Heps (n=1), and Chol-Orgs (n=3) with the full gene name list, used in Figure 2I.

(L) Immunofluorescence staining of Glt1 (Green) and DAPI (Blue) of a Hep-Org section. Scale Bar=20μm.

(M)Comparison of pericentral (PC) and periportal (PP) marker expression in Hep-Orgs compared with primary hepatocytes. Data are represented as mean ±SEM. ** indicates $p<0.01$, *** indicates $p<0.001$.

Figure 10 - Hep-Orgs transdifferentiate into Chol-Orgs in Chol-Org medium

(A)Fluorescent DIC images showing conversion of Tomato+ Hep-Orgs from Albumin-CreERT2; Rosa26-LSL-tdTomato into Chol-Orgs.

(B)DIC images showing typical change of Chol-Orgs in Hep-Medium.

(C)Heatmap of expression profile of RNA sequencing comparing Hep-Orgs with/without Chol-medium exposure and Chol-Orgs with/without Hep-Medium exposure for 10 days. Lane 1 and 3: Hep-Org 3 and Chol-Org 3 also appear in Figure 2I.

Figure 11 - Gene expression analysis of Hep-Orgs and Chol-Orgs by single cell sequencing

(A) t-SNE plot of cells color-coded for expression level of indicated Hepatic markers (Afp, Ahsg, Fgg and Gc) (B), Cholangiocyte marker (Epcam) (C), and cell cycle marker (mKi67 and Mdm2). Combined dataset of Hep-Orgs and Chol-Orgs. Expression is given as normalized log2 value. (D-E) t-SNE plot of cells color-coded for expression level of hepatic marker (Hp and Fgg) (D), Cell cycle (Rps10) (E) in

the Hep-Org dataset. Expression is given as normalized log2 value. (F) Volcano plot showing genes differentially expressed between hepatocytes after PHx vs undamaged PHHs. Every dot represents a gene. Red dots highlight genes that show ≥2 or ≤2 fold change and a significance with p-value < 0.05.

Figure 12 - Establishment, maintenance and characterization of human Hep-Orgs

(A) Key components required for Hep-Org initiation from Fetal-Heps (top) or from PHHs (bottom). One of the main components (EGF, FGF, R-spondin CM, CHIR, A83-01, HGF) was withdrawn from Hep-Medium or Chol-medium was used to compare. Hepatocytes were cultured in different conditions. Data are represented as mean SEM.

(B) Key components for growth of two established Fetal-Orgs at P24, the coding corresponds to Figure 5J. One of the main components of Hep-Medium was withdrawn from Hep-Medium or Chol-medium was compared with Hep-Medium in Hep-Org growth maintain. Data are represented as mean SEM.

(C) PAS staining and (D) β-catenin (membrane labeling) of paraffin section of Fetal-Orgs cultured from human Fetal-Heps (Scale Bar=25 μm).

(E) F-ACTIN Staining of human Chol-Orgs (single confocal plane).

(F) CYP2E1 Staining of Fetal-Orgs (single confocal plane).

(G) Representative image of uptake of rhodamine 123 dye in Chol-Orgs, but not Hep-Orgs.

(H) A1AT secretion measured in supernatant of cultured PHHs (White), PHH-Orgs (White striped), Fetal-Heps (Grey), Fetal-Org (Grey striped) and differentiated Chol-Orgs (Black). Results are indicated as micrograms of albumin per day per million cells. Data are represented as mean ±SEM.

(I) AFP secretion measured in supernatant of cultured PHHs (White), PHH-Orgs (White striped), Fetal-Heps (Grey), Fetal-Org (Grey striped) and differentiated Chol-Orgs (Black). 'Late' is P22 from one well. All coding corresponds to Figure 5J and Figure 12H. Data are represented as mean ±SEM.

Figure 13 - Transcriptional comparison of Hep-Orgs, Chol-Orgs and PHHs

(A)Heatmap of key hepatic gene expression in PHHs, PHH -Orgs, Fetal-Heps, Fetal-Orgs and differentiated Chol-Orgs.

(B)Heatmap of expression of progenitor, cholangiocyte and cell cycle-related genes in PHHs, PHH -Orgs, Fetal-Heps, Fetal-Orgs and differentiated Chol-Orgs.

(C)Principal component analysis (PCA) analysis shows clustering of transcriptomic profiles of Hep-Orgs (PHH-Orgs and Fetal-Orgs) with human hepatocytes (PHHs and Fetal-Orgs).

(D) - (F) t-SNE plot of cells color coded for expression level for hepatocyte marker AFP, RBP4, APOA2 and MT1G (D), COL1A2 and FSTL1 (E), cell cycle related genes PCNA and MKI67 (F). Expression is given as normalized log2 value.

Figure 14 - Engraftment and repopulation by human Hep-Orgs compared with PHHs

(A)AFP expression was reduced in differentiated Fetal-Orgs. Comparison was made between Fetal-Orgs of different passages, before or after differentiation with PHHs and PHH-Orgs.

(B)Graft nodule cross section quantified 90 days after transplantation of human Fetal-Orgs.

(C)Human ALB, hKRT, KRT19 and DAPI immunofluorescent staining of liver section of transplanted FNRG mouse 90 days after transplantation.

(D)Comparison of grafts 30 days after transplantation of PHHs, PHH-Orgs derived from the same donor, Fetal-Heps, and Fetal-Orgs (followed for 30 and 90 days) from the same donor.

DETAILED DESCRIPTION OF THE INVENTION

The method of the invention

According to some embodiments, provided herein is a method for expanding primary hepatocytes *in vitro* (also referred to herein as "the method of the invention"), wherein the method comprises culturing primary hepatocytes in the hepatocyte culture medium of the invention. As will be apparent to the skilled person, the method of the invention for expanding primary hepatocytes may be further used to expand cells and/or hepatocyte organoids generated by the method of the invention, *mutatis mutandis*.

According to some embodiments, the method of the invention is a method for expanding primary hepatocytes *in vitro*, wherein the method comprises culturing primary hepatocytes in a hepatocyte culture medium of the invention.

According to some embodiments, the method of the invention is a method for expanding primary hepatocytes *in vitro*, wherein the method comprises culturing primary hepatocytes in a hepatocyte culture medium, wherein the hepatocyte culture medium comprises:

i. 50 to 500 ng/ml of a Fibroblast Growth Factor (FGF), optionally selected from the group consisting of FGF7, FGF10 and a combination thereof;
ii. a Wnt agonist comprising an R-spondin and at least one glycogen synthase kinase 3 (GSK3) inhibitor;
iii. 5 to 100 ng/ml of epidermal growth factor (EGF);
iv. 5 to 100 ng/ml of hepatocyte growth factor (HGF); and
v. a transforming growth factor beta (TGF-β) inhibitor, comprising an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7 signalling.

According to some embodiments, the method of the invention further comprises a step of isolating the primary hepatocytes from a liver, prior to the step of culturing primary hepatocytes in a hepatocyte culture medium, for example, using any isolation method described herein. Other isolation methods are also known in the art. According to other embodiments, no isolation step is required, and a tissue

explant from a fetal or adult liver comprising primary hepatocytes is cultured in a hepatocyte culture medium.

According to some embodiments, the method of the invention further comprises a step of contacting the primary hepatocytes or isolated primary hepatocytes with an extracellular matrix, as defined herein.

According to some embodiments, the method of the invention is a three-dimensional culture method for expanding primary hepatocytes.

The hepatocyte culture medium (Hep-medium) of the invention

According to some embodiments, the hepatocyte culture medium of the invention comprises:

i. an FGF or an analog, fragment or variant thereof;

ii. a Wnt agonist comprising an Rspondin or an analog, fragment or variant thereof, and a glycogen synthase kinase 3 (GSK3) inhibitor;

iii. epidermal growth factor (EGF) or an analog, fragment or variant thereof;

iv. hepatocyte growth factor (HGF) or an analog, fragment or variant thereof; and

v. a transforming growth factor beta (TGF-β) inhibitor, comprising an inhibitor of ALK4, ALK5 and/or ALK7 signalling.

According to some embodiments, the hepatocyte culture medium of the invention comprises:

i. 50 to 500 ng/ml of an FGF, preferably selected from FGF7 and FGF10;

ii. a Wnt agonist comprising 5-50% (vol/vol) R-spondin conditioned medium and at least one glycogen synthase kinase 3 (GSK3) inhibitor, optionally CHIR99021;

iii. 5 to 100 ng/ml of epidermal growth factor (EGF);

iv. 5 to 100 ng/ml of hepatocyte growth factor (HGF); and

v. a transforming growth factor beta (TGF-β) inhibitor, comprising an inhibitor of ALK4, ALK5 and/or ALK7 signalling, optionally A83-01.

In some embodiments, the hepatocyte culture medium further comprises TGFa. This is particularly advantageous for human hepatocyte culture.

In some embodiments, the hepatocyte culture medium further comprises one or more or all of gastrin, nicotinamide, a ROCK inhibitor, B27 (optionally without vitamin A) and N-acetylcysteine.

In some embodiments, the hepatocyte culture medium further comprises further comprises a cell growth medium, such as, but not limited to, AdDMEM/F12 (by Invitrogen), optionally further comprising Hepes (e.g. 10mM) and/or the Glutamax supplement (Gibco), optionally comprising antibiotics such as penicillin and streptomycin.

According to some embodiments, the hepatocyte culture medium of the invention comprises:

about 100ng/ml FGF7;

about 100ng/ml FGF10;

about 20ng/ml TGFa;

about 50ng/ml EGF;

about 50ng/ml HGF;

B27 (not comprising vitamin A);

about 2 μM A83-01;

about 10μM Y-27632;

about 3μm CHIR99021;

about 10 mM Nicotinamide;

about 10 nM gastrin;

about 1.25mM N-acetylcysteine; and

about 15% R-spondin conditioned medium, optionally wherein the hepatocyte culture medium further comprises a cell growth medium, such as, but not limited to, AdDMEM/F12 (by Invitrogen), optionally further comprising Hepes (e.g. 10mM) and/or the Glutamax supplement (Gibco), optionally comprising antibiotics such as penicillin and streptomycin.

According to some embodiments, the concentrations of components in the hepatocyte culture medium of the invention are such that they enable expansion of primary hepatocytes according to the method of the present invention, optionally further enabling maintenance of key morphological, functional and gene expression properties of primary hepatocytes in the expanded cells.

According to some embodiments, the hepatocyte culture medium of the invention comprises the Hep-medium described in Table 2, wherein the Hep-medium(Human) medium is suitable for expanding human adult or fetal hepatocytes, whereas the Hep-medium(Mouse) medium is suitable for expanding murine hepatocytes.

Table 2 – Hepatocyte culture media for mice/human hepatocytes and differentiation medium for human fetal hepatocytes according to some embodiments of the present invention, as compared to corresponding media for expansion/differentiation of cholangiocytes (and Chol-orgs)

Components	Hep-Medium (Mouse)	Hep-Medium (Human)	Human Fetal -Orgs differentiation medium (DM)	Chol-Medium (Mouse) /Chol-Org expansion medium (EM) (R)	Mouse Chol-Orgs differentiation medium (DM) (R)	Chol-Medium (Human) /Chol-Org expansion medium (EM) (R)	Human Chol-Org isolation medium (R)	Mouse Chol-Orgs differentiation medium (DM) (R)
Advanced DMEM/F-12	+	+	+	+	+	+	+	+
1% penicillin/streptomycin	+	+	+	+	+	+	+	+
1% GlutaMAX	+	+	+	+	+	+	+	+
10 mM HEPES	+	+	+	+	+	+	+	+
B27 supplement (1:50)	(without vitamin A)	(without vitamin A)	(without vitamin A)	Either	Either	Either	Either	with vitamin A
N2 supplement (1:100)	-	-	-	-	-	+	+	+
R-spodin1-conditioned medium	15%	15%	15%	5%	-	10%	10%	-
Noggin-conditioned medium	-	-	-	-	-	-	5%	-
Wnt3a-conditioned medium	-	-	-	-	-	-	30%	-
ChIR99021	3μm	3μm	3μm	-	-	-	-	-
N-acetylcysteine	1.25mM	1.25mM	1.25mM	1 mM	1 mM	1 mM	1 mM	1 mM
nicotinamide	10 mM	10 mM	10 mM	10 mM	-	10 mM	10 mM	-
(recombinant) gastrin	10 nM	10 nM	10 nM	10 nM	10 nM	10 nM	10 nM	10 nM
(recombinant) EGF	50 ng/ml	50 ng/ml	50 ng/ml	50 ng/ml	50 ng/ml	50 ng/ml	50 ng/ml	50 ng/ml
(recombinant) TGFa	-	20ng/ml	20ng/ml	-	-	-	-	-
(recombinant) human FGF7	50 ng/ml	50 ng/ml	50 ng/ml	-	-	-	-	-
(recombinant) human FGF10	50 ng/ml	50 ng/ml	50 ng/ml	100 ng/ml	100 ng/ml	100 ng/ml	100 ng/ml	-
(recombinant) human FGF19	-	-	-	-	-	-	-	100 ng/ml
(recombinant) human HGF	25 ng/ml	25 ng/ml	25 ng/ml	50 ng/ml	-	25 ng/ml	25 ng/ml	25 ng/ml
Forskolin	-	-	-	-	-	10 μM	10 μM	
A83-01	1μM	2 μM	2 μM	-	50 nM	5 μM	5 μM	0.5 μM
DAPT	-	-	-	-	10 μM	-	-	10 μM

dexamethasone	-	-	1 µM	-	3 µM (from day 13 to 15)	-	-	3 µM
Oncostatin M	-	-	10ng/ml	-	-	-	-	-
BMP7	-	-	-	-	-	-	-	25 ng/ml
ROCK inhibitor (Y-27632)	10 µM	10 µM	10 µM	-	-	-	10 µM	-

A cell culture medium that is used in a method of the invention comprises any suitable basal medium, subject to the limitations provided herein. Basal media for cell culture typically contain a large number of ingredients, which are necessary to support maintenance of the cultured cells. Suitable combinations of ingredients can readily be formulated by the skilled person, taking into account the following disclosure. A basal medium for use in the invention will generally comprises a nutrient solution comprising standard cell culture ingredients, such as amino acids, vitamins, lipid supplements, inorganic salts, a carbon energy source, and a buffer, as described in more detail in the literature and below. In some embodiments, the culture medium is further supplemented with one or more standard cell culture ingredient, for example selected from amino acids, vitamins, lipid supplements, inorganic salts, a carbon energy source, and a buffer.

The skilled person will understand from common general knowledge the types of culture media that might be used as the basal medium in the cell culture mediums of the invention. Potentially suitable cell culture media are available commercially, and include, but are not limited to, Dulbecco's Modified Eagle Media (DMEM), Minimal Essential Medium (MEM), Knockout-DMEM (KO-DMEM), Glasgow Minimal Essential Medium (G-MEM), Basal Medium Eagle (BME), DMEM/Ham's F12, Advanced DMEM/Ham's F12, Iscove's Modified Dulbecco's Media and Minimal Essential Media (MEM), Ham's F-10, Ham's F-12, Medium 199, and RPMI 1640 Media. Preferably, the basal medium is advanced-DMEM/F12.

The basal medium may be supplemented with penicillin/streptomycin (e.g. 1%), GlutaMAX (e.g. 1%), HEPES (e.g. 10 mM), B27 supplement (1:50, preferably without vitamin A in the Hep-medium), and optionally with N2 supplement (1:100). Preferably, N2 supplement is excluded from the Hep-medium.

It is furthermore preferred that said cell culture medium is supplemented with a purified, natural, semi-synthetic and/or synthetic growth factor and does not comprise an undefined component such as fetal bovine serum or fetal calf serum. Various different serum replacement formulations are commercially available and are known to the skilled person. Where a serum replacement is used, it may be used at between about 1% and about 30% by volume of the medium, according to conventional techniques.

As will be apparent to the skilled reader, the preferred culture methods of the invention are advantageous because feeder cells are not required. Feeder cell layers are often used to support the culture of stem cells, and to inhibit their differentiation. A feeder cell layer is generally a monolayer

of cells that is co-cultured with, and which provides a surface suitable for growth of, the cells of interest. The feeder cell layer provides an environment in which the cells of interest can grow. Feeder cells are often mitotically inactivated (e.g. by irradiation or treatment with mitomycin C) to prevent their proliferation. The use of feeder cells is undesirable, because it complicates passaging of the cells (the cells must be separated from the feeder cells at each passage, and new feeder cells are required at each passage). The use of feeder cells can also lead to contamination of the desired cells with the feeder cells. This is clearly problematic for any medical applications, and even in a research context, complicates analysis of the results of any experiments performed on the cells. As noted elsewhere herein, the culture media of the invention are particularly advantageous because they can be used to culture cells without feeder cell contact, i.e. the methods of the invention do not require a layer of feeder cells to support the cells whose growth is being sponsored.

Accordingly, the compositions of the invention may be feeder cell-free compositions. A composition is conventionally considered to be feeder cell-free if the cells in the composition have been cultured for at least one passage in the absence of a feeder cell layer. A feeder cell-free composition of the invention will normally contain less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1% feeder cells (expressed as a % of the total number of cells in the composition) or preferably no feeder cells at all.

The culture media used in the invention may comprise serum, or may be serum-free and/or serum-replacement free, as described elsewhere herein. Culture media and cell preparations are preferably GMP processes in line with standards required by the FDA for biologics products and to ensure product consistency.

A culture medium of the invention will normally be formulated in deionized, distilled water. A culture medium of the invention will typically be sterilized prior to use to prevent contamination, e.g. by ultraviolet light, heating, irradiation or filtration. The culture medium may be frozen (e.g. at -20°C or -80°C) for storage or transport. The medium may contain one or more antibiotics to prevent contamination. The medium may have an endotoxin content of less than 0.1 endotoxin units per ml, or may have an endotoxin content less than 0.05 endotoxin units per ml. Methods for determining the endotoxin content of culture media are known in the art.

A preferred cell culture medium is a defined synthetic medium that is buffered at a pH of 7.4 (preferably with a pH 7. 2 - 7.6 or at least 7.2 and not higher than 7.6) with a carbonate-based buffer, while the cells are cultured in an atmosphere comprising between 5 % and 10% CO_2, or at least 5% and not more than 10% CO_2, preferably 5 % CO_2.

The invention also provides a composition or cell culture vessel comprising cells and/or organoids according to any one of the aspects of the invention described above, and a culture medium according to any one of the aspects of the invention described above. For example, such a composition or cell

culture vessel may comprise any number of cells or organoids cultured according to a method of the invention, in a culture medium as described above.

According to a still further aspect of the invention, there is provided a hermetically-sealed vessel containing a culture medium of the invention. In some embodiments, the culture medium is an expansion medium. In some embodiments, the culture medium is a differentiation medium. Hermetically-sealed vessels may be preferred for transport or storage of the culture media, to prevent contamination. The vessel may be any suitable vessel, such as a flask, a plate, a bottle, a jar, a vial or a bag.

The hepatocyte organoids of the invention

According to some embodiments, Hep-orgs are generated from human fetal hepatocytes or adult primary human hepatocytes (PHHs). According to some embodiments, the fetal hepatocytes are from human liver of between 5-30 weeks of gestation, optionally of between 8-25 weeks of gestation, preferably of between 11-20 weeks of gestation.

According to some embodiments, Hep-orgs expanded according to the method of the invention can be expanded for at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50 passages. According to some embodiments, Hep-orgs expanded according to the method of the invention can be expanded for at least 16 passages. According to some embodiments, Hep-orgs expanded according to the method of the invention can be expanded for at least 6, 12, 18, 24 months in culture.

According to some embodiments, Hep-orgs originating from fetal liver cells expanded according to the method of the invention can be expanded for at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50 passages. According to some embodiments, Hep-orgs expanded fetal liver cells according to the method of the invention can be expanded for at least 16 passages. According to some embodiments, Hep-orgs expanded from fetal liver cells according to the method of the invention can be expanded for at least 6, 12, 18, 24 months in culture. According to some embodiments, Hep-orgs expanded from adult primary human hepatocytes according to the method of the invention can be expanded for at least 2, 2.5, 3, 4, 5, 6, 12, 18, 24 months in culture.

According to some embodiments, Hep-orgs comprise a lumina and large cells of hepatocyte morphology. According to some embodiments, Hep-orgs have a larger cell diameter than Chol-orgs. According to some embodiments, cells of human Hep-Orgs have a larger cell diameter than those of Chol-Orgs, and a lower nucleus/cytoplasm ratio. According to some embodiments, the cell diameter/nucleus diameter ratio is 30.08±8.17μm/14.07±1.59 μm for Hep-Orgs from fetal liver cells, 7.54±6.50 μm/11.69±1.64μm for Hep-Orgs from PPHs and 10.42±2.78/7.22±1.55μm for cells of Chol-Orgs. In some embodiments, the Hep-Orgs have a cell diameter of at least 15 μm, at least 20 μm or at least 25 μm. In some embodiments, the Hep-Orgs have a nucleus diameter of at least 5 μm, at least 10 μm or at least 15 μm.

According to some embodiments, human Hep-orgs originating from fetal liver cells (also referred to herein as fetal-orgs) express Alb and HNF4A. According to some embodiments, human Hep-orgs originating from fetal liver cells express Alb, HNF4A and CYP2E1.

According to some embodiments, Hep-orgs comprise bile canaliculi. According to some embodiments, Hep-orgs actively demonstrate functional uptake of Low Density Lipoprotein. According to some embodiments, Hep-orgs demonstrate glycogen accumulation. According to some embodiments, fetal-orgs comprise bile canaliculi. According to some embodiments, fetal-orgs actively demonstrate functional uptake of Low Density Lipoprotein. According to some embodiments, fetal-orgs demonstrate glycogen accumulation.

According to some embodiments, Hep-orgs secrete albumin. According to some embodiments, Hep-orgs secrete albumin in a comparable amount to primary human hepatocytes.

According to some embodiments, Hep-orgs secrete A1AT. According to some embodiments, Hep-orgs secrete A1AT at 25%-50% of the level produced by primary human hepatocytes.

According to some embodiments, Hep-orgs secrete Afp. According to some embodiments, Afp secretion by Hep-orgs decreases over time. According to some embodiments, PHH-orgs re-express Afp.

According to some embodiments, fetal-orgs demonstrate CYP3A4 activity 2-8 lower than that of primary human hepatocytes. According to other embodiments, Hep-orgs derived from primary human hepatocytes demonstrate CYP3A4 activity higher than that of primary human hepatocytes.

According to some embodiments, expression of at genes selected from the following group is comparable in primary human hepatocytes and in Hep-orgs (both from fetal and adult origin): Alb, APOA2, SERPINA1 or a combination thereof.

According to some embodiments, the expression of functional hepatocyte genes, such as, but not limited to, genes related to cytochrome P450 activity, glycogen/lipid metabolism and/or urea cycle, is comparable in Hep-orgs from fetal or adult origin and fetal hepatocytes or adult primary human hepatocytes, respectively.

According to some embodiments, fetal-orgs and/or PHH-orgs comprise at least one of the following cell types:

hepatocytes demonstrating high expression of albumin, SERPINA1 and ASGR1, possibly further expressing AFP, MT1, APOA, RBP4 and FABP1;

cells expressing mesenchymal markers such as COL1A2 and FSTL1, possibly further expressing DCN, VIM, FGFR and TGFBI;

cells expressing liver progenitor markers such as CD24, IL32 and MMP7, optionally rich in mitochondrial gene mRNA;

or a combination thereof.

According to some embodiments, Hep-orgs are able to engraft and repopulate damaged liver tissue. According to some embodiments, Hep-orgs demonstrate significant graft expansion after transplantation.

According to some embodiments, Hep-orgs express fetal hepatocyte marker genes Afp, Alb, Hnf4a, Cyp1a2 and Cyp3a11. According to some embodiments, Hep-orgs express fetal hepatocyte marker Cyp3a11 at a significantly higher level than primary hepatocytes (as seen for example in Fig. 2C). According to some embodiments, Hep-orgs express fetal hepatocyte markers Cyp1a2 and Alb at a significantly lower level than primary hepatocytes (as seen for example in Fig. 2C). According to some embodiments, cytochrome Cyp1a2 activity in Hep-orgs is lower up to three-fold than in primary hepatocytes (as seen for example Fig. 2H).

Hepatocyte organoids (Hep-orgs) vs. Cholangiocyte organoids (Chol-orgs)

According to some embodiments, the hepatocyte organoids of the invention are distinct from organoids which originate from cholangiocytes (also referred to herein as "Chol-orgs") by at least one of the following features: Hep-orgs do not express marker genes typically expressed in cholangiocytes, Hep-orgs express genes which are typically not expressed in cholangiocytes, the compact cell clump (or "bunch of grapes") morphology of Hep-orgs is distinct from the large lumen structure of Chol-orgs, the cells of Hep-orgs demonstrate a hepatocyte like morphology as compared to the cholangiocyte morphology of cells of Chol-orgs, expanding Chol-orgs secrete albumin significantly less than Hep-orgs (about 1000 fold less), Hep-org cells have a larger cell diameter than Chol-org cells, Hep-org cells have a lower nucleus/cytoplasm ration that Chol-org cells, or a combination thereof.

According to some embodiments, Hep-orgs do not demonstrate cholangiocyte function. For example, Hep-orgs are unable to actively transport a substrate of the cholangiocyte surface glycoprotein MDR1 (multidrug resistance protein 1) into their lumen.

According to some embodiments, the hepatocyte organoids of the invention are distinct from Chol-orgs by at least one feature as disclosed in Table 1, optionally by 2, 3, 4, 5, 6, 7, 8 or all the features disclosed in Table 1.

Table 1 – Differences between the Hep-orgs of the invention and Chol-orgs according to some embodiments

		Hep-Orgs	Chol-Orgs
Establis	Isolation	Two-step collagenase perfusion (IV) with 3-5 minutes digestion to	Digestion takes >45 minutes with Collagenase XI+Dispase.

		keep fragile hepatocytes alive, low speed Centrifugation.	Human biopsy: collagenase-accutase digestion for 30 minutes.
	Cell Origin	Alb+ murine hepatocytes.	Human or murine Epcam+ duct cells
		Fresh or cryoperserved fetal-Hepatocytes or cryopreserved PHHs.	
	Clonogenicity	Around 1%	28.4%±3.2%
Basic Characterization	**Morphology**	Dense, bunch-of-grape-like organoids. Typical Hepatocyte morphology and structure (Transmission EM).	Cystic organoids with large lumen and lined by flat cells. Cholangiocyte morphology and structure (Transmission EM).
	Growth speed	Fetal-Orgs have a doubling time around 5-7 days, with passaging 1:3 per 7-10 days. PHH-Orgs are kept 50-75 days with 1-2 passages.	Doubling time around 60 hours, with passage ratio of 1:5-7 per week.
	Expression	High expression of hepatic markers such as ALB, A1AT, and HNF4A similar with hepatocytes	High expression of cholangiocyte markers EPCAM, KRT19 and KRT7
Function	**In vitro Assay**	Gene expression profile similar to hepatocytes during normal culture expansion. Almost each cell in Hep-Orgs shows LDL uptake, Glycogen storage, lipid droplet secretion, Albumin Secretion, Cytochrome 450 metabolism etc.	Without hepatocyte differentiation, Chol-Orgs show hardly any glycogen storage or LDL-uptake capacity and have high uptake of rhodamine 123 indicating biliary function.
	Transplantation	Engraftment and significant graft expansion of Fetal-Orgs from fetal hepatocytes in chronic liver damage model with 400 ug/ml hAlb in circulation after 3 months. PHH-Orgs proliferate at the same speed as PHHs 60 days after transplantation.	Low level engraftment in acute liver damage model.

Compositions of the invention

According to some embodiments, there is provided a composition comprising a culture medium of the invention and a liver tissue explant. According to some embodiments, there is provided a composition comprising a culture medium of the invention and one or more primary hepatocyte. According to some embodiments, there is provided a composition comprising a culture medium of the invention and one or more expanded hepatocyte. According to some embodiments, there is provided a composition comprising a culture medium of the invention and a hepatocyte organoid. Any of the compositions of the invention may further comprise an extracellular matrix as described herein.

There is also provided a composition comprising a culture medium of the invention and an extracellular matrix as described herein.

The invention also provides a culture medium supplement that can be used to produce a culture medium as disclosed herein. A 'culture medium supplement' is a mixture of ingredients that cannot itself support stem cells, but which enables or improves stem cell culture when combined with other cell culture ingredients. The supplement can therefore be used to produce a functional cell culture medium of the invention by combining it with other cell culture ingredients to produce an appropriate medium formulation. The use of culture medium supplements is well known in the art.

Pharmaceutical compositions of the invention

According to some embodiments, the invention provides a pharmaceutical composition comprising the components of any culture medium as described herein and a pharmaceutically acceptable diluent and/or excipient.

According to some embodiments, the invention provides a pharmaceutical composition comprising one or more expanded hepatocyte obtained by a method of the invention. According to some embodiments, the invention provides a pharmaceutical composition comprising one or more hepatocyte organoid obtained by a method of the invention. According to some embodiments, the invention provides a pharmaceutical composition comprising one or more hepatocyte from an organoid obtained by a method of the invention. The pharmaceutical composition may be formulated so that it is suitable for administration to a patient in need thereof. The pharmaceutical composition may further comprise a scaffold. Examples of suitable scaffolds for cellular transplantation therapy are well known in the art. A scaffold provides a two-dimensional or three dimensional network. Suitable synthetic materials for such a scaffold comprise polymers selected from porous solids, nanofibers, and hydrogels such as, for example, peptides including self-assembling peptides, hydrogels composed of polyethylene glycol phosphate, polyethylene glycol fumarate, polyacrylamide, polyhydroxyethyl methacrylate, polycellulose acetate, and/or co-polymers thereof (see, for example, Saha et al., 2007. Curr Opin Chem Biol. 11(4): 381–387; Saha et al., 2008. Biophysical Journal 95: 4426–4438; Little et al., 2008. Chem. Rev 108, 1787–1796).

Extracellular matrix (ECM)

According to some embodiments, the method of the present invention further comprises contacting the primary hepatocytes and/or the hepatocyte organoids to be expanded with an extracellular matrix (also referred to herein as "ECM"). The ECM is preferably an exogenous ECM. In some embodiments the extracellular matrix is three-dimensional ("3D ECM"). In other embodiments, it is contemplated that the extracellular matrix is in suspension with the culture medium. According to some embodiments, the method of the invention provides a method of expanding primary hepatocytes and/or hepatocyte organoids in culture, the method comprising culturing the primary hepatocytes and/or hepatocyte organoids in contact with an ECM in the presence of the hepatocyte culture medium of the invention.

According to some embodiments, the primary hepatocytes and/or hepatocyte organoids to be expanded by the method of the invention are contacted with the ECM prior to incubation with the culture medium of the invention. According to some embodiments, the ECM comprises the hepatocyte culture medium of the invention.

"In contact" means a physical or mechanical or chemical contact, which means that for separating said resulting organoid or population of cells from said extracellular matrix a force needs to be used. According to some embodiments, contacting refers to seeding the cells on top of and/or embedding the cells in the 3D ECM. According to preferable embodiments, the primary hepatocytes and/or hepatocyte organoids to be expanded by the method of the invention are embedded within a non-solid 3D ECM, followed by culturing the cells and the 3D ECM in the hepatocyte culture medium of the invention. According to preferable embodiments, the primary hepatocytes and/or hepatocyte organoids are embedded in the 3D ECM. The hepatocyte culture medium of the invention may be diffused into an extracellular matrix (ECM).

Any suitable extracellular matrix may be used with the method of the invention. According to some embodiments, the ECM used in accordance with the invention may be naturally occurring ECM, a commercial ECM mimicking naturally occurring ECM and/or synthetic ECM. Naturally occurring ECM is secreted by connective tissue cells and may be composed of a variety of polysaccharides, water, elastin, and glycoproteins, wherein the glycoproteins may comprise, for example, collagen, entactin (nidogen), fibronectin, and laminin. Different types of naturally occurring ECM are known, comprising different compositions including different types of glycoproteins and/or different combination of glycoproteins. Said ECM can be provided, for example, by culturing ECM-producing cells. Alternatively, ECM mimicking naturally occurring ECM may be commercially provided. Examples of commercially available extracellular matrices are extracellular matrix proteins (Invitrogen), basement membrane preparations from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells (e.g. Matrigel™ (BD Biosciences)) and Basement Membrane Extract (e.g. Cultrex BME™, R&D systems). Alternatively, a synthetic extracellular matrix material, such as ProNectin (Sigma Z378666) may be used.

An example of an ECM for use in a method of the invention comprises at least one glycoprotein, such as laminin. An optional ECM for use in a method of the invention comprises at least two distinct glycoproteins, such as two different types of collagen or a collagen and laminin. A further optional ECM comprises laminin, entactin, and collagen IV. A further preferred ECM is provided by Matrigel™ (BD Biosciences), which comprises laminin, entactin, and collagen IV. In some embodiments the extracellular matrix is a laminin-containing extracellular matrix such as Matrigel™ (BD Biosciences).

In some embodiments, the hepatocyte culture medium is placed on top of the ECM. The culture medium can then be removed and replenished as and when required. In some embodiments, the culture medium is replenished every 1, 2, 3, 4, 5, 6 or 7 days. If components are "added" or "removed" from

the media, then this can in some embodiments mean that the media itself is removed from the ECM and then a new media containing the "added" component or with the "removed" component excluded is placed on the ECM. In some embodiments the hepatocyte culture medium of the invention is in contact with an extracellular matrix.

The method of the invention may further comprise passaging the organoids obtained using the culture method. In some embodiments, one week to 10 day old organoids are removed from the extracellular matrix and mechanically dissociated into small fragments before being transferred to fresh extracellular matrix. The skilled person would know how to split the organoids in order to passage them so that they can multiply without exceeding the concentration limit for their container. In some embodiments, passaging is performed for at least 2 months, for example for 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18, 20, 24 or more months. In some embodiments, passaging is performed in 1:4 to 1:8 split ratios once per week for at least 2 months, for example for 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18, 20, 24 or more months. In some embodiments, the method comprises passaging the organoids more than 6 times, e.g. more than 7, 8, 9, 10, 12, 15, 18, 20, 25, 30 times. The passaging interval can be adapted as necessary. Suitable examples are twice per week, once per week, once every 10 days, once every two weeks, for example once every 7-10 days. The split of the culture medium can be adapted as necessary. Suitable examples are 1:3-1:10 dilutions, e.g. 1:3-1:9, 1:4-1:8, 1:4-1:6 dilutions. According to some embodiments, the hepatocyte culture medium of the invention induces or promotes the survival and/or proliferation of cells during at least 42, 50, 75, 100, 125, 150, 175, 200, 250, 300, 365 days of culture. Proliferation can be assessed using techniques known in the art such as BrdU staining, Edu staining, Ki67 staining and the use of growth curves assay can be done.

Isolation of primary hepatocytes

According to some embodiments, the method of the invention comprises isolating the primary hepatocytes to be expanded. The primary hepatocytes cells may be obtained by any suitable method. According to some embodiments, the primary hepatocytes are isolated from a biopsy or from a tissue fragment.

In some embodiments, primary hepatocytes are isolated by collagenase digestion, for example, as described in the examples and in Dorell et al., 2008 (Hepatology. 2008 Oct;48(4):1282-91. Surface markers for the murine oval cell response. Dorrell C, Erker L, Lanxon-Cookson KM, Abraham SL, Victoroff T, Ro S, Canaday PS, Streeter PR, Grompe M). In some embodiments, collagenase digestion is performed on a tissue biopsy. In some embodiments, collagenase and accutase digestion are used to obtain the epithelial stem cells for use in the invention.

In some embodiments, the starting primary hepatocyte to be expanded is a single cell. A single cell suspension comprising primary hepatocytes can be mechanically generated by mechanical disruption. Alternatively, a population of primary hepatocytes may be used as the starting point. Any one of a number of physical methods of separation known in the art may be used to select the cells of the

invention and distinguish these from other cell types. Such physical methods may involve FACS and various immuno-affinity methods based upon makers specifically expressed by the cells of the invention.

In a further embodiment, there is provided a method for obtaining a hepatocyte organoid comprising expanding primary hepatocytes in the hepatocyte culture medium of the invention as described herein. Following culturing, the method may further comprise obtaining and/or isolating one or more hepatocyte organoid or cells derived therefrom.

Additional definitions

Cells taken directly from live tissue, i.e. freshly isolated cells, are also referred to as primary cells. Accordingly, the term "primary hepatocytes", as used herein, refers to hepatocytes which have been isolated from liver tissue, including, but not limited to, hepatocytes from human adult liver, hepatocytes from a human fetus or hepatocytes from a murine liver. According to some embodiments, primary hepatocytes isolated from human fetus to be used with the method of the invention, is isolated from a fetus at weeks 5-30, optionally 10-20 of gestation.

Primary hepatocytes to be expanded by the method of the present invention may be isolated by any suitable method known in the art. In some embodiments, the primary hepatocytes for use in the methods of invention are isolated from a liver biopsy. In some embodiments, primary hepatocytes are isolated by collagenase digestion, for example, as described in the examples and in Dorell et al., 2008 (Hepatology. 2008 Oct;48(4):1282-91. Surface markers for the murine oval cell response. Dorrell C, Erker L, Lanxon-Cookson KM, Abraham SL, Victoroff T, Ro S, Canaday PS, Streeter PR, Grompe M). In some embodiments, collagenase digestion is performed on a liver biopsy. In some embodiments, collagenase and accutase digestion are used to obtain the primary hepatocytes for use in the invention. According to some embodiments, the primary hepatocytes for use in the methods of the invention are isolated using a two-step collagenase perfusion, as described in the examples herein below.

As used herein, the term "expanding" as it relates to primary hepatocytes and the hepatocyte organoids of the invention refers to proliferation of the cells. According to some embodiments, while the method of the invention provides expansion of primary hepatocytes and/or the resulting hepatocyte organoids *in vitro*, the hepatocyte organoids of the invention are capable of further expanding when transplanted to a liver of a subject *in vivo*.

As used herein, the terms "culturing" and "in culture" refers to the maintenance of cells in an in vitro culture. As known to the skilled person, maintenance of cells in culture may include periodically changing growth medium (such as the hepatocyte culture medium of the invention) and/or transfer to a different growth plate/flask/matrix.

The terms "Epidermal growth factor" and "EGF" as used herein are used interchangeably and refer to any suitable EGF, for example, EGF (Peprotech). EGF may also comprise active analogs, fragments and variants of EGF. By "active" it is meant it activates the EGF receptor with comparable or improved activity relative to EGF.

The terms "transforming growth factor alpha", "TGF-alpha" and TGFa" are used herein interchangeably, and may also comprise active analogs, fragments and variants of TGFa. By "active" it is meant it activates the EGF receptor with comparable or improved activity relative to TGFa. TGFa is a member of the EGFR ligand family and binds EGFR like EGF, but produces a quantitatively and qualitatively distinct cellular response compared to EGF. The present inventors have shown that it is advantageous to include both EGF and TGFa in the hepatocyte medium of the invention.

The terms "Fibroblast growth factor" and "FGF" as used herein are used interchangeably and refer to any suitable FGF, for example, FGF7 (e.g. Peprotech) or FGF10 (e.g. Peptrotech). FGF may also comprise active analogs, fragments and variants of FGF. By "active" it is meant it activates the FGF receptor with comparable or improved activity relative to FGF. FGF7 and FG10 bind to the FGFR2 receptor and, more specifically, to the IIIb isoform. Other FGFs that binds to this receptor and which could therefore be used as alternatives to FGF7 and/or FGF10 include FGF1, FGF3, and FGF22. Therefore in some embodiments, the FGF is selected from one or more of FGF7, FGF10, FGF1, FGF3 and FGF22, and active analogs, fragments or variants thereof.

The terms "hepatocyte growth factor" and "HGF" as used herein are used interchangeably and refer to any suitable HGF, for example, HGF (Peptrotech). HGF may also comprise active analogs, fragments and variants of HGF. By "active" it is meant it activates the HGF receptor (the protein product of the c-Met oncogene) with comparable or improved activity relative to HGF.

The Wnt signalling pathway is defined by a series of events that occur when the cell-surface Wnt receptor complex, comprising a Frizzled receptor, LRP and LGR, is activated, usually by an extracellular signalling molecule, such as a member of the Wnt family. This results in the activation of Dishevelled family proteins which inhibit a complex of proteins that includes axin, GSK-3, and the protein APC to degrade intracellular β-catenin. The resulting enriched nuclear β-catenin enhances transcription by TCF/LEF family transcription factors. As used herein, the term "Wnt agonist" refers to an agent that activates transcription mediated by the TCF/LEF family of transcription factors in a cell. Wnt agonists are therefore selected from true Wnt agonists that bind and activate the Wnt receptor complex (including any and all of the Wnt family proteins), an inhibitor of intracellular β-catenin degradation, a GSK-3 inhibitor (such as CHIR99021) and activators of TCF/LEF. According to some embodiments, the GSK3 inhibitor is "CHIR99021". "CHIR99021" refers to 6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile, CT9902; CAS Number 252917-06-9; PubChem Substance ID 329825639.

"Rspondin" binds to Lgr5 and enhances Wnt signalling. Rspondin is therefore an Lgr5 agonist and a Wnt agonist. Any suitable Rspondin may be used in the hepatocyte culture medium of the invention. According to some embodiments the Rspondin may be selected from one or more of Rspondin 1, Rspondin 2, Rspondin 3 and Rspondin 4 or active analogs, fragments or variants thereof. By "active" it is meant it enhances Wnt signalling via Lgr5 with comparable or improved activity relative to Rspondin 1. According to a preferred embodiment, the Rspondin is Rspondin 1, Rspondin 2, Rspondin 3 or Rspondin 4. In one particular embodiment, the Rspondin is Rspondin 1. Rspondin may be provided in the form of Rspondin conditioned media. For example, about 5% to about 50%, about 10% to about 30% or about 15%. In some embodiments, Rspondin analogs, fragments or variants, or Lgr5 antibodies may be used instead of Rspondin. Examples are known in the art by the skilled person in the field.

As used herein, a "TGF-beta inhibitor" (also referred to herein as an "inhibitor of TGF-beta signalling" of "a transforming growth factor beta (TGF-β) inhibitor") is an inhibitor that reduces the activity of the TGF-beta pathway. The TGF-beta superfamily ligands comprise bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs), anti-müllerian hormone (AMH), activin, nodal and TGF-betas. In general, Smad2 and Smad3 are phosphorylated by the ALK4, 5 and 7 receptors in the TGF-beta/activin pathway. By contrast, Smad1, Smad5 and Smad8 are phosphorylated as part of the bone morphogenetic protein (BMP) pathway. The TGF-beta inhibitor according to the invention reduces the activity of the TGF-beta signalling pathway, preferably the signalling pathway that acts via Smad2 and/or Smad3, more preferably the signalling pathway that acts via activin receptor like kinases 4, 5 and/or 7 (ALK4, ALK5 and/or ALK7). According to some embodiments, no more than one TGF beta inhibitor is present in the hepatocyte culture medium, wherein the one inhibitor is an inhibitor of activin receptor like kinases 4, 5 and/or 7 (ALK4, ALK5 and/or ALK7). In other embodiments, more than one TGF beta inhibitor is present in the hepatocyte culture medium, e.g. 2, 3, 4 or more. According to some embodiments, the TGF-beta inhibitor within the hepatocyte culture medium of the invention comprises a small molecule inhibitor selected from the group consisting of the following inhibitors which are defined in Table 3: A83-01, SB-431542, SB-505124, SB-525334, LY 364947, SD-208, SJN 2511 and a combination thereof. According to a preferable embodiment, the TGF-beta inhibitor within the hepatocyte culture medium of the invention comprises A83-01, optionally at a concentration of about 2μM.

Table 3: Small-molecule TGF-beta inhibitors according to some embodiments

Inhibitor	Targets	IC50 (nM)	Mol Wt	Name	Formula
A83-01	ALK5 (TGF-βR1)	12	421.52	3-(6-Methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide	C25H19N5S
	ALK4	45			
	ALK7	7.5			
SB-431542	ALK5	94	384.39		C22H16N4O3
	ALK4				

	ALK7			4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]benzamide	
SB-505124	ALK5	47	335.4	2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3Himidazol-4-yl)-6-methylpyridine hydrochloride hydrate	C20H21N3O2
	ALK4	129			
SB-525334	ALK5	14.3	343.42	6-[2-(1,1-Dimethylethyl)-5-(6-methyl-2-pyridinyl)-1H-imidazol-4-yl]quinoxaline	C21H21N5
SD-208	ALK5	49	352.75	2-(5-Chloro-2-fluorophenyl)-4-[(4-pyridyl)amino]pteridine	C17H10ClFN6
LY-36494	TGR-βRI	59	272.31	4-[3-(2-Pyridinyl)-1H-pyrazol-4-yl]-quinoline	C17H12N4
	TGF-βRII	400			
	MLK-7K	1400			
SJN-2511	ALK5	23	287.32	2-(3-(6-Methylpyridine-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine	C17H13N5

According to some embodiments, the method of the invention comprises a hepatocyte medium further comprising N-acetylcysteine. "N-acetylcysteine" refers to $HSCH_2CH(NHCOCH_3)CO_2H$ (PubChem CID 12035), and is available commercially as a culture medium supplement (e.g. from Sigma A9165; PubChem Substance ID 24891402).

According to some embodiments, the method of the invention does not comprise a cAMP pathway activator. A cAMP pathway activator is any suitable activator which increases the levels of cAMP in a cell, including, for example, an adenylyl cyclase activator, such as forskolin or cholera toxin. Other examples include cAMP analogs, for example 8-bromo-cAMP. A further example of a cAMP pathway activator is NKH477 (e.g. catalogue no. Tocris 1603).

As used herein, the terms "Alpha-fetoprotein", "Afp", "α-fetoprotein", "alpha-1-fetoprotein", "alpha-fetoglobulin" or "alpha fetal protein" are used interchangeably and refer to the gene or protein product thereof, according to context. As known to the skilled person, Afp is typically expressed at a high degree in fetal liver during development.

As used herein, "hepatic marker" includes but is not necessarily limited to Alb, Hnf4a, Hpx, Hp, Fgg Afp, Ahsg and Gc.

As used herein, the terms "partial hepatectamy" and "PHx" are used interchangeably and refer to removal of a part of a liver.

General

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art.

The term "comprising" encompasses "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term "about" in relation to a numerical value *x* is optional and means, for example, $x \pm 10\%$.

The word "substantially" does not exclude "completely" *e.g.* a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

Unless specifically stated, a process or method comprising numerous steps may comprise additional steps at the beginning or end of the method, or may comprise additional intervening steps. Also, steps may be combined, omitted or performed in an alternative order, if appropriate.

Various embodiments of the invention are described herein. It will be appreciated that the features specified in each embodiment may be combined with other specified features, to provide further embodiments. In particular, embodiments highlighted herein as being suitable, typical or preferred may be combined with each other (except when they are mutually exclusive).

EXAMPLES

Example 1 – Methods

The following methods are used throughout the examples provided herein.

Mice

All animal experiments were performed after institutional review by the Animal Ethics Committee of the Royal Netherlands Academy of Arts and Sciences (KNAW) with project license of AVD8010020151 and research protocol HI16.10.05 and HI16.1002. Albumin- CreERT2 and Axin2-CreERT2 mice were described before (Schuler et al., 2004; van Amerongen et al., 2012). Rosa26-LSL-tdTomato mice were crossed with Albumin-CreERT2 or Axin2-CreERT2 mice and their offspring were used for lineage tracing. Generation and genotyping methods have been previously described (Schuler et al., 2004; van Amerongen et al., 2012). Mice carrying Cre and Rosa alleles aged 8-24 weeks were subjected to a single intraperitoneal injection of 0.2mg per 25 g of body weight tamoxifen (Sigma, T548). Mice were collected after 5-7 days after induction. Both male and female mice were used. For PHx, surgery was performed as described (Greene and Puder, 2003). Mice were sacrificed 2.5-3 days post PHx (Greene and Puder, 2003; Yokoyama et al., 1953). For human cell transplantations, *Fah-/-* NOD *Rag1-/- Il2rgnull* (FNRG) female mice were used at the Rockefeller University under IACUC protocol 15814.

Cell lines

293t-HA-Rspon1-Fc cells were used as previously described to generate conditional medium of R-spondin1. Cells were cultured in a humidified 5% CO2 atmosphere. Commercial human primary hepatocytes were obtained from Thermo Scientific (HMCS10).

Patients and Clinical Specimens

Human fetal livers were obtained from Susana M. Chuva de Sousa Lopes in Leiden University Medical Center. Human liver biopsies were obtained from patients undergoing surgery at University Medical Centre Utrecht Hospital and Rotterdam and University Medical Center. Human cryopreserved fetal and primary human hepatocytes were from Rockefeller University and Lonza Company. The use of samples for research was approved by ethical committees and informed consent were obtained from donors when appropriate.

Isolation of primary mouse hepatocytes

Hepatocytes were isolated from mice by two-step collagenase perfusion (Li et al., 2010b). Briefly, after placing catheter into the portal vein, the inferior vena cava was cut and the liver was perfused at 5-7ml/min with pre-warmed Perfusion Medium for 10 minutes. Then, perfusion was performed with pre-warmed Digestion Medium including Type IV collagenase and Ca2+ at 5 ml/min for 3-5 minutes. After dissociation, cells were filtered through a 70μm filter. Hepatocytes were further separated and purified by centrifugation at low speed (50g, 1-3min) and Percoll gradient centrifugation was optimally performed as described before (Broutier et al., 2016; Huch et al., 2013).

Organoid Culture of Hepatocytes

Isolated hepatocytes were filtered by 70μm filter, washed twice with cold AdDMEM/F12, counted and mixed with MatrigelR in suspension plates (Greiner). 20,000-50,000 cells were used per well of a 24 well plate. After MatrigelR was solidified, Hep-Medium was added. Hep- Medium consists of AdDMEM/F12 (Thermo Scientific, with Hepes, GlutaMax and Penicillin- Streptomycin) plus 15% RSPO1 conditioned medium (home-made), B27 (minus vitamin A), 50ng/ml EGF (Peprotech), 1.25mM N-acetylcysteine (Sigma), 10 nM gastrin (Sigma), 3μM CHIR99021 (Sigma), 25ng/ml HGF (Peprotech), 50ng/ml FGF7 (Peprotech), 50ng/ml FGF10 (Peprotech), 1μM A83-01 (Tocris), 10 mM Nicotinamide (Sigma), and 10μM Rho Inhibitor Y-27632 (Calbiochem). 14 days after seeding, organoids were mechanically fragmented and re- seeded into new MatrigelR. During culturing, medium was refreshed at most every three days. Organoids are usually passaged with a split ratio of 1:3 every 7-10 days.

Human Hep-Org Culture

Fetal hepatocytes were isolated from human fetal tissue by the two-step collagenase perfusion method and isolated by 5min centrifugation at 100g. Red blood cells were removed. Hepatocytes were isolated

from human adult liver by two steps of collagenase digestion and cells were filtered through a 70µm filter and collected by 5min centrifugation at 100g. Different fractions were washed with Advanced DMEM/F12. 10,000 cryopreserved fetal (11-20 weeks), pediatric (age=0.6 years) and adult hepatocytes or freshly isolated cells were mixed with human Hep-Medium and MatrigelR (with a ratio of 1:3) and seeded per 24 wells. After solidification, medium was added. Human Hep-Medium: AdDMEM/F12 (Thermo Scientific, with Hepes, GlutaMax and Penicillin-Streptomycin) plus 15% RSPO1 conditioned medium (home-made), B27 (minus vitamin A), 50ng/ml EGF (Peprotech), 1.25mM N-acetylcysteine (Sigma), 10 nM gastrin (Sigma), 3µM CHIR99021 (Sigma), 50ng/ml HGF (Peprotech), 100ng/ml FGF7 (Peprotech), 100ng/ml FGF10 (Peprotech), 2 µM A83-01 (Tocris), 10 mM Nicotinamide (Sigma), 10µM Rho Inhibitor Y-27632 (Calbiochem) and 20ng/ml TGFa. For Fetal-Org differentiation, Human Hep-Medium was supplemented with 10ng/ml Oncostatin M and 1µM Dexamethasone (Differentiation Medium, DM). During culturing, medium was refreshed every 2-3 days. Organoids are usually passaged with a split ratio of 1:3 every 7-8 days until P10-15 and 1:3 every 14 days from P15 onwards. Chol-Orgs were cultured and passaged as previously described (Broutier et al., 2016). Cell number of organoids growth was calculated by CellTiter- Glo® 3D Cell Viability Assay with a standard curve made from known numbers of primary hepatocytes.

RNA isolation and qRT-PCR

RNA isolation of organoids, tissues, and primary cells were performed with RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Samples with low amount of RNA were lysed in TRIzol (Invitrogen). RNA was reverse transcribed with M-MLV Reverse Transcriptase, RNase H Minus (Promega). qPCR analysis was performed with SYBR Green Mixture (Bio-rad Laboratories) in 96 or 384 q-PCR machine (Bio-rad Laboratories). Primers for qPCR were designed using NCBI Primer-BLAST.

mRNA Sequencing and Analysis

RNA-sequencing libraries were prepared based on the DESeq2 method (Hashimshony et al., 2016; Love et al., 2014). Briefly, total mRNA was isolated by TRIzol or RNeasy Mini Kit (Qiagen) and reverse transcribed with Ambion kit. In vitro transcription was performed using 1-5 ng cDNA as template and RNA was reverse transcribed into sequencing library. Samples were sequenced on an Illumina HiSeq 2000 instrument. Sequencing data analysis was performed using the DESeq2 package (CIT) in R environment. Paired reads were then quantified and normalized. Sample variability was visualized by Principal Component Analysis (PCA). Differential gene expression analysis was performed by DeSeq2 package and visualized by heatmap. Correlation analysis and visualization was performed with Corrplot package. All data analysis and visualization were performed suing R studio.

Single Cell Sequencing and Analysis

Organoids were trypsinized and tissue was perfused with collagenase into single cells. DAPI was used for live/dead cell discrimination. Single, live cells were sorted in 384-well plates using an Aria II cell

sorter (BD bioscience). Sequencing library were prepared accordingly to CEL- seq2 methods (Hashimshony et al., 2016). Briefly, after sorting into 384 plates, cells were lysed at 65°C for 5 minutes and then RNAs were reverse transcribed and pooled before in vitro transcription. Illumina sequencing libraries were prepared using the TruSeq small RNA primers (Illumina) and sequenced paired-end at 75 bp read length on Illumina NextSeq500. All data analysis was performed using RaceID2 algorithm. All RNA sequencing data is accessible through GEO series number GSE111301 in the NCBI Gene Expression Omnibus

Immunohistochemistry, Immunofluorescence, Whole Mount Staining and Microscopy

For section immunofluorescence, organoids were isolated from MatrigelR, fixed in 2% paraformaldehyde in 4°C overnight, washed, and embedded into paraffin blocks. Sections were cut and hydrated before staining. Sections were boiled with citrate (pH=6.0), permeablilized in PBS supplemented with 0.2% triton X-100 (PBST) and blocked with 2% normal donkey serum (Jackson ImmunoResearch) or Power Block™ Universal Blocking Reagent (BioGenex) for 1h at RT. Primary antibodies were then incubated at 4°C overnight. Subsequently, after wash with PBS, sections were incubated with secondary antibodies, stained with DAPI and then embedded using Vertashield (Vector labs). Images were captured on Sp8 confocal microscope (Leica) and processed using Photoshop CS4 or Image J software. Cell size of liver organoids were measured on organoid sections co-stained with member-labelling and DAPI by Image J software.

For Immunohistochemistry, after sections were made and hydrated, they were incubated with blocking buffer with H2O2 for 15 min and boiled with citrate (pH=6.0). After cooling down, sections were treated with pre-blocking buffer and incubated with primary antibodies at 4°C overnight. Sections were incubated with secondary antibodies and DAB stained. Sections were enclosed with Pertex and images were taken on a DM4000 microscope (Leica).

For whole mount staining, organoids were harvested using cell recovery solution (Corning) and fixed in 4% paraformaldehyde at 4°C for 30 min. Organoids were then washed with PBT (PBS, 0.1% Tween), permeablilized in 0.5% PBST, blocked with 2% normal donkey serum in PBS or Power Block™ Universal Blocking Reagent (BioGenex) for 1h at RT and incubated overnight with primary antibodies. The next day, organoids were washed with PBT and incubated overnight at 4 °C with secondary antibodies, Alexa-fluor-647 Phalloidin (both from Thermo Fischer Scientific) and DAPI (Invitrogen). Organoids were optically cleared in a glycerol- based clearing solution for 10 min prior to imaging. Organoid imaging was performed on Sp8 confocal microscope (Leica) or a Zeiss LSM 880 using a 25x oil immersion objective. Images were processed using Photoshop CS4 or Image J software and Imaris imaging software was used for 3D rendering of images.

Microarray

Total RNA was isolated from liver 3 days after 2/3 PHx and from undamaged liver. RNA was amplified, labelled and pooled for microarray analysis at the University of Hong Kong facility.

Universal mouse reference RNA (Agilent) was differentially labelled and hybridized to the tissue. The data for the microarray analysis is deposited at Gene Expression Omnibus under the accession number GSE110292.

Flow cytometry (FACS)

For Albumin+ hepatocyte or Axin2+ hepatocyte sorting, hepatocytes were isolated by the two step collagenase perfusion method and stained with DAPI. Single live Tomato+ cells were sorted and collected into 15ml tubes or 384 wells plates for culture, mRNA or single cell sequencing. For ploidy sorting of hepatocytes, cells were stained with Hoechst 34580 (Invitrogen) at 37°C for 15 minutes and 2n, 4n, or 8n cells were separately collected as described before (Duncan et al., 2010).

Transmission EM

For Transmission EM, organoids were grown in MatrigelR on 3 mm diameter and 200 microm depth standard flat carriers for high pressure freezing and immediately cryoimmobilized using a Leica EM high-pressure freezer (equivalent to the HPM10), and stored in liquid nitrogen until further use. They were freeze-substituted over 3 days at –90 °C in anhydrous acetone containing 2% osmium tetroxide and 0.1% uranyl acetate at –90 °C for 72 hours and warmed to room temperature, 5° per hour (EM AFS-2, Leica, Vienna, Austria). The samples were kept for 2h at 4°C and 2h more at room temperature. After several acetone rinses (4 x 15 min), samples were infiltrated with Epon resin for 2 days (3:1-3h; 2:2-3h; 3:1-overnight; pure resin- 6h +overnight+6h+overnight+3h). Alternatively, chemical fixation with 1.6% glutaraldehyde was performed. The fixation was followed by dehydration in acetone and then embedding in Epon resin as explained. Ultrathin sections were observed in a Tecnai Spirit T12 Electron Microscope equipped with an Eagle CCD camera (Thermo Fisher Scientific, The Netherlands).

Functional Analysis of Hep-Orgs and Chol-Orgs

Hep-orgs of late passages were used for functional analysis. To access glycogen storage, we used periodic acid-Schiff (PAS, Sigma) staining. LDL uptake was detected with DiI-Ac-LDL (Biomedical Technologies). MDR1-mediated transport of rhodamine 123 was detected over a 10-15 minutes incubation. Mouse and Human albumin secretion was detected with Bethyl Elisa Kit and P450 activity was tested by Promega Kit. Human α1-Antitrypsin and human alpha-Fetoprotein (AFP) were detected by Elisa kit from Assaypro and R&D. All experiments were followed manufacturers' instructions.

Transplantation

Human Fetal-Orgs were used after differentiation in DM for 5-7 days on 2D collagen coated plates. Before transplantation, organoids were harvested and digested with trypsin digestion to produce single cells, filtered through at 70 μm cell strainer (BD Bioscience), washed and viable cell numbers were calculated by trypan blue exclusion. Hep-Orgs (Fetal-Orgs and PHH-Orgs) cells after digestion were maintained at 4°C in cold Hep-Medium until transplantation. 100,000 to 300,000 per

mouse were injected intrasplenically into female FNRG mice preconditioned with one 70mg/kg dose of retrorsine, OSM, and 1x109 genomic equivalents of an adenoviral vector expressing human HGF (Agilent). ALB and AFP levels were quantified by ELISA in blood serum obtained every 1-2 weeks after transplantation. Mice were sacrificed at various times (around 30 days and 90 days) after transplantation and liver sections were stained for human markers (hALB and NuMA).

Statistical Analysis

Data was compared between two groups of samples using the unpaired, two-tailed Student's t-test. Error bar presented as mean+/- standard deviation or SEM and p value were calculated.

GSEA Analysis

Gene Set Enrichment Analysis (GSEA, Broad Institute) was used to classify different gene sets. Genes were ranked according to the expression level (up-regulated or down-regulated at least 5 folds) after partial hepatectomy. Then the bulk mRNA sequencing data of Hep-Orgs and Primary-Heps was submitted to GSEA list to pre-ranked modes.

Example 2 – Murine Hepatocyte organoids (Hep-Orgs) generated from single mature hepatocytes

Primary hepatocytes have been isolated from wildtype adult C57BL/6 mouse liver by two-step collagenase perfusion and the cells were suspended in MatrigelR (Figure 1A). A variety of small molecules and biologicals were tested for the ability to support growth, including Wnt agonists such as R-spondin1 and CHIR99021, EGF, FGF7 and FGF10, HGF and the TGF-β inhibitor A83-01 (Figure 8A). During the first week of culture, small organoids emerged from Matrigel-embedded hepatocytes in some of the culture conditions. This allowed refining the conditions, eventually resulting in robust organoid growth (Figure 8A and 8B) in the optimized Hep-Medium (Table 2). Unlike Chol-Orgs, Hep-Orgs were compact with a typical 'bunch-of-grapes appearance (Figure 1B in comparison to Figure 1D: Table 1 gives an overview of all comparisons). Plating efficiency was about 0.5%-1.0% of mature hepatocytes (Figure 1C). This contrasted with the high plating efficiency of biliary epithelial cells (up to 30% of purified cholangiocytes) (Huch et al., 2015; Li et al., 2017), but was similar to that of organoids derived from stomach, colon, or prostate (Bartfeld et al., 2015; Drost et al., 2016; Sato et al., 2011). The organoids expanded to a diameter of ~400μm within 15-20 days and could be passaged by mechanical disruption at a ratio of 1:3 every 7-10 days. Growth speed tended to slow down after 2-3 months (Figure 8C). Organoids could also be obtained from BALB/c and C57BL/6 x BALB/c mice (Figure 8D).

Figure 1E gives a confocal image section stained for the adhesion junction marker *β*-catenin (yellow) and the proliferation marker mKi67 (red), revealing the absence of the large lumen seen in murine Chol-Orgs (Huch et al., 2013 and Figure 2B). Typical hepatocyte morphology was revealed by transmission EM (Figure 1F).

To probe the cell-of-origin, we isolated hepatocytes from *Albumin-CreERT2*; *Rosa26-LSL- tdTomato* mice. After tamoxifen induction, ~99% of albumin-secreting hepatocytes were labelled, whereas no cells of the biliary epithelium showed recombination (Figure 8E). Single, sorted tomato-labelled hepatocytes generated fully labelled organoids, implying that the Hep- Orgs arise from single mature, albumin-expressing hepatocytes (Figure 1G).

Hepatocytes in liver lobules differentially express zonation markers along the pericentral (PC)-to-periportal vein (PP) axis (Bahar Halpern et al., 2017; Burke et al., 2009; Grun et al., 2015). During homeostasis, expression of the Wnt target gene Axin2 marks hepatocytes adjacent to the PC that are the main source of homeostatic self-renewal (Wang et al., 2015). To investigate the zonal origin of Hep-Orgs, we isolated mature hepatocytes from mice carrying *Axin2-CreERT2* and *Rosa26-LSL-tdTomato* alleles one week after Tamoxifen induction by FACS. Axin2+ hepatocytes displayed much higher plating efficiencies than Axin2- hepatocytes (Figure 8F). Control Epcam+ cholangiocytes generated essentially no Hep-Orgs in Hep- Medium (Figure 8G). Additionally, we sorted hepatocytes of different ploidy states (2n, 4n and 8n) as described (Duncan et al., 2010). Tetraploid hepatocytes, common in adult mouse liver, surprisingly yielded a comparable plating efficiency to diploid cells, while octaploid cells did not grow out (Figure 8H).

Example 3 – Hepatocyte organoids (Hep-Orgs) retain key functions and gene expression profiles of hepatocytes

Hep-Orgs as generated in Example 2 were then analyzed by immunofluorescence and immuno-histochemical staining. Hep-Orgs showed strong albumin expression, yet were negative for the bile duct markers Krt19 or Krt7 (Figure 2A and 2B, Figure 9A and 9C). H&E, E-cadherin and β-catenin staining of paraffin sections revealed a characteristic organization of the Hep-Orgs (Figure 2A and Figure 9B). Hep-Org cells were larger in size (Figure 2A and 9B). Quantitative PCR (qPCR) was performed for the hepatocyte markers Alb, Hnf4a, Cyp1a2, and Cyp3a11, the fetal hepatocyte marker Afp, cholangiocytes/progenitor marker Krt19, and progenitor markers Tbx3 and Sox9 (Figure 2C and Figure 2D). Expression of these markers in Hep-Orgs closely resembled that of primary mouse hepatocytes (Primary-Hep). Yet, the former re-expressed the fetal hepatocyte gene Afp, a feature of hemi-hepatectomy (Engelhardt et al., 1976; Sell et al., 1974).

Hep-Orgs showed strong periodic acid-Schiff (PAS) staining indicative of glycogen accumulation (Figure 2E). Low-density lipoprotein uptake (LDL) was readily visualized by fluorescent probes (Figure 2F). Albumin secretion of Hep-Orgs (Passage 1 (P1) and P3) was only 2-4 fold lower compared to primary hepatocytes. Of note, expanding Chol-Orgs secrete at least 1000 fold less Albumin, and differentiated Chol-Orgs at least 10 fold less (Huch et al., 2013) (Figure 2G). Cytochrome activity (Cyp1a2) was also only 2-3 fold lower compared with primary hepatocytes (Figure 2H).

Bulk mRNA sequencing was performed for Hep-Orgs and Chol-Orgs from isolates of three different mice and compared to primary hepatocyte RNA. Figure 2I represents a heat-map of the expression for

~40 hepatocyte genes, 10 cholangiocyte/progenitor genes and a number of proliferation markers (Full gene list given in Figure 9K). Markers of hemi-hepatectomy response (Afp, cell cycle genes) were high in Hep-Orgs, but not in primary hepatocytes. Notably, genes involved in hepatocyte functions such as Cytochrome P450 activity, glycogen metabolism, lipid metabolism, steroid metabolism, urea cycle and complement activation all displayed similar expression profiles between Hep-Orgs and primary hepatocytes (Figure 9D- 9J). Hep-Orgs expressed PC marker to a higher extent than PP markers (Figure 9L and 9M).

These data demonstrate that Hep-Orgs display crucial functional aspects of primary hepatocytes. The hepatocyte organoids of the invention have distinct gene expression profiles compared to transdifferentiated cholangiocytes.

Example 4 – Hepatocyte organoids (Hep-Orgs) recapitulate hepatocyte proliferation upon partial hepatectamy (PHx)

Gene expression patterns over time were assessed by mRNA sequencing for two murine Hep-Org cultures (M1 and M2). These remained remarkably similar over time as visualized by PCA plot and assessed at P1, P3 and P7 (Figure 3A). The heat-map of main hepatic markers such as Alb, Hnf4a, Hpx indicated their stable expression during Hep-Orgs passaging, while Krt7 and Epcam were not re-expressed (Figure 3B).

While Hep-Orgs display crucial functional aspects of primary hepatocytes, they obviously differ from these hepatocytes by being in cycle and by expressing fetal markers such as Afp and Alb (Figure 3B). PHx drives hepatocytes into proliferation, peaking at the third day after damage (Michalopoulos, 2010). To compare gene expression profiles of Hep-Orgs to the *in vivo* transcriptional state of proliferating hepatocytes, we isolated hepatocytes by collagenase digestion at Day 3 post PHx as well as control undamaged hepatocytes and performed mRNA sequencing. Hep-Orgs clustered more closely with PHx hepatocytes than with undamaged hepatocytes or Chol-Orgs (Figure 3C). A gene set of the top-100 genes differentially expressed between PHx samples and control undamaged hepatocytes was compared to Hep-Orgs by gene set enrichment analysis (GSEA). Multiple genes were up-regulated in proliferating hepatocytes after partial-hepatectomy that were also activated in the Hep-Orgs; gene enrichment was found in Hep-Orgs of P1, P3 and P7 (Figure 3D). In addition, we independently determined differential gene expression between PHx and control undamaged liver by microarray analysis and from this analysis we generated a gene set list including genes that changed at least 5-fold. Significant enrichment was again observed by GSEA. Genes that were down-regulated in proliferating post-PHx hepatocytes were repressed in mouse Hep-Orgs. Conversely, upregulated genes in PHx samples were enriched in Hep-Orgs (Figure 3D).

These data indicate that Hep-Orgs have similar gene expression profiles to proliferating hepatocytes.

Example 5 – Hepatocyte organoids (Hep-Orgs) can undergo transdifferentiation into cholangiocytes

It was observed that some progenitor markers like Krt8/18 and Spp1 were upregulated during liver regeneration post-PHx. An in vivo study has described hepatocyte-to-biliary epithelial cell conversion after injury (Yanger et al., 2013). Similarly, cholangiocarcinomas can be derived from hepatocytes upon Notch and AKT activation (Fan et al., 2012; Sekiya and Suzuki, 2014). When culturing primary hepatocytes from Albumin-CreERT2; Rosa26-LSL-tdTomato mice, cystic organoids occasionally formed when CHIR was removed. Indeed, when Tomato-positive organoids were cultured in Chol-medium for more than 10 days, Krt7/19 were strongly induced while Alb and Hnf4a expression were gradually reduced (Figure 10A and 10C). Inversely, transferring Chol-Orgs into Hep-Medium did reduce the Krt7/19 expression but did not induce expression of hepatocyte-lineage markers. (Figure 10B and 10C).

Example 6 – Single cell mRNA analysis of hepatocyte organoids

A single cell mRNA sequencing has been performed on Hep-Orgs and Chol-Orgs (Figure 4A). 384 cells were sequenced from each and the results were analyzed by RaceID2 (Grun et al., 2015). After filtering for cells with > 4000 transcripts, a total of 186 cells from Hep-Orgs and 253 cells from Chol-Orgs were retained for analysis. Analysis of combined datasets from Hep-Orgs and Chol-Orgs was performed and gene expression was visualized by t-SNE (Figure 4B). Consistently, Alb, Ahsg, Afp, Fgg and Gc were only highly expressed in Hep-Orgs population while Epcam and Krt7 were highly expressed in Chol-Orgs (Figure 4C-4D, and Figure 11A and 11B). Both organoid types contained proliferating cells (Figure 11C).

Clustering analysis was then performed on cells derived only from the Hep-Orgs, to address their cellular composition (Figure 4E). Five different clusters were identified. Cluster 1 represented non-cycling mature hepatocytes (Figure 4F and 11D shows high albumin and hepatic marker expression). Clusters 2 and 3 expressed lower levels of albumin, high levels of hepatocyte progenitor markers such as Spp1 (Liu et al., 2015) and represented cycling hepatocyte progenitor cells (Figure 4G and Figure 11E). Cluster 4 expressed high levels of cycling markers and lacked mature markers, identifying it as a yet more primitive cycling cell population. In some cells of Cluster 5, we noted expression of cholangiocyte-lineage markers such as Krt7 (Figure 4H), suggestive of a trans-differentiation event.

197 post PHx hepatocytes and 291 undamaged hepatocytes by single cell sequencing were also retained for analysis after filtering for >3000 transcripts per cell. The top 100 differentially expressed genes between these were grouped in a gene set that was then used for GSEA of Hep-Orgs (Figure 11F). Hep-Orgs were more comparable to proliferating, post- PHx hepatocytes than to control hepatocytes (Figure 4I). We used violin plots to visualize gene expression in undamaged hepatocytes, in PHx hepatocytes and in cells from Hep-Orgs. As expected, cell cycle-related and ribosome synthesis genes were significantly increased in both Hep-Org cells and in post-PHx hepatocytes (Figure 4J). Typical

non-cell-cycle-related genes that were up-regulated after partial-hepatectomy showed concordant expression in Hep-Orgs. These included Afp, Lcn2, Actg1, Fabp5, Clu, Ly6d, Mt2, S100a11, Stmn1, Tubb6, Cdkn1a, and Dynll1 (Figure 4J).

Example 7 – Establishment and characterization of hepatocyte organoids (Hep-Orgs) from human hepatocytes

The Hep-Org medium was optimized for clonogenicity and expansion of human hepatocytes (Hep-Medium) (Figure 12A and 12B). We isolated human fetal liver cells from donor embryos of 11-20 weeks of gestation. Human fetal Hep-Orgs (Fetal-Orgs) could be established from 7 out of 8 fetal donor tissues, each displaying the typical grape-like structure (Figure 5A). Five of these could be expanded for >16 passages and were still growing exponentially at a split ratio of 1:3 every 7-10 days at the time of manuscript resubmission. The longest culture is currently at Passage 28 (fetal origin: 18 weeks post gestation, 11 months of culture). We also established Hep-Orgs (PHH-Orgs) from cryopreserved pediatric and adult primary human hepatocytes (PHHs). These appeared to be more limited in their expansion times (2-2.5 months) yet yielded organoids of very similar composition when compared to the fetal cultures with plating efficiency of around 1% (Figure 5A and 5B). Unlike Chol-Orgs, human Hep-Orgs contained small lumina and consisted of large cells of hepatocyte morphology (Figure 5C, Figure 12C-12E and Table 1). Human Hep-Orgs have a larger cell diameter than Chol-Orgs, and a lower nucleus/cytoplasm ratio (cell diameter/nucleus diameter: 30.08±8.17µm/14.07±1.59 µm for Fetal-Orgs, 27.54±6.50 µm/11.69±1.64µm for PHH-Orgs and 10.42 ±2.78/7.22 ±1.55 µm for Chol-Orgs). Transmission EM revealed an abundance of glycogen particles in the cytoplasm. Typical liver hepatocyte features included nuclei with prominent nucleoli with fibrillar centers and de-condensed chromatin, large numbers of mitochondria with few and short cristae, and individual cisternae of RER organized around them. Also, Golgi apparatus saccule stacks, bile canaliculi, tight junctions, peroxisomes, lysosomes, multi-vesicular bodies and autophagic vacuoles were present in human Hep-Orgs, closely resembling those of hepatocytes (Figure 5D and 5E).

Fetal-Orgs abundantly expressed ALB and HNF4A, while a significant portion of cells expressed CYP2E1 (Figure 5F and Figure 12F). Striking networks of bile canaliculi were revealed by MRP2 staining (Figure 5G). DiI-Ac-LDL and strong periodic acid-Schiff (PAS) staining in Fetal-Orgs indicated functional LDL uptake and confirmed glycogen accumulation (Figure 5H and 5I). Hep-Orgs did not demonstrate cholangiocyte function: Rhodamine123, a fluorescent substrate for the cholangiocyte surface glycoprotein multidrug resistance protein-1 (MDR1), was actively transported into the lumen of Chol-Orgs but not of Hep-Orgs (Figure S5G). Notably, albumin secretion by Hep-Orgs (6 fetal donors, 1 PHH donor) was comparable to that of PHHs (Figure 5J). A1AT secretion by Hep-Orgs was 25%-50% of the level produced by PHHs (Figure 12H). While ALB slightly increased with increasing Fetal- Org passage number (compare Fetal-Orgs 2 and 3 with Fetal-Orgs 2 late and 3 late in Figure 5J), AFP secretion decreased over time (Figure 12I). CYP3A4 activity in Hep-Orgs

derived from PHHs was higher than that of PHHs, while activity in Fetal-Orgs was 2-8 fold lower than that of PHHs (Figure 5K).

Bulk mRNA sequencing was performed to compare Fetal-Orgs of different passages and PHH-Orgs to Fetal-Heps and PHHs. The heatmap represents a broad array of hepatocyte genes such as ALB, APOA2 and SERPINA1 in Fetal-Orgs and PHH-Orgs that remained comparable to PHHs over multiple passages (Figure 6A). Expression levels of functional hepatocyte genes (cytochrome P450 activity, glycogen/lipid metabolism and urea cycle) all displayed comparable expression levels between late passage Fetal-Orgs and PHH-Orgs with Fetal-Heps/PHHs (Figure 13A). These levels were much higher than those of hepatocyte-like cells in Chol-Orgs after differentiation. By contrast, progenitor/cholangiocyte markers like EPCAM, SOX9, KRT8/18 and KRT7/19 remained higher in Chol-Orgs in differentiation medium (DM), when compared to Hep-Orgs (Figure 13B). The PCA plot underscored the difference between Chol-Orgs in DM and Hep-Orgs. It is noted that PHH-Orgs re-express AFP (Figure 6A) which makes PHH-Orgs closer to Fetal-Heps than to PHHs in the PCA plot (Figure 13A and Figure 13C).

We then performed single-cell mRNA sequencing on 384 cells derived from Fetal-Orgs and 384 cells from human Chol-Orgs. After applying filtering criteria of a minimum of 4000 detected transcripts/cell, 161 and 197 cells were retained for analyses, respectively. Clustering analysis by RaceID2 on the combined Fetal-Orgs and Chol-Orgs datasets revealed six main clusters, as visualized in a t-SNE map (Figure 6B and 6C). Cluster i-iv cells almost exclusively derived from Fetal-Orgs. Marker expression analysis revealed that cluster i represented hepatocytes, marked for instance by high Albumin, SERPINA1 and ASGR (Figure 6D-6E and Figure 13D). Cluster ii was defined by mesenchymal markers such as COL1A2 and FSTL1 (Figure 13E). Cluster iii and iv expressed liver progenitor markers such as CD24 and IL32, while some cells were rich in mitochondrial gene mRNAs that are also highly expressed during liver regeneration. The large clusters v and vi expressed (early) cholangiocyte markers such as KRT19 and EPCAM (Figure 6F). Indeed, almost all cells in these two clusters were derived from Chol-Orgs. Both Fetal-Orgs and Chol-Orgs cells expressed markers of proliferation (Figure 6G and Figure 13F).

Example 8 –Hepatocyte organoids (Hep-Orgs) from human hepatocytes can engraft and repopulate damaged liver

We addressed whether human Hep-Orgs are able to engraft and repopulate damaged liver tissue. Since transplantability generally correlates with hepatocyte maturity, we defined a differentiation medium (DM, containing dexamethasone and oncostatin M) to increase maturation of Fetal-Orgs (Kamiya et al., 2001) (Figure 14A). Fetal-Org line 2 (Passage 16) cells were seeded on collagen-coated plates for 5-7 days in DM (7-10 days). Subsequently, organoids were transplanted as single cells into immunodeficient *Fah-/- NOD Rag1-/- Il2rg-/-* (FNRG) mice (Aini et al., 2014; Billerbeck et al., 2016; Grompe, 2017) by splenic injection.

For the first 30 Days after transplantation, human ALB in mouse circulation remained stable and was clearly detectable in all mice that received Fetal-Orgs (Figure 7D). Initial engraftment of small clusters of 1-2 cells was confirmed by human ALB and NuMa staining (Billerbeck et al, 2016) (Figure 7A). Fetal-Heps of the same donor that were transplanted in parallel as positive control showed continuous increase in hAlb in the same time period and had formed bigger clusters at day 30 post transplantation. (Fig.7D and 14D). After day 30, however, the organoid graft started to proliferate more rapidly and expanded at the same rate as primary cells. 90 days after transplantation, serum hALB of Fetal-Orgs had risen 200-fold to more than 200 ug/ml on average. Clusters had grown significantly and demonstrated ongoing proliferation as confirmed by Ki67 staining and quantitative histology (Figure 7B and 14B). The repopulating grafts ('nodules') stained positive for ALB, MRP2 and CYP2E1, which indicated their functional maturity. (Figure 7C) Almost none of the transplanted cell retained expression of AFP (fetal hepatocyte marker) and no KRT19 (cholangiocyte marker) could be detected within the graft (Figure 7C and Figure 14C). The same experiment was performed with an organoid culture grown from one pediatric PHH donor. Since we had access to hepatocytes and Hep-Orgs from the same donor, we were again able to compare engraftment side-by-side. Similar to Fetal-Orgs, mature organoid grafts showed an initial lag phase before proliferating at the same rate as primary hepatocyte controls (Fig. 7D and 14D). Mature primary cells and organoids clearly outperformed their fetal counterparts in engraftment level and graft proliferation, which demonstrates the regenerative potential of Hep-Orgs and the faithful conservation of basic tissue features (e.g. transplantability) in organoid culture. In summary, our data show that Hep-Orgs are able to successfully repopulate damaged livers and demonstrate significant graft expansion after transplantation.

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CLAIMS

1. A method for expanding hepatocytes *in vitro*, wherein the method comprises culturing hepatocytes in a hepatocyte culture medium, wherein the hepatocyte culture medium comprises:
   i. 50 to 500 ng/ml of a Fibroblast Growth Factor (FGF), optionally selected from the group consisting of FGF7, FGF10 and a combination thereof;
   ii. a Wnt agonist comprising an R-spondin and at least one glycogen synthase kinase 3 (GSK3) inhibitor;
   iii. 5 to 100 ng/ml of epidermal growth factor (EGF);
   iv. 5 to 100 ng/ml of hepatocyte growth factor (HGF); and
   v. a transforming growth factor beta (TGF-β) inhibitor, comprising an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7 signalling pathway.
2. The method of claim 1, wherein the R-spondin is selected from the group consisting of: R-spondin 1, R-spondin 2, R-spondin 3, R-spondin 4, an active fragment or variant thereof, and a combination thereof.
3. The method of claim 2, wherein the R-spondin is provided as conditioned medium and is at a concentration of 5%-50%, 5%-40%, 5%-35%, 10%-30%, 10%-20%, 12%-17%, or preferably about 15% (vol/vol) within the hepatocyte culture medium.
4. The method of claim 3, wherein the R-spondin in said R-spondin conditioned medium is R-spondin 1, optionally at a concentration of about 15%.
5. The method of any one of the preceding claims, wherein the GSK3 inhibitor is at a concentration of 1 to 10 μM, 0.5 to 5 μM, 1 to 5 μM, 2 to 4 μM, or preferably about 3 μM.
6. The method of claim 5, wherein the GSK3 inhibitor is selected from the group consisting of: CHIR99021, SB216763, TWS119, 5-bromoindole, SB415286, BIO, AZD2858, AZD1080, AR-A014418, TDZD-8, LY2090314, BIO-acetoxime, 1-Azakenpaullone and a combination thereof.
7. The method of claim 6, wherein the GSK3 inhibitor is CHIR99021, optionally in a concentration of about 3 μM.
8. The method of any one of the preceding claims, wherein the hepatocyte culture medium comprises FGF7 at a concentration of 50 to 400 ng/ml, 50 to 300 ng/ml, 75 to 200 ng/ml, 80 to 150 ng/ml or preferably about 100 ng/ml.
9. The method of claim 8, the hepatocyte culture medium comprises FGF10 at a concentration of 50 to 400 ng/ml, 50 to 300 ng/ml, 75 to 200 ng/ml, 80 to 150 ng/ml or preferably about 100 ng/ml.

10. The method of any one of claims 8 or 9, wherein the culture medium comprises FGF10 and FGF7.
11. The method of any one of the preceding claims, wherein the EGF is at a concentration of 10 ng/ml to 100 ng/ml, 20 ng/ml to 100 ng/ml, 20 ng/ml to 80 ng/ml, 40 ng/ml to 80 ng/ml, or preferably about 50 ng/ml.
12. The method of any one of the preceding claims, wherein the HGF is at a concentration of 10 ng/ml to 100 ng/ml, 20 ng/ml to 100 ng/ml, 20 ng/ml to 80 ng/ml, 40 ng/ml to 80 ng/ml, or preferably about 50 ng/ml.
13. The method of any one of the preceding claims, wherein the TGF-β inhibitor is at a concentration of 1 to 10 μM, 0.5 to 5 μM, 1 to 4 μM, 1 to 3 μM, or preferably about 2 μM.
14. The method of claim 13, wherein the TGF-β inhibitor is an inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7 selected from the group consisting of: A83-01, SB-431542, SB-505124, EW-7197, LY-2157299, GW6604 and a combination thereof.
15. The method of claim 14, wherein the inhibitor of activin receptor-like kinase ALK4, ALK5 and/or ALK7 is A83-01, optionally at a concentration of about 2 μM.
16. The method of any one of the preceding claims, wherein the hepatocyte culture medium further comprises nicotinamide, optionally at a concentration of 1 to 100 mM.
17. The method of claim 16, wherein the nicotinamide is at a concentration of 1 to 50 mM, 1 to 30 mM, 1 to 20 mM, 5 to 20 mM, 5 to 15 mM, or preferably about 10 mM.
18. The method of any one of the preceding claims, wherein the hepatocyte culture medium further comprises gastrin, optionally at a concentration of 1 to 100 nM.
19. The method of claim 18, wherein the gastrin is at a concentration of 1 to 50 nM, 1 to 30 nM, 1 to 20 nM, 5 to 20 nM, 5 to 15 nM, or preferably about 10 nM.
20. The method of any one of the preceding claims, wherein the hepatocyte culture medium further comprises transforming growth factor alpha (TGF-α).
21. The method of claim 20, wherein the TGF-α is at a concentration of 1 to 50 ng/ml, 5 to 35 ng/ml, 10 to 30 ng/ml, 15 to 25 ng/ml, or preferably about 20 ng/ml.
22. The method of any one of the preceding claims, wherein the hepatocyte culture medium further comprises a Rho-associated protein kinase (ROCK) inhibitor; optionally wherein the ROCK inhibitor is selected from the group consisting of: Y-27632, GSK429286A, Fasudil, Thiazovivin, Rho Kinase Inhibitor IV and a combination thereof; optionally wherein the ROCK inhibitor is Y-27632.
23. The method of claim 22, wherein the ROCK inhibitor is at a concentration of 1 to 50 μM, 1 to 30 μM, 1 to 20 μM, 5 to 20 μM, 5 to 15 μM, or preferably about 10 μM.
24. The method of any one of the preceding claims, wherein the hepatocyte culture medium further comprises N-acetylcysteine.

25. The method of claim 24, wherein the N-acetylcysteine is at a concentration of 0.5 to 5 mM, 0.5 to 4 mM, 1 to 4 mM, 1 to 2 mM, 1 to 1.5 mM, or preferably about 1.25mM.
26. The method of any one of the preceding claims, wherein the hepatocyte culture medium further comprises B27, optionally wherein the B27 does not comprise vitamin A.
27. The method of any one of the preceding claims, wherein the hepatocyte culture medium further comprises a cell growth medium, optionally wherein the cell growth medium comprises antibiotics, optionally penicillin and streptomycin; wherein the cell growth medium is optionally AdDMEM/F12, optionally comprising Hepes and/or Glutamax.
28. The method of any one of the preceding claims, wherein the hepatocyte culture medium does not comprise a cAMP pathway activator.
29. The method of any one of the preceding claims, wherein the hepatocytes are primary hepatocytes, preferably isolated cells from liver tissue.
30. The method of claim 29, wherein the method further comprises isolating the primary hepatocytes from a liver tissue, optionally wherein the liver tissue is an explant or a liver sample from a subject to be administered with the expanded hepatocytes and/or wherein isolating comprises collagenase digestion.
31. The method of any one of the preceding claims, wherein the hepatocytes are cultured in contact with a three dimensional extracellular matrix (ECM) in the presence of the hepatocyte culture medium.
32. The method of claim 31, wherein the three dimensional extracellular matrix comprises the hepatocyte culture medium, optionally at a medium:matrix ratio of 1:1, 1:2, or preferably 1:3.
33. The method of any one of claims 31 and 32, wherein the three dimensional extracellular matrix is selected from the group consisting of: a synthetic ECM, a natural ECM and a combination thereof; optionally wherein the three dimensional ECM comprises Matrigel™.
34. The method of any one of the preceding claims, wherein the hepatocytes comprise cells selected from the group consisting of: fetal hepatocytes, adult hepatocytes and a combination thereof.
35. The method of claim 34, wherein the hepatocytes are albumin-expressing hepatocytes, optionally wherein the cultured hepatocytes are diploid or tetraploid.
36. The method of any one of the preceding claims, wherein at least part of the expanded cells form one or more hepatocyte organoids.
37. The method of claim 36, wherein the hepatocyte organoids are capable of being further expanded in culture for at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45 or 50 passages.

38. The method of claim 36, wherein the hepatocyte organoids are capable of being further expanded in culture for at least 3, 4, 5, 6, 10, 12, 15, 18, 20 or 24 months.

39. The method of any one of claims 36-38, wherein the hepatocyte organoids comprise a lumen.

40. The method of any one of claims 36-39, wherein the hepatocyte organoids comprise characteristics of whole liver and primary hepatocyte selected from the group consisting of: glycogen accumulation, functional uptake of Low Density Lipoprotein (LDL), nuclei comprising prominent nucleoli having a fibrilar centre and de-condensed chromatin, large numbers of mitochondria with few and short cristae, bile canaliculi, tight junctions, peroxisomes, lysosomes, multi-vesicular bodies, autophagic vacuoles and a combination thereof.

41. The method of any one of claims 36-40, wherein the hepatocyte organoids secrete at least one of albumin, alpha-1 antitrypsin (A1AT), alpha-fetoglobulin (Afp) and a combination thereof.

42. The method of any one of claims 36-41, wherein hepatocyte organoids demonstrate gene expression comparable to that of the primary hepatocytes for genes selected from the group consisting of: albumin, Apolipoprotein A2 (APOA2), Serpin family member 1 (SERPINA1) and a combination thereof.

43. The method of any one of claims 36-42, wherein the hepatocyte organoids expand exponentially, optionally wherein the expansion is at a ratio of about 1:3 every 7-10 days.

44. The method of any one of claims 36-43, wherein the hepatocyte organoids have a plating efficiency of at least 0.5%, 0.6%, 0.7%, 0.8%, 0.9% or 1%.

45. The method of any one of claims 36-44, wherein the hepatocyte organoids do not express marker genes typically expressed in cholangiocytes and/or wherein the hepatocyte organoids express genes which are typically not expressed in cholangiocytes.

46. A hepatocyte organoid obtainable or obtained by any one of claims 36-45 and/or cells, optionally single cells, derived therefrom.

47. The hepatocyte organoid of claim 46 and/or the cells derived therefrom for use in medicine or diagnostics.

48. The hepatocyte organoid of claim 46 and/or the cells derived therefrom for use in screening the potential effect of a substance on hepatocytes *in-vivo*, optionally for use in screening for a therapeutic or a toxic effect.

49. The hepatocyte organoid of claim 46 and/or the cells derived therefrom for use in treating a disease or condition which is caused by and/or associated with damaged hepatocytes in the liver of a subject.

50. The hepatocyte organoid of claim 49 and/or the cells derived therefrom, wherein the disease or condition is selected from the group consisting of: Hepatocellular Carcinoma,

Alagille Syndrome, Alpha- 1- Antitrypsin Deficiency, Autoimmune Hepatitis, Biliary Atresia, Chronic Hepatitis, Cancer of the Liver, Cirrhosis, Liver Cysts, Fatty Liver Disease, Galactosemia Gilbert's Syndrome, Primary Biliary Cirrhosis, Hepatitis A, Hepatitis B, Hepatitis C, Primary Sclerosing Cholangitis, Reye's Syndrome, Sarcoidosis, Tyrosinemia, Type I Glycogen Storage Disease, Wilson's Disease, Neonatal Hepatitis, Non-alchoholic SteatoHepatitis, Porphyria, Hemochromatosis, Progressive familial intrahepatic cholestasis, Glycogen storage disease type III, Tyrosinemia, Deoxyguanosine kinase deficiency, Pyruvate carboxylase deficiency, Congenital dyserythropoietic anemia, Polycystic Liver Disease Polycystic Kidney Disease, Alpha-1 antitrypsine deficiency, Ureum cycle defects, Organic acidemiea, lysosomal storage diseases, Fatty Acid Oxydation Disorders, and a combination thereof.

51. The hepatocyte organoid of claim 46 and/or the cells derived therefrom for use in a method for repairing a liver which underwent damage and/or underwent at least partial hepatectamy (HPx), the method comprising transplanting the hepatocyte organoid and/or the single cells derived therefrom into the liver.
52. The hepatocyte organoid and/or the cells derived therefrom according to claim 51, wherein the hepatocyte organoid was expanded from fetal hepatocytes and wherein the method for repairing a liver further comprises differentiating the hepatocyte organoid, optionally wherein differentiating the hepatocyte organoid is prior to transplanting the hepatocyte organoid and/or the cells derived therefrom.
53. The hepatocyte organoid and/or the cells derived therefrom according to claim 52, wherein differentiating the hepatocyte organoid expanded from fetal hepatocytes comprises culturing the hepatocyte organoid in a differentiation medium, optionally wherein the differentiation medium comprises dexamethasone and oncostatin M.
54. The hepatocyte organoid and/or the cells derived therefrom according to claim 53, wherein the differentiation medium comprises the hepatocyte culture medium of any one of claims 1 to 28 supplemented by dexamethasone and oncostatin M, optionally wherein the dexamethasone is at a concentration of about 1 μM and/or the oncostatin M is at a concentration of about 10 ng/ml.
55. A hepatocyte culture medium according to any one of claims 1 to 28.
56. The hepatocyte culture medium of any one of claims 1-28 and 55, comprising:
about 100ng/ml FGF7;
about 100ng/ml FGF10;
about 20ng/ml TGFa;
about 50ng/ml EGF;
about 50ng/ml HGF;
B27 (not comprising vitamin A);

about 2 μM A83-01;

about 10μM Y-27632;

about 3μm CHIR99021;

about 10 mM Nicotinamide;

about 10 nM gastrin;

about 1.25mM N-acetylcysteine; and

about 15% R-spondin conditioned medium.

Figure 1

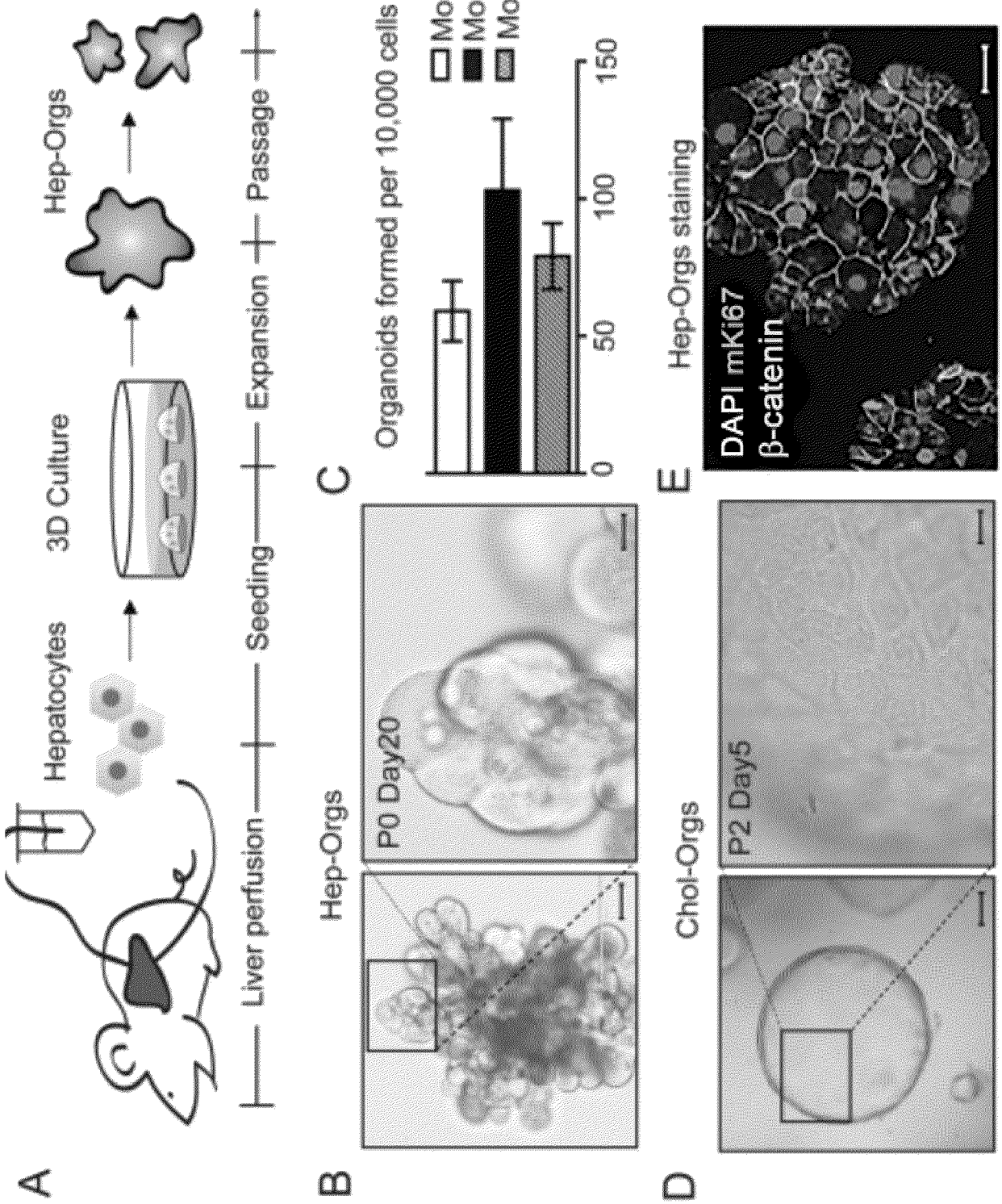
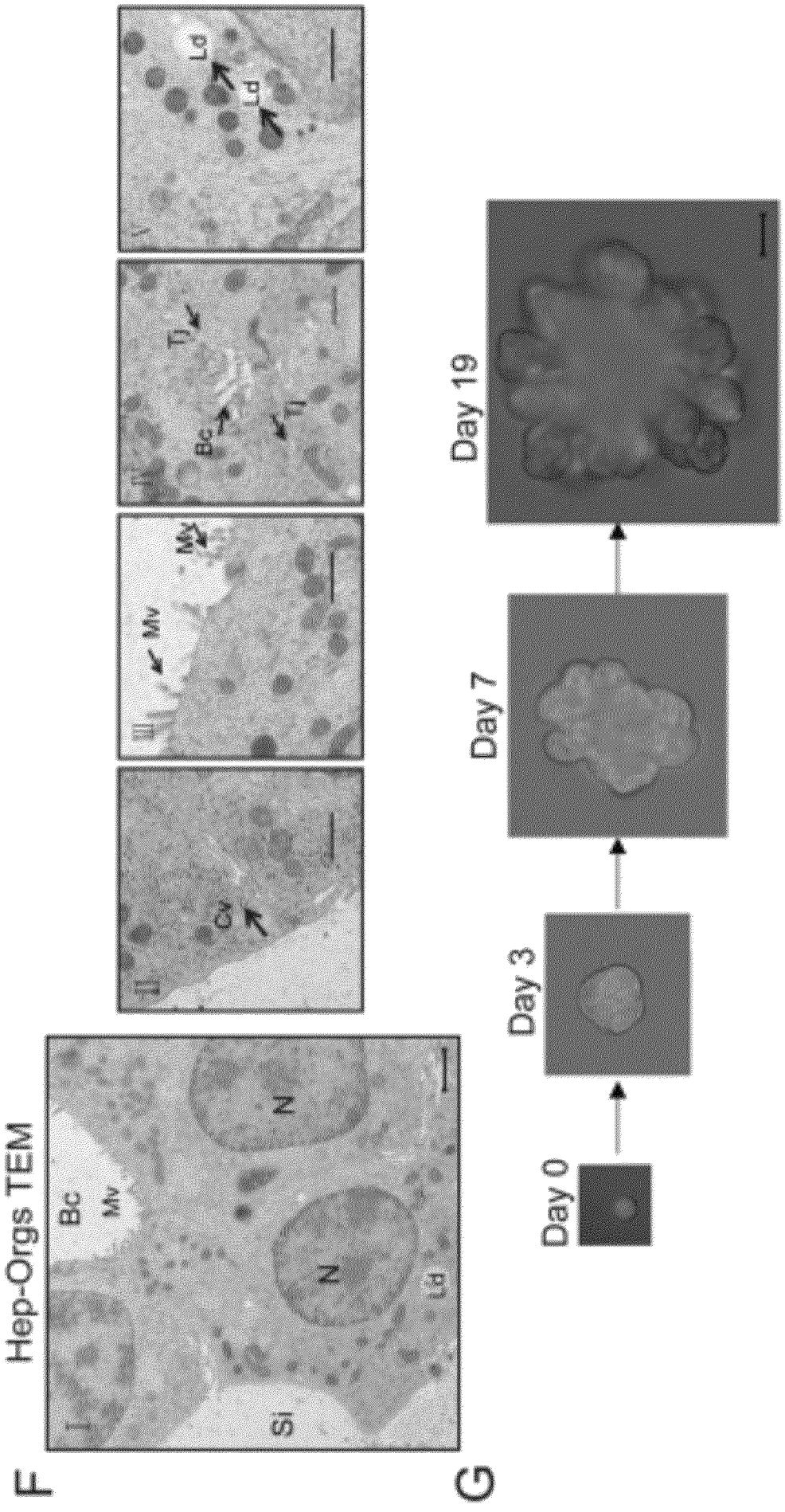

A
Hepatocytes
3D Culture
Hep-Orgs
Liver perfusion
Seeding
Expansion
Passage
B
Hep-Orgs
P0 Day20
C
Organoids formed per 10,000 cells
Mouse1
Mouse2
Mouse3
0
50
100
150
D
Chol-Orgs
P2 Day5
E
Hep-Orgs staining
DAPI mKi67
β-catenin

Figure 1 cont.



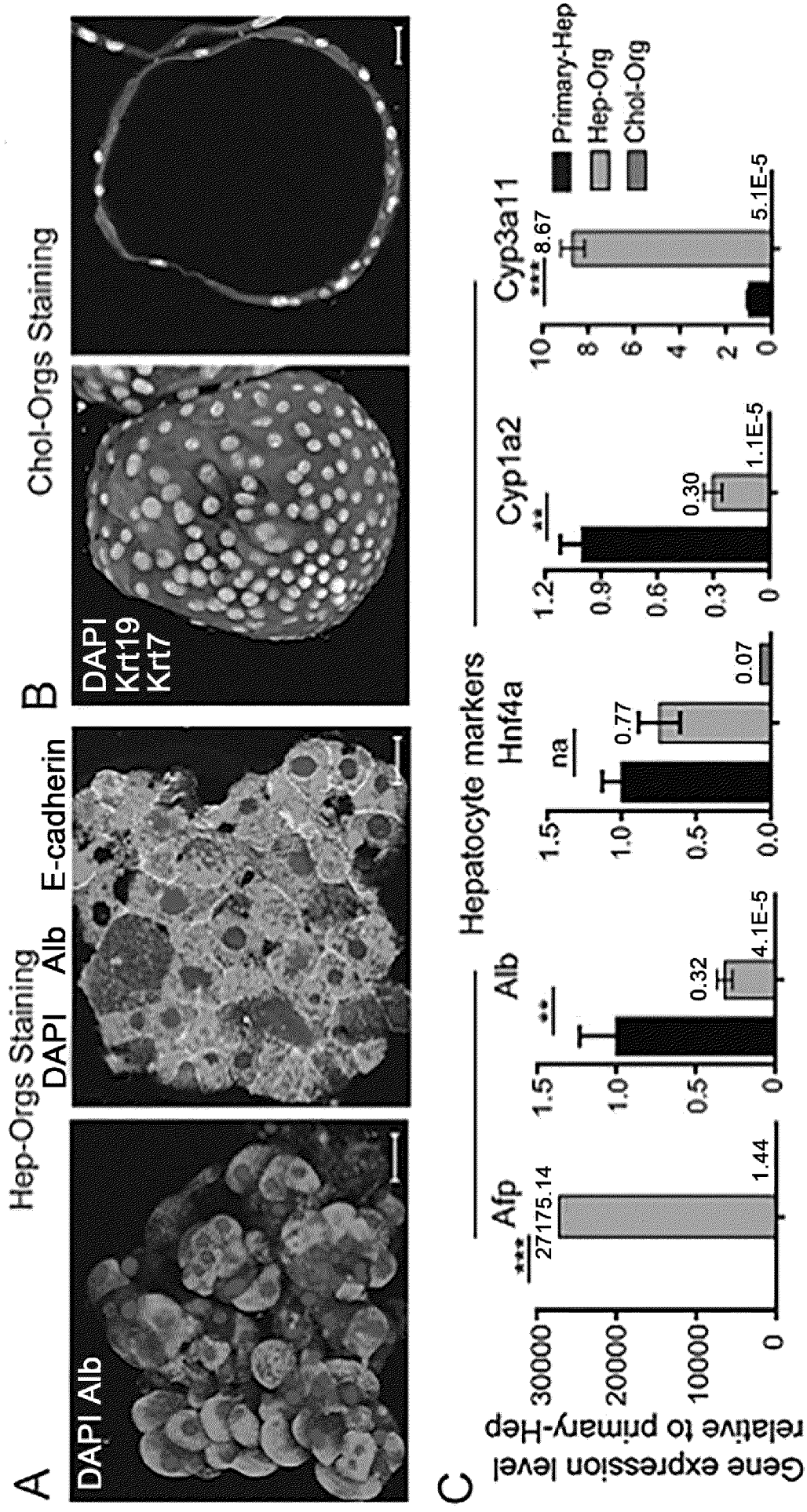

A
Hep-Orgs Staining
DAPI Alb
E-cadherin
DAPI Alb
B
Chol-Orgs Staining
DAPI
Krt19
Krt7
C
Gene expression level relative to primary-Hep
Hepatocyte markers
Afp
Alb
Hnf4a
Cyp1a2
Cyp3a11
Primary-Hep
Hep-Org
Chol-Org
***
**
na
27175.14
1.44
0.32
4.1E-5
0.77
0.07
0.30
1.1E-5
8.67
5.1E-5

Figure 2

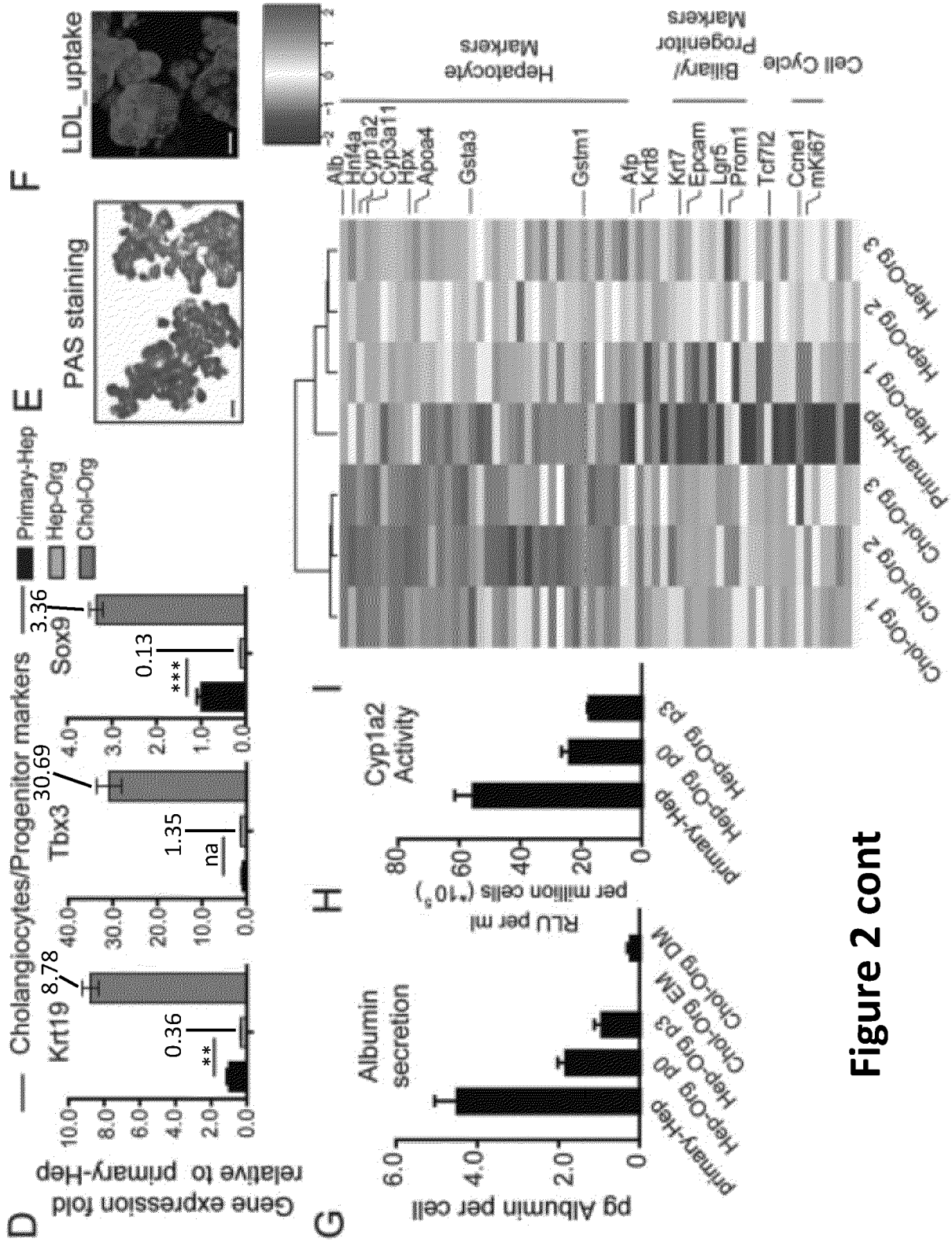


Figure 2 cont

Figure 3

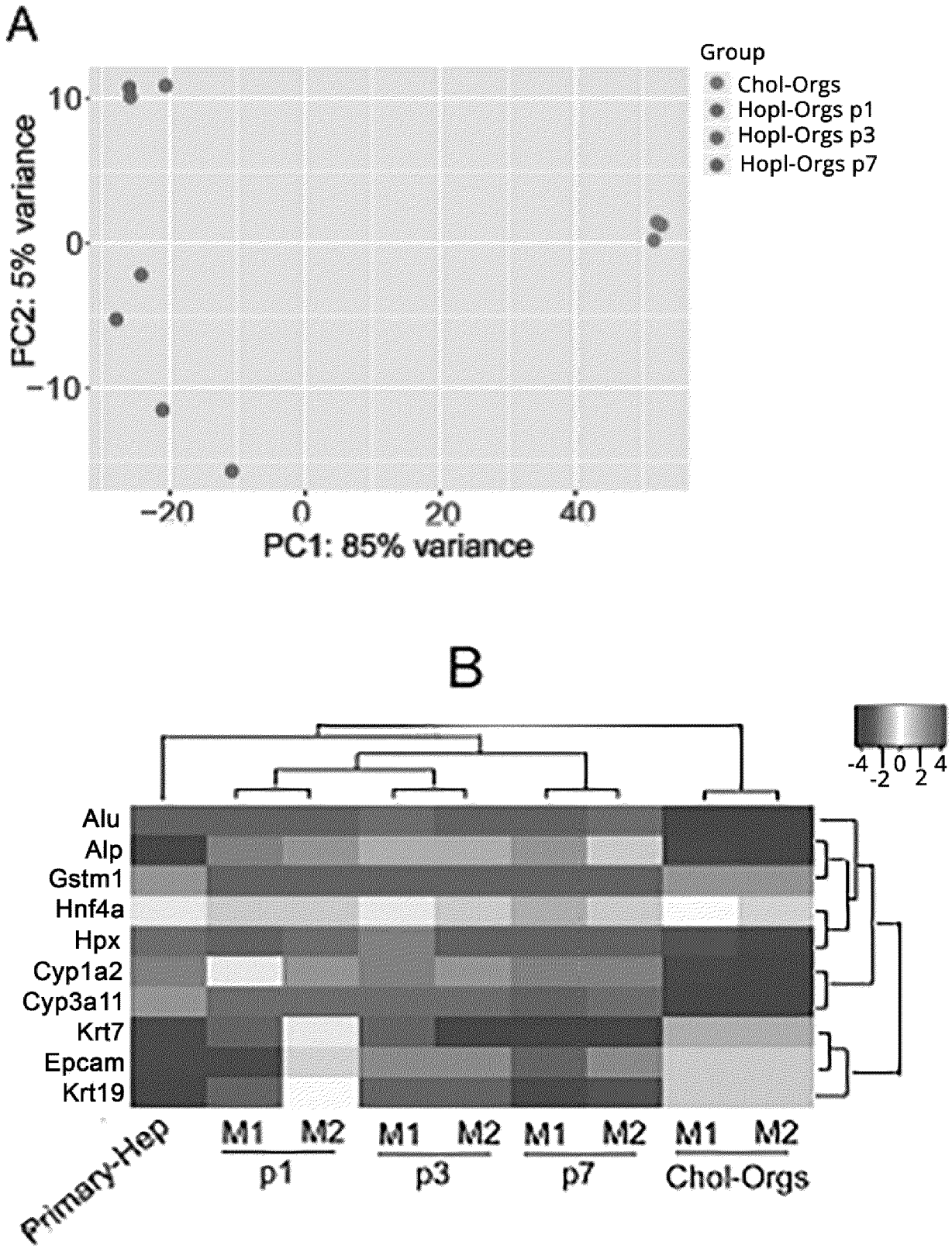

A
Group
Chol-Orgs
Hopl-Orgs p1
Hopl-Orgs p3
Hopl-Orgs p7
10
0
−10
FC2: 5% variance
−20
0
20
40
PC1: 85% variance
B
-4
-2
0
2
4
Alu
Alp
Gstm1
Hnf4a
Hpx
Cyp1a2
Cyp3a11
Krt7
Epcam
Krt19
Primary-Hep
M1
M2
p1
p3
p7
Chol-Orgs

Figure 3 cont

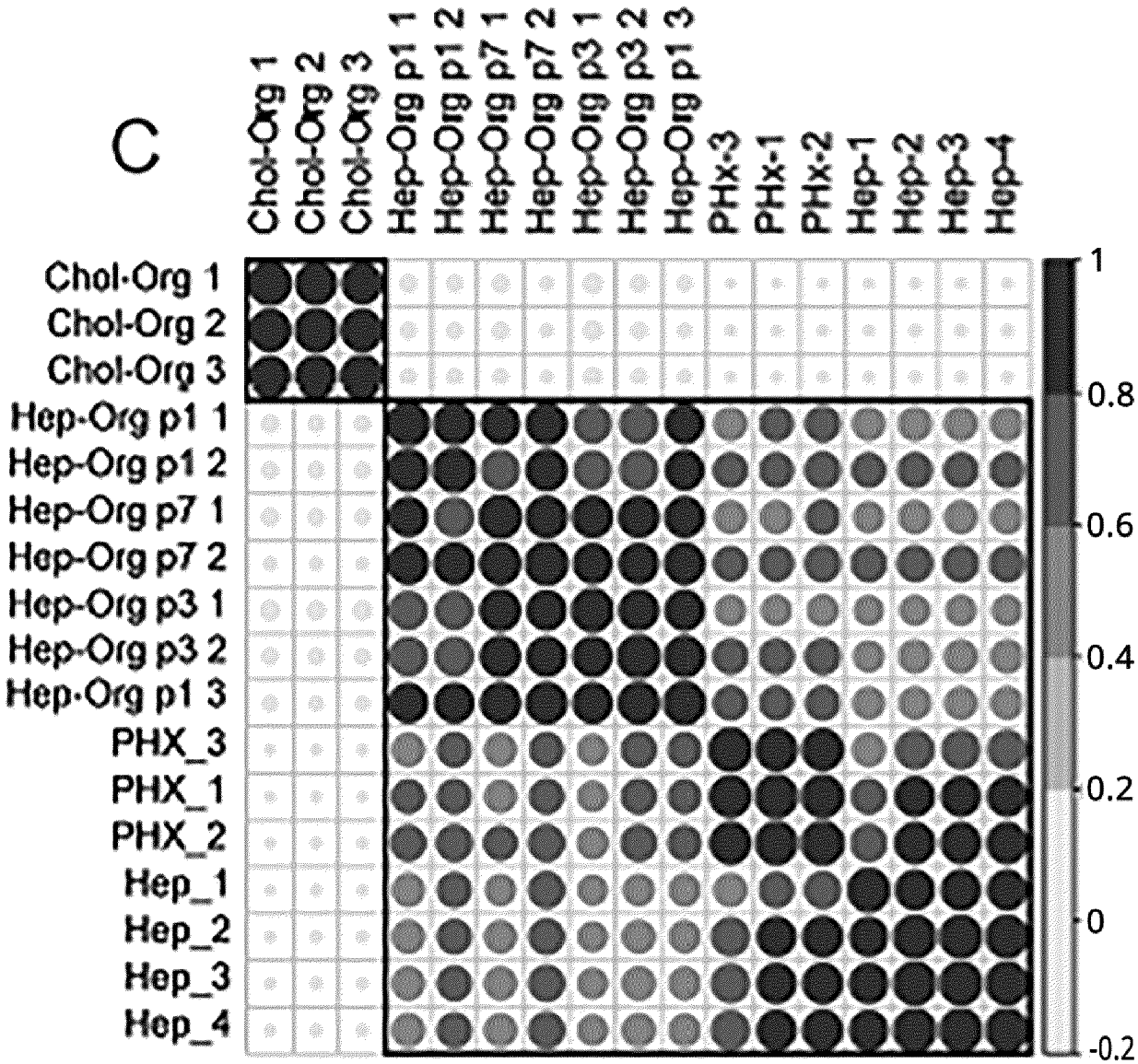
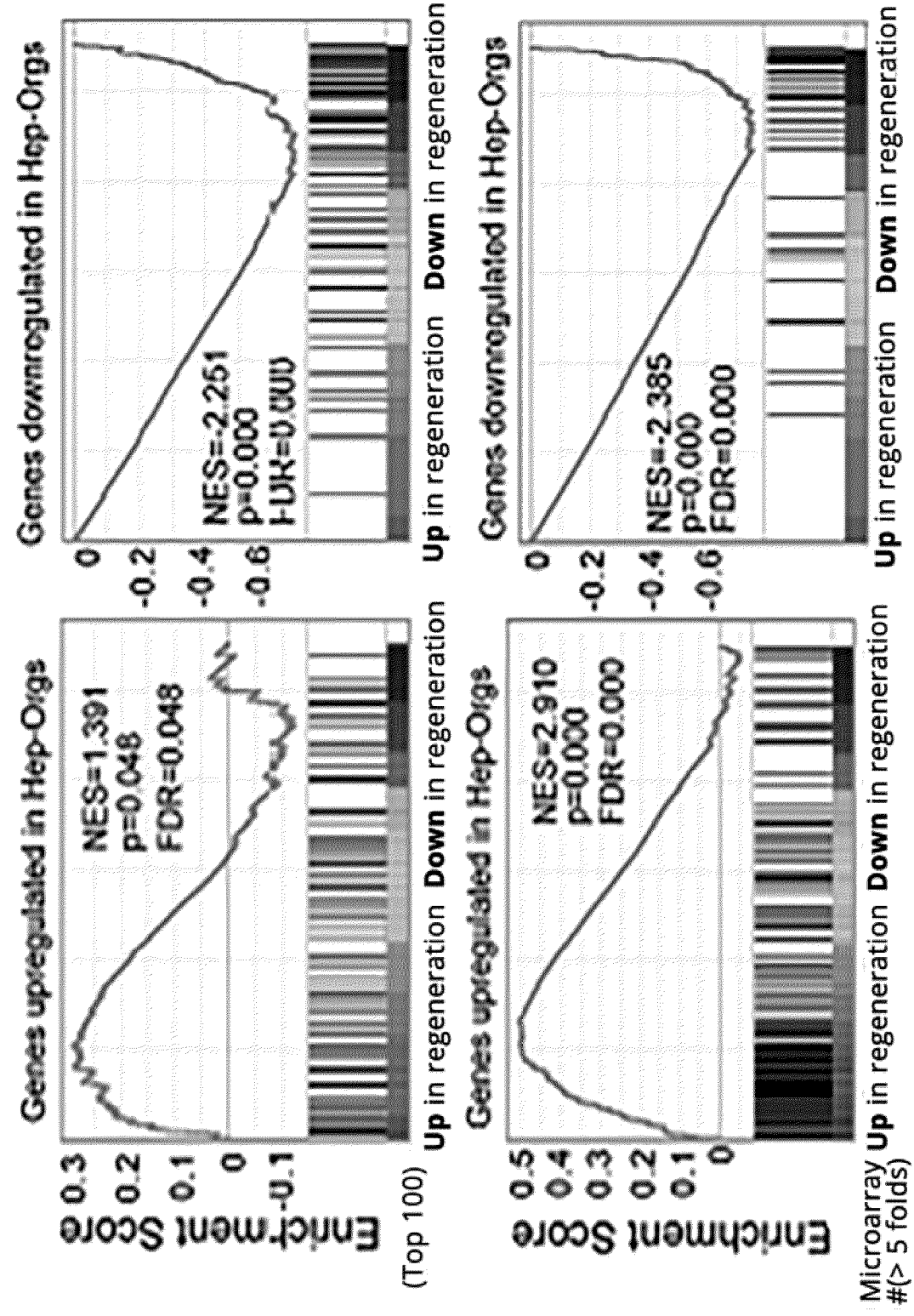


Figure 3 cont



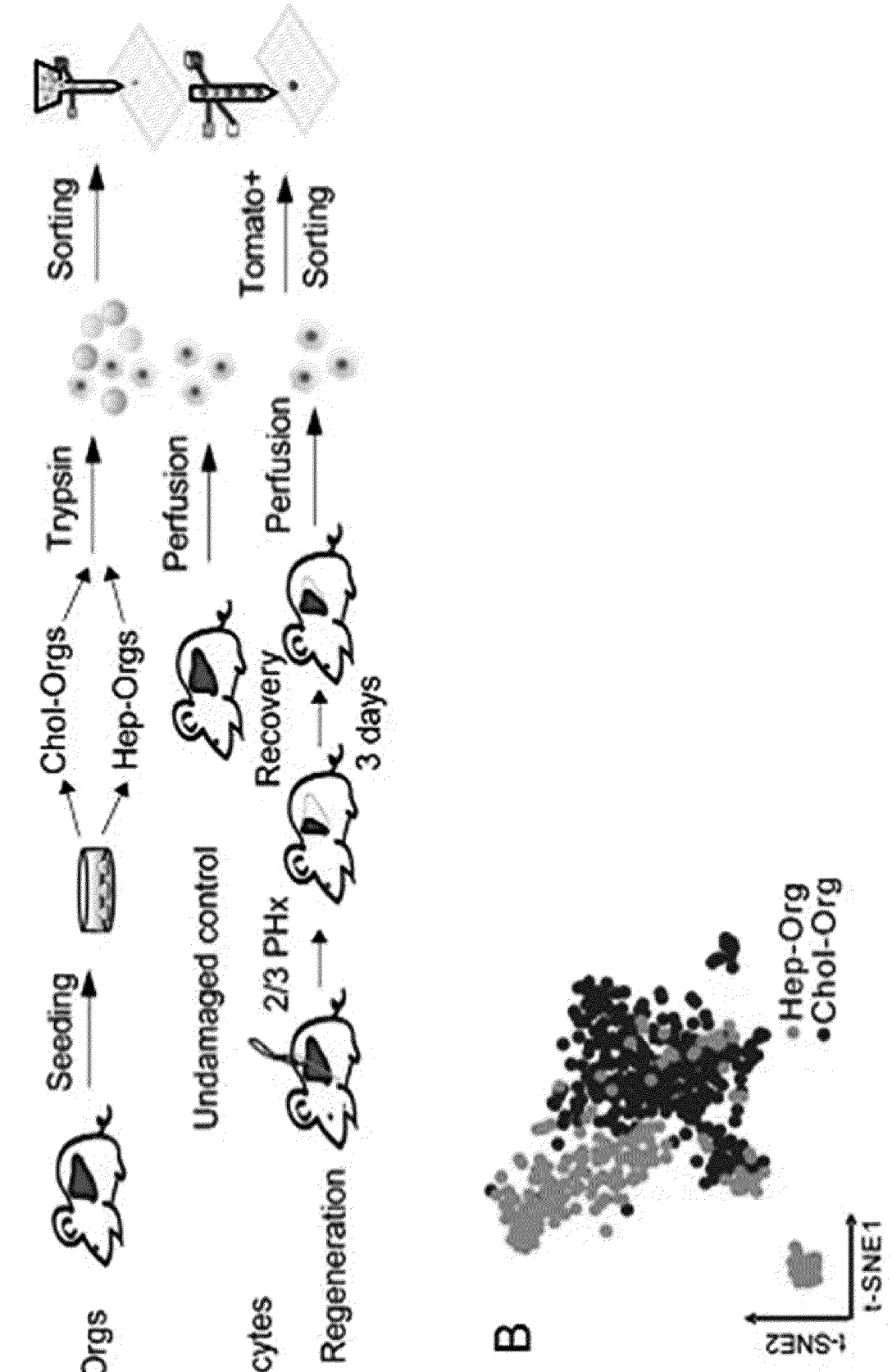
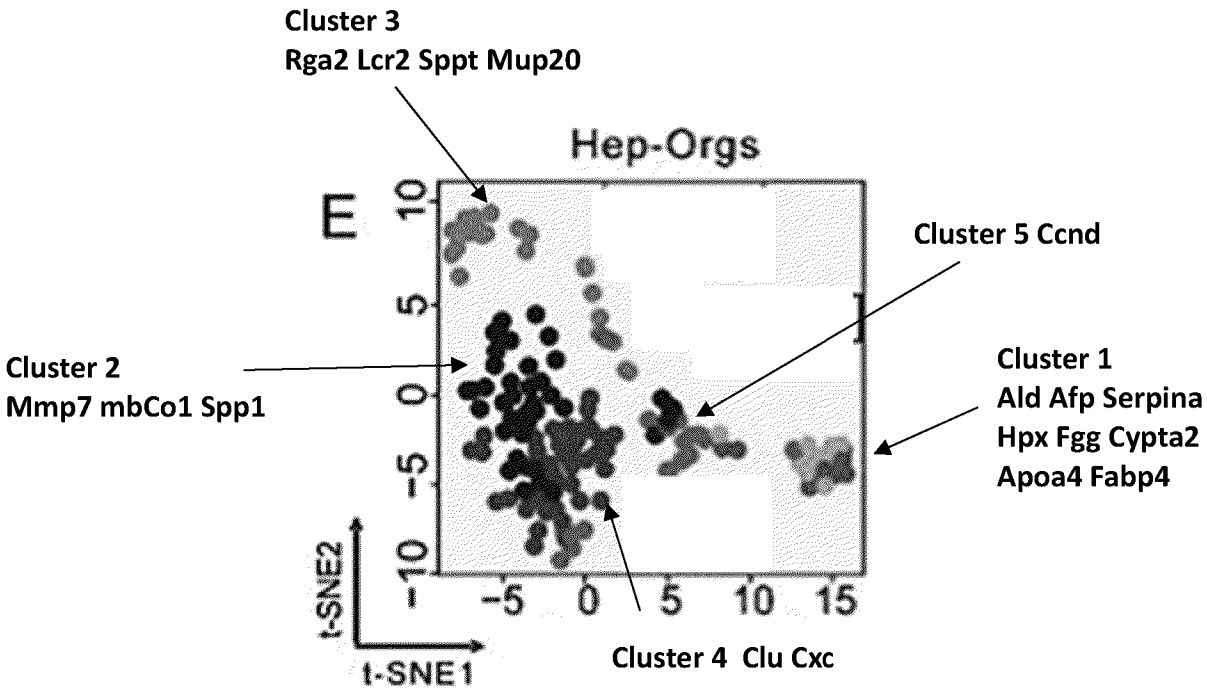

A
Liver Orgs
Seeding
Chol-Orgs
Hep-Orgs
Trypsin
Sorting
Hepatocytes
Regeneration
2/3 PHx
Undamaged control
Recovery
3 days
Perfusion
Perfusion
Tomato+
Sorting
B
Hep-Org
Chol-Org
t-SNE2
t-SNE1

Figure 4


Cluster 3
Rga2 Lcr2 Sppt Mup20
Hep-Orgs
E
Cluster 5 Ccnd
Cluster 2
Mmp7 mbCo1 Spp1
Cluster 1
Ald Afp Serpina
Hpx Fgg Cypta2
Apoa4 Fabp4
10
5
0
−5
−10
−5
0
5
10
15
t-SNE2
t-SNE1
Cluster 4 Clu Cxc

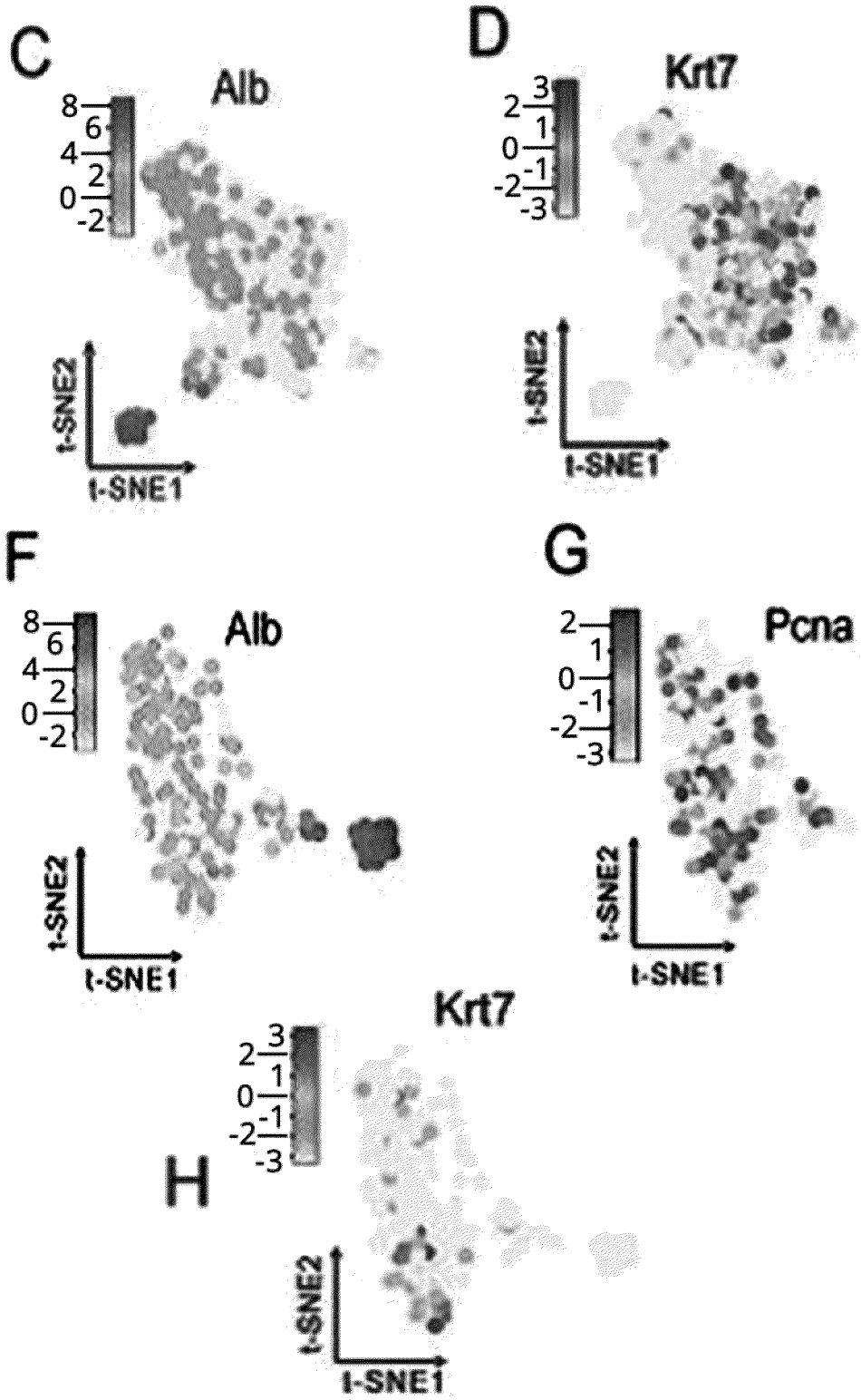


Figure 4 cont

I

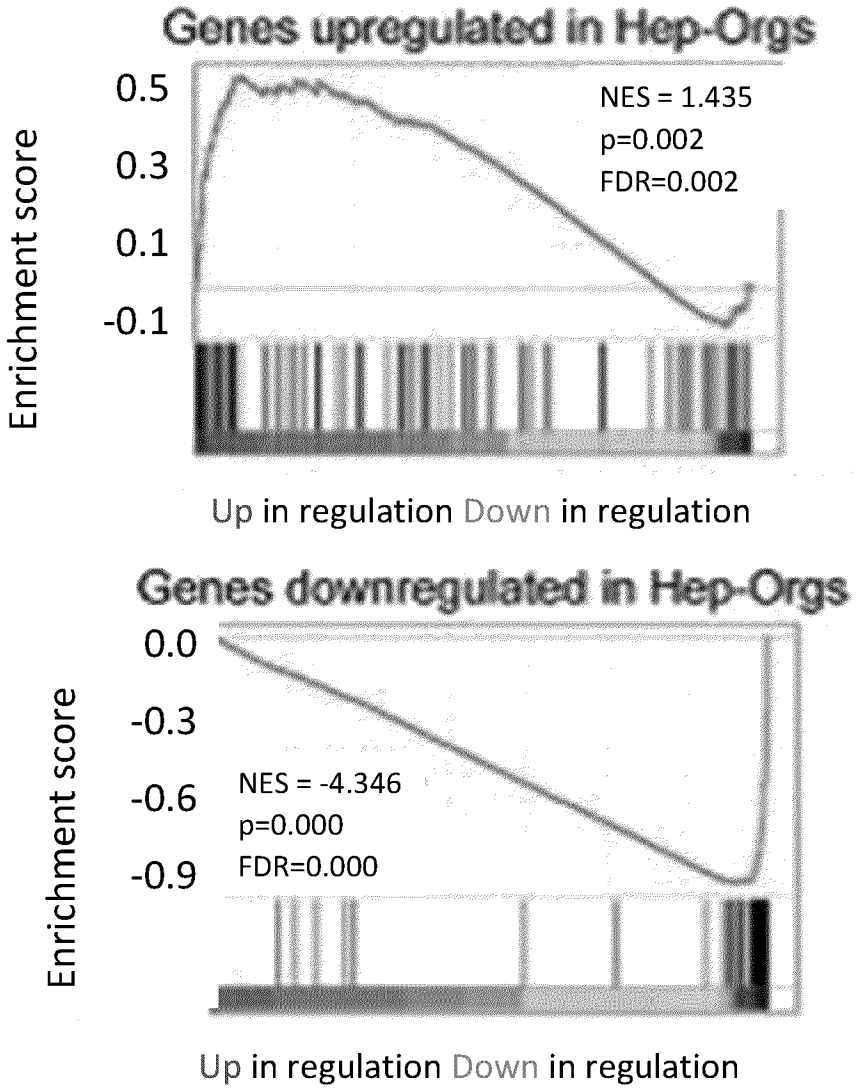


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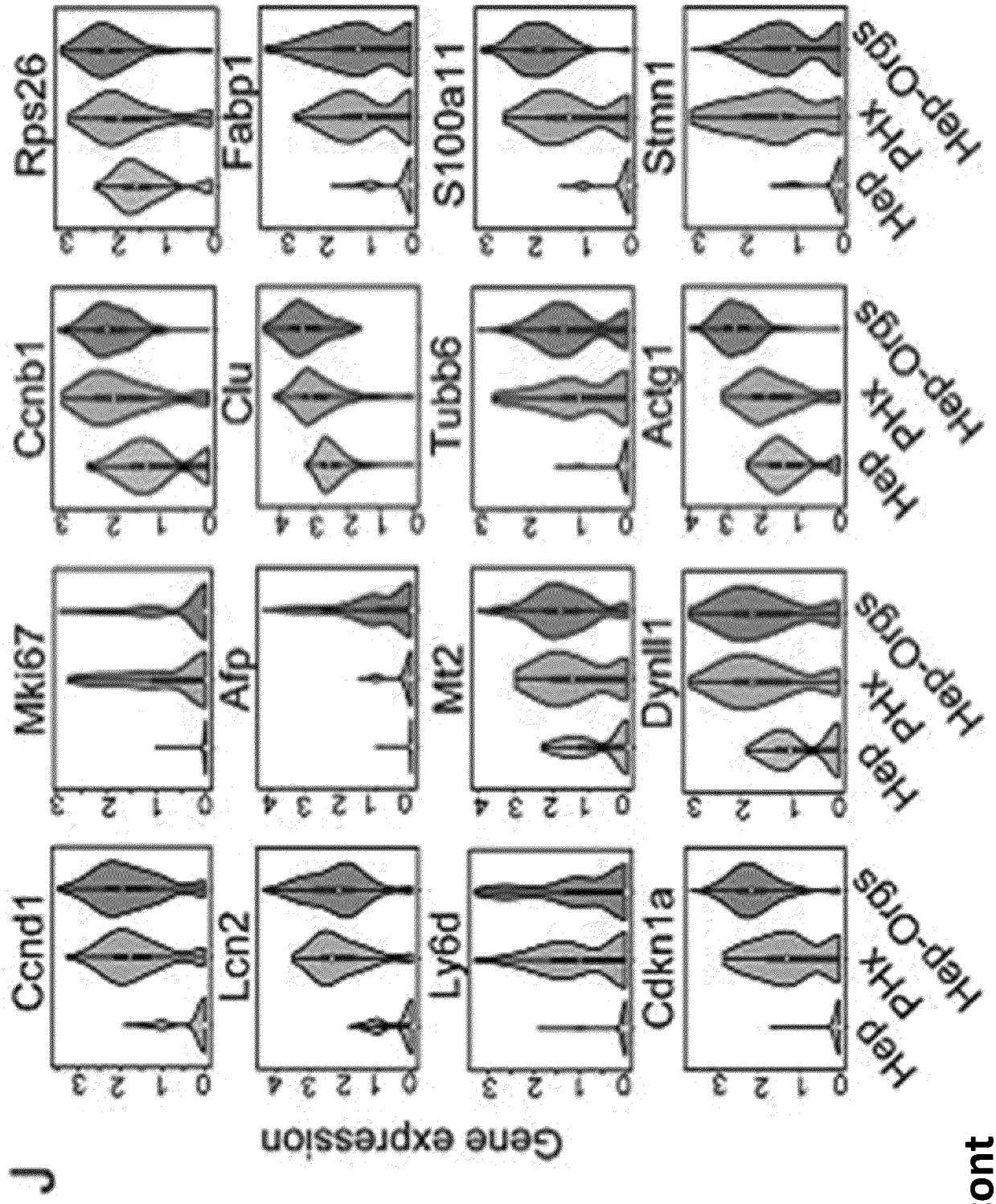
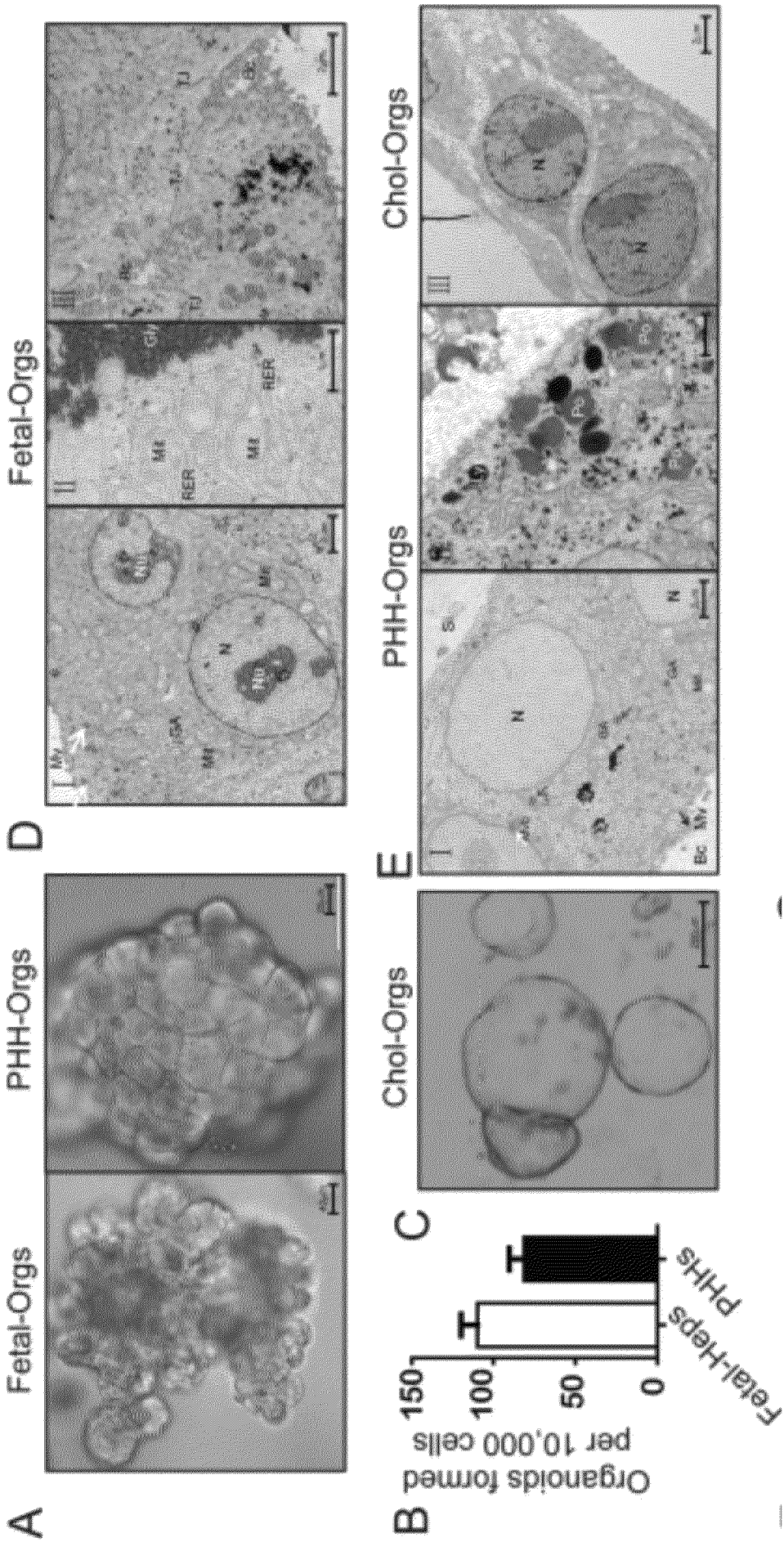


Figure 4 cont

Figure 5


A
Fetal-Orgs
PHH-Orgs
B
Organoids formed per 10,000 cells
150
100
50
0
Fetal-Heps
PHHs
C
Chol-Orgs
D
Fetal-Orgs
E
PHH-Orgs
Chol-Orgs

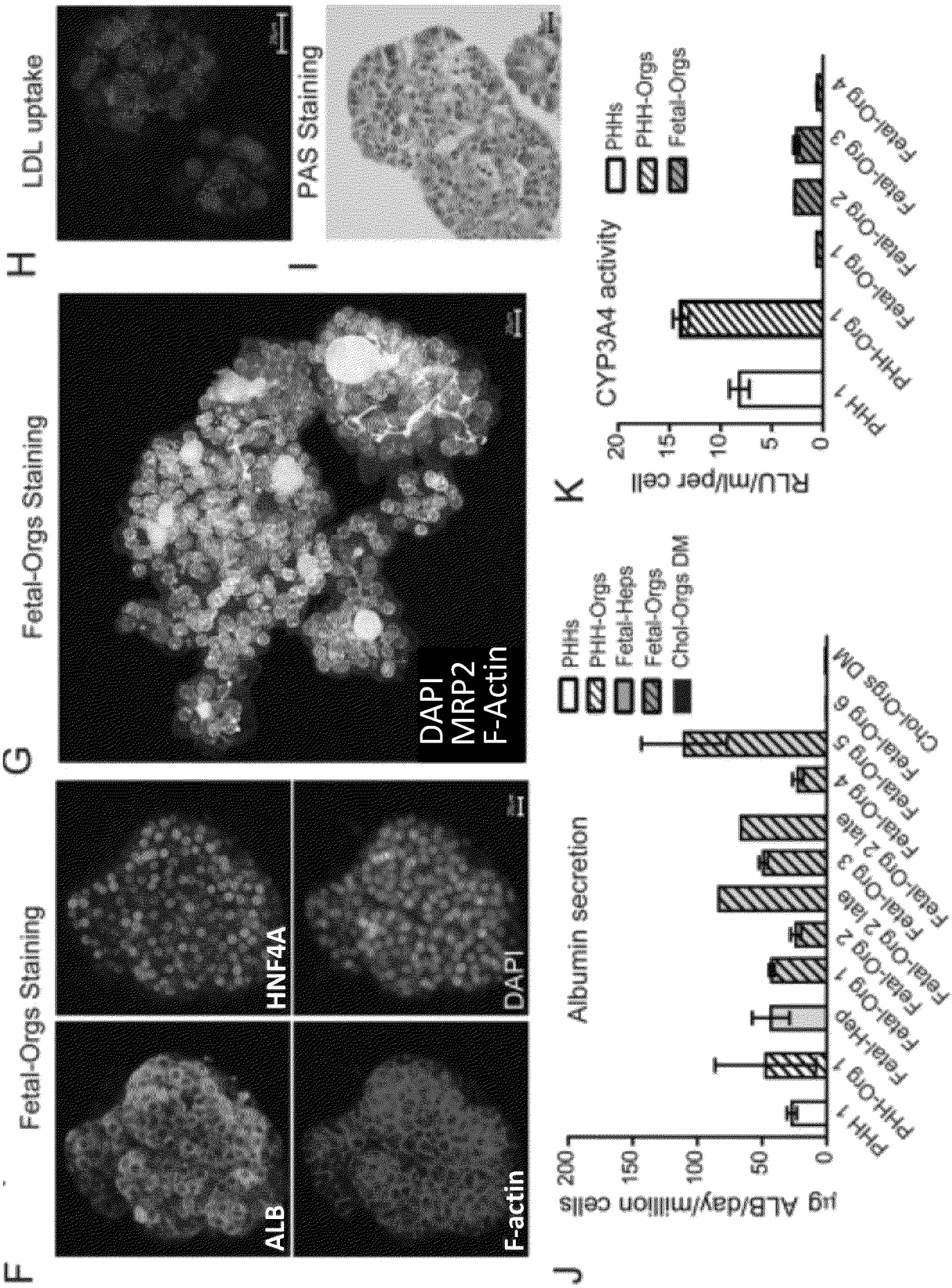


Figure 5 cont

Figure 6

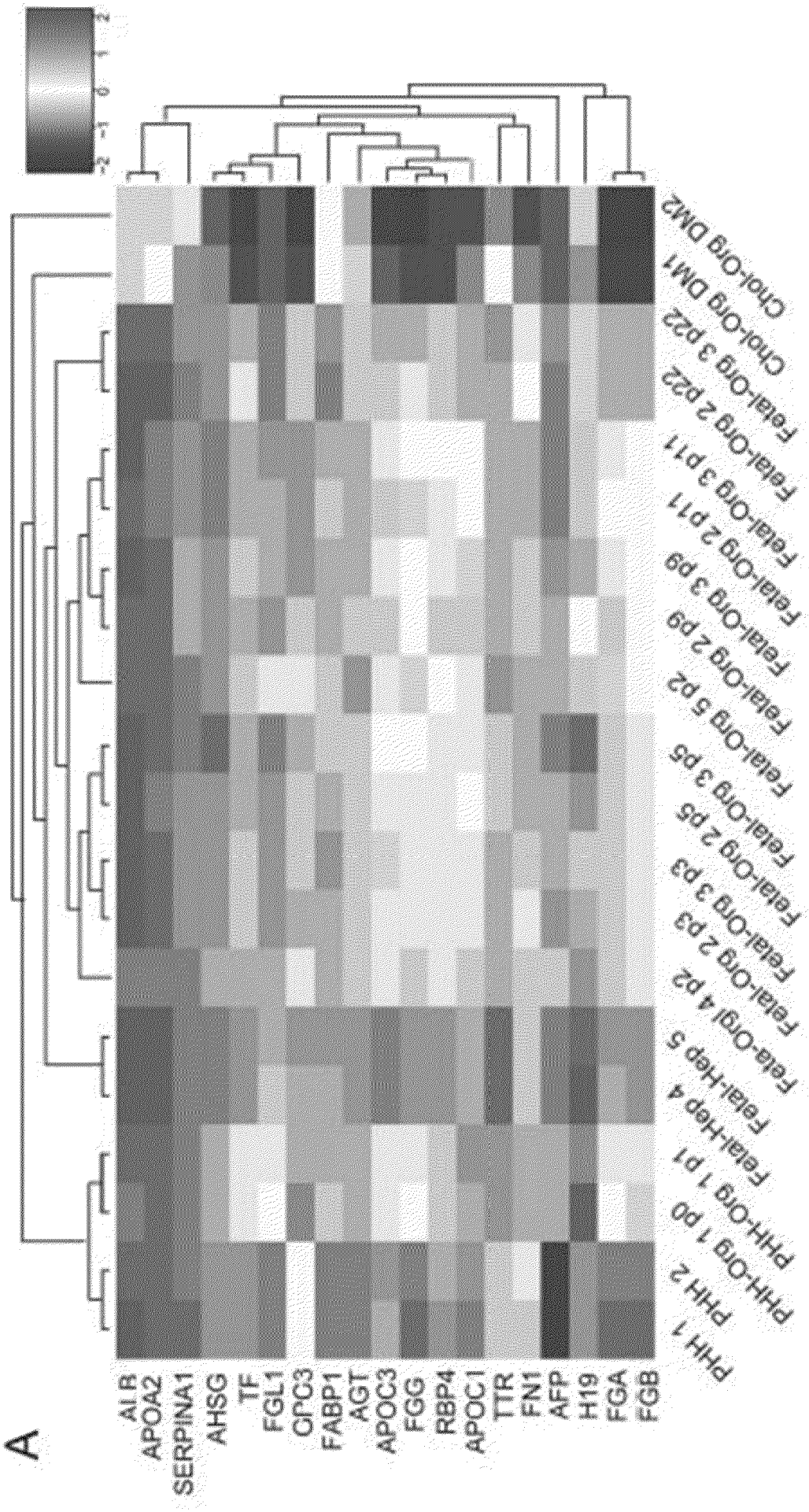
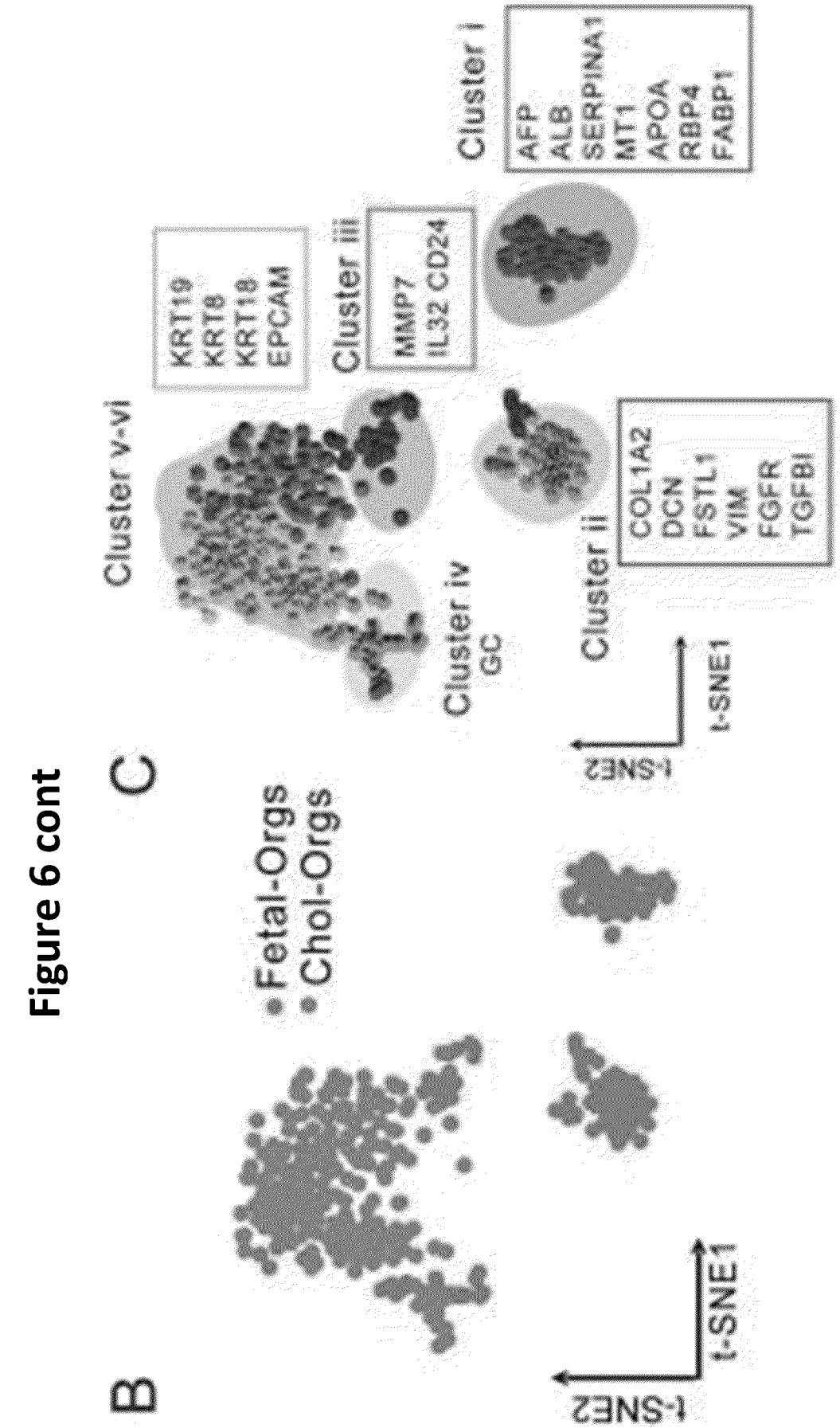

A
ALB
APOA2
SERPINA1
AHSG
TF
FGL1
CPC3
FABP1
AGT
APOC3
FGG
RBP4
APOC1
TTR
FN1
AFP
H19
FGA
FGB
PHH 1
PHH 2
PHH-Org 1 p0
PHH-Org 1 p1
Fetal-Hep 4
Fetal-Hep 5
Feta-Org 4 p2
Fetal-Org 2 p3
Fetal-Org 3 p3
Fetal-Org 2 p5
Fetal-Org 3 p5
Fetal-Org 5 p2
Fetal-Org 2 p9
Fetal-Org 3 p9
Fetal-Org 2 p11
Fetal-Org 3 p11
Fetal-Org 2 p22
Chol-Org 3 p22
Chol-Org DM1
Chol-Org DM2

Figure 6 cont



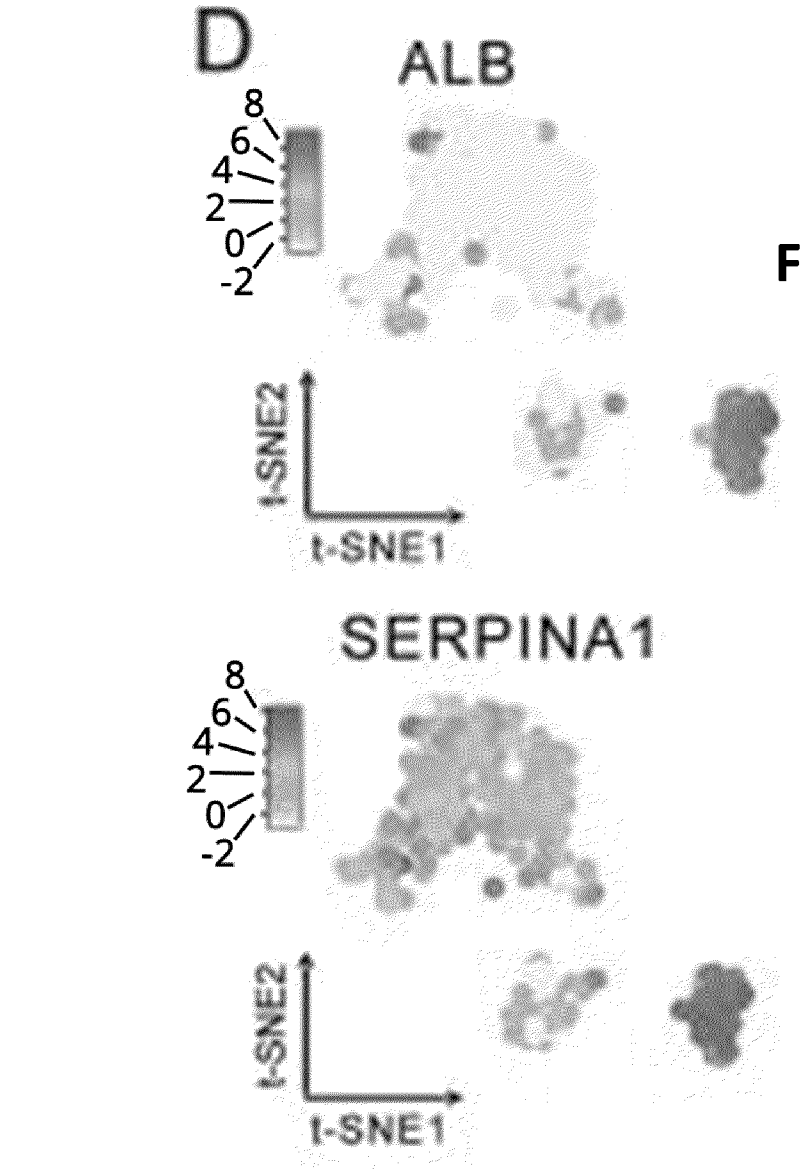


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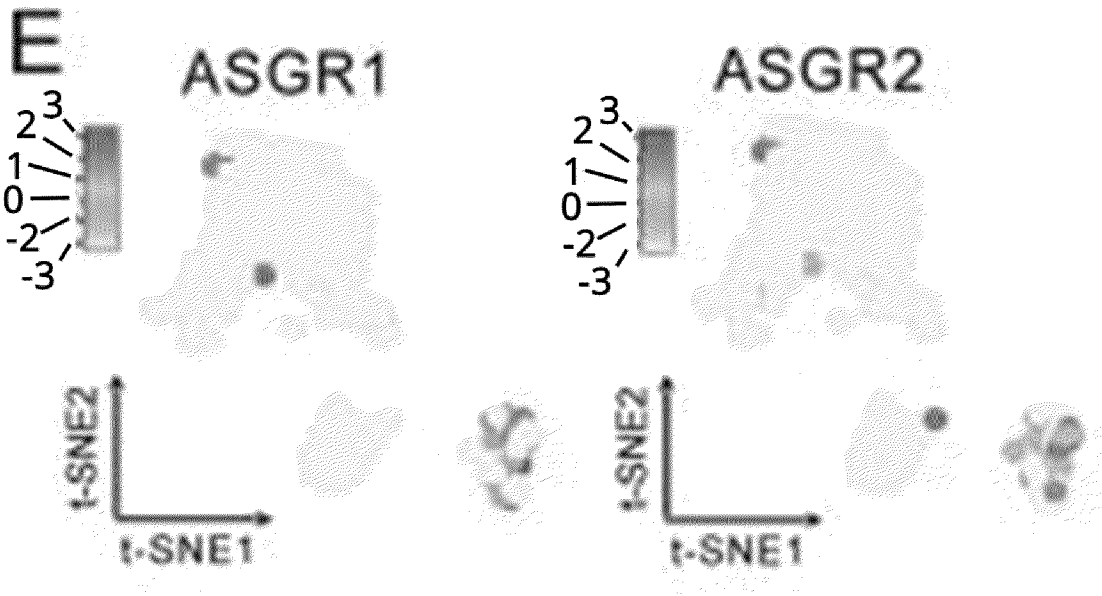
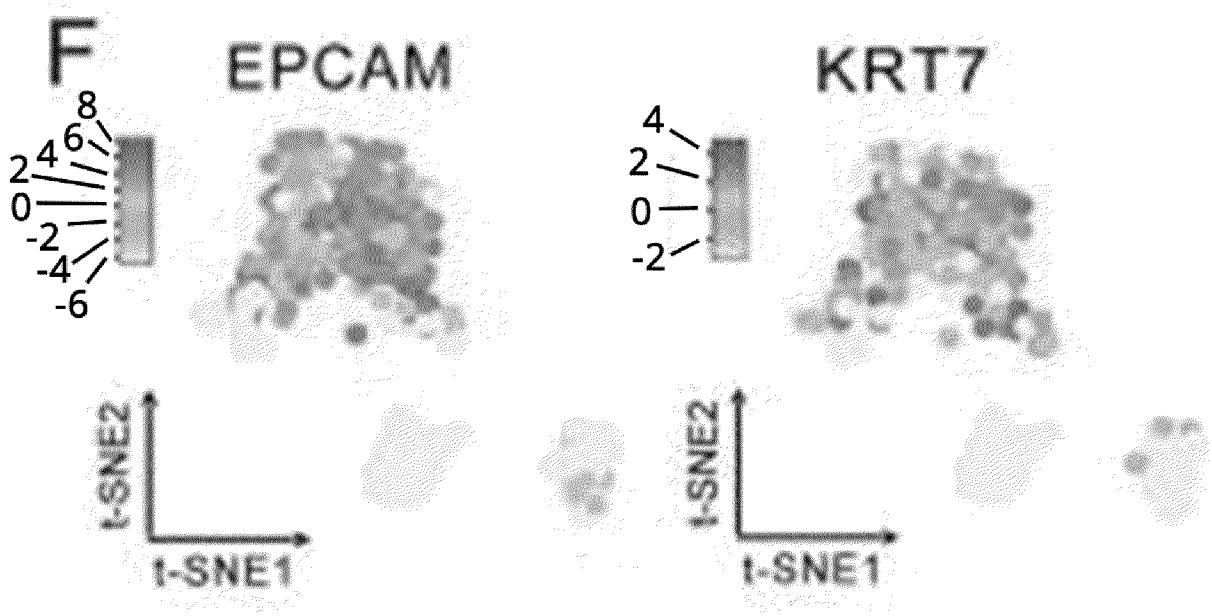
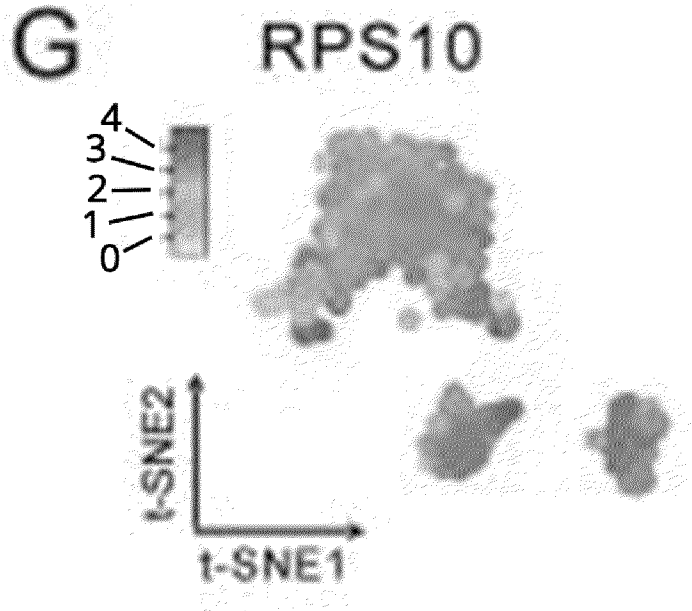


Figure 6 cont





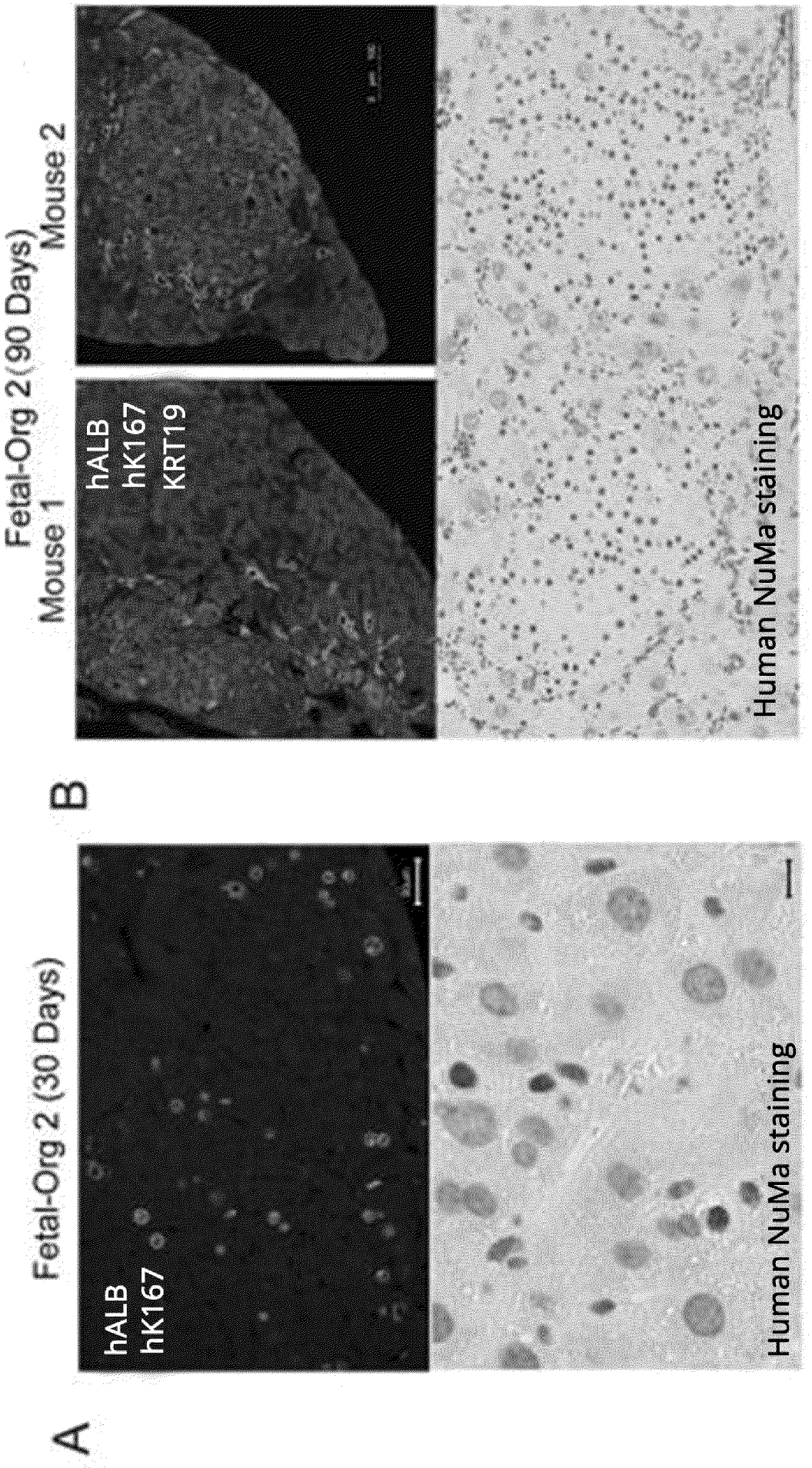


Figure 7A and 7B

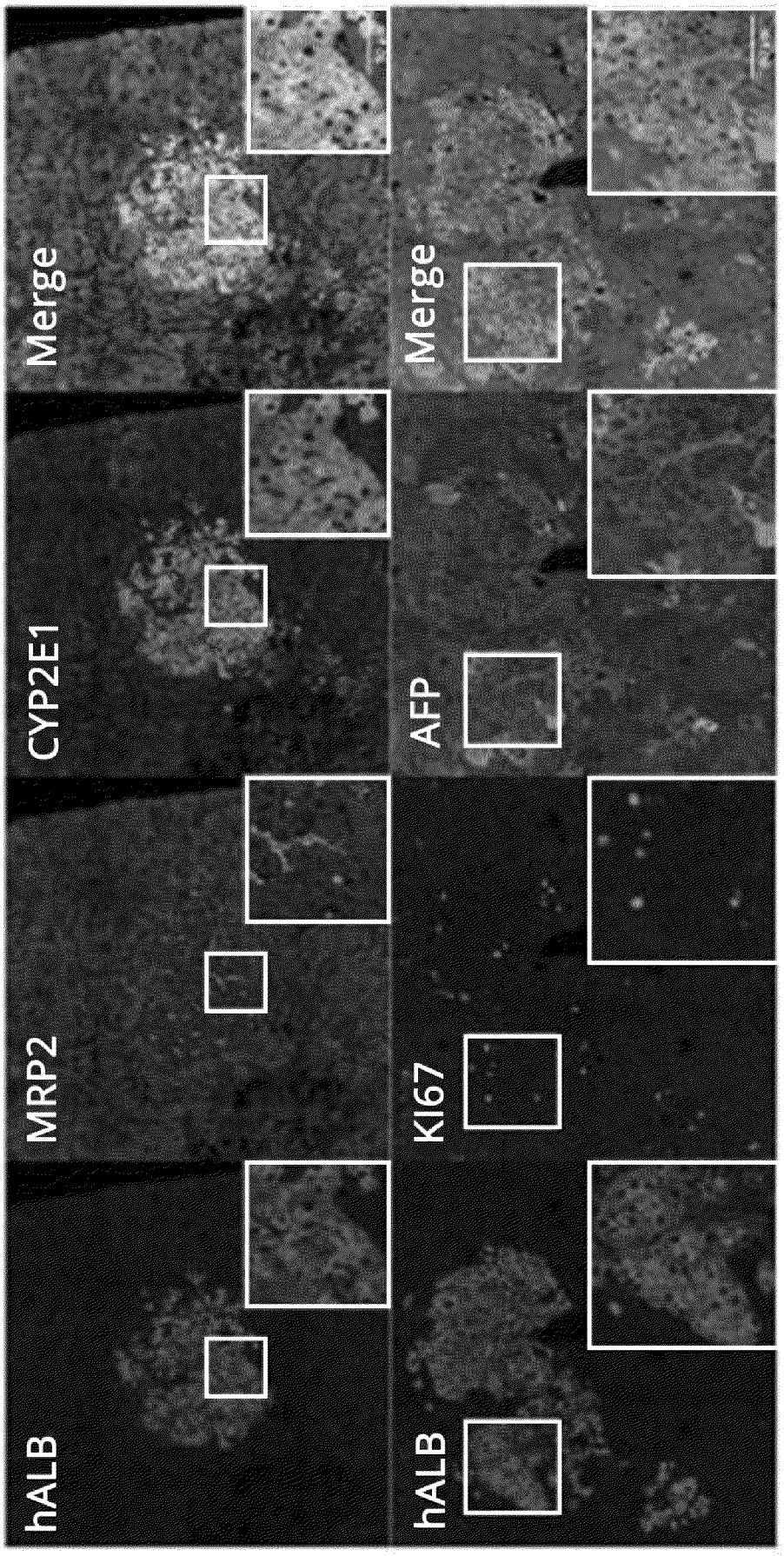


Figure 7 cont

Figure 7 cont

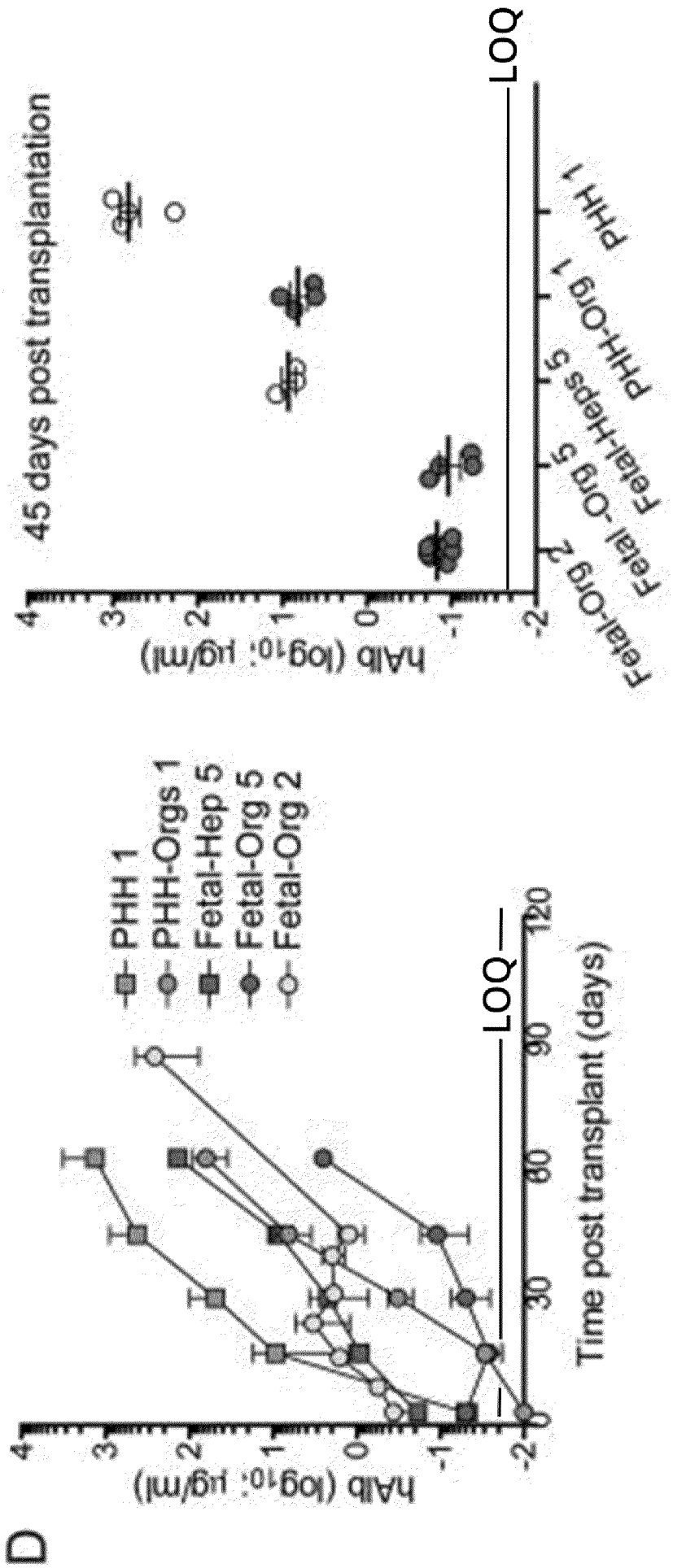
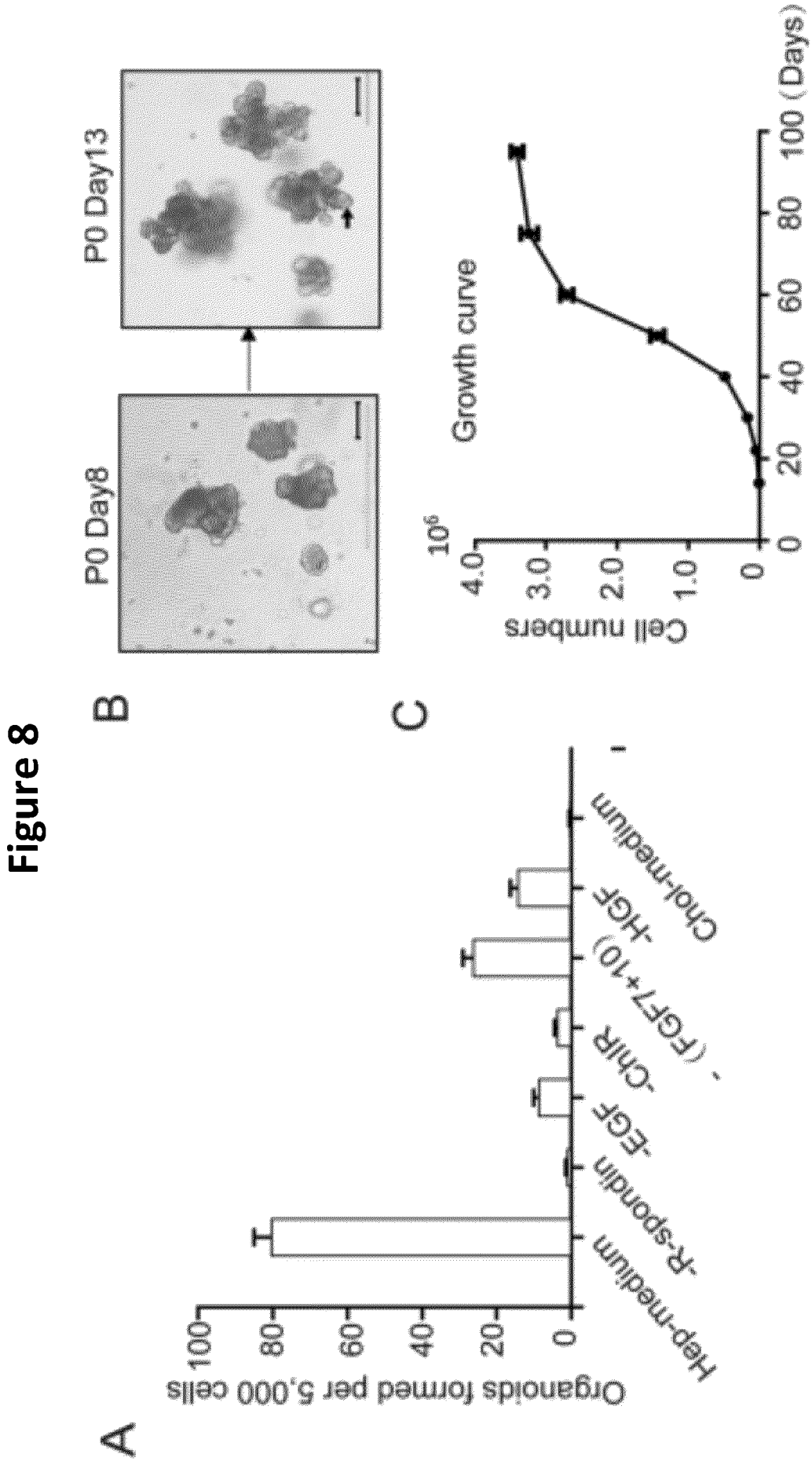


Figure 8


A
Organoids formed per 5,000 cells
0
20
40
60
80
100
Hep-medium
-R-spondin
-EGF
-Chir
-(FGF7+10)
-HGF
Chol-medium
B
P0 Day8
P0 Day13
C
Growth curve
Cell numbers
10^6
0
1.0
2.0
3.0
4.0
0
20
40
60
80
100 (Days)

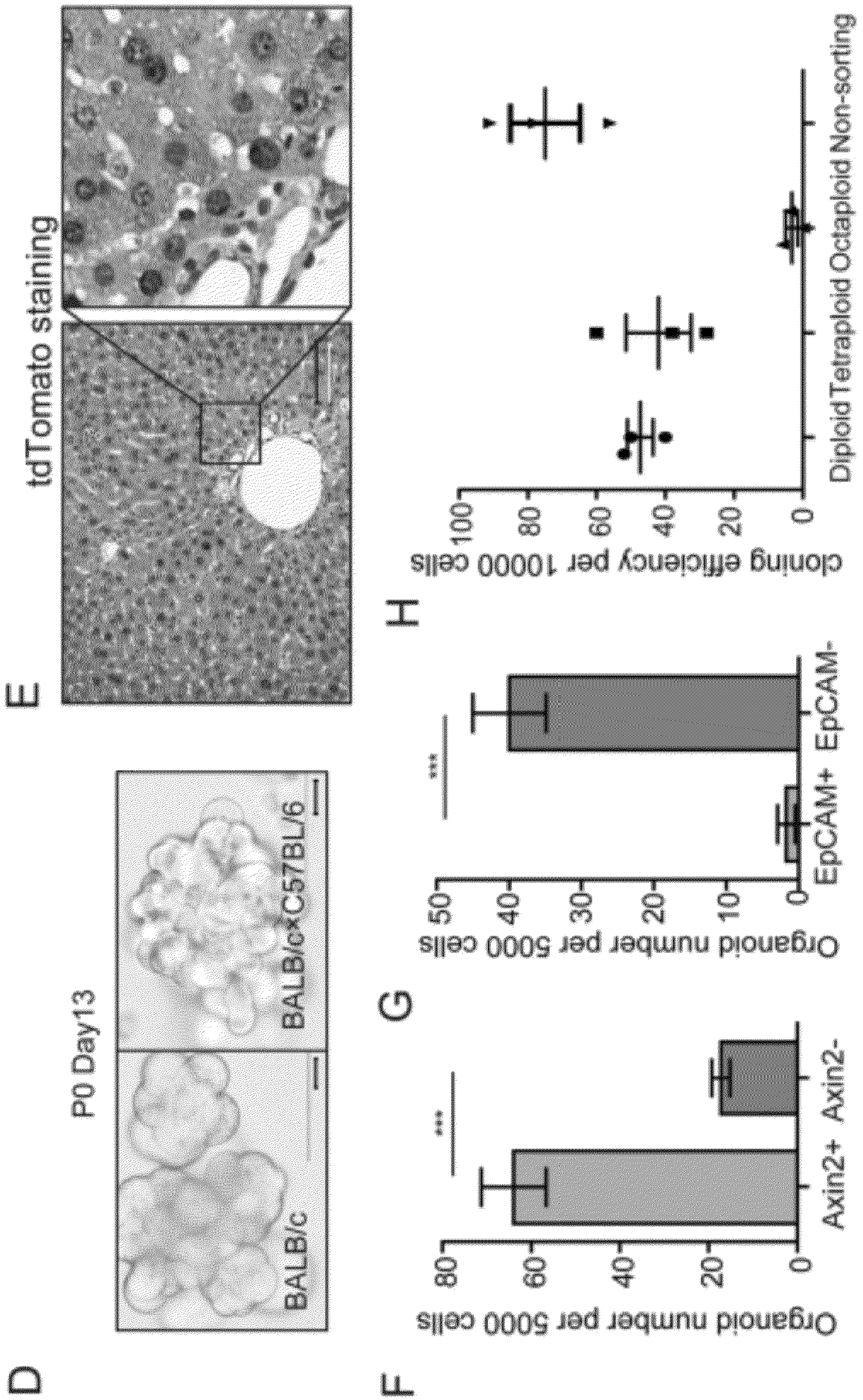


Figure 8 cont

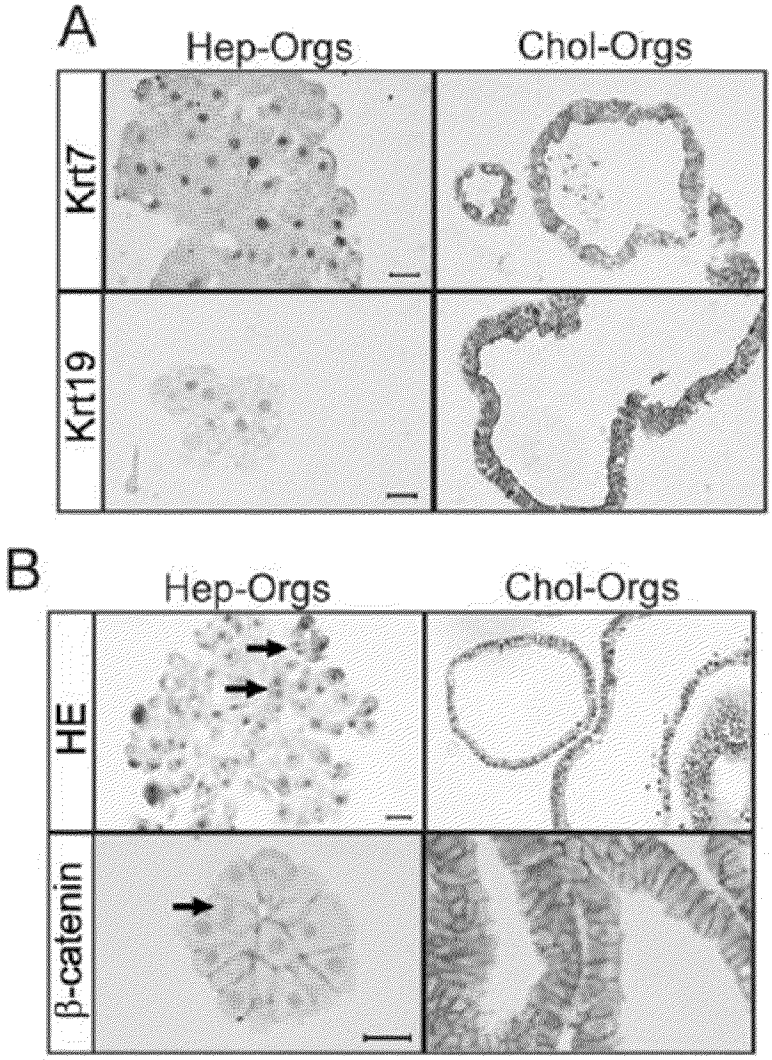

A
Hep-Orgs
Chol-Orgs
Krt7
Krt19
B
Hep-Orgs
Chol-Orgs
HE
β-catenin
C
Chol-Orgs Staining
DAPI
Epcam
F-actin

Figure 9

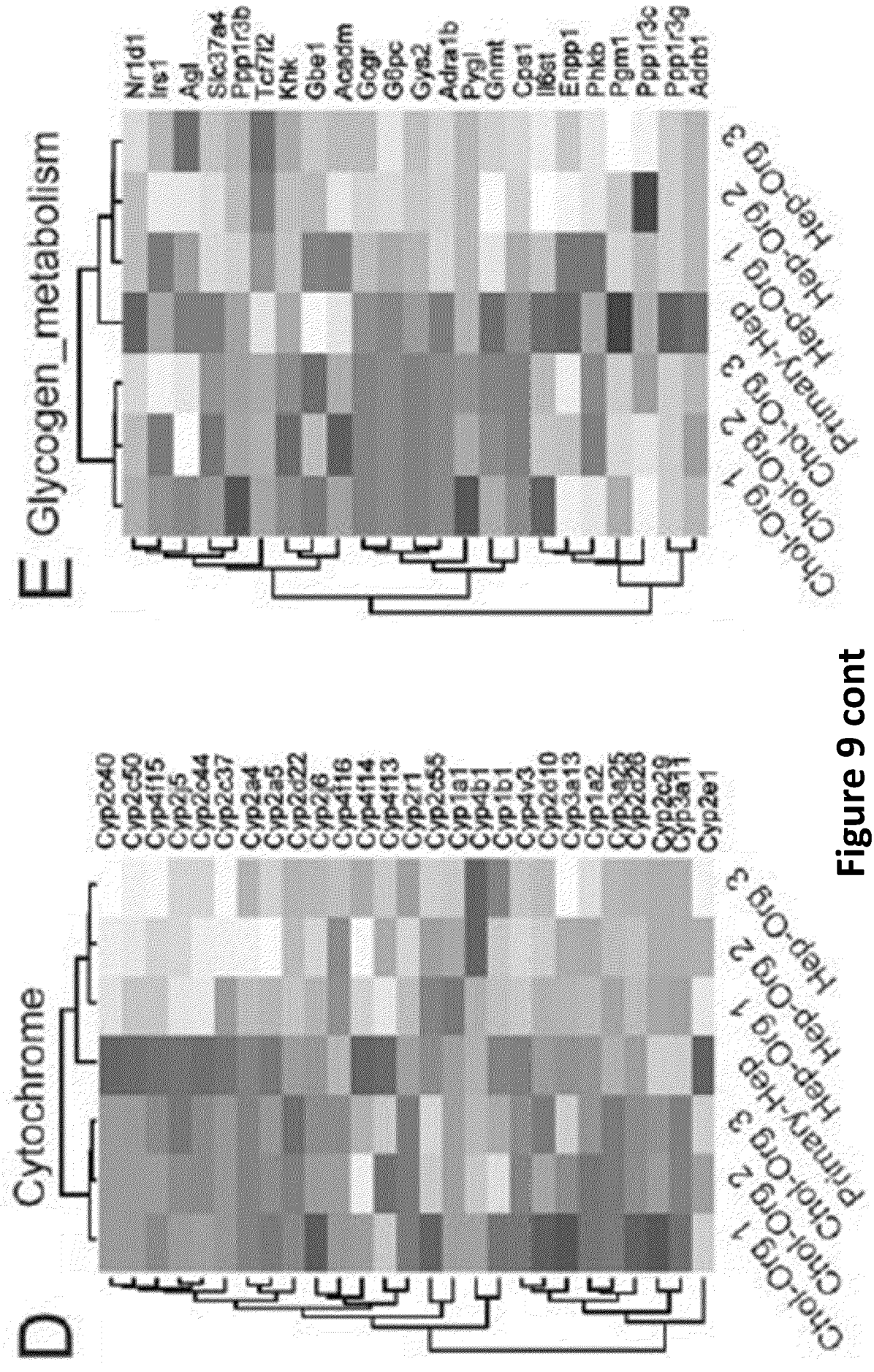


Figure 9 cont

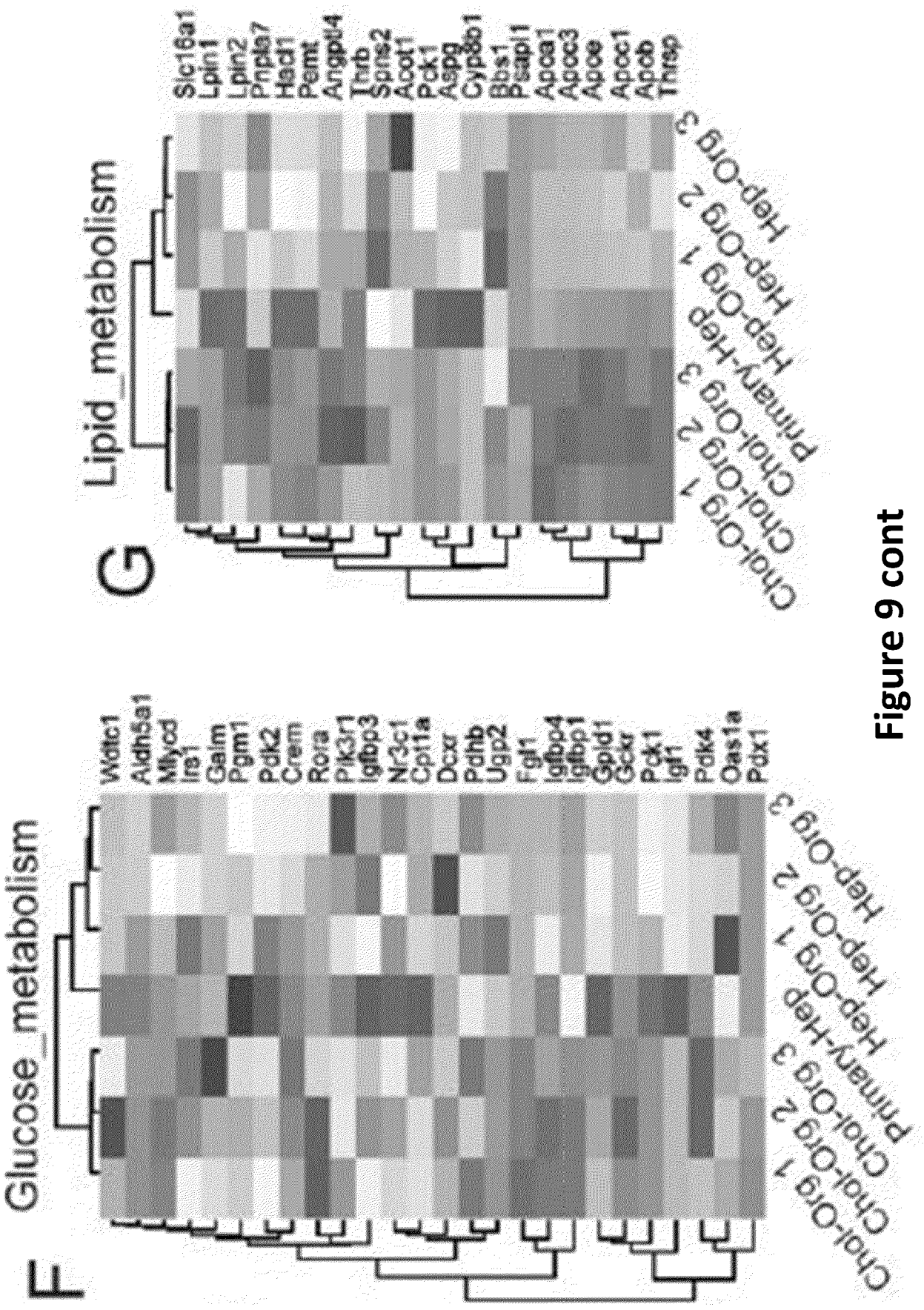
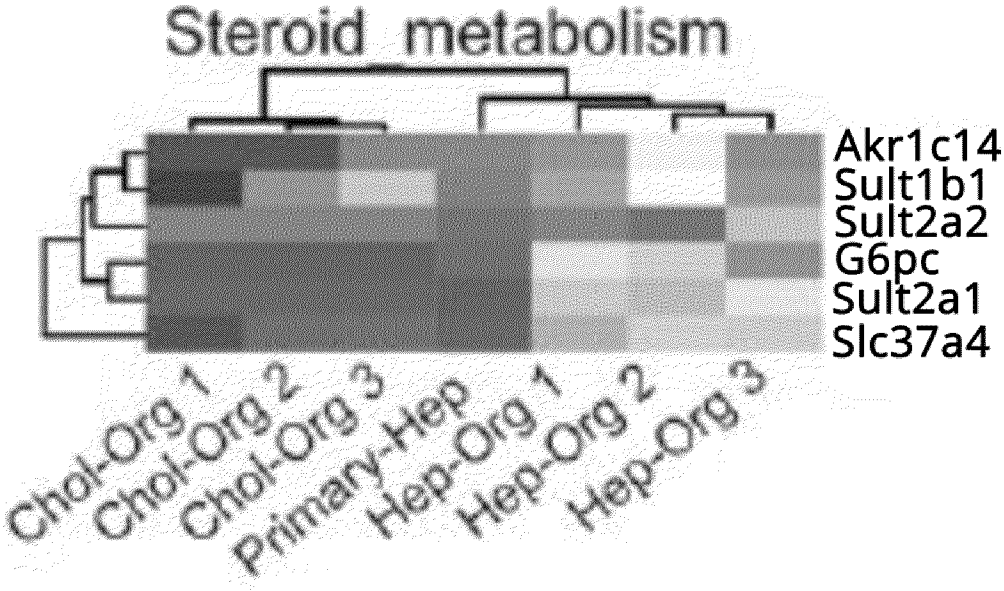


Figure 9 cont

Figure 9 cont

J



L

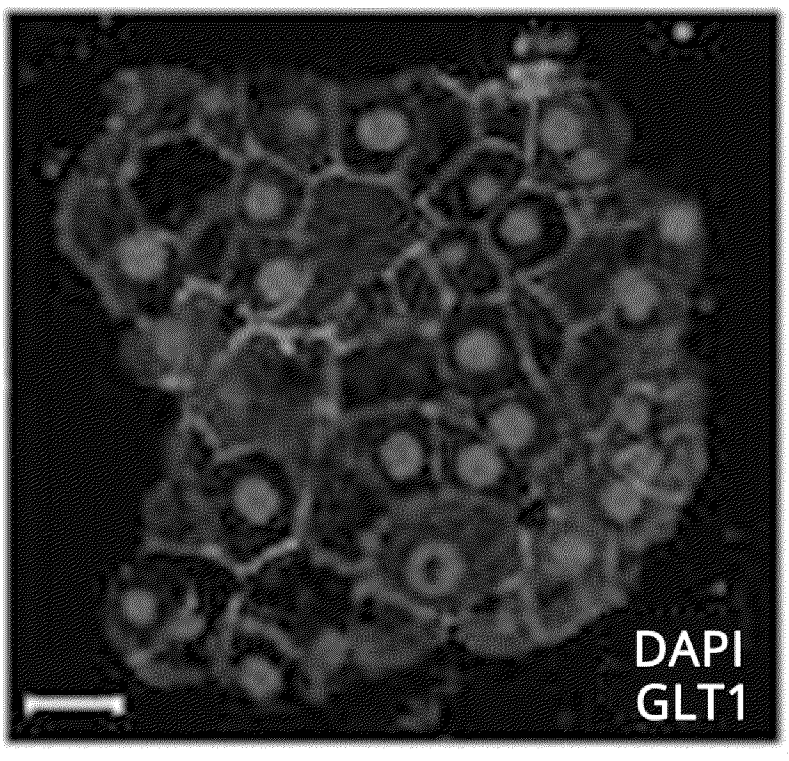
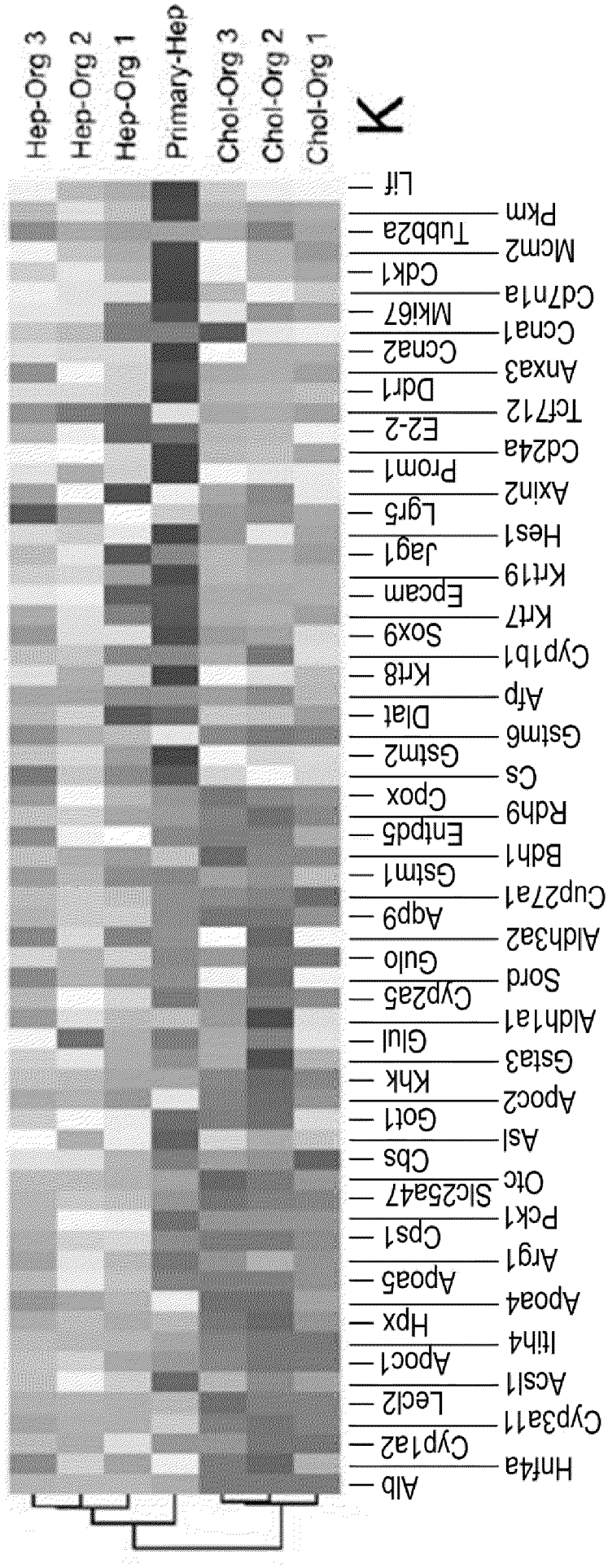


Figure 9 cont



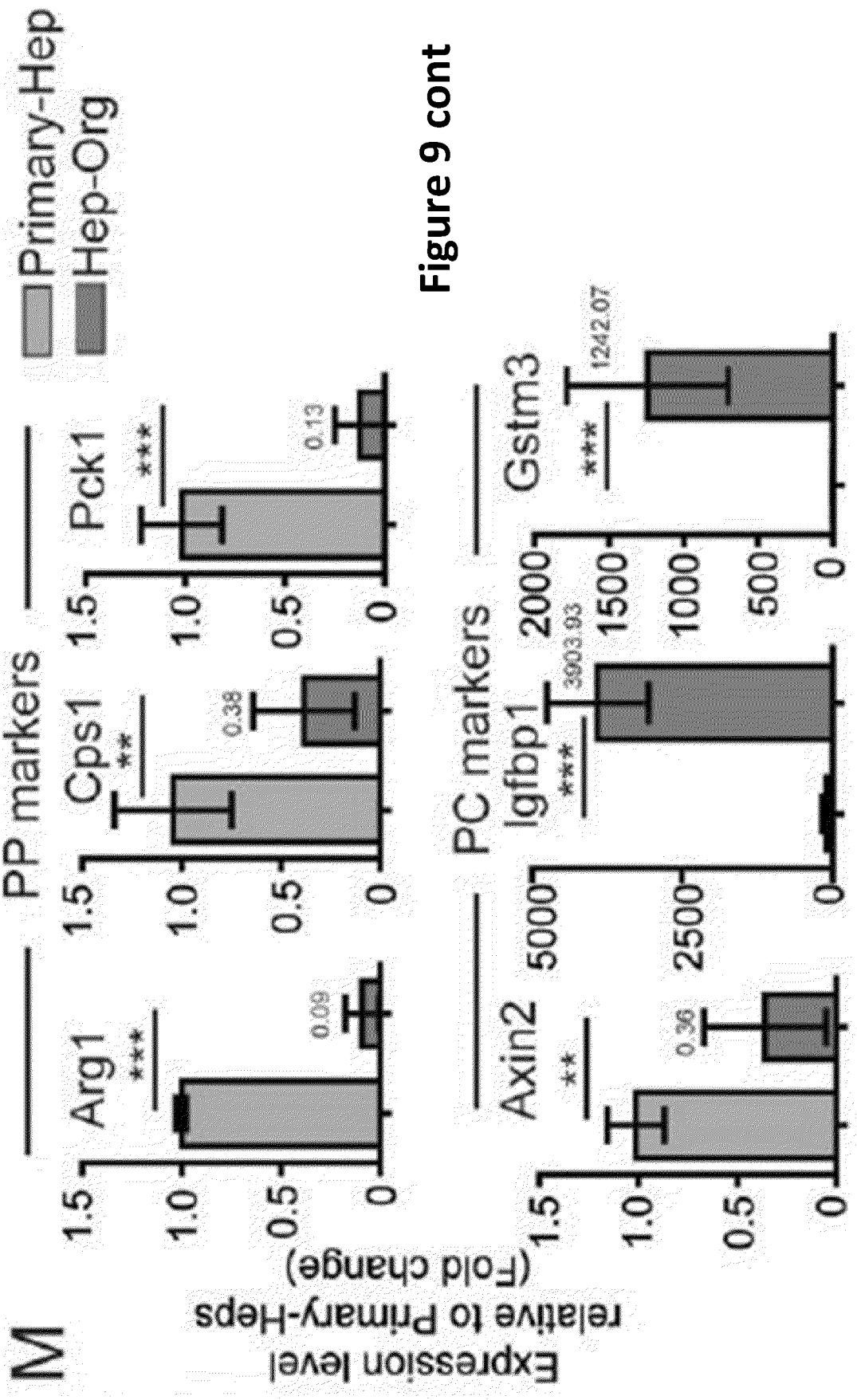


Figure 9 cont

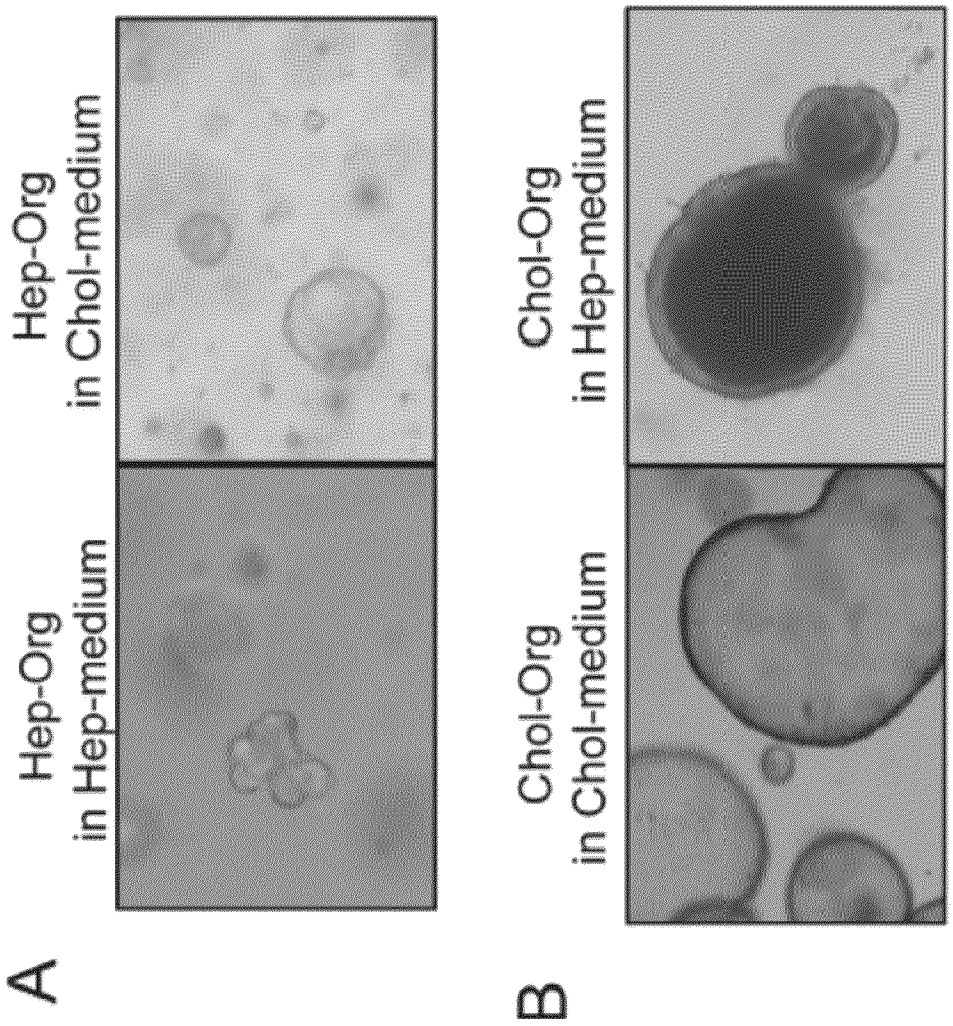

A
Hep-Org
in Hep-medium
Hep-Org
in Chol-medium
B
Chol-Org
in Chol-medium
Chol-Org
in Hep-medium

Figure 10

C

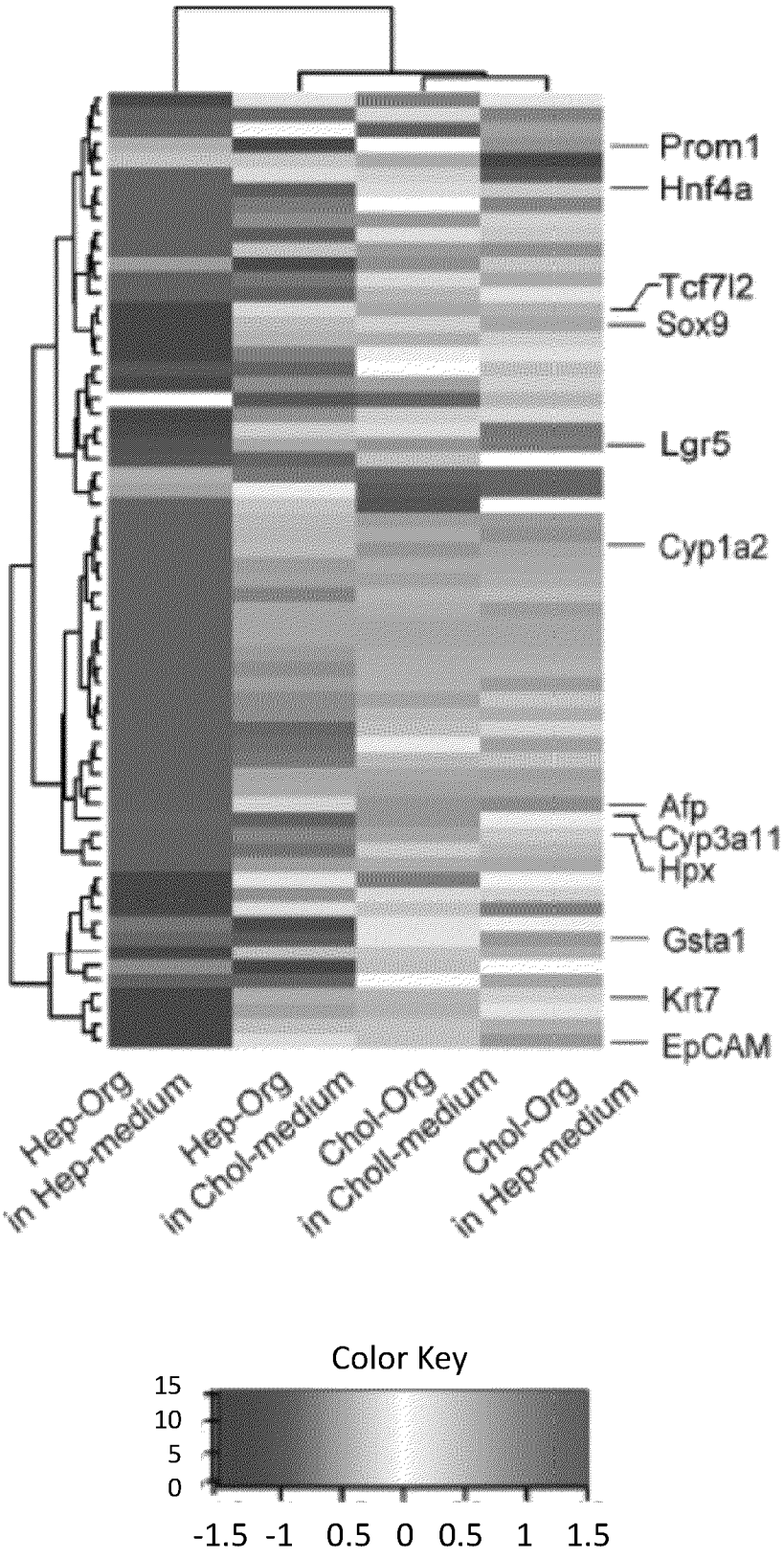

Prom1
Hnf4a
Tcf7l2
Sox9
Lgr5
Cyp1a2
Afp
Cyp3a11
Hpx
Gsta1
Krt7
EpCAM
Hep-Org in Hep-medium
Hep-Org in Chol-medium
Chol-Org in Chol-medium
Chol-Org in Hep-medium
Color Key
15
10
5
0
-1.5 -1 0.5 0 0.5 1 1.5

Figure 11

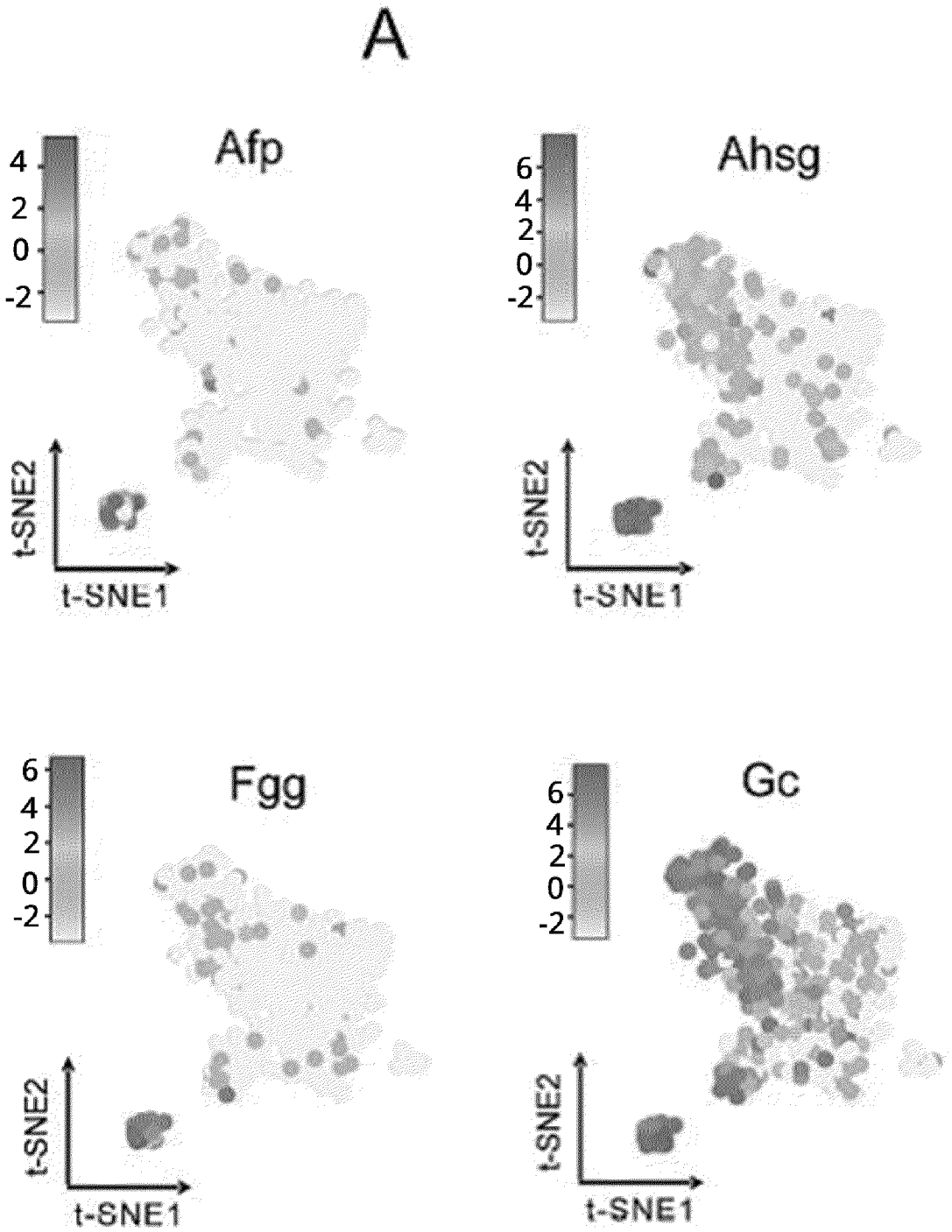

A
Afp
4
2
0
-2
t-SNE2
t-SNE1
Ahsg
6
4
2
0
-2
t-SNE2
t-SNE1
Fgg
6
4
2
0
-2
t-SNE2
t-SNE1
Gc
6
4
2
0
-2
t-SNE2
t-SNE1

Figure 11 cont.

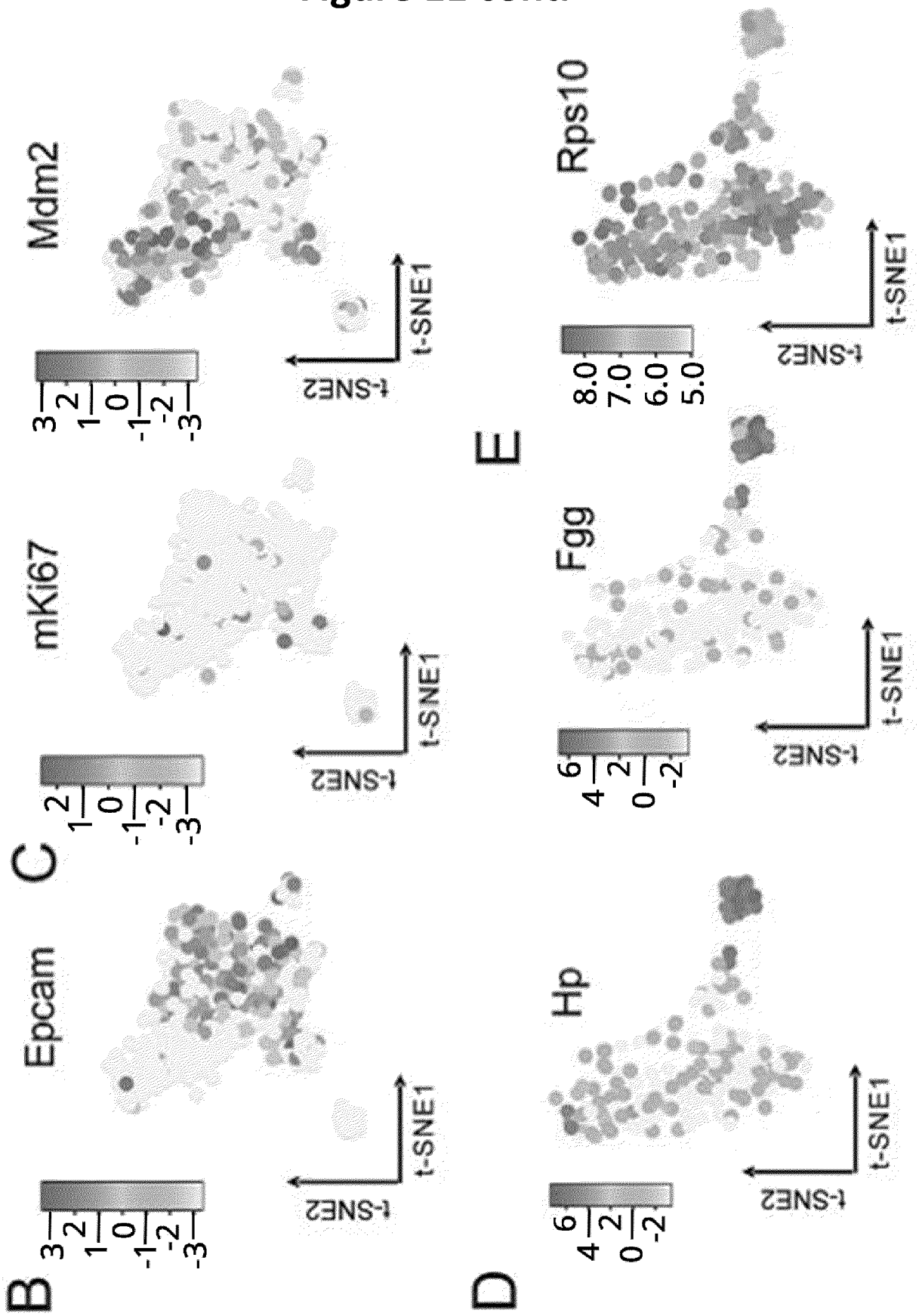


Figure 11 cont.

F

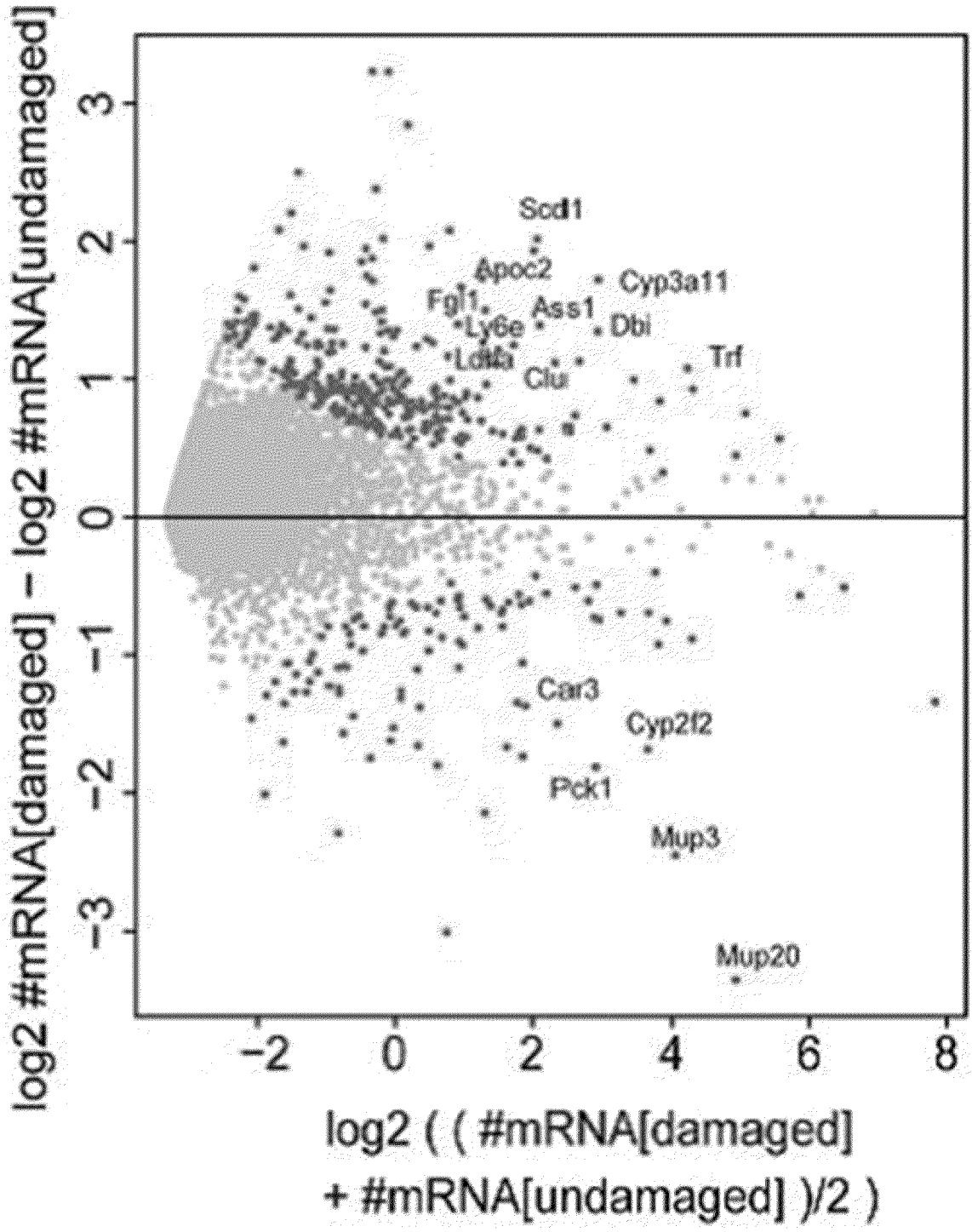
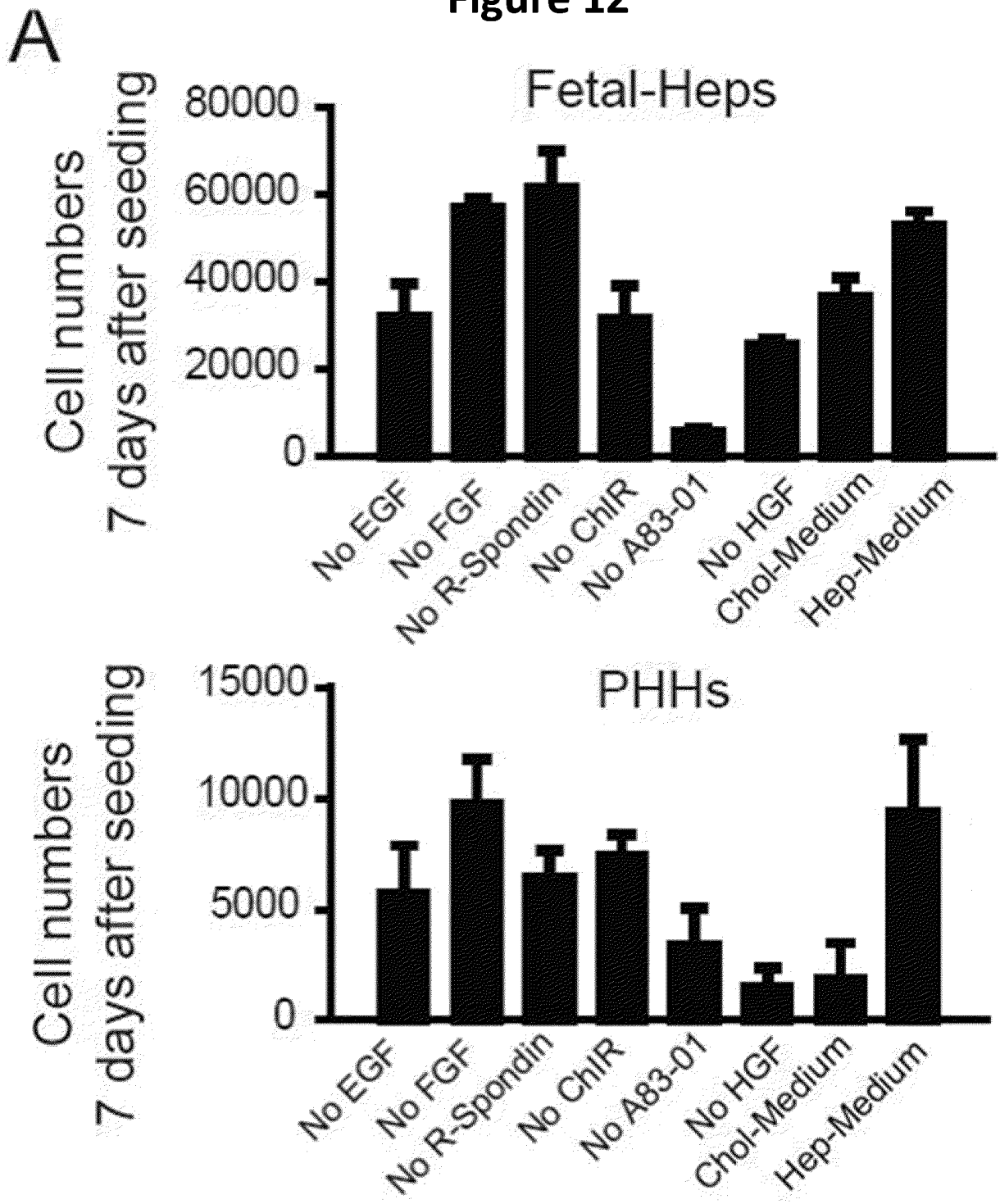


Figure 12


A
Fetal-Heps
Cell numbers
7 days after seeding
80000
60000
40000
20000
0
No EGF
No FGF
No R-Spondin
No ChIR
No A83-01
No HGF
Chol-Medium
Hep-Medium
PHHs
Cell numbers
7 days after seeding
15000
10000
5000
0
No EGF
No FGF
No R-Spondin
No ChIR
No A83-01
No HGF
Chol-Medium
Hep-Medium

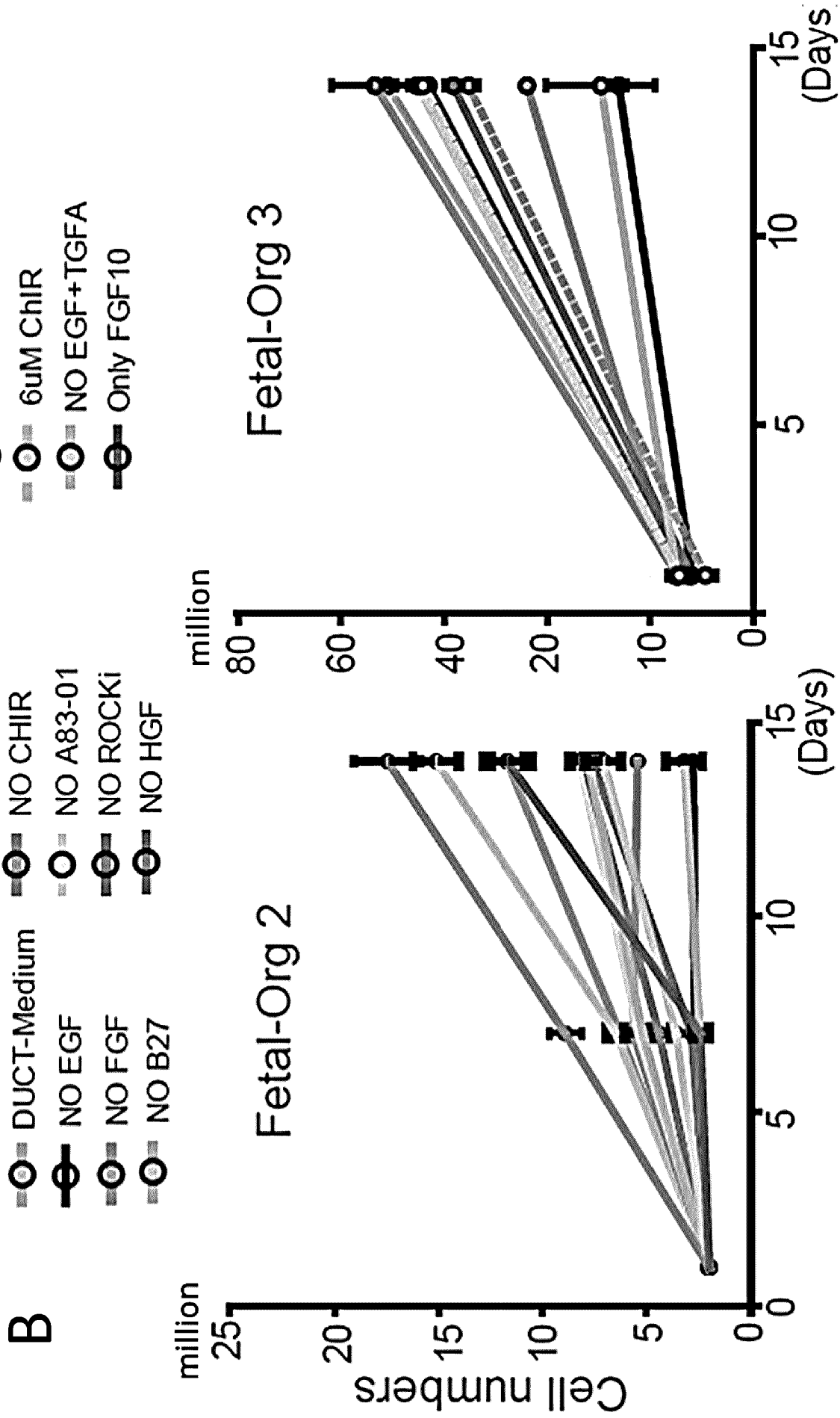


Figure 12 cont

Figure 12 cont

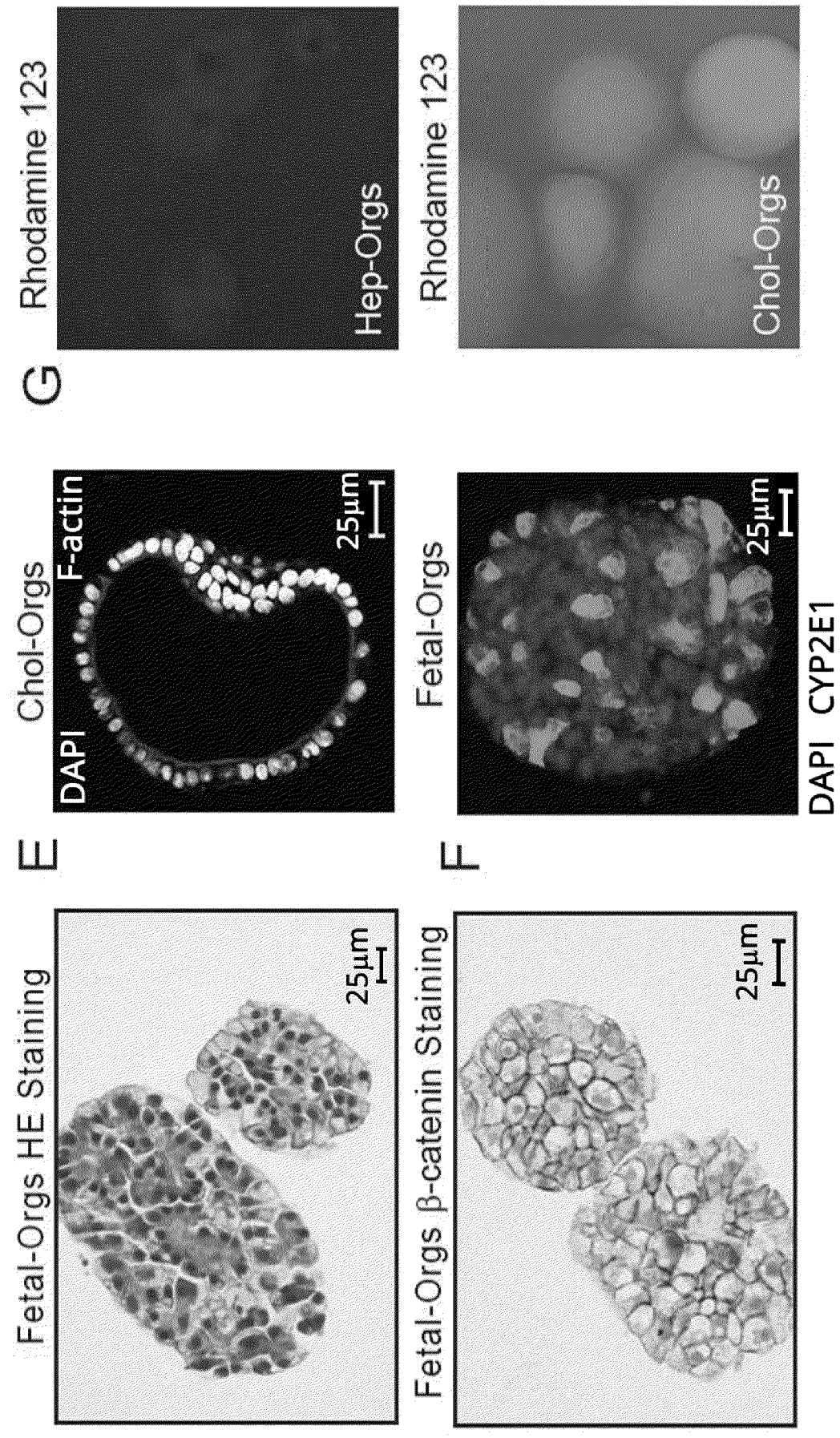


Figure 12 cont

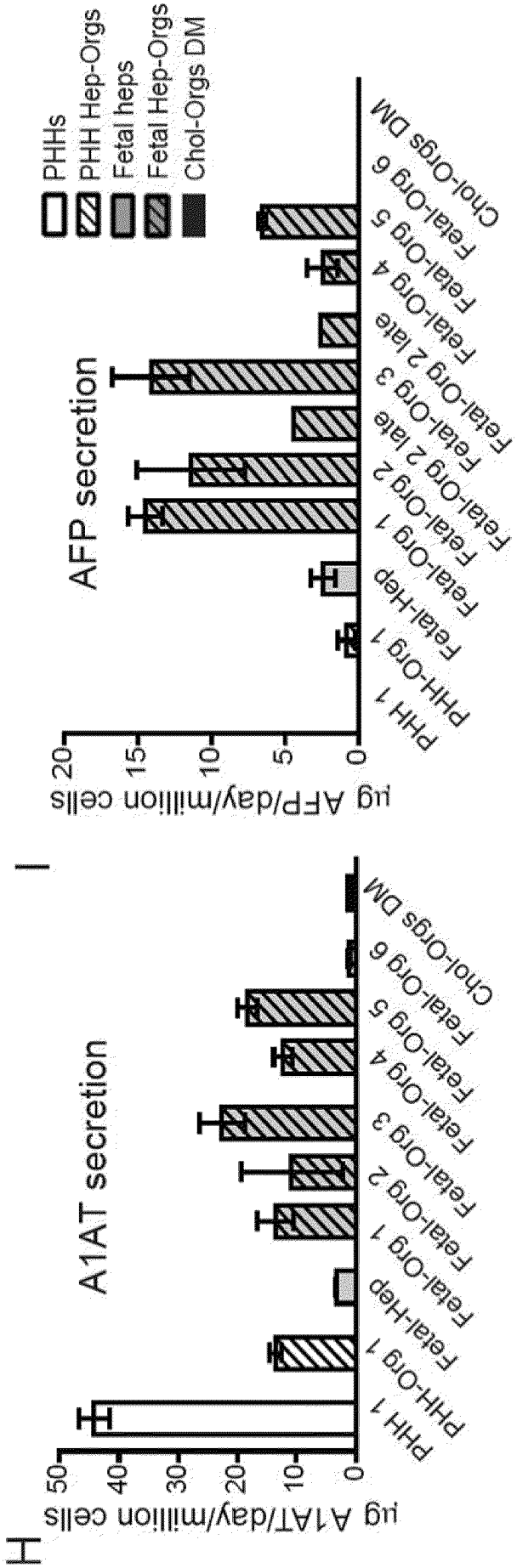


Figure 13

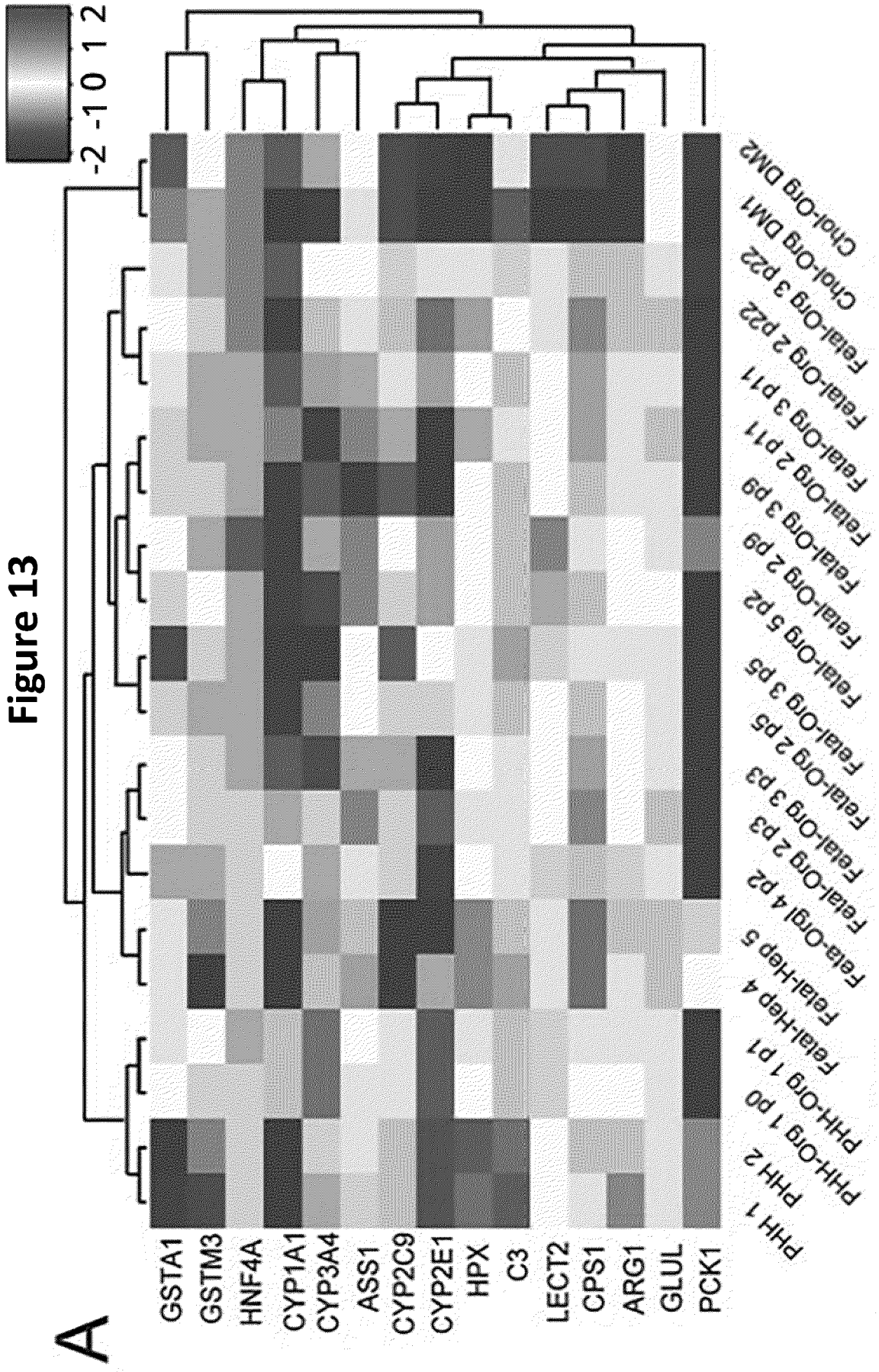

A
-2 -1 0 1 2
GSTA1
GSTM3
HNF4A
CYP1A1
CYP3A4
ASS1
CYP2C9
CYP2E1
HPX
C3
LECT2
CPS1
ARG1
GLUL
PCK1
PHH 1
PHH 2
PHH-Org 1 p0
PHH-Org 1 P1
Fetal-Hep 4
Fetal-Hep 5
Feta-Org1 4 p2
Fetal-Org 2 p3
Fetal-Org 3 p3
Fetal-Org 2 p5
Fetal-Org 3 p5
Fetal-Org 5 p2
Fetal-Org 2 p9
Fetal-Org 3 p9
Fetal-Org 2 p11
Fetal-Org 3 p11
Fetal-Org 2 p22
Fetal-Org 3 p22
Chol-Org DM1
Chol-Org DM2

Figure 13 cont

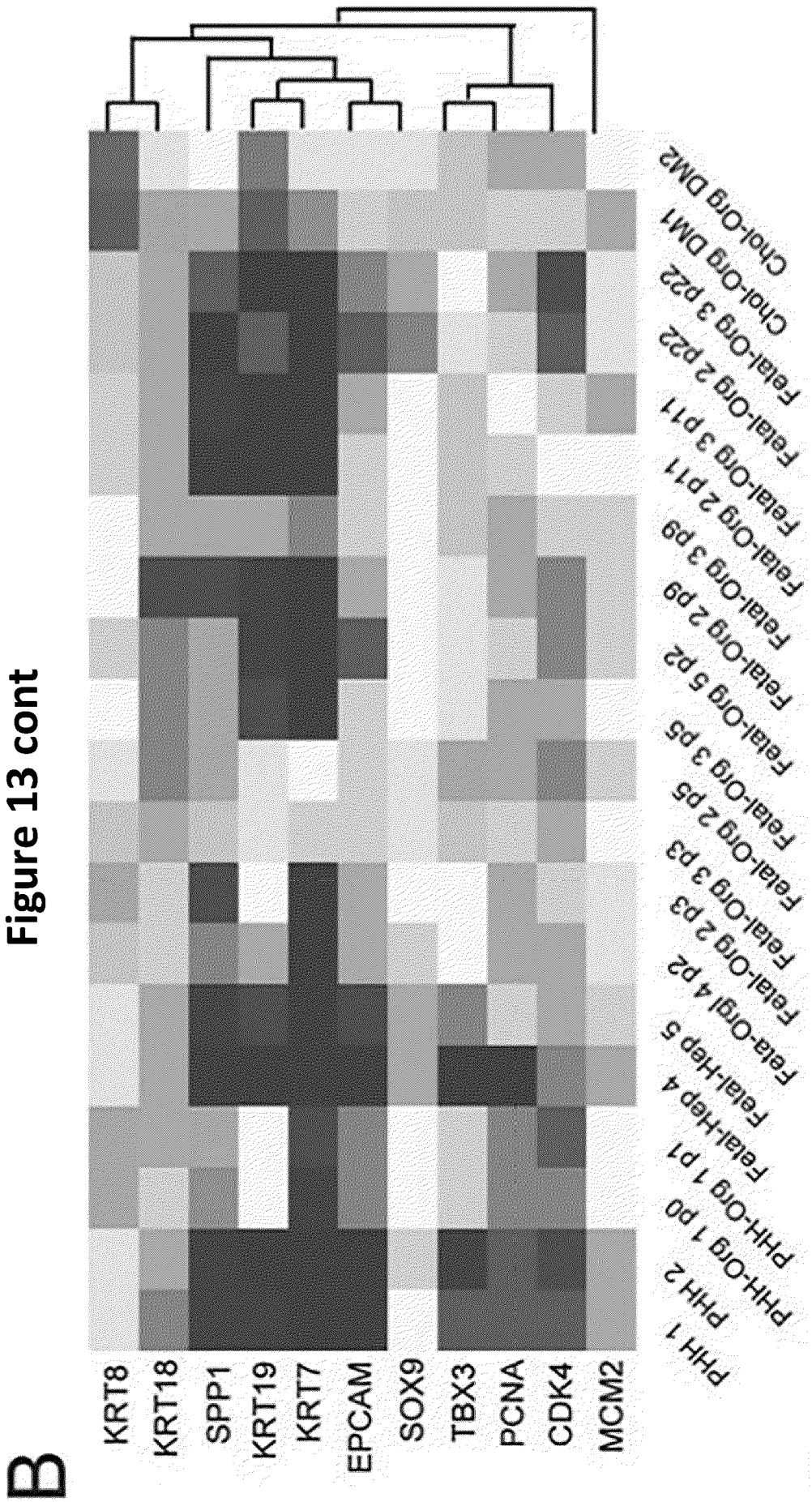


Figure 13 cont.

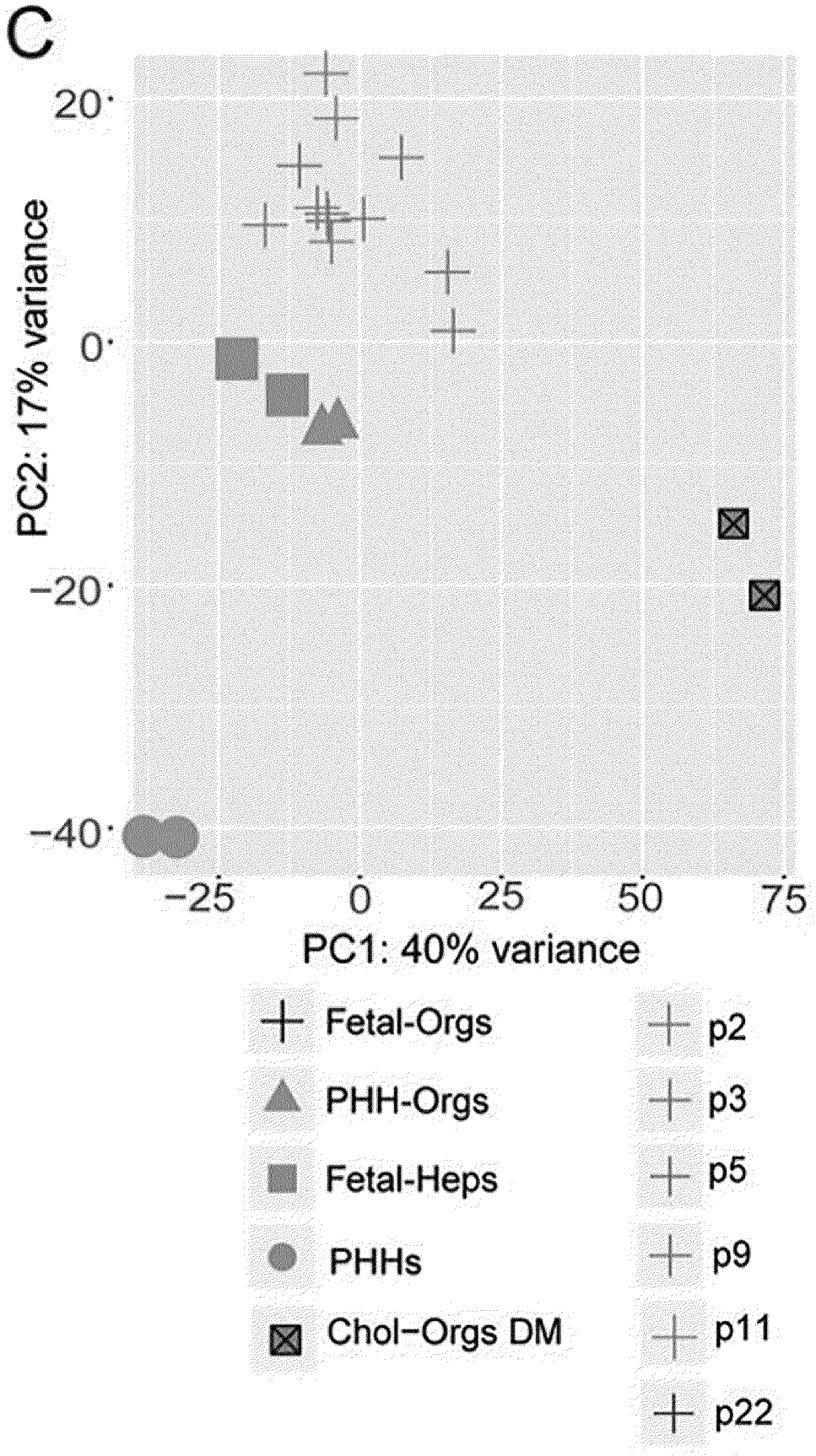


Figure 13 cont

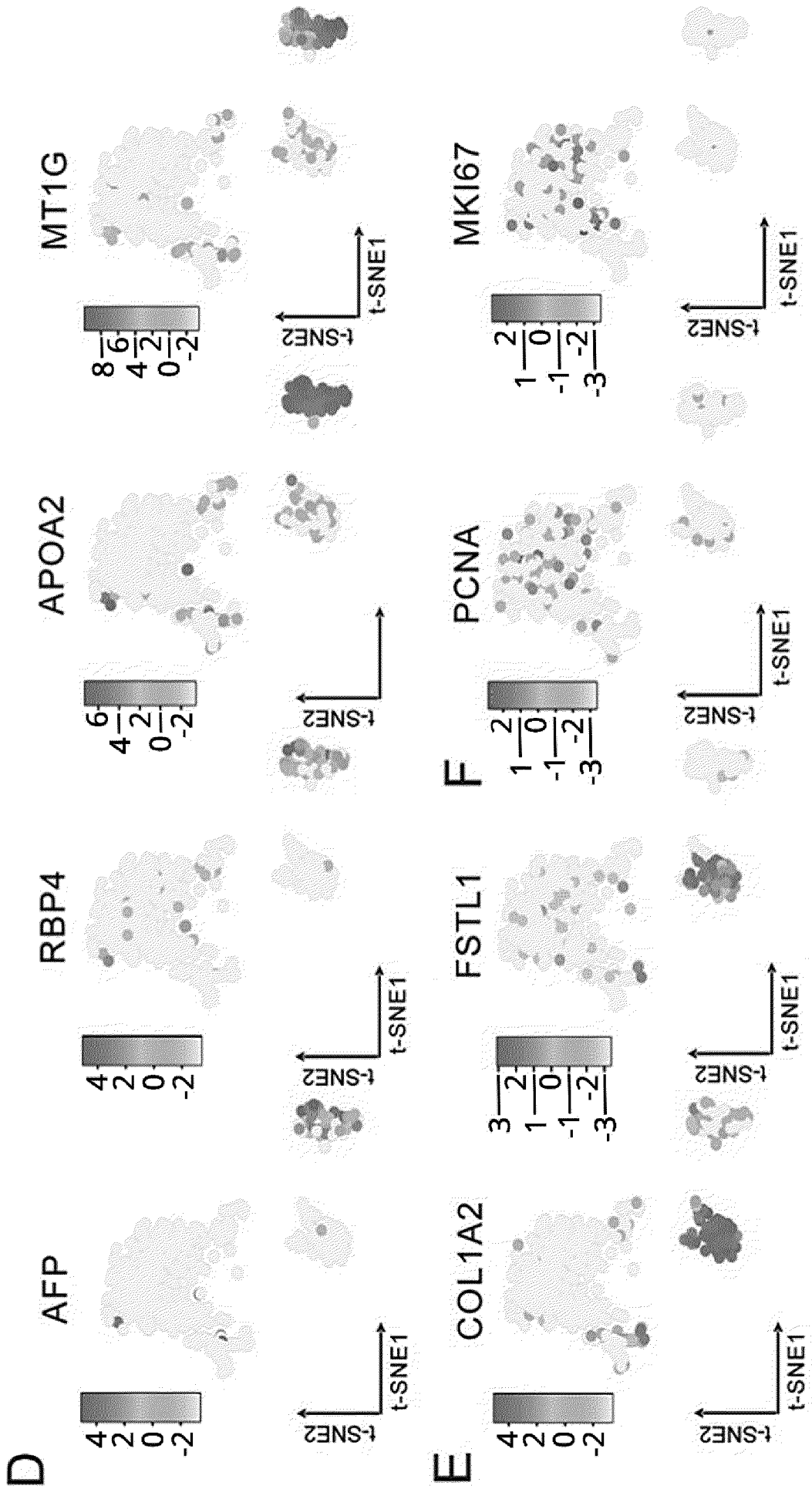
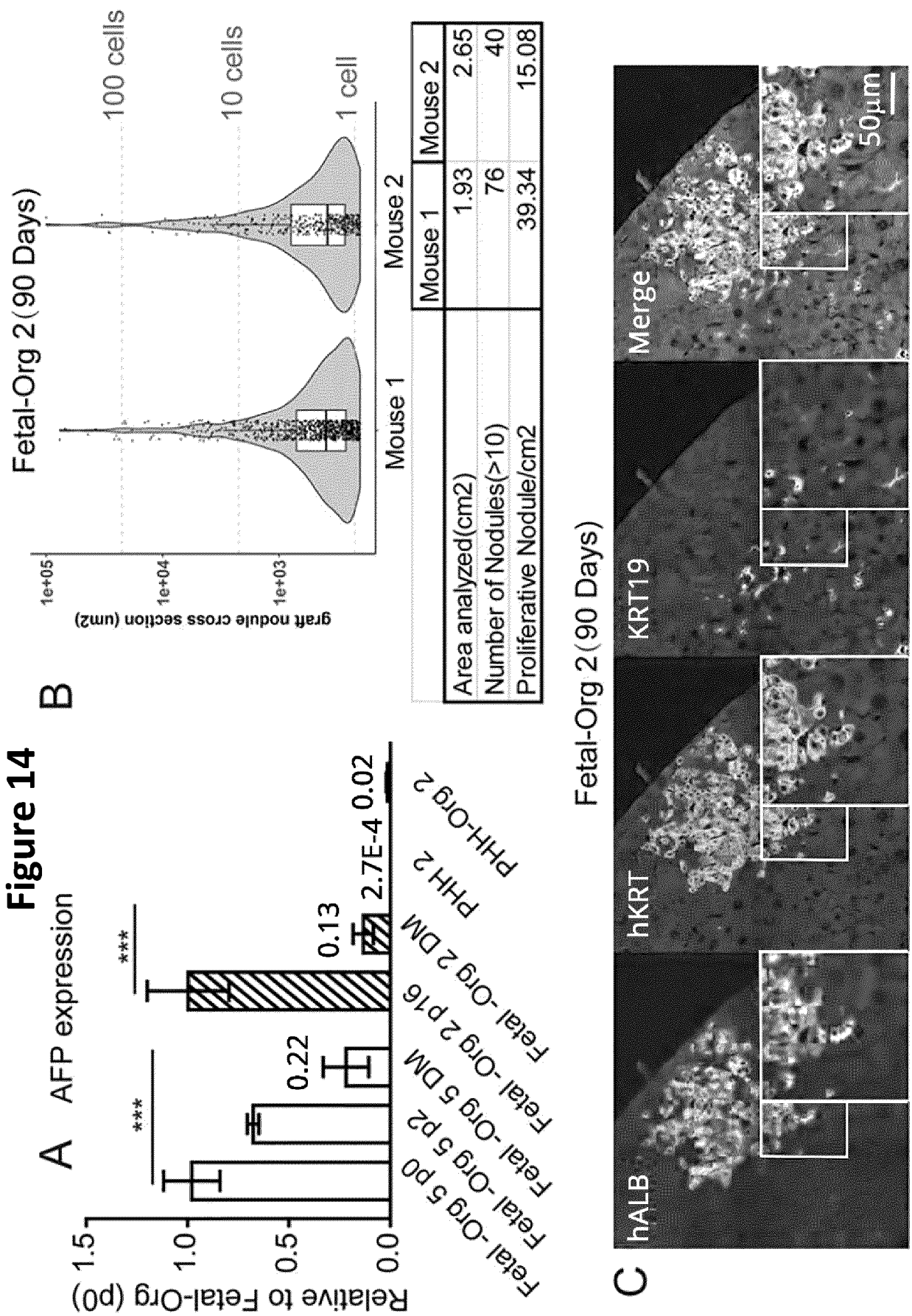


Figure 14


A
AFP expression
Relative to Fetal-Org (p0)
1.5
1.0
0.5
0.0
***
***
0.22
0.13
2.7E-4
0.02
Fetal -Org 5 p0
Fetal -Org 5 p2
Fetal -Org 5 DM
Fetal -Org 2 p16
Fetal -Org 2 DM
PHH 2
PHH-Org 2
B
Fetal-Org 2 (90 Days)
100 cells
10 cells
1 cell
graft nodule cross section (um2)
1e+05
1e+04
1e+03
Mouse 1
Mouse 2
Mouse 1
Mouse 2
Area analyzed(cm2) 1.93 2.65
Number of Nodules(>10) 76 40
Proliferative Nodule/cm2 39.34 15.08
C
Fetal-Org 2 (90 Days)
hALB
hKRT
KRT19
Merge
50µm

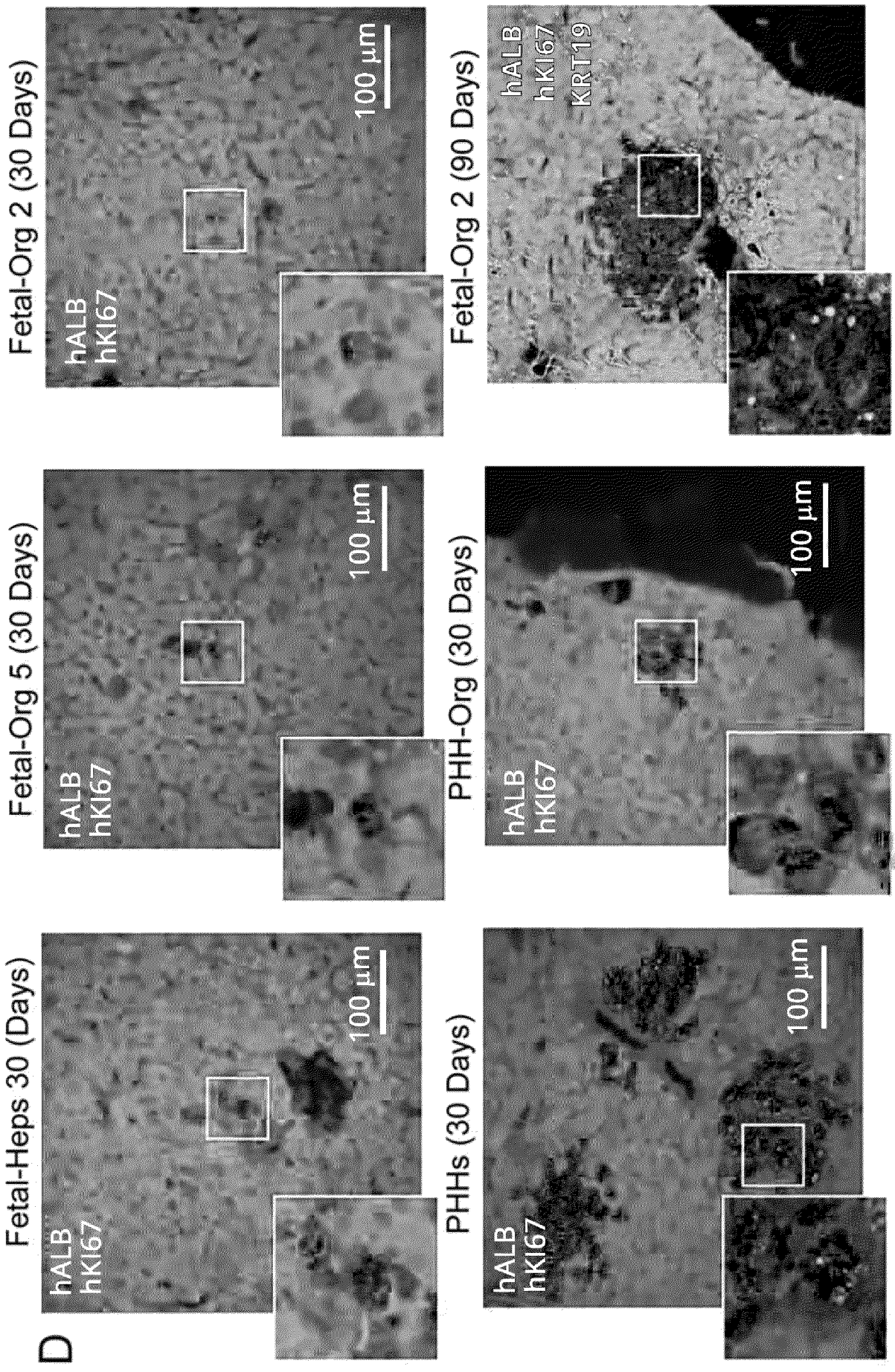


Figure 14 cont

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/082618

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/071 A61K35/407 A61P1/16
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/036119 A1 (THE SECOND MILITARY MEDICAL UNIV [CN]) 1 March 2018 (2018-03-01)	1,2,5-7, 11-15, 22,23, 27, 29-35,55
Y	the whole document paragraph [0100] example 1	3,4, 8-10, 16-21, 24-26, 28,56

X	US 2017/191030 A1 (HUCH ORTEGA MERITXELL [NL] ET AL) 6 July 2017 (2017-07-06)	36-54
Y	the whole document page 43 - page 44; example 12 page 46; example 16	3,4, 8-10, 16-21, 24-26, 28,56

	-/--	

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[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 January 2020	11/02/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Zuber Perez, C

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/082618

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2018/066233 A1 (HUCH ORTEGA MERITXELL [NL] ET AL) 8 March 2018 (2018-03-08)	46-54
Y	the whole document page 4, paragraph 0043 - paragraph 0047 "Wnt agonists"; page 6 - page 7 page 29; example 7	3,4, 8-10, 16-21, 24-26, 28,56

X	WO 2017/149025 A1 (KONINKLIJKE NEDERLANDSE AKADEMIE VAN WETENSCHAPPEN [NL]) 8 September 2017 (2017-09-08)	46-54
Y	the whole document example 11 page 110; table 2	3,4, 8-10, 16-21, 24-26, 28,56

X	WO 2017/048193 A1 (AGENCY FOR SCIENCE TECH AND RES [SG]) 23 March 2017 (2017-03-23)	46-54
Y	the whole document example 11 page 110; table 2	3,4, 8-10, 16-21, 24-26, 28,56

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/082618

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		TR 201910320 T4	21-08-2019
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WO 2017149025 A1	08-09-2017	AU 2017228002 A1	30-08-2018
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