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(54) Title: BILIARY ORGANOIDS

(57) Abstract: This invention relates to culture methods that allow the efficient long-term expansion of biliary progenitor cells, in the form of organoids. The methods comprise culturing a population of primary liver cells in a biliary progenitor expansion medium comprising epidermal growth factor (EGF), a TGFβ inhibitor, a non-canonical Wnt signalling potentiator and a ROCK inhibitor, to produce an expanded population of biliary progenitor cells. The biliary progenitor cells are bipotent and may be further differentiated into hepatocytes and cholangiocytes. Culture methods, cell populations and uses thereof are provided.

Biliary Organoids

Field

The present invention relates to the *in vitro* generation of organoids comprising biliary progenitor cells, for example for use in modelling liver disease or development, drug screening and regenerative medicine.

Background

The liver is unique as an organ in the broad spectrum of its functions during adult life (Gille *et al.*, 2010). These functions include iron, vitamin and mineral homeostasis, and the detoxification of alcohol, drugs and other chemicals circulating in the bloodstream. The liver also synthesises bile for the digestion of fat and secretes blood clotting factors and serum proteins such as albumin (Alb) that represents the most abundant protein in the plasma. Finally, the liver plays an essential metabolic activity by storing glycogen and lipids (Gille *et al.*, 2010). Most of these activities are managed by hepatocytes which constitute over 80% of the liver mass (Blouin, Bolender and Weibel, 1977). The cholangiocytes from the biliary tree also have an essential role in collecting waste, processing bile acids and possibly in hepatic regeneration after injury (Deng *et al.*, 2018). Importantly, disorders affecting functions of these two cell types can be life-threatening and organ transplantation remains the only treatment for end stage liver diseases. However, the number of organ donors has remained constant for the past 10 years while the demand for liver transplantation has more than doubled in the meantime (Hopkinson and Allen, 2017). This situation is likely to worsen in the foreseeable future due to the pandemic of liver disease associated with obesity and non-alcoholic fatty liver disease (Younossi *et al.*, 2016). Importantly, understanding liver organogenesis, especially the mechanisms by which hepatocytes and cholangiocytes are generated during development, could help to develop alternative regenerative approach such as cell-based therapies and also in vitro systems for disease modelling and drug screening.

Studies in the mouse and human have clearly established that hepatocytes and cholangiocytes originate from bi-potential progenitors, such as hepatoblasts (Yang *et al.*, 2017)(Shiojiri *et al.*, 2001). More precisely, hepatoblasts represent a proliferative population first detected in the liver bud at 5 weeks post conception in humans. These progenitors persist until 20 weeks of development playing a crucial role in liver organogenesis. They are characterised by their capacity to express markers specific for both hepatocytes and cholangiocytes such as Epithelial Cell Adhesion Molecule (EPCAM), Albumin and Alpha-Fetoprotein (AFP). Importantly, the signalling pathways involved in hepatoblast self-renewal and differentiation remain to be fully uncovered as contradicting reports have suggested that Wnt signalling could block or promote differentiation of hepatoblasts toward hepatocytes and cholangiocytes in the mouse embryo (Decaens *et al.*, 2008; Tan *et al.*, 2008). On the other hand, TGFβ has been shown to direct differentiation of hepatoblasts toward the biliary lineage. However, such mechanisms have not been confirmed in human and other pathways may be involved (Clotman *et al.*, 2005). Of note, transplantation of isolated human hepatoblasts have shown that these cells can colonise the rat adult liver in hepatic failure models thereby demonstrating their interest for regenerative medicine applications (Oertel *et al.*, 2003). Thus, derivation of human hepatoblasts may provide a unique platform not only to study human liver development but also to produce cells with clinical interests. However, the development of robust protocols to grow human hepatoblasts *in vitro* have remained elusive, thereby limiting the study and utilisation of these progenitors.

It has been proposed that other bipotent hepatic cells may also produce hepatocytes and cholangiocytes during foetal development (Simper-Ronan et al Development 2006 133: 4269-4279). These cells may be maintained in the adult liver and may play a key function in regeneration.

Organoid technology consists of growing stem cells, and more broadly a combination of progenitor and epithelial cells, in three-dimensional culture conditions. This technology was first developed using intestinal stem cells (Sato *et al.*, 2011) and then was rapidly applied to a diversity of adult organs including the liver, pancreas, prostate, mammary gland, biliary three and lungs (Huch and Koo, 2015). In addition, similar culture conditions have been combined with differentiation of human Pluripotent Stem Cells (hPSCs) to generate brain, kidney, gut and liver organoids which mimic the cellular complexity and architecture of native organs. The interest of organoids has been broadly demonstrated for basic studies, disease modelling (Schwank *et al.*, 2013), drug screening (van de Wetering *et al.*, 2015), and also regenerative medicine applications (Cruz-Acuña *et al.*, 2017). Nonetheless, this technology has been more rarely applied to foetal tissues in the context of developmental studies. Indeed, primary organoids have only been derived so far from the foetal gut and lung tips. Interestingly, the resulting cells display capacity of differentiation in vitro (Kraiczy *et al.*, 2017) and in vivo after co-culture with mesenchymal cells (Yui *et al.*, 2018).

Summary

The present inventors have developed culture methods that allow the efficient long-term expansion of biliary progenitor cells, in the form of organoids. These biliary progenitor cells have not been previously described and may differentiate into cholangiocytes and hepatocytes. Populations of biliary progenitor cells expanded as described herein may be useful for example in regenerative medicine.

A first aspect of the invention provides a method for producing an expanded population of biliary progenitor cells *in vitro* comprising:

(i) providing a population of primary liver cells and;

(ii) culturing the population in a biliary progenitor expansion medium comprising epidermal growth factor (EGF), a TGFβ inhibitor, a non-canonical Wnt signalling potentiator and a ROCK inhibitor, to produce an expanded population of biliary progenitor cells.

Preferably the primary liver cells are human primary liver cells.

Preferably, the population of primary liver cells is cultured in three-dimensional culture in the biliary progenitor expansion medium. The biliary progenitor cells in the population may form organoids in the expansion medium during expansion.

In some embodiments, the method may further comprise disrupting the organoids to produce a population of isolated biliary progenitor cells. The isolated biliary progenitor cells may be further cultured in the expansion medium to expand or propagate the population.

In some embodiments, the biliary progenitor cells may be further differentiated, for example into cholangiocytes or hepatocytes.

A second aspect of the invention provides an isolated population of biliary progenitor cells produced by a method according to the first aspect or hepatocytes or cholangiocytes differentiated therefrom.

A third aspect of the invention provides a biocompatible scaffold comprising an isolated population of the second aspect.

A fourth aspect provides a method of treating a liver disease comprising administering an isolated population of the second aspect or a scaffold of the third aspect to an individual in need thereof.

A fifth aspect of the invention provides a method of screening comprising;

contacting an isolated population of the second aspect or a scaffold of the third aspect with a test compound, and;

determining the effect of the test compound on the population or scaffold and/or the effect of the population or scaffold on the test compound.

Preferably, the population contacted with the test compound is in the form of organoids.

A sixth aspect of the invention provides a kit for the production of biliary progenitor cell organoids comprising a biliary progenitor expansion medium that comprises epidermal growth factor (EGF), a TGFβ inhibitor, non-canonical Wnt signalling potentiator, and a ROCK inhibitor.

Other aspects and embodiments of the invention are described in more detail below.

Brief Description of the Figures

Figure 1 shows the derivation and characterisation of Fetal Biliary Organoids. 1A shows a schematic representation of the process of derivation of organoid from human foetal liver. B) Representative brightfield images of ductal organoids derived using Cholangiocyte-Organoid Medium- CO-M) (FBO, left) (i), Huch et al 2015 (Liver Stem Cell Organoids - LSCO, center) (ii), and Sampaziotis et al 2017 (Extrahepatic Cholangiocytes Organoids - ECO, right) (iii). C) Immunofluorescent staining of FBO using antibodies to Cytokeratin-19 (KRT19, green), Cytokeratin-7 (KRT7, red), Alpha-1-antitrypsin (A1AT, red) and SRY-box 9 (SOX9, yellow). D) Gene expression in primary fetal liver (PFL, n=3), primary adult hepatocytes (PAH n=6 – three donors, six plates), LSCO (n = 8, p5-11), ECO (n = 5, p4-18) and FBO (n = 9, p3-15) for the genes albumin (ALB), alpha-fetoprotein (AFP), KRT19, and alpha-1-antitrypsin (SERPINA1). Data derived using qPCR shown as mean +/- SEM relative to housekeeping gene Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0). E) tSNE plot of scRNAseq data derived from FBO (n=2 lines, 3152 cells), demonstrating two primary clusters of cells labelled Cholangiocyte Progenitors (CoP, n = 2270) and Cholangiocytes (Co, n = 882). F) Feature plot derived from the tSNE in E, displaying levels of gene expression for each gene listed, with darker red points representing higher gene expression in that cell, and lighter points demonstrating lower gene expression. G) Violin plots of gene expression for the markers in F across the cells in each cluster; CoP or Co. H) Heatmap demonstrating the comparison of the top 20 differentially expressed genes within principal component 1 that separates the two clusters, CoP and Co. The colour scale used is yellow=high, purple=low.

Figure 2 shows the basic characterisation of Hepatoblast Organoid (HO). 2A qPCR gene expression data comparing HO (n=25, p3-15, 9 donors) FBO (n=9, p3-15, 2 donros), PFL (n=3 donors) and PAH (n = 6, 3 donors, 6 plates) for the genes listed above. Expression is relative to housekeeping gene RPLP-0, mean+/- SEM. B) Representative brightfield images of HO (scale bar = 400uM, top, 200uM, bottom). C) Immunofluorescent staining of HO for HNF4a (green, top left), AFP (red, top right), ALB (yellow, bottom left), and overlay image (bottom right). Nuclear staining (blue) was performed using Hoechst dye. C) Concentration of secreted proteins in the culture medium of HO (n=16, 11 donors, p2-15) and FBO (n=4, p11-15, 2 donors) after 48 hours of freshly applied medium as detected by ELISA and normalised to cell number. D) CYP3A5/7 and CYP3A4 activity measured by chemiluminescent assay (relative luminescence units, RLU) with luciferin PFBE (top) and IPA (bottom), normalised to cell number for HO (n=3, p14), FBO, (n=2, p14), and PAH (n=6).

Figure 3 shows the characterisation of Primary Fetal Liver (PFL) 3A Sections of an 8 post-conceptional week (pcw) primary fetal liver, stained using haematoxylin and eosin (H&E, far left), immunohistochemistry for AFP (center left), KRT19 (center right), and hepatocyte marker Hep Par-1 (HEPPAR1) (far right). B) tSNE plot derived from whole primary fetal liver scRNAseq data (n=2, 6pcw, 1406 cells) demonstrating independent clustering into six groups, labelled according to cellular identity (stellate, hepatoblast, haematopoietic stem/progenitor cells (HSPC), megakaryocytes, lymphocytes, and Kupffer cells). C) Heatmap comparing the top 10 differentially expressed genes differentiating each cluster as identified on the tSNE from B. D) Violin plots displaying markers distinct to each cluster of primary fetal liver cells. E) Feature plots displaying cellular gene expression for each gene listed, based upon the tSNE in E, with darker points representing higher gene expression in that cell, and lighter points demonstrating lower gene expression.

Figure 4 shows a comparison of HO and FBO to primary foetal liver. 4A) tSNE analysis based on scRNAseq data for HO (n=2 lines, p8, 1973 cells) and PFL hepatoblast (n=2 lines, 308 cells) demonstrating independent clustering based on original cellular identity (B) Feature plots displaying cellular gene expression for each gene listed, based upon the tSNE in A, with darker colored points representing higher gene expression in that cell, and lighter points demonstrating lower gene expression. C) Violin plots demonstrating the expression of key hepatic markers across each cellular population of PFL-hepatoblasts and HO-hepatoblasts. D) Principal component analysis of scRNAseq data from PFL-hepatoblasts (red), HO (green), CoP (turquoise) and Co (purple). E) Violin plots of scRNAseq data from PFL hepatoblasts, HO, CoP and Co, demonstrating the respective hepatoblast and cholangiocytic profiles. F) Heatmap comparing the top genes for each cluster identity for PFL hepatoblast, HO, CoP and Co. Yellow = higher expression, purple = lower expression.

Detailed Description

This invention relates to the *in vitro* expansion of primary biliary progenitor cells using a biliary progenitor expansion medium comprising epidermal growth factor (EGF), a TGFβ inhibitor, a non-canonical Wnt signalling potentiator, and a ROCK inhibitor. Biliary progenitor cells (also referred to herein as cholangiocyte progenitors or “CoPs”) are a previously unreported cell type. The *in vitro* production of expanded biliary progenitor populations as described herein may be useful for example in modelling liver development, drug screening and regenerative medicine, for example in the treatment of liver disease.

Biliary cells are cells from the epithelium of biliary tissue, which is a monolayer covering the luminal surface of the biliary tree, including the bile duct and gall bladder. Biliary cells play important roles in bile secretion and electrolyte transport *in vivo* and may include cells of the cholangiocyte lineage including cholangiocytes and progenitors thereof. Biliary progenitors are bipotent biliary cells that are distinct from foetal hepatoblasts and are capable of further differentiation into cholangiocytes and hepatocytes.

The biliary progenitors described herein may be expanded from a population of immature liver cells. Immature liver cells may include neonatal and prenatal liver cells. The population of immature liver cells may comprise immature biliary cells.

Immature liver cells may be primary cells isolated from a sample of immature liver tissue. The sample of immature liver tissue may comprise or consist of immature biliary tissue, for example from the immature biliary epithelium. In some embodiments, a sample of immature liver tissue may be obtained from an individual of 2 years or less, 1 year or less 6 months or less or 1 month or less (e.g. a neonate (Tolosa et al (2014) Cell Transplant. 23 (10) 1229-1242)). For example, neonatal liver cells may be employed. In other embodiments, immature liver cells may be obtained from a sample of foetal liver tissue pre-birth, for example a sample of tissue having a gestational age of 5 weeks or more, 6 weeks or more, 8 weeks or more or 12 weeks or more, for example to 5 to 20 weeks, or 6 to 12 weeks. Suitable samples of foetal liver tissue may for example be obtained from patients following elective terminations. Immature liver cells may express hepatic markers, such as ALB, AFP and EpCAM. In other embodiments, a sample of immature liver tissue may be obtained from individual of more than 2 years old, for example an adult, who has undergone liver injury. Liver injury has been shown to induce ductal plate reactions in liver tissue that comprise immature liver cells.

Immature liver cells may be isolated from primary liver tissue derived from heathy individuals or from patients with known pathology to enable disease modelling. Biliary progenitors derived from an individual with a liver disorder, such as a biliary disorder, may be used to generate expanded populations of biliary progenitors which display a genotype or phenotype associated with the liver disorder.

The immature liver cells may be isolated from a sample of immature liver tissue using any convenient technique. For example, a sample of immature liver tissue may be dissociated into a single cell suspension by enzymatic treatment, for example with collagenase and hyaluronidase, and the suspension sorted for EpCAM positive cells using anti-EpCAM coated microbeads.

Immature liver cells for use as described herein may be mammalian cells, for example human, mouse or rat cells, preferably human.

Populations of biliary progenitors may be expanded from the immature liver cells, preferably immature biliary cells. Immature biliary cells may correspond to biliary cells in immature liver tissue, for example foetal, prenatal or neonatal biliary cells, or cells mature liver tissue following injury .

The biliary progenitors are expanded in a biliary progenitor expansion medium. This is a cell culture medium that supports the proliferation of biliary progenitors in the form of organoids (referred to herein as "foetal biliary cell organoids" or FBOs).

The biliary progenitor expansion medium is a nutrient medium which comprises epidermal growth factor (EGF), a TGFβ inhibitor, a non-canonical Wnt signalling potentiator, a canonical Wnt potentiator and a ROCK inhibitor.

Epidermal Growth Factor (EGF; NCBI GeneID: 1950, nucleic acid sequence NM_001178130.1 GI: 296011012; amino acid sequence NP_001171601.1 GI: 296011013) is a protein factor which stimulates cellular growth, proliferation and cellular differentiation by binding to an epidermal growth factor receptor (EGFR). EGF may be produced using routine recombinant techniques or obtained from commercial suppliers (e.g. R&D Systems, Minneapolis, MN; Stemgent Inc, USA). Suitable concentrations of EGF for expanding cholangiocyte organoids as described herein may be readily determined using standard techniques. For example, the expansion medium may comprise 2 to 500ng/ml EGF, preferably about 20ng/ml.

A TGFβ inhibitor is a compound that reduces, blocks or inhibits TGFβ signalling through the TGFβRI and TGFβRII receptors. Suitable TGFβ inhibitors include A83-01 3-(6-Methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide), D4476 (4-[4-(2,3-Dihydro-1,4-benzodioxin-6-yl)-5-(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide), GW788388 (4-[4-[3-(2-Pyridinyl)-1*H*-pyrazol-4-yl]-2-pyridinyl]-*N*-(tetrahydro-2*H*-pyran-4-yl)-benzamide), IN1130 (3-[[5-(6-Methyl-2-pyridinyl)-4-(6-quinoxalinyl)-1*H*-imidazol-2-yl]methyl]benzamide), LY364947 (4-[3-(2-Pyridinyl)-1*H*-pyrazol-4-yl]-quinoline), SB525334 (6-[2-(1,1-Dimethylethyl)-5-(6-methyl-2-pyridinyl)-1*H*-imidazol-4-yl]quinoxaline), SB431542 (4-(5-Benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1H-imidazol-2-yl)-benzamide hydrate; Sigma, Tocris Bioscience, Bristol UK), SB-505124 (2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride) and soluble protein factors, such as lefty (e.g. human lefty 2: NP_003231.2 GI:27436881), cerberus (e.g. human Cerberus 1: NP_005445.1 GI:4885135) and follistatin (e.g. human foistatin: NP_006341.1 GI:5453652). Suitable TGFβ inhibitors are available from commercial suppliers. In some embodiments, the TGFβ inhibitor may be A8301, for example at 10 to 1000μM, preferably about 50μM.

A non-canonical Wnt signalling potentiator is a compound that stimulates, promotes or increases the activity of the non-canonical Wnt signalling pathway. The non- canonical Wnt signalling pathway is a β-catenin-independent pathway involved in tissue polarity and morphogenetic processes in vertebrates (Komiya, Y. & Habas, R. *Organogenesis* 4, 68–75 (2008); Patel, V. *et al.. Hum. Mol. Genet.* 17, 1578–1590 (2008); Strazzabosco, M. & Somlo, S. *Gastroenterology* 140, (2011).) Components of the non- canonical Wnt signalling pathway include Wnt4, Wnt5a, Wnt11, LRP5/6, Dsh, Fz, Daam1, Rho, Rac, Prickle and Strabismus. Suitable methods for determining the activity of the non- canonical Wnt/PCP signalling pathway are well known in the art and include ATF-2-based reporter assays (Ohkawara et al (2011) Dev Dyn 240 (1) 188-194) and Rho-associated protein kinase (ROCK)-based assays. A non- canonical Wnt signalling potentiator may selectively potentiate non-canonical Wnt signalling or more preferably, may potentiate both the non-canonical Wnt signalling and the canonical Wnt signalling pathway (i.e. a Wnt signalling agonist).

Preferred non- canonical Wnt signalling potentiators include the Wnt signalling agonist R-spondin. R-spondin is a secreted activator protein with two cysteine-rich, furin-like domains and one thrombospondin type 1 domain that positively regulates Wnt signalling pathways. Preferably, R-spondin is human R-spondin. R-spondin may include RSPO1 (GeneID 284654 nucleic acid sequence reference NM_001038633.3, amino acid sequence reference NP_001033722.1), RSPO2 (GeneID 340419 nucleic acid sequence reference NM_001282863.1, amino acid sequence reference NP_001269792.1), RSPO3 (GeneID 84870, nucleic acid sequence reference NM_032784.4, amino acid sequence reference NP_116173.2) or RSPO4 (GeneID 343637, nucleic acid sequence reference NM_001029871.3, amino acid sequence reference NP_001025042.2).

R-spondin is readily available from commercial sources (e.g. R&D Systems, Minneapolis, MN). Suitable concentrations of R-spondin for expanding cholangiocytes as described herein may be readily determined using standard techniques. For example, the expansion medium may comprise 50ng/ml to 5µg/ml R-spondin, preferably about 500ng/ml.

A ROCK inhibitor is a compound that reduces, blocks or inhibits Rho kinase (ROCK) (Liao et al (2007) J. Cardiovasc Pharmacol. 50 (1) 17-24). Suitable ROCK inhibitors include fasudil, Y39983 (4-[(1*R*)-1-Aminoethyl]-*N*-1*H*-pyrrolo[2,3-*b*]pyridin-4-ylbenzamide dihydrochloride), azabenzimidazole-aminofurazans and Y-27632 (trans-4-[(1R)-1-Aminoethyl]-N-4-pyridinylcyclohexanecarboxamide) and are available from commercial suppliers. For example, the hepatoblast expansion medium may comprise 1 to 100µM Y-27632, preferably about 10 µM.

The biliary progenitor expansion medium may be devoid of growth factors other than the epidermal growth factor (EGF), TGFβ inhibitor, non-canonical Wnt signalling potentiator, and ROCK inhibitor. For example, the biliary progenitor expansion medium may consist of a basal medium supplemented with epidermal growth factor (EGF), TGFβ inhibitor, non-canonical Wnt signalling potentiator, and ROCK inhibitor.

Suitable biliary cell expansion media may include CO-M (Table 1).

Preferably, the immature liver cells are cultured in the hepatocyte expansion medium in three-dimensional culture in the methods described above. For three-dimensional culture, the expansion medium further comprises a scaffold matrix which supports the growth and proliferation of cells in 3-dimensions and allows the biliary cells to assemble into organoids. For example, the expansion medium may comprise or consist of the scaffold matrix and the nutrient medium.

Suitable scaffold matrices are well-known in the art and include hydrogels, such as collagen, collagen/laminin, compressed collagen (e.g. RAFT™, Lonza), alginate, agarose, complex protein hydrogels, such as Base Membrane Extracts, and synthetic polymer hydrogels (Gjorevski et al Nature (2016) 539 560-564), such as polyglycolic acid (PGA) hydrogels and crosslinked dextran and PVA hydrogels (e.g. Cellendes Gmbh, Reutlingen DE), inert matrices, such as porous polystyrene, and isolated natural ECM scaffolds (Engitix Ltd, London UK).

The scaffold matrix may be chemically defined, for example a collagen or densified collagen hydrogel, or non-chemically defined, for example a complex protein hydrogel. Preferably, the scaffold matrix in the expansion medium is a complex protein hydrogel. Suitable complex protein hydrogels may comprise extracellular matrix components, such as laminin, collagen IV, enactin and heparin sulphate proteoglycans. Complex protein hydrogels may also include hydrogels of extracellular matrix proteins from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. Suitable complex protein hydrogels are available from commercial sources and include Matrigel™ (Corning Life Sciences) or Cultrex™ BME 2 RGF (Amsbio™ Inc). For example, the expansion medium may comprise 66% Matrigel™.

The biliary progenitor expansion medium may comprise or consist of a scaffold matrix and a nutrient medium supplemented with epidermal growth factor (EGF), a TGFβ inhibitor, a non-canonical Wnt signalling potentiator, such as R-spondin, and a ROCK inhibitor.

A nutrient medium may comprise a basal medium. Suitable basal media include Iscove's Modified Dulbecco's Medium (IMDM), Ham's F12, Advanced Dulbecco's modified eagle medium (DMEM) or DMEM/F12 (Price et al Focus (2003), 25 3-6), Williams E (Williams, G.M. et al Exp. Cell Research, 89, 139-142 (1974)), and RPMI-1640 (Moore, G.E. and Woods L.K., (1976) Tissue Culture Association Manual. 3, 503-508.

In some embodiments, DMEM/F12 medium may be preferred.

The basal medium may be supplemented with a media supplement, such as B27 (ThermoFisher Scientific) and/or one or more additional components, for example L-glutamine or substitutes, such as L-alanyl-L-glutamine (e.g. Glutamax™), nicotinamide, N-acetylcysteine, buffers, such as HEPES, and antibiotics such as penicillin and streptomycin. For example, the basal medium may be supplemented with 2% (v/v) B27, 20mM nicotinamide, 2mM N=acetylcysteine, 1% L-alanyl-L-glutamine, 1% HEPES, 1% penicillin and 1% streptomycin.

The nutrient medium may be a chemically defined basal nutrient medium. A chemically defined medium is a nutritive solution for culturing cells which contains only specified components, preferably components of known chemical structure. A chemically defined medium is devoid of undefined components or constituents which include undefined components, such as feeder cells, stromal cells, serum, serum albumin and complex extracellular matrices, such as Matrigel™. A chemically defined medium may be humanised. A humanised chemically defined medium is devoid of components or supplements derived or isolated from non-human animals, such as Foetal Bovine Serum (FBS) and Bovine Serum Albumin (BSA), and mouse feeder cells. Conditioned medium includes undefined components from cultured cells and is not chemically defined.

Suitable chemically defined nutrient media are well-known in the art and include DMEM/F12 supplemented with B27, nicotinamide, N-acetylcysteine, L-alanyl-L-glutamine, HEPES, penicillin and streptomycin.

The biliary progenitors may form organoids in the expansion medium. The biliary progenitor organoids may be disrupted as required, such that the expanded population comprises individual cells.

The biliary progenitors may be cultured in the expansion medium for multiple passages. For example, the biliary progenitors may be cultured for 25 or more, 30 or more, 40 or more or 50 or more passages. A passage may take 7-14 days, preferably about 10 days.

The biliary progenitors may be passaged by digesting the scaffold matrix, harvesting organoids by centrifugation and disrupting the organoids into individual biliary cells. The biliary progenitors may be re-suspended and cultured as described above in the expansion medium where they reform into organoids.

Suitable techniques for cell culture are well-known in the art (see, for example, Basic Cell Culture Protocols, C. Helgason, Humana Press Inc. U.S. (15 Oct 2004) ISBN: 1588295451; Human Cell Culture Protocols (Methods in Molecular Medicine S.) Humana Press Inc., U.S. (9 Dec 2004) ISBN: 1588292223; Culture of Animal Cells: A Manual of Basic Technique, R. Freshney, John Wiley & Sons Inc (2 Aug 2005) ISBN: 0471453293, Ho WY et al J Immunol Methods. (2006) 310:40-52, Handbook of Stem Cells (ed. R. Lanza) ISBN: 0124366430) Basic Cell Culture Protocols' by J. Pollard and J. M. Walker (1997), 'Mammalian Cell Culture: Essential Techniques' by A. Doyle and J. B. Griffiths (1997), 'Human Embryonic Stem Cells' by A. Chiu and M. Rao (2003), Stem Cells: From Bench to Bedside' by A. Bongso (2005), Peterson & Loring (2012)Human Stem Cell Manual: A Laboratory Guide Academic Press and 'Human Embryonic Stem Cell Protocols' by K. Turksen (2006). Media and ingredients thereof may be obtained from commercial sources (e.g. Gibco, Roche, Sigma, Europa bioproducts, R&D Systems). Standard mammalian cell culture conditions may be employed for the above culture steps, for example 37°C, 21% Oxygen, 5% Carbon Dioxide. Media is preferably changed every two days and cells allowed to settle by gravity

The population of biliary progenitors may be expanded 10^{10} fold or more, 10^{20} fold or more, 10^{30} fold or more, 10^{40} fold or more or 10^{50} fold or more as organoids in the expansion medium as described herein.

The population of biliary progenitors proliferates in the expansion medium and assembles into organoids. Organoids are three-dimensional multicellular assemblies or cysts that comprise a layer of the biliary cells linked by tight junctions which surrounds an interior lumen and separates it from the external environment. The morphology and physical characteristics of biliary progenitor organoids may be determined by standard microscopic procedures.

Biliary cell may express one or more of epithelial cellular adhesion molecule (EpCAM), gamma-glutamyl transferase (GGT), cystic fibrosis transmembrane conductance regulator (CFTR), and cytokeratin-19 (KRT19).

The expression of cell markers may be determined by any suitable technique, including immunocytochemistry, immunofluorescence, RT-PCR, immunoblotting, fluorescence activated cell sorting (FACS), and enzymatic analysis.

The biliary progenitors in the expanded population may display long term stability. For example, the biliary progenitors may be maintained in culture for at least 6 months without DNA copy number or other genetic abnormalities and with stable, preferably homogeneous, expression of biliary markers, such as EpCAM,

GGT, CTFR and KRT19. The presence of genetic abnormalities may be determined for example by comparative genomic hybridisation (CGH).

The biliary progenitors in the expanded population may display high proliferative potential. For example, the biliary cells may display a doubling time of 2-4 days, for example about 3 days.

A population of biliary progenitors expanded as described herein may comprise cholangiocyte progenitors and cholangiocytes. Biliary progenitors may express one or more, preferably all of AFP, APOE, AMBP, SOX4, CD24, CLDN6, APOA2, DLK1, LCN15, KRT19, SOX9, alpha-1-antitrypsin (SERPINA1), APOB, and TTR. The markers IGF2, AFP and DLK1 are specific to biliary progenitors and may be useful in separating biliary progenitors from other cell types such as cholangiocytes. Other biliary progenitor markers may include LCN1, FGB, APOA1, CA4, VTN, DUSP5, LAPTM4B, AMBP, CLDN6, MDK, and BEX1.

In some embodiments, biliary progenitors expanded as described herein may be further isolated and/or purified, for example from other cell types, including more differentiated cells, such as cholangiocytes. This may be performed, for example, using standard cell sorting techniques, such as flow cytometry. For example, the presence of biliary progenitor markers may be used to isolate cholangiocyte progenitors.

The population of biliary progenitors, whether in the form of organoids or individual cells, may be free or substantially free from other cell types i.e. the population of biliary progenitors may be homogeneous or substantially homogeneous. For example, the population may contain, 80% or more, 90% or more, 95% or more, 98% or more or 99% or more biliary cells, following culture in the medium. Preferably, the population of biliary progenitors is sufficiently free of other cell types that no purification is required.

Biliary progenitors expanded as described herein, may be cultured or maintained using standard mammalian cell culture techniques or subjected to further manipulation or processing. In some embodiments, the cell populations produced as described herein may be stored, for example by lyophilisation and/or cryopreservation. The biliary progenitors may be stored as organoids, sub-organoid assemblies or individual cells. Suitable storage methods are well known in the art. For example, the biliary cells may be suspended in a cryopreservation medium (for example, Cellbanker™ (AMS Biotechnology Ltd, UK) and frozen, for example at -70°C or below.

In some embodiments, biliary progenitors expanded by the methods described above may be used directly, for example in regenerative medicine applications. In other embodiments, the biliary progenitors may be further differentiated *in vitro.*

The biliary progenitors may be differentiated into cholangiocytes by culturing in a cholangiocyte differentiation medium. Culturing in the cholangiocyte differentiation medium may increase the expression of cholangiocytic markers, such as KRT19, and reduce the expression of biliary progenitor markers. For example, cholangiocytes produced as described herein may express cholangiocytic markers and not biliary progenitor markers.

Cholangiocytes may express one or more, preferably all of KRT7, KRT19, KRT20, MUC5B, CTFR, CEACAM6, CYSTM1, ADIRF, MMP1, ADIRF, LYZ, GPX2, TESC, CTSE, S100A14, ANXA10, TFF1, TFF2, LGALS4, and AQP5. The markers KRT7, MMP1, CYSTM1 and REG4 are specific to cholangiocytes and may be useful in separating cholangiocytes from biliary progenitors. Other cholangiocyte markers may include TFF1, TFF2, TSPAN8, NEAT1, LGALS4, ANXA10, S100A14, CTSE, TESC, GPX2, CEACAM6, LYZ, ADIRF, and S100A6. The absence of biliary progenitor markers and/or the presence of cholangiocyte markers may be used to isolate cholangiocytes.

The biliary progenitors may be differentiated into hepatocytes by culturing in a hepatocyte differentiation medium. Culturing in the differentiation medium may increase the expression of hepatocytic markers, such as ASGR1, CYP3A4, albumin (ALB), alpha-1-antitrypsin (SERPINA1), APOH, APOM, APOC1, CYP1A1, RBP4, HP, AHSG and TTR, and reduce the expression of biliary progenitor markers. For example, hepatocytes produced as described herein may express hepatocytic markers and not biliary progenitor markers.

The absence of biliary progenitor markers and/or the presence of hepatocytic markers may be used to isolate hepatocytes.

The population of biliary progenitors produced as described herein, or cholangiocytes or hepatocytes differentiated therefrom, may be admixed with other reagents, such as buffers, carriers, diluents, preservatives, and/or pharmaceutically acceptable excipients. Suitable reagents are described in more detail below. A method described herein may comprise admixing the population with a therapeutically acceptable excipient to produce a therapeutic composition. The admixed hepatic cells may be in the form of organoids, sub-organoid assemblies or individual cells.

In some embodiments, the biliary progenitors produced as described herein or cholangiocytes or hepatocytes differentiated therefrom, may be useful in therapy. For therapeutic applications, the biliary progenitors, cholangiocytes or hepatocytes are preferably clinical grade cells. Populations of biliary progenitors, cholangiocytes or hepatocytes, for use in treatment are preferably produced from immature liver cells, such as foetal or neonatal liver cells as described herein using chemically defined media. The biliary progenitors, cholangiocytes or hepatocytes may be in the form of organoids, sub-organoid assemblies or individual cells, depending on the specific application.

The population of biliary progenitors, cholangiocytes or hepatocytes may be transplanted, infused or otherwise administered into the individual. Suitable techniques are well known in the art.

In some preferred embodiments, the biliary progenitors, cholangiocytes or hepatocytes, produced as described herein may be admixed with a biocompatible scaffold.

A biocompatible scaffold may be seeded with biliary progenitors, cholangiocytes or hepatocytes produced as described above. For example, individual biliary progenitors or sub-organoid assemblies of biliary progenitors may be injected on or into a scaffold or mixing into the scaffold during the manufacturing process. The scaffold containing the biliary progenitors may then be cultured in expansion medium, such that

the biliary progenitors populate the scaffold. The biliary progenitors may proliferate within the scaffold and assemble into organoids.

Suitable biocompatible scaffolds may include hydrogels, such as fibrin, chitosan, glycosaminoglycans, silk, fibrin, fibronectin, elastin, collagen, glycoproteins such as fibronectin, or polysaccharides such as chitin, or cellulose collagen, collagen/laminin, densified collagen, alginate, agarose, complex protein hydrogels, such as Base Membrane Extracts, bio-organic gels, and synthetic polymer hydrogels, such as polylactic acid (PLA) polyglycolic acid (PGA), polycapryolactone (PCL) hydrogels, crosslinked dextran and PVA hydrogels (e.g. Cellendes Gmbh, Reutlingen DE), inert matrices, such as porous polystyrene, polyester, soluble glass fibres porous polystyrene, and isolated natural ECM scaffolds, for example decellularized gall bladder and bile duct scaffolds (Engitix Ltd, London UK). The scaffold may be biodegradable.

The size or shape of the scaffold is dependent on the intended application. Suitable scaffold shapes may for example include patches, sheets and tubes, including straight and branched tubes, with diameters up to for example 10-12 mm.

Biliary progenitors cultured within a biocompatible scaffold organize into a functional hepatic tissue, for example functional hepatocytic or biliary tissue. For example, biliary progenitors, or cholangiocytes or hepatocytes produced therefrom cultured within a biocompatible scaffold may organize into a functional biliary epithelium that displays one or more properties of the biliary epithelium.

An aspect of the invention provides an isolated population of biliary progenitors, produced by a method described above or cholangiocytes or hepatocytes produced therefrom. The cells may be in the form of organoids, sub-organoid clusters or individual cells.

A population of cells generated as described herein may be substantially free from other cell types. For example, the population may contain 70% or more, 80% or more, 85% or more, 90% or more, or 95% or more cells, following culture in the expansion medium. The presence or proportion of biliary progenitors in the population may be determined through the expression of biliary progenitor markers as described above.

Preferably, the population of biliary progenitors, or cholangiocytes or hepatocytes produced therefrom is sufficiently free of other cell types that no purification is required. If required, the population of cells or organoids may be purified by any convenient technique, including FACS.

In some embodiments, the biliary progenitors, or cholangiocytes or hepatocytes produced therefrom, may be engineered to express a heterologous protein, for example a marker protein, such as GFP, or an enzyme and/or to reduce or prevent expression of one or more endogenous protein, for example proteins associated with immunogenicity, such as HLA antigens.

The population of biliary progenitors, or cholangiocytes or hepatocytes produced therefrom may be within a biocompatible scaffold. Another aspect of the invention provides a biocompatible scaffold comprising an isolated population of biliary progenitors, produced by a method described herein or cholangiocytes or hepatocytes produced therefrom. Suitable scaffolds are described above.

Another aspect of the invention provides a collection of isolated populations of biliary progenitors as described herein or cholangiocytes or hepatocytes produced therefrom, wherein each population in the collection comprise a different set of tissue markers. For example, each population in the collection may have a different antigenic profile or HLA type. This may be useful in matching a population in the collection to a recipient individual without generating a host immune response.

Aspects of the invention also extend to a pharmaceutical composition, medicament, drug or other composition comprising biliary progenitors produced as described herein or cholangiocytes or hepatocytes produced therefrom in solution or in a biocompatible scaffold, and a method of making a pharmaceutical composition comprising admixing such cells with a pharmaceutically acceptable excipient, vehicle, carrier or biodegradable scaffold, and optionally one or more other ingredients.

A pharmaceutical composition containing biliary progenitors produced as described herein or cholangiocytes or hepatocytes produced therefrom may comprise one or more additional components. Pharmaceutical compositions may comprise, in addition to the cells, a pharmaceutically acceptable excipient, carrier, buffer, preservative, stabiliser, anti-oxidant, or other material well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the activity of the cells. The precise nature of the carrier or other material will depend on the route of administration.

Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, tissue or cell culture media, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

The composition may be in the form of a parenterally acceptable aqueous solution, which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride, Ringer's Injection, or Lactated Ringer's Injection. A composition may be prepared using artificial cerebrospinal fluid.

Another aspect of the invention provides a method of treatment of a liver disease comprising administering a population of biliary progenitors as described herein or cholangiocytes or hepatocytes produced therefrom to an individual in need thereof.

Another aspect of the invention provides a population of biliary progenitors produced as described herein or cholangiocytes or hepatocytes produced therefrom for use in a method of treatment of a liver disease in an individual in need thereof comprising administering the population to the individual.

Another aspect of the invention provides the use of a population of biliary progenitors, produced as described herein or cholangiocytes or hepatocytes produced therefrom in the manufacture of a medicament for use in the treatment of a liver disease.

The biliary progenitors, or cholangiocytes or hepatocytes produced therefrom may be in the form of organoids, sub-organoid assemblies or clusters or individual cells.

A liver disease is a condition in which liver tissue in an individual is damaged, defective or otherwise dysfunctional, for example, disorders characterised by damage to or destruction of liver tissue, or aberrant liver tissue. Liver disease may include hepatitis (e.g. hepatitis A, B, C, D, E, G or K), cirrhosis, fibrosis, hepatocellular carcinoma, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis (NASH), drug induced liver injury (DILI), alcoholic liver disease, autoimmune liver disease or an inherited metabolic disorder such as Alpha 1 Antitrypsin deficiency, a Glycogen Storage Disease, for example Glycogen Storage Disease Type 1a, Familial Hypercholesterolemia, Hereditary Tyrosinaemia, Crigler Najjar syndrome, ornithtine transcarbamylase deficiency, or factor IX deficiency or other haemophilia, haemochromatosis, Wilson's disease, Dubin-Johnson syndrome, familial amyloidosis, and Refsum's disease.

The liver disease may be a biliary disorder. A biliary disorder is a condition in which the biliary tissue in an individual is damaged, defective or otherwise dysfunctional, for example, disorders characterised by damage to or destruction of bile ducts, aberrant bile ducts or the absence of bile ducts. Biliary disorders may include biliary tissue injury, ischaemic strictures, traumatic bile duct injury and cholangiopathies, for example inherited, developmental, autoimmune and environment-induced cholangiopathies, such as Cystic Fibrosis associated cholangiopathy, drug induced cholangiopathy, Alagille Syndrome, polycystic liver disease, primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), AIDS associated cholangiopathy, disappearing bile duct syndrome, biliary cancer, ductopenias such as adult idiopathic ductopenia, post-operative biliary complications, and biliary atresia.

In some embodiments, a population of biliary progenitors, or cholangiocytes or hepatocytes produced therefrom may be administered to the individual in solution. The administration of a population of cells, in solution may be useful for example in the treatment of liver diseases, such as ductopenias, including ischaemic ductopenia, congenital ductopenia, such as alagille syndrome, metabolic ductopenia, complex diseases, such as intrahepatic PSC and PBC, drug induced ductopenia, vanishing bile duct syndrome and conditions affecting the intrahepatic biliary tree, as well as acute liver injury and chronic liver disease as described above.

In other embodiments, a population of biliary progenitors, or cholangiocytes or hepatocytes produced therefrom may be administered to the individual within a biocompatible scaffold. For example, a scaffold populated with cells may be administered to the individual. For example, the administration of a population of bile progenitors in a scaffold may be useful for example in the treatment of biliary atresia, biliary strictures, traumatic or iatrogenic biliary injury and conditions affecting the extrahepatic biliary tree

Biliary progenitors, or cholangiocytes or hepatocytes produced therefrom, in solution or in scaffolds may be implanted into a patient by any technique known in the art (e.g. Lindvall, O. (1998) Mov. Disord. *13*, Suppl. 1:83-7; Freed, C.R., et al., (1997) Cell Transplant, *6*, 201-202; Kordower, et al., (1995) New England Journal of Medicine, *332*, 1118-1124; Freed, C.R.,(1992) New England Journal of Medicine, 327, 1549-1555, Le Blanc et al, Lancet 2004 May 1;363(9419):1439-41). In particular, cell suspensions may be injected or infused into the bile duct, gallbladder, portal vein, liver parenchyma, peritoneal cavity or spleen of a patient.

A hepatic cell suspension may be administered intravenously, intraperitoneally or via an endoscopic retrograde cholangio-pancreatography (ERCP) or percutaneous cholangiography (PTC). A scaffold populated with hepatic cells may be administered to the individual by surgical implantation.

Administration of a composition in accordance with the present invention is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors.

A composition comprising biliary progenitors produced as described herein or cholangiocytes or hepatocytes produced therefrom may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Populations of isolated biliary progenitors, produced as described herein or cholangiocytes or hepatocytes produced therefrom, may be useful in modelling the interaction of test compounds with the cells, for example in toxicity screening, modelling biliary disorders or screening for compounds with potential therapeutic effects.

Another aspect of the invention provides the use of a population of biliary progenitors, produced as described herein or cholangiocytes or hepatocytes produced therefrom for disease modelling and study of pathogenesis of liver diseases, including biliary disorders.

Cells for use in modelling and screening may be in the form of organoids (biliary progenitor organoids), sub-organoid clusters or individual cells (biliary progenitors) produced, for example by disruption of organoids.

A method of screening a compound may comprise;

contacting a population of biliary progenitors, produced as described herein or cholangiocytes or hepatocytes produced therefrom with a test compound, and;

determining the effect of the test compound on said cells and/or the effect of said cells on the test compound.

The proliferation, growth, apoptosis or viability of the biliary progenitors, cholangiocytes or hepatocytes , protein production, metabolic activity of key enzymes, expression of stress response genes, or the ability of the hepatoblasts to perform one or more cell or organoid functions may be determined in the presence relative to the absence of the test compound.

A decrease in proliferation, growth, viability or ability to perform one or more cell or organoid functions is indicative that the compound has a toxic effect and an increase in growth, viability or ability to perform one or more cell or organoid functions is indicative that the compound has an ameliorative effect on the biliary progenitors, cholangiocytes or hepatocytes.

Gene expression may be determined in the presence relative to the absence of the test compound. For example, the expression of one or more biliary marker genes may be determined, for example one or more marker genes listed above. Combined decrease in expression is indicative that the compound has a toxic effect or can modify the functional state of the hepatic cells. Gene expression may be determined at the nucleic acid level, for example by RT-PCR, or at the protein level, for example, by immunological techniques, such as ELISA, or by activity assays. Cytochrome p450 assays, for example, luminescent, fluorescent or chromogenic assays are well known in the art and available from commercial suppliers.

The metabolism, degradation, or breakdown of the test compound by the biliary progenitors, hepatocytes, or cholangiocytes, may be determined. In some embodiments, changes in the amount or concentration of test compound and/or a metabolite of said test compound may be determined or measured over time, either continuously or at one or more time points. For example, decreases in the amount or concentration of test compound and/or increases in the amount or concentration of a metabolite of said test compound may be determined or measured. In some embodiments, the rate of change in the amount or concentration of test compound and/or metabolite may be determined. Suitable techniques for measuring the amount of test compound or metabolite include mass spectrometry.

This may be useful in determining the *in vivo* half-life, toxicity, efficacy or other in vivo properties of the test compound.

38. Other aspects of the invention relate to kits and their use for production of expanded populations as described herein.

A kit for the production of an expanded population of biliary progenitors may comprise a biliary progenitor expansion medium comprising epidermal growth factor (EGF), TGFβ inhibitor, a non-canonical Wnt signalling potentiator, and a ROCK inhibitor.

Suitable biliary cell expansion media are described in more detail above.

A kit may further comprise a scaffold matrix, such as Matrigel™. The scaffold matrix may be provided as part of the expansion medium or may be provided separately.

The expansion medium may be formulated in deionized, distilled water. The expansion medium will typically be sterilized prior to use to prevent contamination, *e.g.* by ultraviolet light, heating, irradiation or filtration. The one or more media may be frozen (*e.g.* at -20°C or -80°C) for storage or transport. The one or more media may contain one or more antibiotics to prevent contamination.

The kit may further comprise a dissociation buffer to dissociate immature liver cells from sample tissue. Suitable buffers include Hanks Buffered Saline Solution (HBSS) supplemented with Liberase DH (Roche Applied Science) and Hyaluronidase (Sigma-Aldrich).

The kit may further comprise cryopreservation solution. Suitable cryopreservation media are described above.

The one or more media may be a 1x formulation or a more concentrated formulation, *e.g.* a 2x to 250x concentrated medium formulation. In a 1x formulation each ingredient in the medium is at the concentration intended for cell culture, for example a concentration set out above. In a concentrated formulation one or more of the ingredients is present at a higher concentration than intended for cell culture. Concentrated culture media are well known in the art. Culture media can be concentrated using known methods *e.g.* salt precipitation or selective filtration. A concentrated medium may be diluted for use with water (preferably deionized and distilled) or any appropriate solution, *e.g.* an aqueous saline solution, an aqueous buffer or a culture medium.

The one or more media in the kit may be contained in hermetically-sealed vessels. 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.

Other aspects of the invention provide the use of a biliary progenitor expansion medium for the production of an expanded population of biliary progenitors.

Other aspects and embodiments of the invention provide the aspects and embodiments described above with the term "comprising" replaced by the term "consisting of" and the aspects and embodiments described above with the term "comprising" replaced by the term "consisting essentially of".

It is to be understood that the application discloses all combinations of any of the above aspects and embodiments described above with each other, unless the context demands otherwise. Similarly, the application discloses all combinations of the preferred and/or optional features either singly or together with any of the other aspects, unless the context demands otherwise.

Modifications of the above embodiments, further embodiments and modifications thereof will be apparent to the skilled person on reading this disclosure, and as such, these are within the scope of the present invention.

All documents and sequence database entries mentioned in this specification are incorporated herein by reference in their entirety for all purposes.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Experimental

Materials and Methods

Processing of human foetal liver tissue

Primary human foetal tissue was obtained from patients undergoing elective terminations (REC-96/085), at gestational ages reported in table 1. The liver was dissected from the abdominal cavity and placed into a solution containing Hanks Buffered Saline Solution (HBSS) supplemented with 1.07 Wünsch units/ml

Liberase DH (Roche Applied Science) and 70U/ml Hyaluronidase (Sigma-Aldrich), and placed on a microplate shaker at 37°C, 750rpm, for 15 minutes. The sample was subsequently washed three times in HBSS using centrifugation at 400g for five minutes each time. The single cell suspension could then be sorted for EPCAM/CD326 positive cells using CD326 microbeads (Miltenyi), according to the manufacturer's guidelines.

Establishment of cholangiocyte organoids

The single cell suspension was resuspended in cholangiocyte organoid media (CO-M) for FBO. To the resuspended cells was added an equal volume of Growth Factor Reduced Phenol Free Matrigel (Corning), and the mixture pipetted into 48 well plates (20uL per well). The plates were placed at 37°C for fifteen minutes to allow the mixture to set, and subsequently 200uL of fresh HO-media applied to each well.

Establishment of ductal organoids using "Liver stem cell organoid" and "extrahepatic cholangioctye organoid" conditions

Both protocols were applied to dissociated foetal liver tissue as per author's guidelines (Huch *et al.*, 2015; Sampaziotis *et al.*, 2017).

Maintenance of cell lines

The respective culture medium was changed every 48-72 hours, and organoids mechanically passaged every 7-10 days. Organoids were passaged by scraping the gel away from the plate, pipetting the resulting solution into 1.5ml tubes, and pipetting the solution up and down to break individual organoids into pieces. If a precise cell number was required, the organoids can alternatively be processed to a single cell solution as described below, and then re-plated at the required dilution in the 55% Matrigel-medium solution.

Dissociation of FBO

Organoids established in culture were dissociated to single cell suspensions for splitting/ single cell sequencing/ cell counting by first removing the media from each well and replacing with Cell Recovery Solution (Corning). The organoids in the Matrigel could then be scraped off the plate and placed on ice for 30 minutes to remove the Matrigel. The organoids should then be washed with PBS, and placed in TrypLE (ThermoFisher Scientific) for 15 mins at 37°C. Cells should finally be washed three times in PBS or basal media.

Imaging of organoids

The medium was removed and replaced with 4% Paraformaldehyde solution for 20 minutes at room temperature to fix the organoids in situ, followed by three washes in PBS (each for five minutes). Fixed organoids could then be stored in PBS for later Immunohistochemistry. Immunohistochemistry was performed by permeabilizing and blocking with 0.3% Triton X-100 (Sigma-Aldrich), and 10% donkey serum (Biorad) for three hours at room temperature, and then incubated overnight at 4°C, with the primary antibody in 1% donkey serum. Samples were then washed with three one-hour PBS washes, followed by incubation with the secondary antibody for one hour at room temperature. The samples were finally washed three times in PBS, with 1:10000 dilution Hoechst 33258 (ThermoFisher Scientific) added to the first wash. Imaging was performed

Culture of primary hepatocytes

Primary hepatocytes were purchased from Biopredic International (Rennes, France). Hepatocytes were isolated from human liver resects from three donors (two male and one female), and met the manufacturer's quality control requirements in regards to viability, confluency and functionality, which was assessed by Phase I and II dependent enzymatic activity. Cells were purchased as monolayer cultures and maintained in William's E (Gibco) supplemented with 1% Glutamine (Gibco), 1% Penicillin-streptomycin (Gibco), 700nM Insulin (Sigma) and 50μM Hydrocortisone (Sigma) for no longer than 48h upon receipt.

qPCR analysis

RNA was extracted from cells using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instruction. cDNA was synthesised from RNA using SuperScript II (Invitrogen) according to the manufacturer's instructions. qPCR was performed using SensiMix SYBR low-ROX (Bioline) and 150 nM forward and reverse primers (Sigma-Aldrich; primers listed in supplementary materials). All samples were run in technical duplicates and gene expression calculated relative to housekeeping gene Ribosomal Protein Lateral Stalk Subunit P0 (RPLP-0).

Single Cell RNA-sequencing

Primary Foetal Liver or organoids were dissociated to a single cell solution by the respective methods described above. The single cell solution from each was then processed using the Chromium single cell controller and reagents according to the manufacturer's guidelines (10X genomics).

Sequencing was performed on the HiSeq4000 sequencing system, and the subsequent raw data was aligned and used to generate matrices using the Cell Ranger platform (version 2.1.0). Basic analysis of the data was performed in R using the Seurat, ggplot2, and Monocle packages.

Assessment of genomic stability

Genomic stability of the organoids was assessed using the CytoScan 750k array, performed by the Medical Genetics Service at Cambridge University Hospitals.

Determination of cell line gender

Cell line gender was assessed by assessing RNA expression of the SRY gene (male), or Xist gene (female) based upon qPCR and/or single cell RNA sequencing analysis

Flow cytometry

Organoids were dissociated to single cell solution as described above. The single cells were then fixed and permeabilised in FixPerm (ThermoFisher Scientific) for 20 minutes, then washed three times in PBS before resuspension in PermWash (ThermoFisher Scientific). Blocking was performed using 10% donkey serum (Biorad) in PBS for 30 minutes. Primary antibodies were diluted 1:100 in 1% donkey serum in PBS and incubated with the cell suspension at room temperature for 1 hour. After three washes in PBS, cells were incubated with secondary antibodies diluted 1:1000 in 1% donkey serum in PBS. Data were collected using Cyan ADP flow cytometer, and analysed using FlowJo X.

Assessment of cell proliferation rates

Organoids were extracted and dissociated to single cells as described above. The cells were then counted using the countess II automated cell counter (Bio-Rad), and the final number adjusted according to the number of wells used and the volume of resuspension.

Cytochrome P-450 assays

P450 Glo (Promega) assays were used according to manufacturer's guidelines. Luciferin IPA (CYP3A4 specific), and Luciferin PFBE (CYP3A7 & CYP3A5 > CYP3A4), were added to media and incubated at 37°C for four hours, before using detection assay as per the manufacturer's instruction. Luminescence RLU was then corrected for cell number.

Serum protein analysis

Albumin, alpha-fetoprotein, apolipoprotein-B, and alpha-1-antitrypsin were detected in media by enzyme linked immunosorbent assay (performed by core biomedical assay laboratory, Cambridge University Hospitals). Concentrations were normalised to cell number.

Bodipy assay

BODIPY 493/503 (ThermoFisher Scientific) was diluted 1:1000 in the organoid culture media, and applied to organoids for 30 minutes. After this time the organoids were washed with fresh media, and imaged in situ using a fluorescent microscope.

Freezing/ thawing of HO/FBO

Organoids were extracted from Matrigel using cell recovery solution as described above. The organoids were then washed in PBS and resuspended in Cell Culture Freezing Medium (ThermoFisher Scientific), placed in cryogenic vials (ThermoFisher Scientific), and cooled to -80oC using a "Mr. Frosty" Freezing Container (ThermoFisher Scientific). Organoids could then be kept long term in liquid nitrogen. To thaw, cryovials were kept at 4oC until the freezing medium had melted. Organoids were then washed three times in PBS to remove any residual freezing medium, and then replated as above.

Statistical analyses

Unless otherwise stated, statistical analyses of direct comparisons of two groups were performed using t-test with Welch correction. Those comparisons labelled * represent $P<0.05$, ** $p<0.01$, *** $P<0.001$, and **** $p<0.0001$.

Results

Adult liver organoid culture conditions allow the derivation of biliary organoids from foetal hepatic tissue.

Organoids have been previously derived from the adult liver using two approaches. The first method consists of deriving adult stem cells from the intrahepatic biliary epithelium using WNT, Noggin and Rock-inhibitor for the first three days of in vitro culture (Huch *et al.*, 2015). The resulting liver stem cell organoids (LSCO) mainly express biliary markers while displaying a limited capacity to differentiate into hepatocytes (Huch *et al.*, 2015). The second method derived similar cells from the extra-hepatic biliary epithelium (Sampaziotis *et al.*, 2017). These Extra-hepatic Cholangiocytes Organoids or ECOs closely resemble cholangiocytes and

can't differentiate into any other hepatic lineage. Based on these reports, we decided to assess whether these protocols could be applied to foetal tissues and give rise to hepatoblasts. For that, human foetal livers were carefully dissected from 5-10 post conceptional weeks (PCW) embryos and then dissociated into single cells which were then grown in 3D conditions as described by the respective protocols(Huch *et al.*, 2015; Sampaziotis *et al.*, 2017). After 5 days of culture, we observed the appearance of spheres of cells resembling adult biliary organoids with no specific morphological features (Fig 1B). Of note, these biliary organoids could be grown over prolonged period of time (>20 passages) while maintaining their characteristic morphology. In parallel, we also developed alternative culture conditions using a new basic medium supplemented with R-Spondin, EGF, A83-01, and Y-27632. These conditions were subsequently used to generate fetal biliary organoids (FBO) that could be directly compared to hepatoblast organoids (see below), avoiding interference with divergent conditions which could affect experimental outcomes. As expected, the fetal biliary organoids (FBO) express key biliary markers at both bulk and single cell RNA expression level; including Epithelial cellular adhesion molecule (EPCAM), gamma-glutamyl transferase (GGT), cystic fibrosis transmembrane conductance regulator (CFTR), and cytokeratin-19 (KRT19) (Fig 1D-H). To further confirm their identity, we performed single cell RNA-Sequencing (scRNA-seq) analysis on 2 FBO cell lines using 10X technology (Fig 1E-G). Gene expression analyses confirm that FBOs are made of cells expressing biliary markers such as KRT19, but also low levels of hepatoblast genes such as AFP (Fig. 1D). Interestingly, tSNE analyses revealed that a least one FBO cell line contains two distinct populations of cells expressing KRT19. Indeed, one population appears to be positive for traditional cholangiocytes markers such as KRT19 and SOX9, but also hepatoblast markers such as alpha-1-antitrypsin (SERPINA1), AFP and TTR (although not albumin). The other group co-express high levels of KRT19 with more mature markers such as KRT7, CFTR, and CFTR. Thus, FBO organoids seem to contain a combination of cholangiocyte progenitors (CoP) and more differentiated cholangiocytes (Co). Heat map visualisation of genes differentially expressed between CoP and Co identify additional markers specific for the two populations (CoP: APOA2, APOE, DLK1 and LCN15; Co: KRT20, CEACAM6, CYSTM1, and ADIRF). Interestingly, a number of CoP specific genes are expressed at the protein level in the liver and intrahepatic bile duct while most genes marking Co were expressed in biliary cells. Further, GO term analyses show that genes differentially expressed are involved in processes related to. Visualization of gene expression with cells ordered in pseudotime revealed temporal sequence of gene expression events between CoP and Co, and many cells at intermediate stages (Fig 6F). Interestingly, genes known to be expressed in hepatoblast (AFP, APOB, APOE) disappear during the progression from CoP to Co, whilst genes known to mark functional activity of Co (CFTR, AQP5) increase simultaneously. Thus, pseudotime inference suggests that KRT7/CFTR cholangiocytes could indeed differentiate from AFP/CK19 CoP. Considered together, these results demonstrate that culture conditions originally developed to grow hepatic adult cells allow the isolation of cholangiocytes progenitors which have the capacity to further differentiate into cholangiocytes. Interestingly, such biliary progenitors have not been described previously and suggest the existence of a previously unknown intermediate state of differentiation between hepatoblasts and cholangiocytes.

Single cell analyses confirm the presence of hepatoblasts in the early human foetal liver

We defined the gene expression profile of hepatic cells in vivo using scRNA-seq. For that, we isolated 2 foetal livers at 6 pcw, performed single cell dissociation, and analysed the transcriptome of 1406 cells using 10X technology. tSNE analysis demonstrated independent clustering of non-parenchymal cell types including Kupffer Cells (CD68, FCGR3A, CD33, ITGAM, SPI1) Stellate Cells (COL1A1, ACTA2, VIM, NES),

hematopoietic stem/progenitor cells (PTPRC, SPN, MYB, KLF1, TFRC), lymphocytes (LSP1, CD52, CD37, CD48) and megakaryocytes (NRGN, PPBP, ITGA2B, GP1BA, GP6), as well as a homogeneous population of hepatoblasts (ALB, AFP, KRT8, KRT18, EPCAM, SERPINA1, TTR) (Figure 3B).

Interestingly, these analyses could not identify biliary cells suggesting that these cells could be extremely rare at this stage of development and that large scale scRNA-seq analyses will be necessary to establish a complete atlas of the human foetal liver. Nonetheless, our data already provide interesting preliminary information into the landscape of the liver at this stage and demonstrate the early colonization of the liver by a diversity of cell types including immune cells such as tissue resident macrophages and dendritic cells. Our data also confirm the production of MK cells by HSC in the liver and reinforce previous reports. Finally, the presence of stellate cells is also intriguing and suggest that these cells could colonise the liver earlier than initial thought and thus could have a function in early organogenesis. The population of hepatoblast cells showed great similarity between the biological replicates and showed a homogeneity in their gene expression signature, demonstrating the global ability of the liver parenchyma at this stage to expand the hepatoblast population. Importantly, this population of hepatoblasts did express a spectrum of known hepatic markers including albumin, alpha-fetoprotein, TTR and SERPINA1 and also new genes which were not identified previously such as RBP4, AHSG, APOH, APOM or FABP1. Interestingly, a majority of these genes are expressed at the protein level in adult hepatocytes based on the protein atlas with the exception of SPINK1 and FGB (Table S1). Based on this information, hepatoblast could be defined by the combined expression of ALB, SPINK1, AFP, SERPINA1, CYP3A7. Taken together, these data show that the foetal human liver contains a diversity of cell types including a homogeneous population of hepatoblast.

Hepatoblast organoids form a homogeneous population that closely resembles the in vivo counterpart

We compared HO hepatoblast cells to PFL hepatoblast cells and the FBO. The Co and CoP populations maintain their distinct clustering, to each other and to the hepatoblasts, with the most differentially expressed genes confirming the different identities of the hepatoblasts (SERPINA1, APOB, ALB, ASGR1, TF), CoP (SOX4, CD24, CLDN6) and Co (KRT7, KRT20, MUC5B). Thus, HO and CoP clearly represent different types of progenitors despite sharing the expression of key genes such as AFP. Further detailed tSNE analyses refines these observations by showing that HO and primary hepatoblast can be distributed in separate clusters. Nonetheless, heat map visualisation reveals that HO and primary hepatoblasts broadly express the same markers but at different levels (fig 4). Principal component analysis (PCA) demonstrated that HO and PH are highly similar, expressing similar levels of functional genes such as transferrin (TF), fatty acid binding protein (FABP1), and albumin (ALB). The genes driving differences between the two populations included higher expression of genes from the Methallothionein sub-family of genes (MT1H, MT1A, MT1G, MT1E, MT1F, MT2A, MT1X) and also higher expression of some markers of immaturity such as AFP, whilst the HO had higher levels of expression of functional enzymes such as CYP1A1 and CYP2E1, and metabolic proteins such as fatty acid synthase (FASN) and fatty acid desaturase (FADS1). GO term analyses show that these genes mainly correspond to hepatic function.

The above experiments demonstrate the derivation of the first immature biliary organoid model. These models offer an exciting opportunity to study human foetal liver development in vitro long term and in three dimensions, allowing assessment of the behaviour and characteristics of a previously poorly understood cell types and a previously undiscovered intermediate population, that have been difficult to investigate until now.

The genetic and transcriptomic stability along with the high proliferative capacity of the organoids also allows for large scale expansion of a single biological sample, thus enabling in depth analysis of the system and allowing complex research questions to be investigated in the same system over long periods. Similarly, the homogeneity and high potential for cell expansion enables the potential of generating large scale biobanks of tissue that can be selectively utilised.

The ability to generate cells that could be expanded and transplanted into tissue matched recipients could represent an exciting opportunity to manage patients requiring liver transplant. The efficiency of organoid generation alongside a ready supply of tissue offers the potential of rapidly generating a large-scale biobank of hepatoblast organoids that could cover the majority of variations in tissue markers such as HLA. The ability of the organoids to produce serum albumin and differentiate to daughter cell types present an exciting prospect of developing a future bridge therapy for end stage biliary disease, restoring some level of hepatic function through tissue matched donor organoids.

Overall, this new organoid system represents an exciting system to model development, cell fate decisions, and opportunities for regenerative medicine.

Reagent	HO-M	CO-M	Stock concentration	Final concentration
Basal medium:				
DMEM/F12	500ml	500ml		
Glutamax	5ml	5ml	100%	1% (by vol)
HEPES	5ml	5ml	100%	1% (by vol)
Penicillin/streptomycin	5ml	5ml	100%	1% (by vol)
B27	10ml	10ml	100%	2% (by vol)
Nicotinamide	10ml	10ml	1M	20mM
N-acetylcysteine	2ml	2ml	0.5M	2mM
Complete medium:				
Basal medium	19ml	19ml		
DMEM	-	25ml		
Wnt3a	25ml	-	100%	50% (by vol.)
R-spondin	5ml	5ml	100%	10% (by vol.)
EGF	25uL	25ul	100ug/ml	50ng/ml
A8301	50uL	50uL	50mM	50uM
Y27632	50ul	50ul	10mM	10uM

Table 1

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Claims

1. A method for producing an expanded population of biliary progenitors *in vitro* comprising:

(i) providing a population of primary immature liver cells and;

(ii) culturing the population in a biliary progenitor expansion medium comprising epidermal growth factor (EGF), a TGFβ inhibitor, a non-canonical Wnt signalling potentiator, and a ROCK inhibitor, to produce an expanded population of biliary progenitors.

2 .A method according to claim 1 wherein the population of primary immature liver cells comprises biliary cells.

3. A method according to any one of the preceding claims wherein the expanded population forms organoids in the expansion medium.

4. A method according to any one of the preceding claims wherein primary immature liver cells are foetal or neonatal liver cells.

5. A method according to any one of claims 1 to 3 wherein primary immature liver cells are isolated from mature liver tissue following injury.

6. A method according to any one of the preceding claims wherein immature liver cells are human liver cells.

7. A method according to any one of the preceding claims wherein the biliary progenitor expansion medium is a chemically defined medium consisting of a basal medium, epidermal growth factor (EGF), a TGFβ inhibitor, a non-canonical Wnt signalling potentiator, and a ROCK inhibitor.

8. A method according to any one of the preceding claims wherein the TGFβ inhibitor is A8301.

9. A method according to any one of the preceding claims wherein the non-canonical Wnt signalling potentiator is R-spondin.

10. A method according to according to any one of the preceding claims wherein the ROCK inhibitor is Y-27632.

11. A method according to any one of the preceding claims wherein the canonical Wnt potentiator is Wnt3a.

12. A method according to any one of the preceding claims comprising differentiating the biliary progenitors into cholangiocytes.

13, A method according to any one of claims 1 to 11 comprising differentiating the biliary progenitors into hepatocytes.

14. An isolated population of biliary progenitors, hepatocytes or cholangiocytes produced by a method according to any one of claims 1 to 13.

15 An isolated population according to claim 14 wherein the cells are biliary progenitors and said biliary progenitors express the markers LCN1, FGB, TTR, APOA1, CA4, VTN, DUSP5, LAPTM4B, AMBP, CLDN6, MDK, APOE, BEX1, IGF2, AFP and DLK1

16. A biocompatible scaffold comprising an isolated population according to claim 14 or 15.

17. A method of treating a liver disease comprising administering an isolated population according to claim 14 or 15 or a scaffold according to claim 16 to an individual in need thereof.

18. An isolated population according to claim 14 or 15 or a scaffold according to claim 16 for use in a method of treating a liver disease in an individual in need thereof.

19. A method of screening comprising;

contacting an isolated population according to claim 14 or 15 or a scaffold according to claim 16 with a test compound, and;

determining the effect of the test compound on the population or scaffold and/or the effect of the population or scaffold on the test compound.

20. A method according to claim 19 wherein the population is contacted with the test compound is in the form of organoids.

21. A kit for the *in vitro* expansion of biliary progenitors comprising a biliary progenitor expansion medium that comprises epidermal growth factor (EGF), a TGFβ inhibitor, non-canonical Wnt signalling potentiator, and a ROCK inhibitor.

22. Use of a biliary progenitor expansion medium to expand biliary progenitors in vitro, wherein the biliary progenitor expansion medium comprises epidermal growth factor (EGF), a TGFβ inhibitor, non-canonical Wnt signalling potentiator, and a ROCK inhibitor.

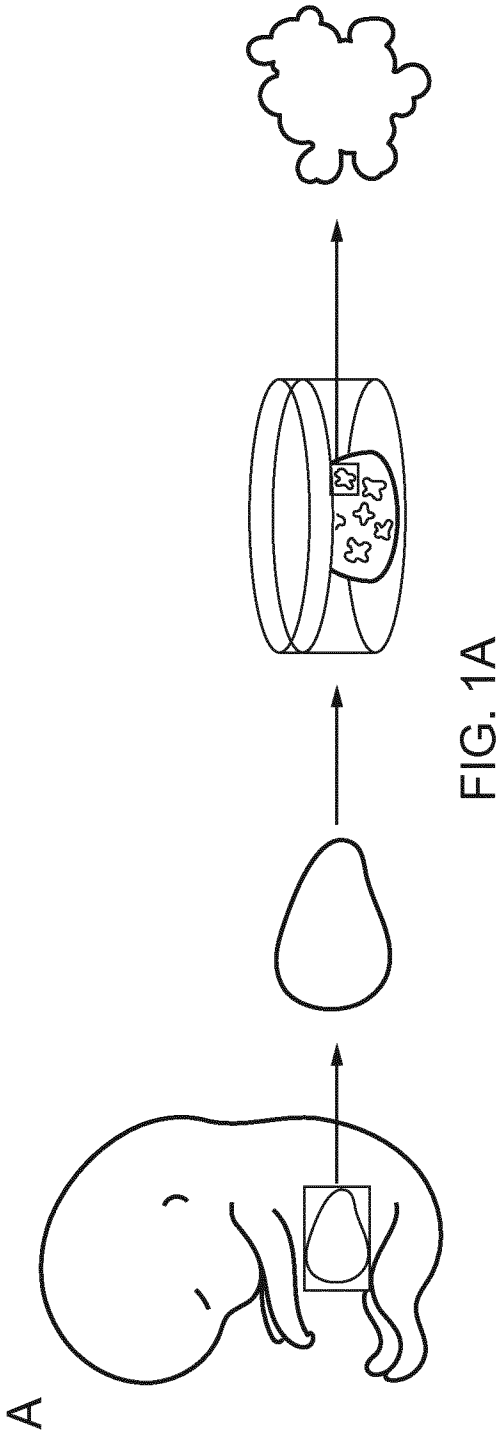

FIG. 1A

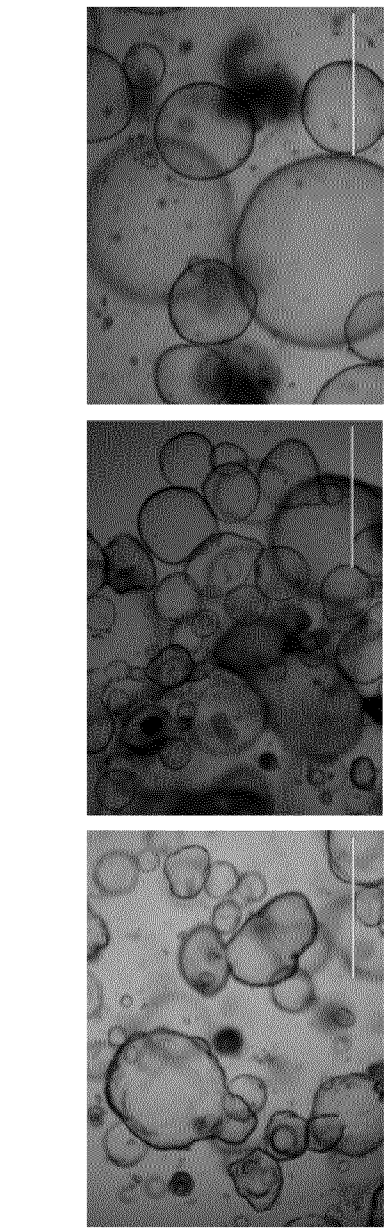

FIG. 1B

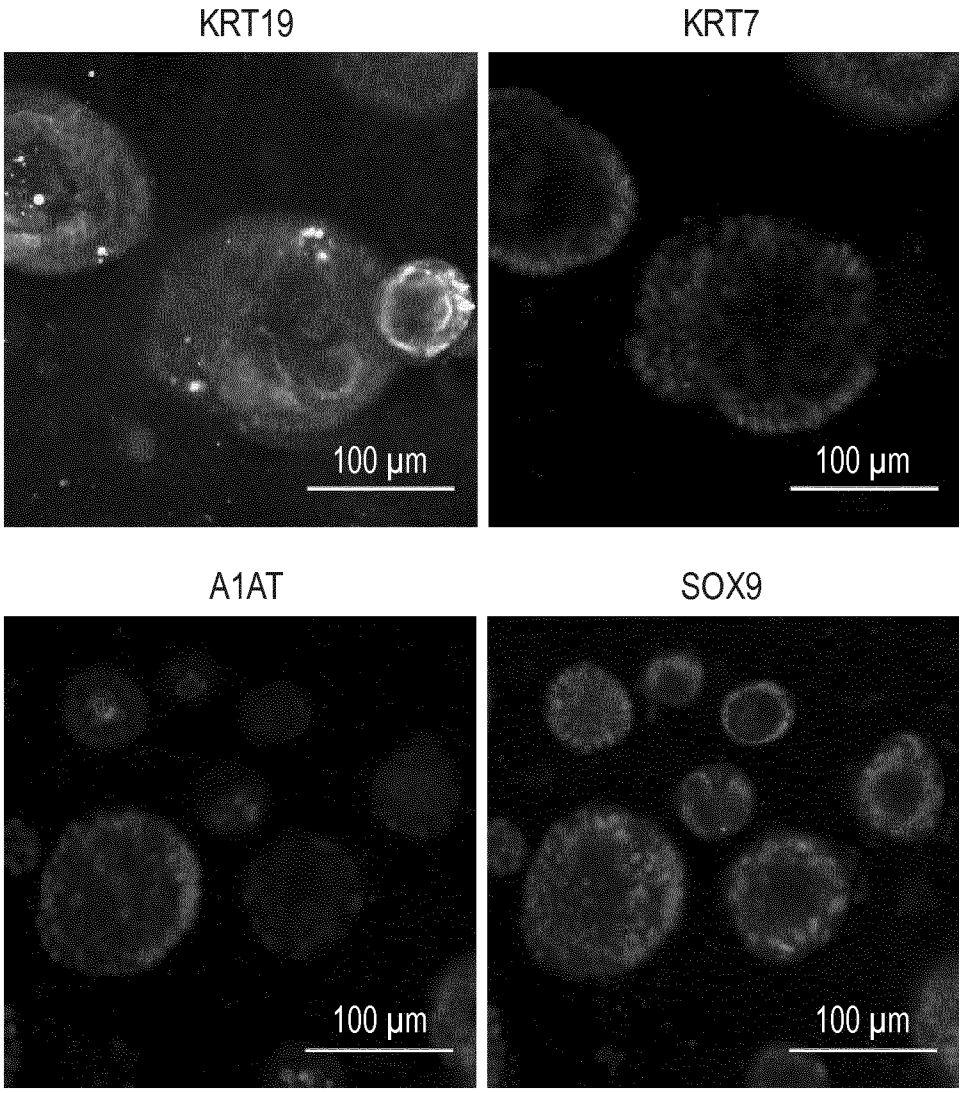

FIG. 1C

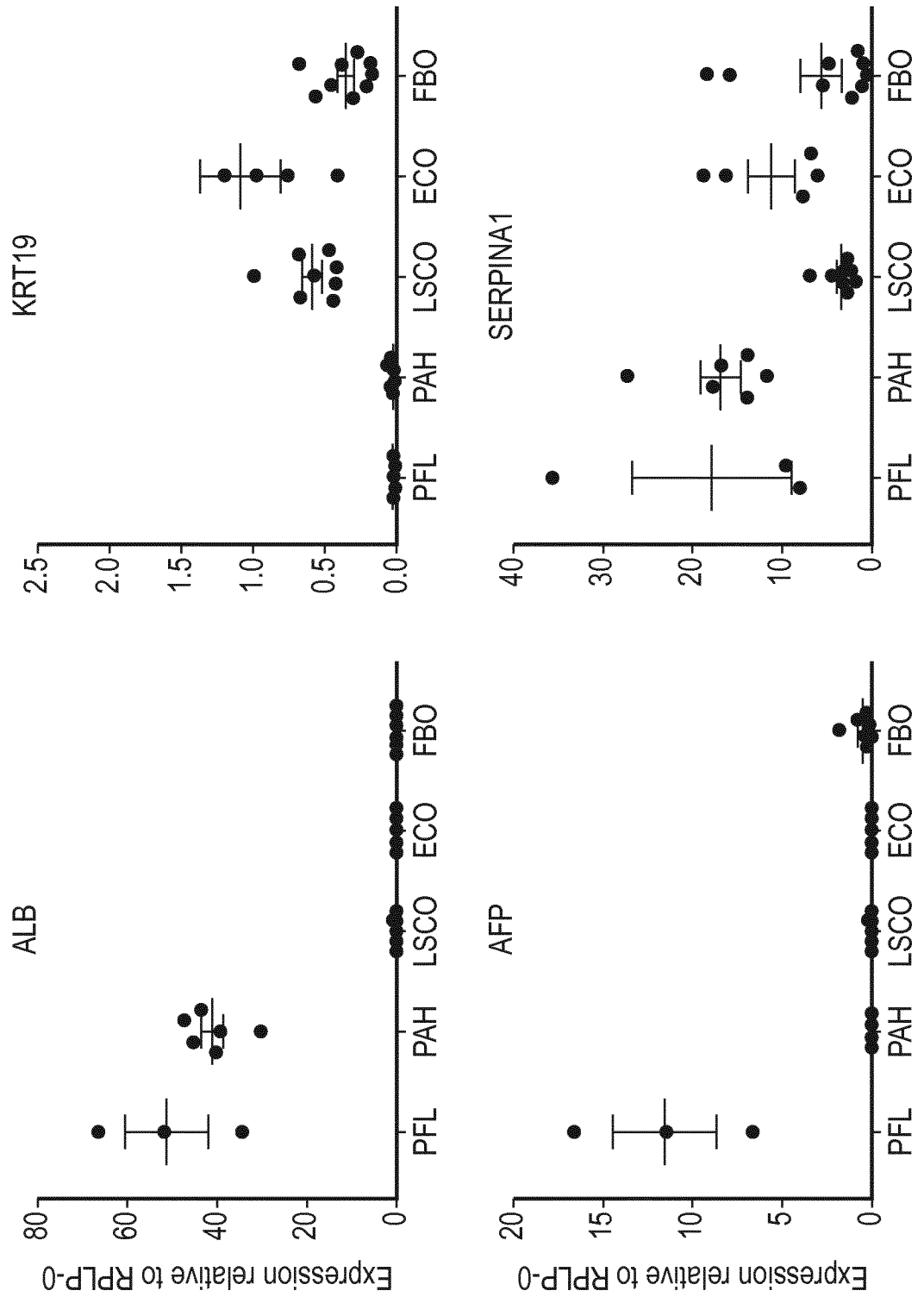
KRT19
SERPINA1
ALB
AFP
Expression relative to RPLP-0
PFL
PAH
LSCO
ECO
FBO

FIG. 1D



FIG. 1E

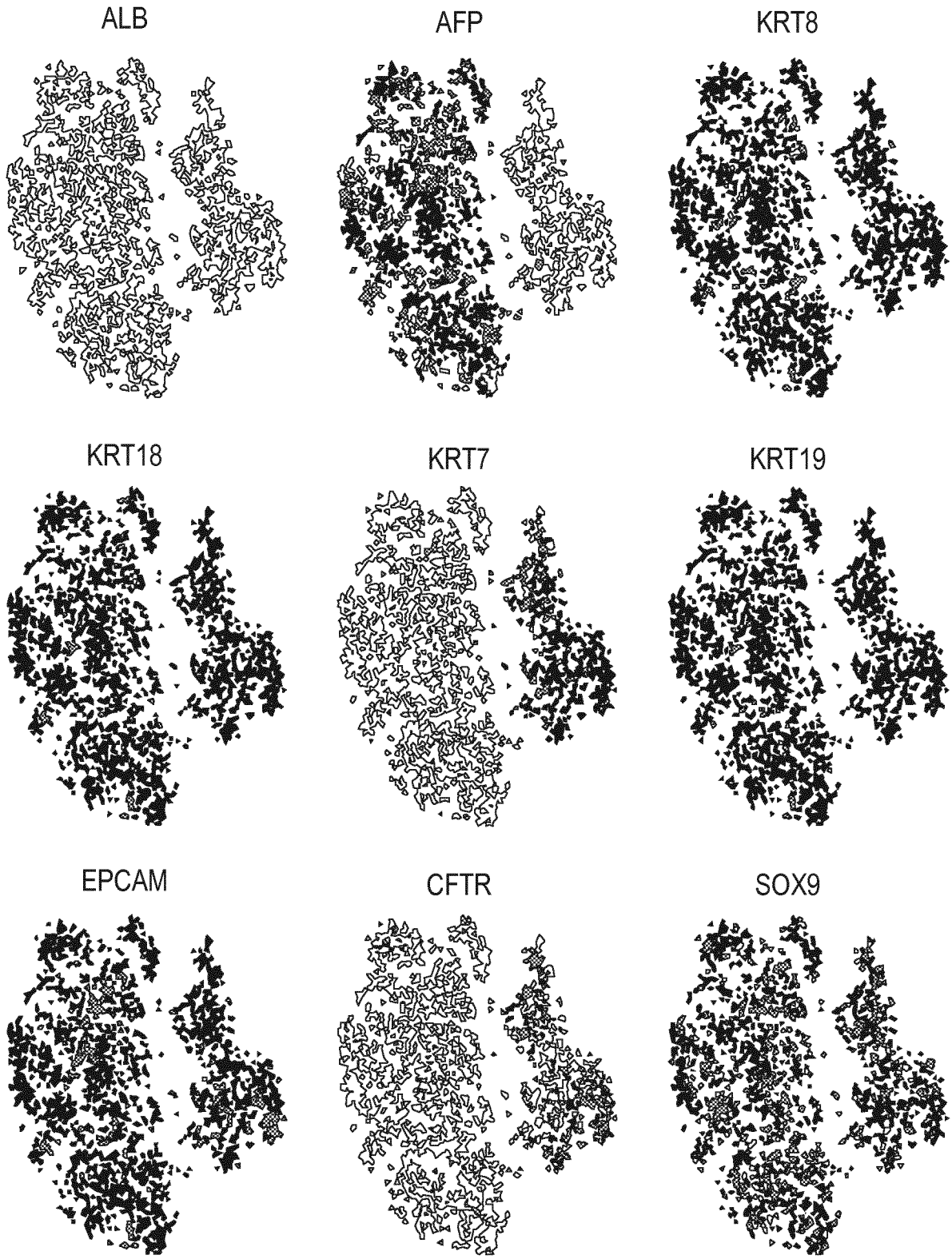

FIG. 1F

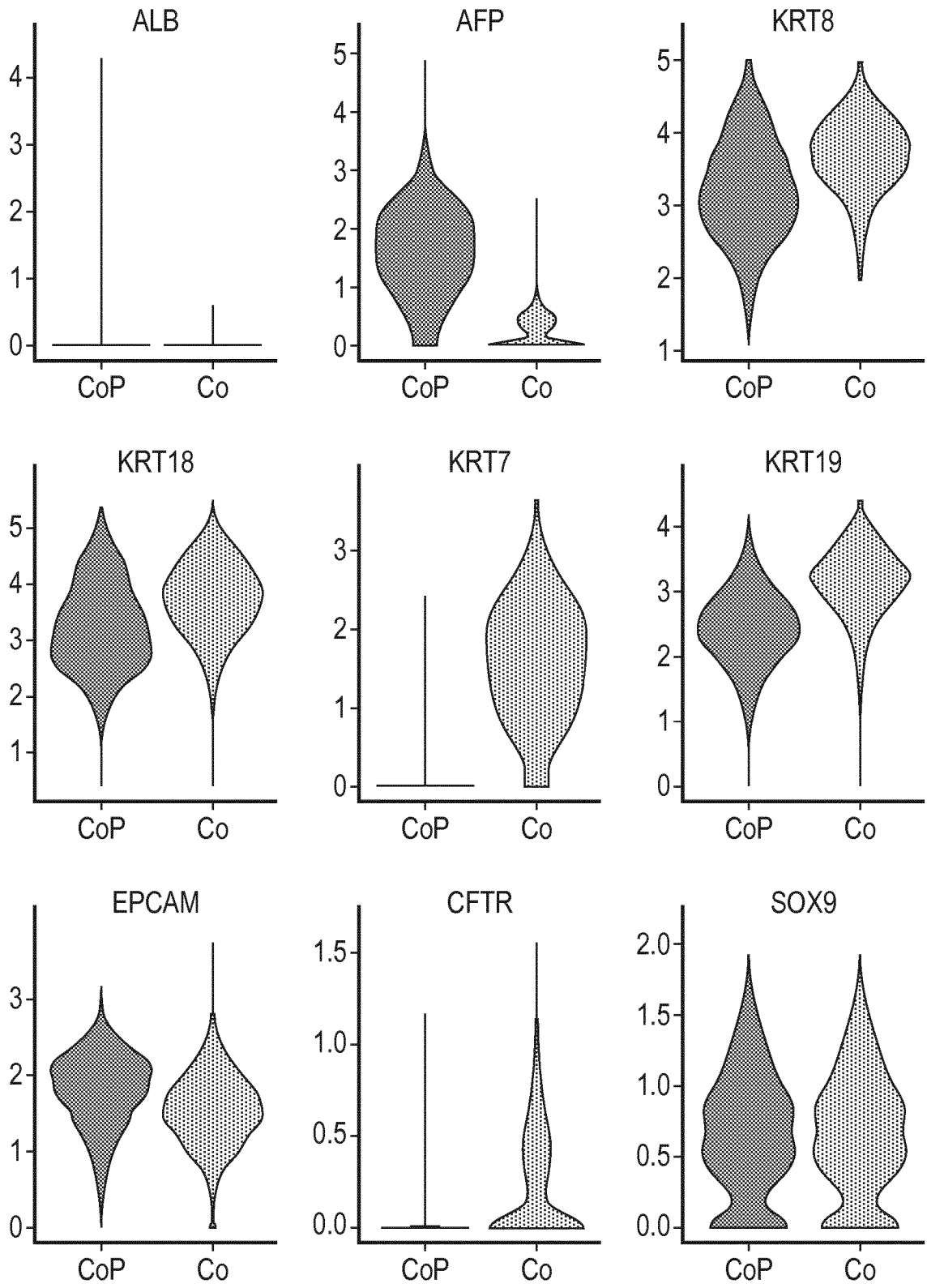

FIG. 1G

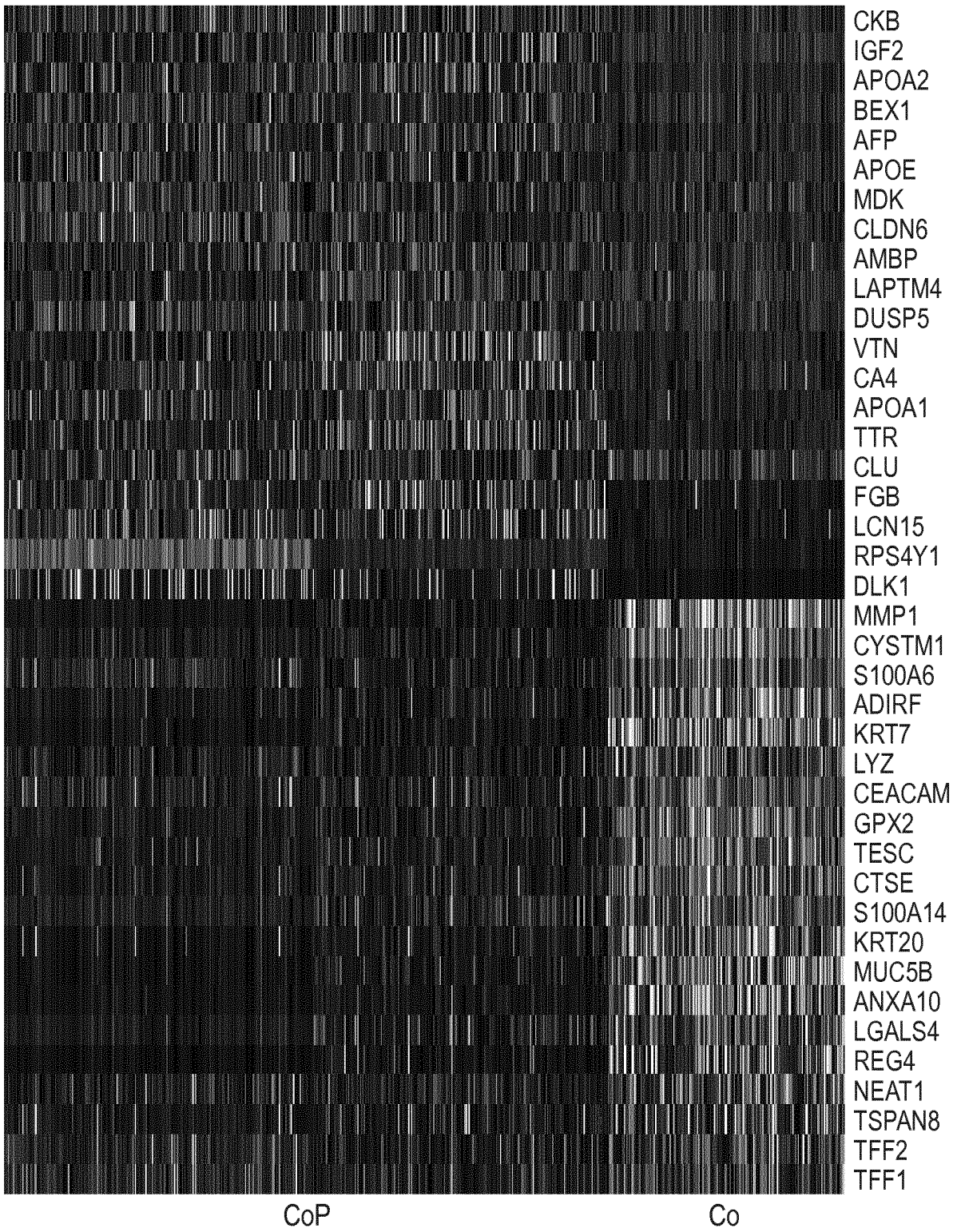
CKB
IGF2
APOA2
BEX1
AFP
APOE
MDK
CLDN6
AMBP
LAPTM4
DUSP5
VTN
CA4
APOA1
TTR
CLU
FGB
LCN15
RPS4Y1
DLK1
MMP1
CYSTM1
S100A6
ADIRF
KRT7
LYZ
CEACAM
GPX2
TESC
CTSE
S100A14
KRT20
MUC5B
ANXA10
LGALS4
REG4
NEAT1
TSPAN8
TFF2
TFF1
CoP
Co

FIG. 1H

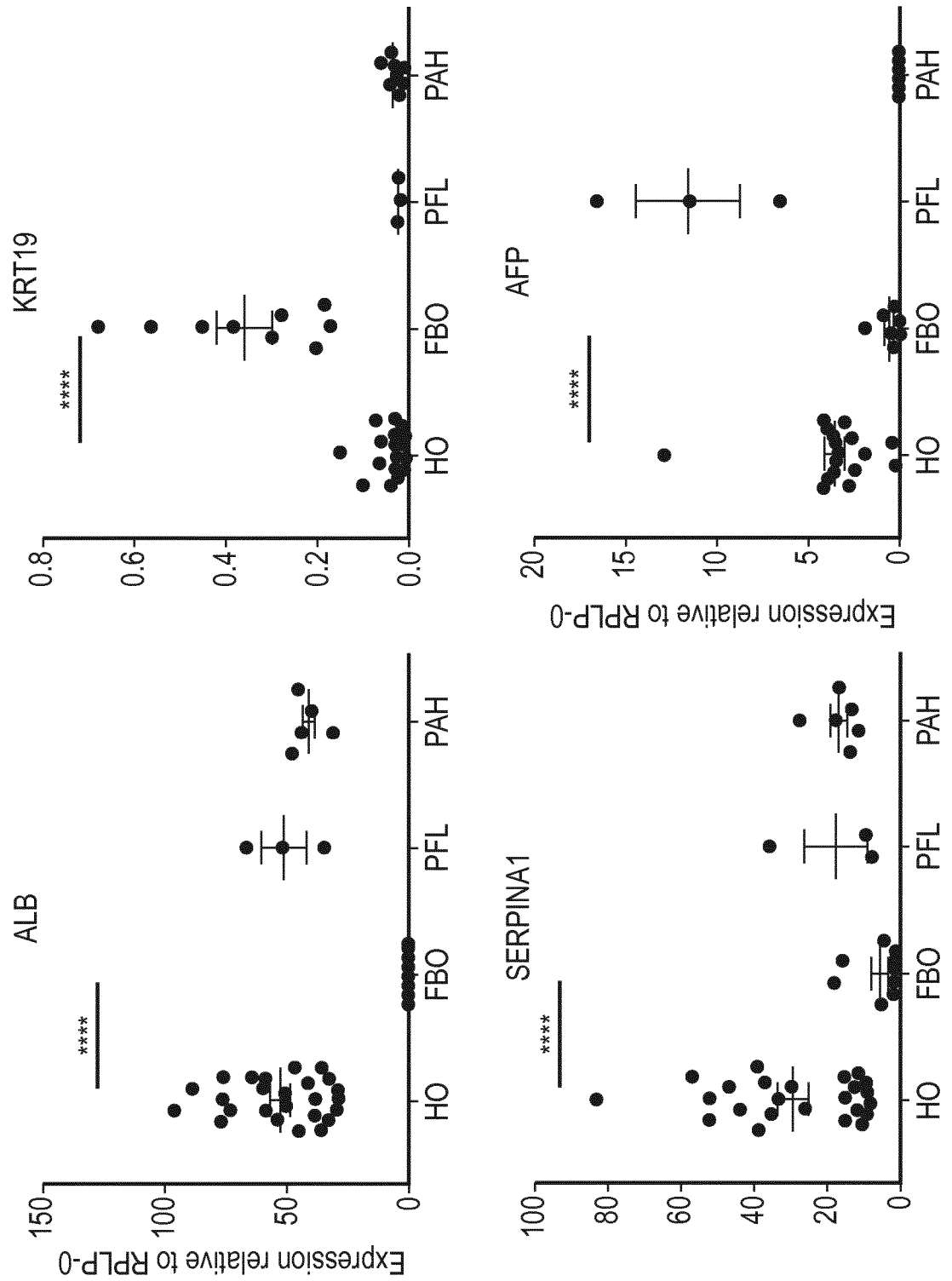
KRT19
AFP
ALB
SERPINA1
Expression relative to RPLP-0
****
HO
FBO
PFL
PAH
0.0
0.2
0.4
0.6
0.8
0
5
10
15
20
0
50
100
150
0
20
40
60
80
100

FIG. 2A

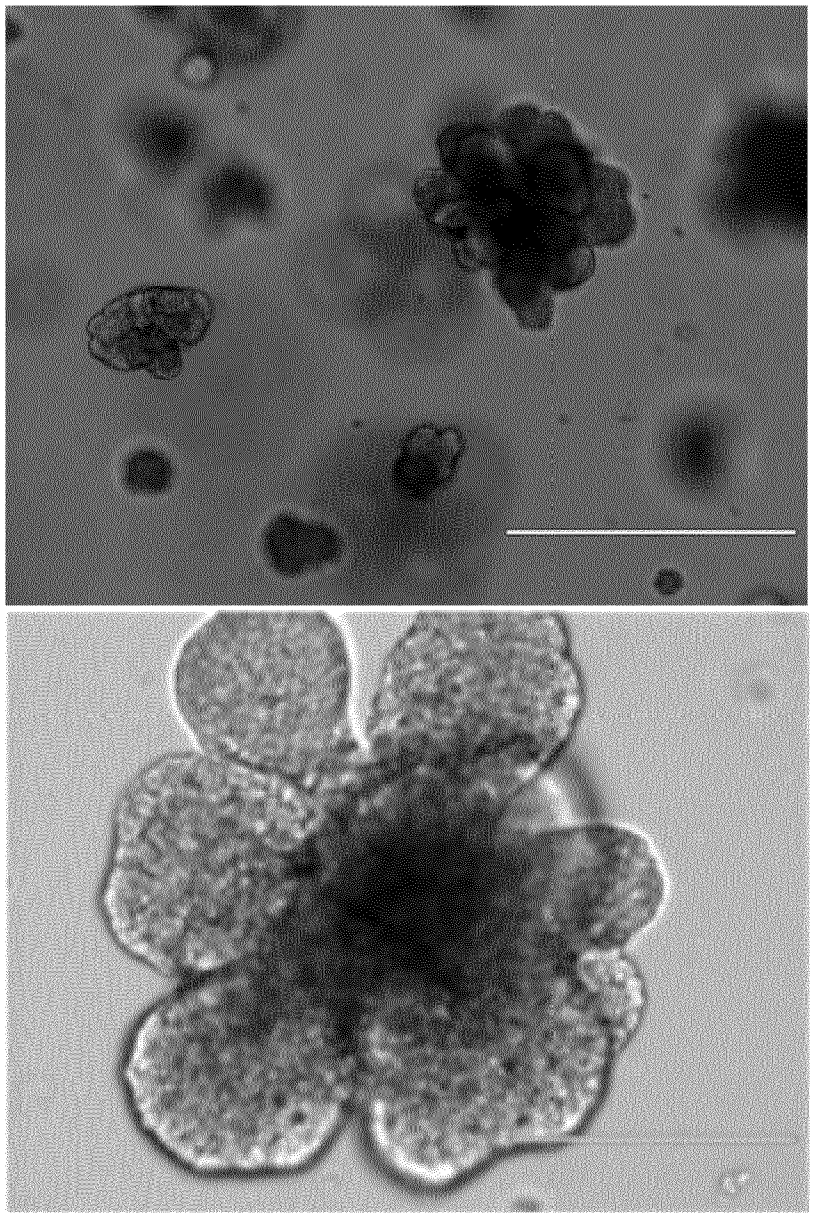

FIG. 2B

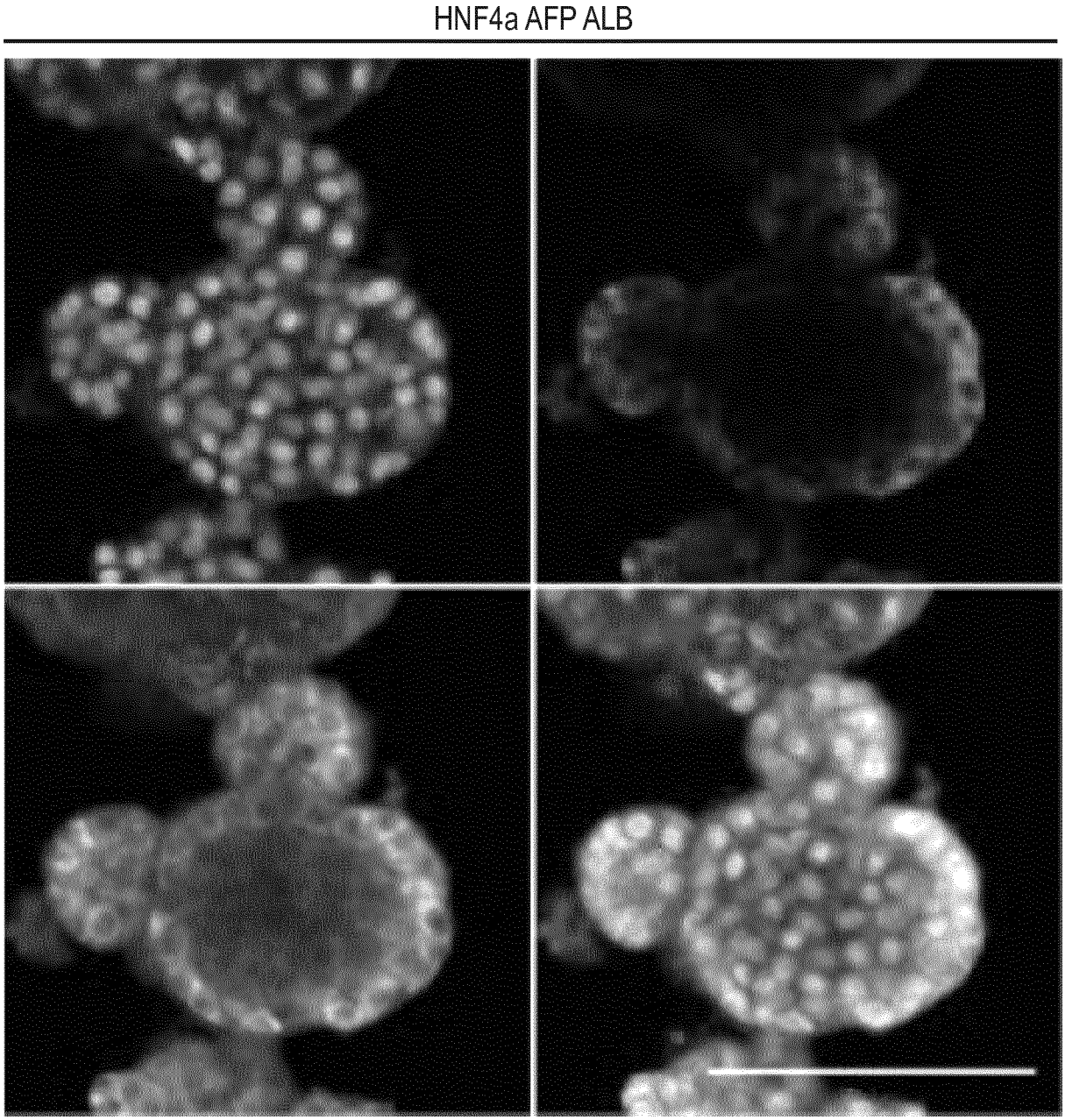

FIG. 2C

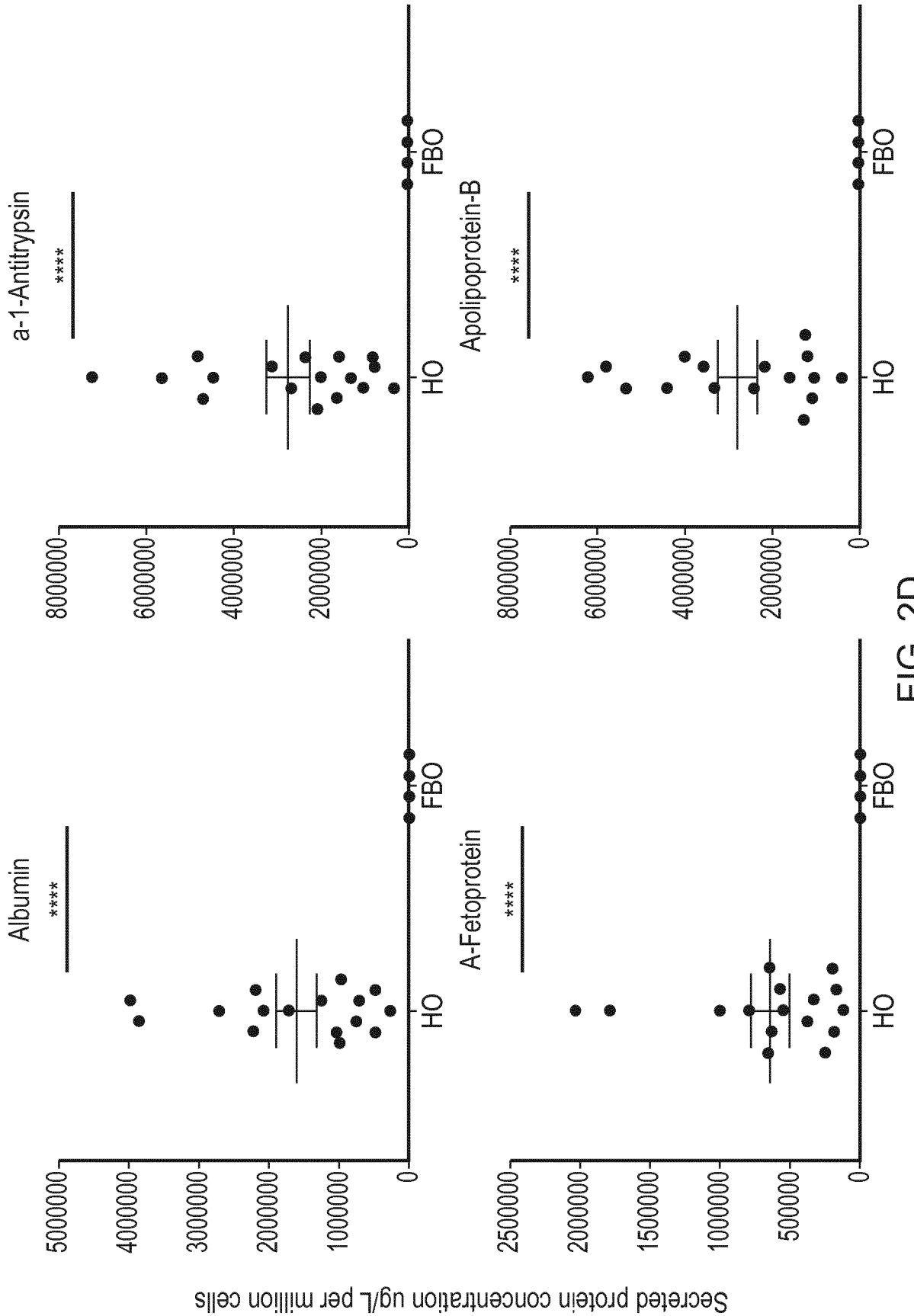
a-1-Antitrypsin
Apolipoprotein-B
Albumin
A-Fetoprotein
****
HO
FBO
0
2000000
4000000
6000000
8000000
1000000
3000000
5000000
500000
1500000
2500000
Secreted protein concentration ug/L per million cells

FIG. 2D

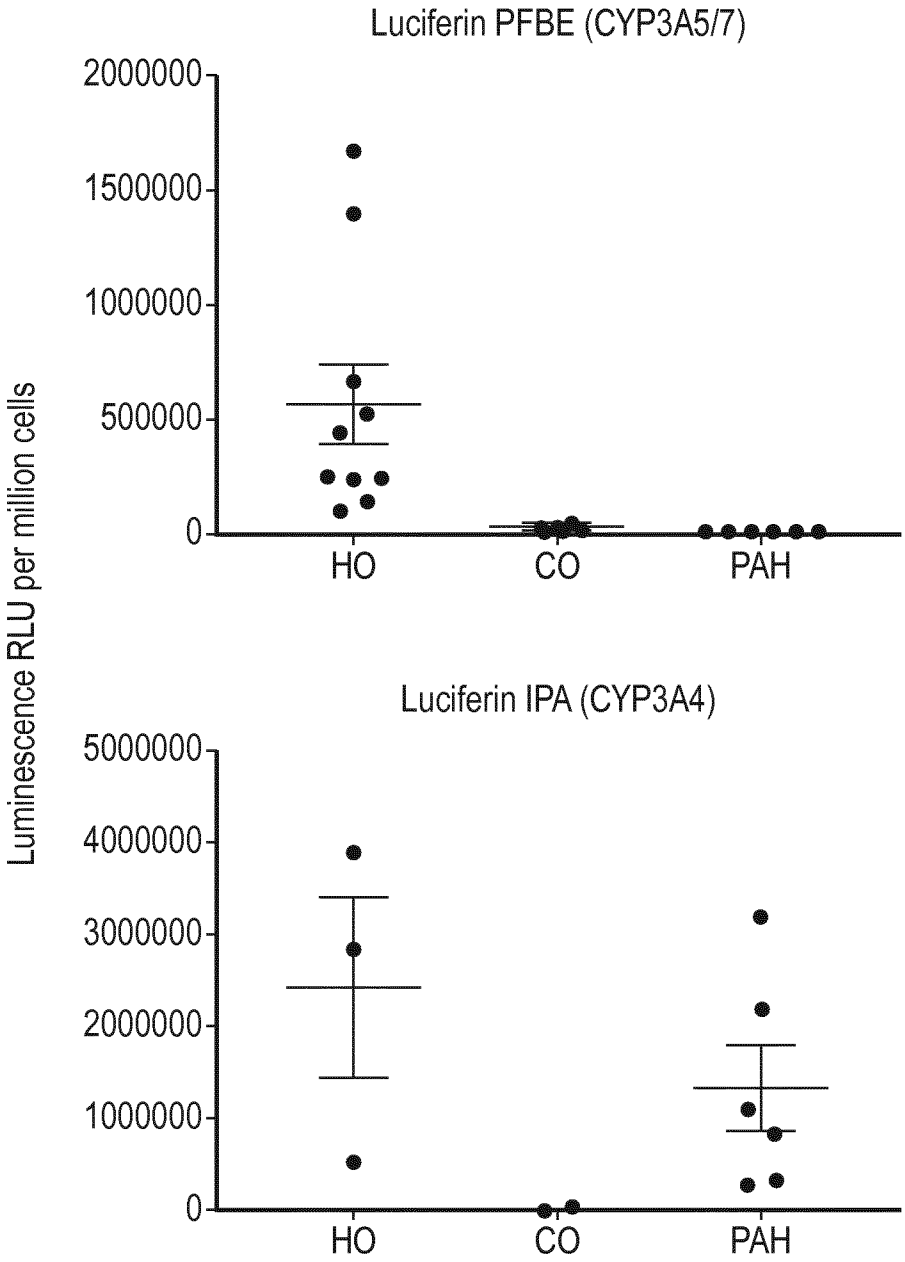
Luciferin PFBE (CYP3A5/7)
2000000
1500000
1000000
500000
0
HO
CO
PAH
Luciferin IPA (CYP3A4)
5000000
4000000
3000000
2000000
1000000
0
HO
CO
PAH
Luminescence RLU per million cells

FIG. 2E

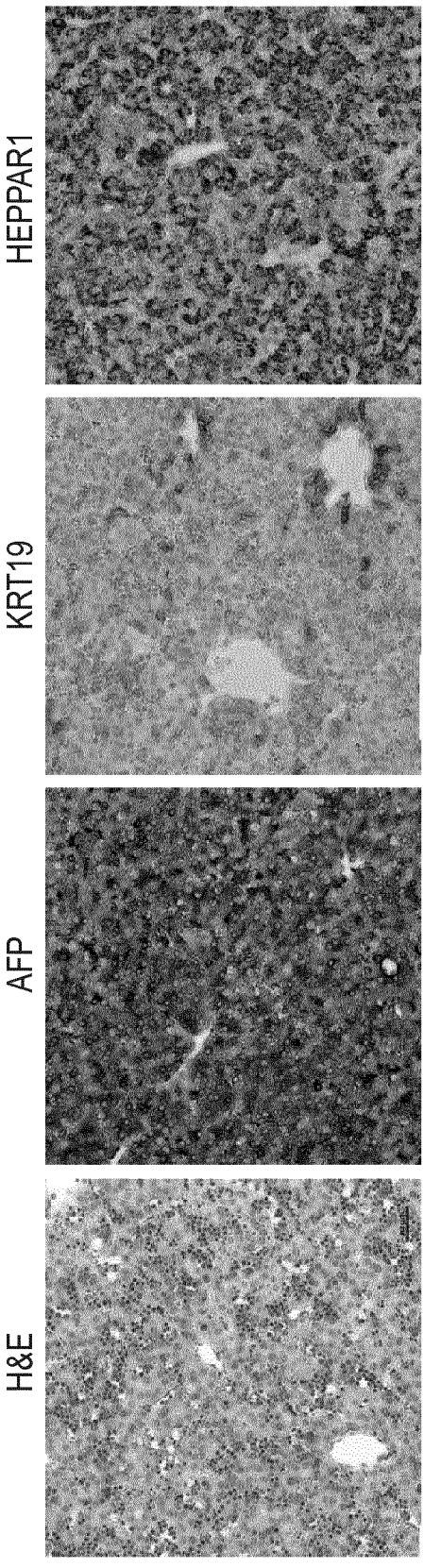

FIG. 3A

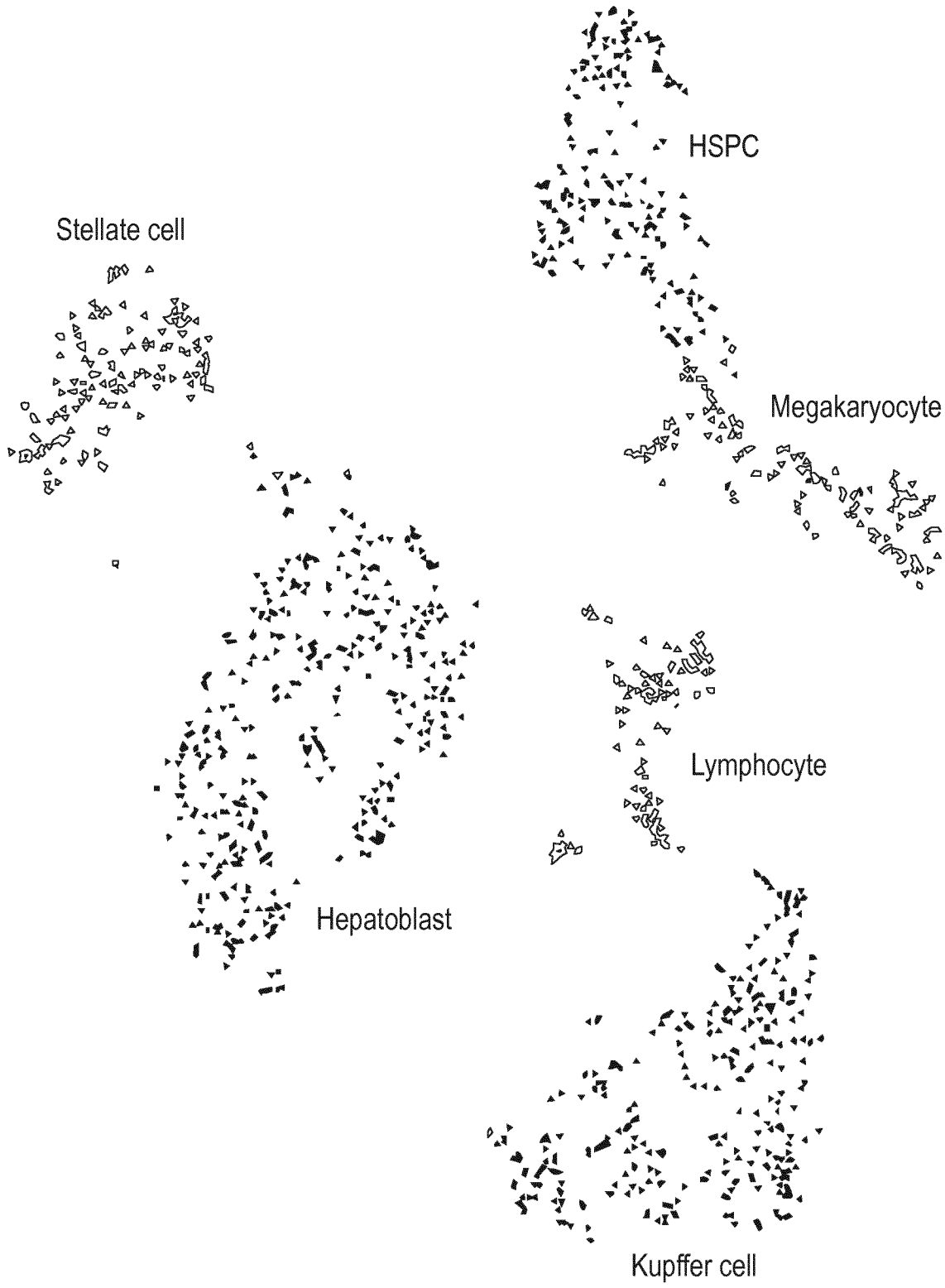
HSPC
Stellate cell
Megakaryocyte
Lymphocyte
Hepatoblast
Kupffer cell

FIG. 3B

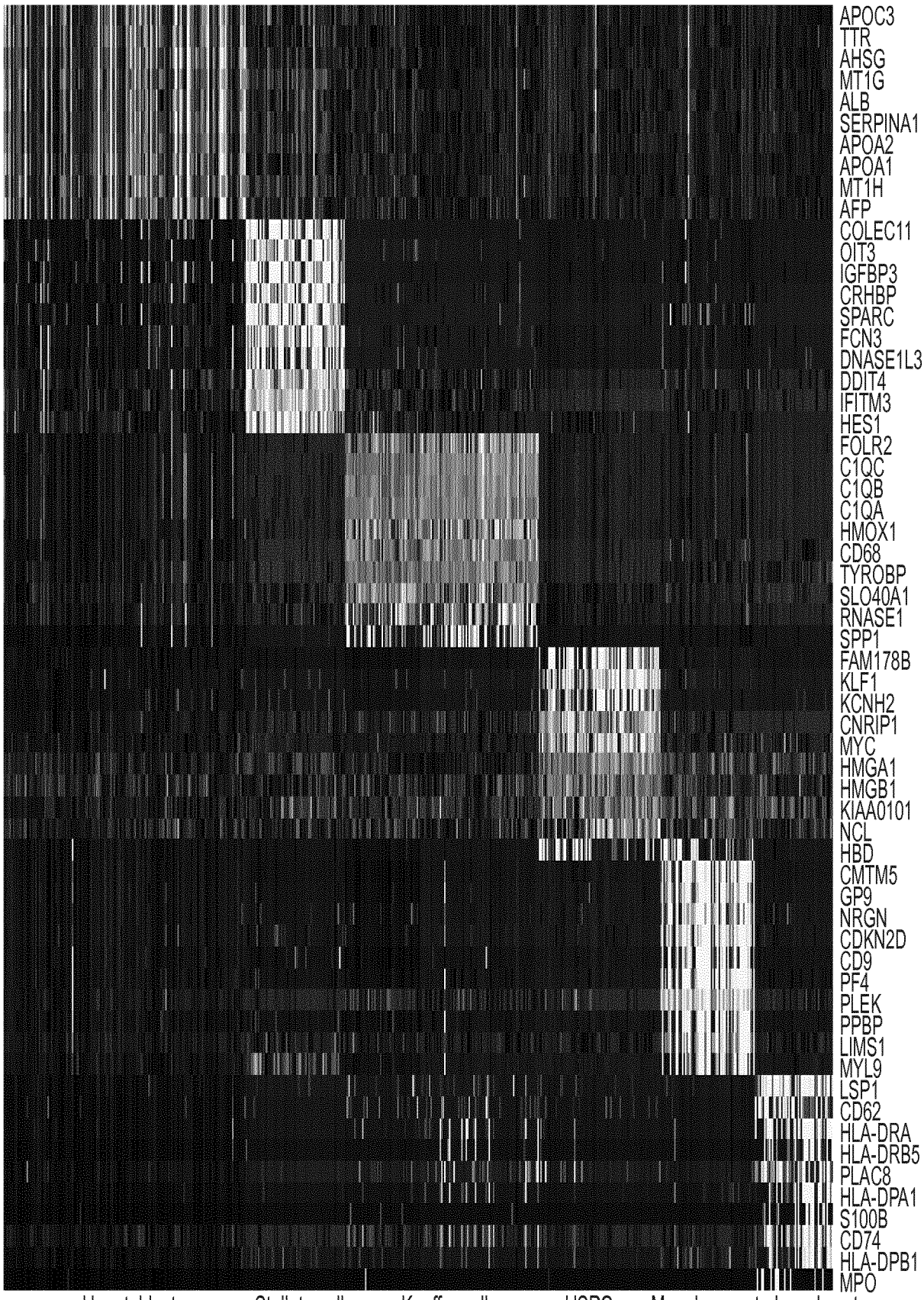
APOC3
TTR
AHSG
MT1G
ALB
SERPINA1
APOA2
APOA1
MT1H
AFP
COLEC11
OIT3
IGFBP3
CRHBP
SPARC
FCN3
DNASE1L3
DDIT4
IFITM3
HES1
FOLR2
C1QC
C1QB
C1QA
HMOX1
CD68
TYROBP
SLO40A1
RNASE1
SPP1
FAM178B
KLF1
KCNH2
CNRIP1
MYC
HMGA1
HMGB1
KIAA0101
NCL
HBD
CMTM5
GP9
NRGN
CDKN2D
CD9
PF4
PLEK
PPBP
LIMS1
MYL9
LSP1
CD62
HLA-DRA
HLA-DRB5
PLAC8
HLA-DPA1
S100B
CD74
HLA-DPB1
MPO
Hepatoblast
Stellate cell
Kupffer cell
HSPC
Megakaryocyte
Lymphocyte

FIG. 3C

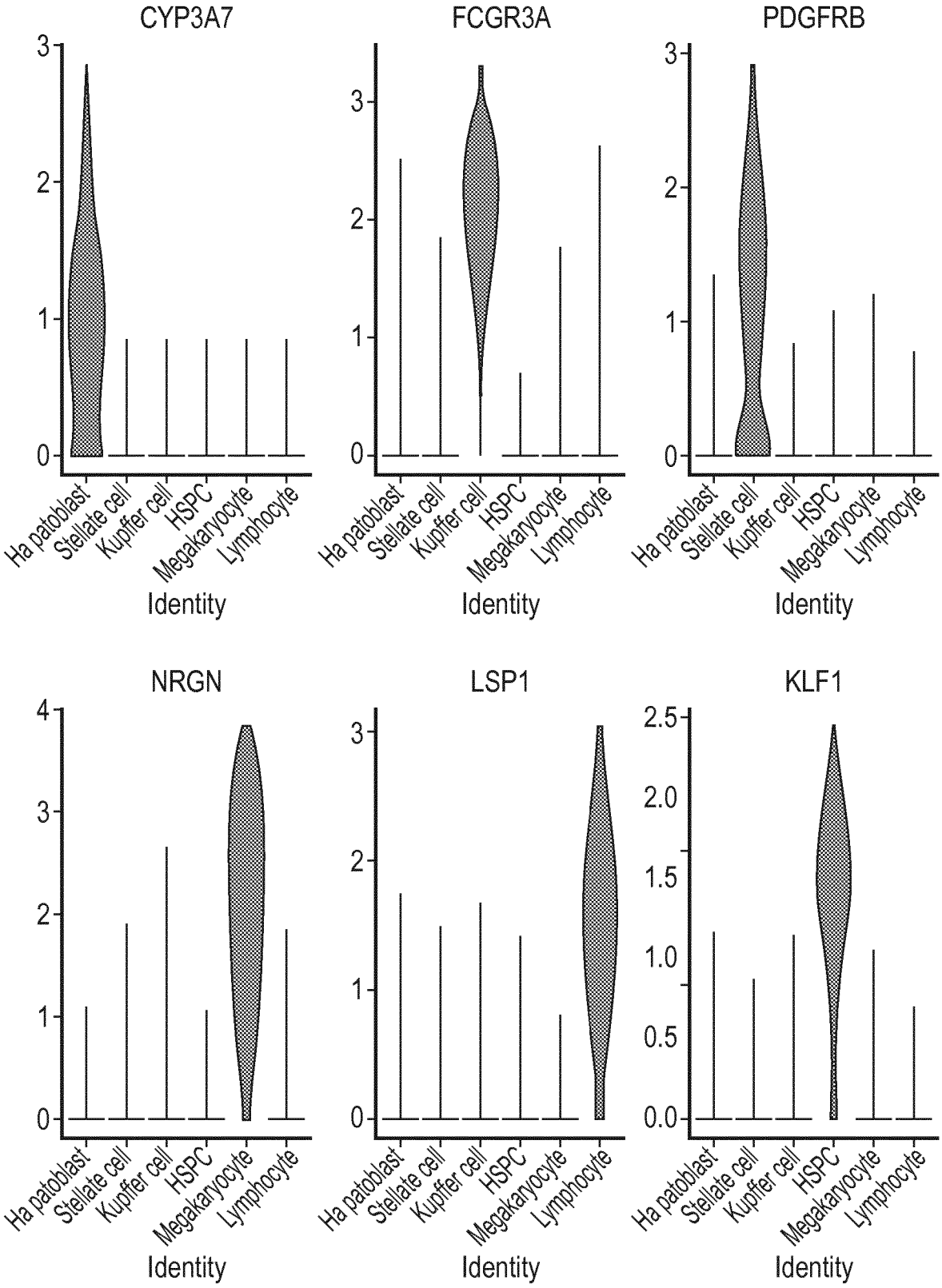
CYP3A7
FCGR3A
PDGFRB
NRGN
LSP1
KLF1
Ha patoblast
Stellate cell
Kupffer cell
HSPC
Megakaryocyte
Lymphocyte
Identity

FIG. 3D

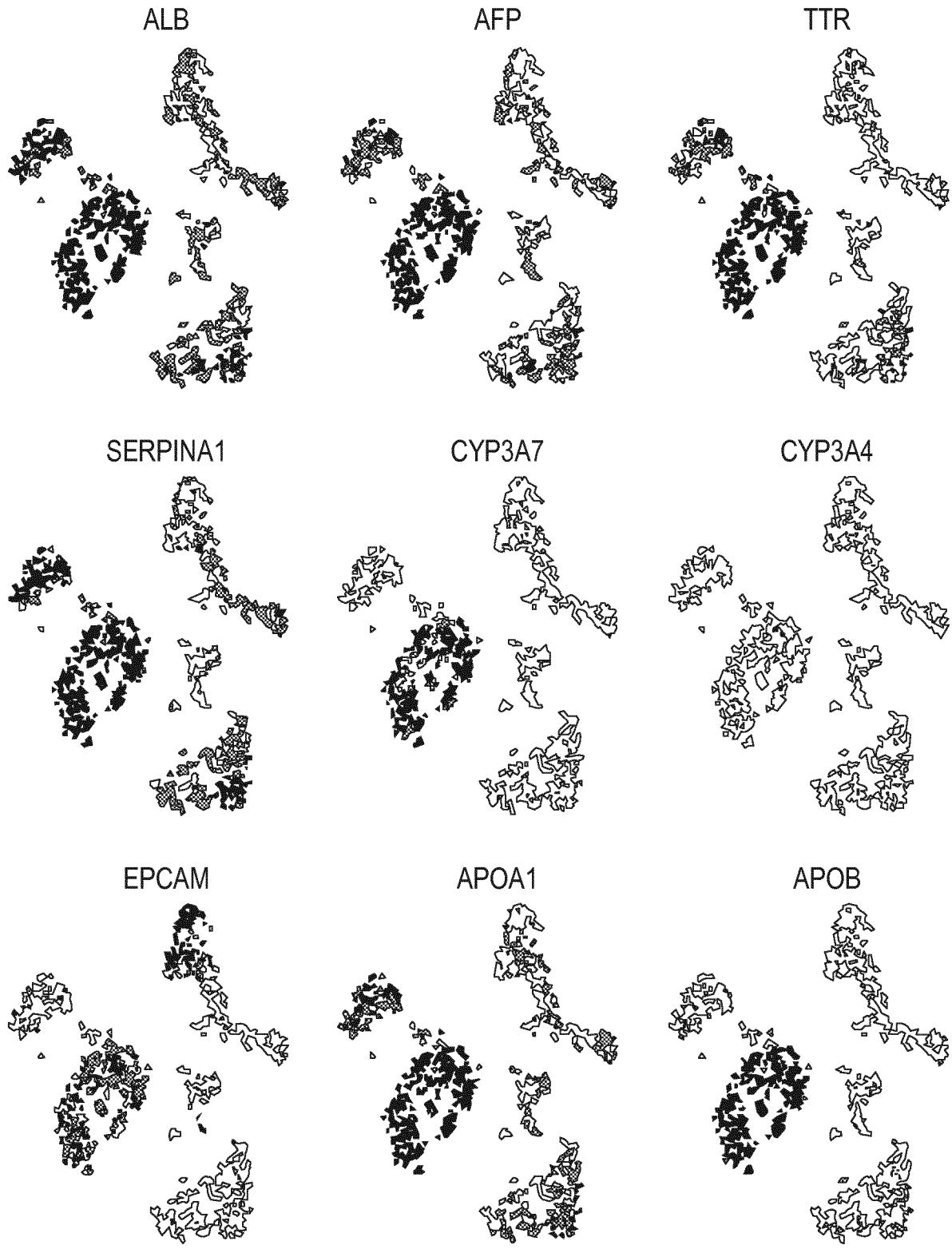

FIG. 3E



FIG. 4A

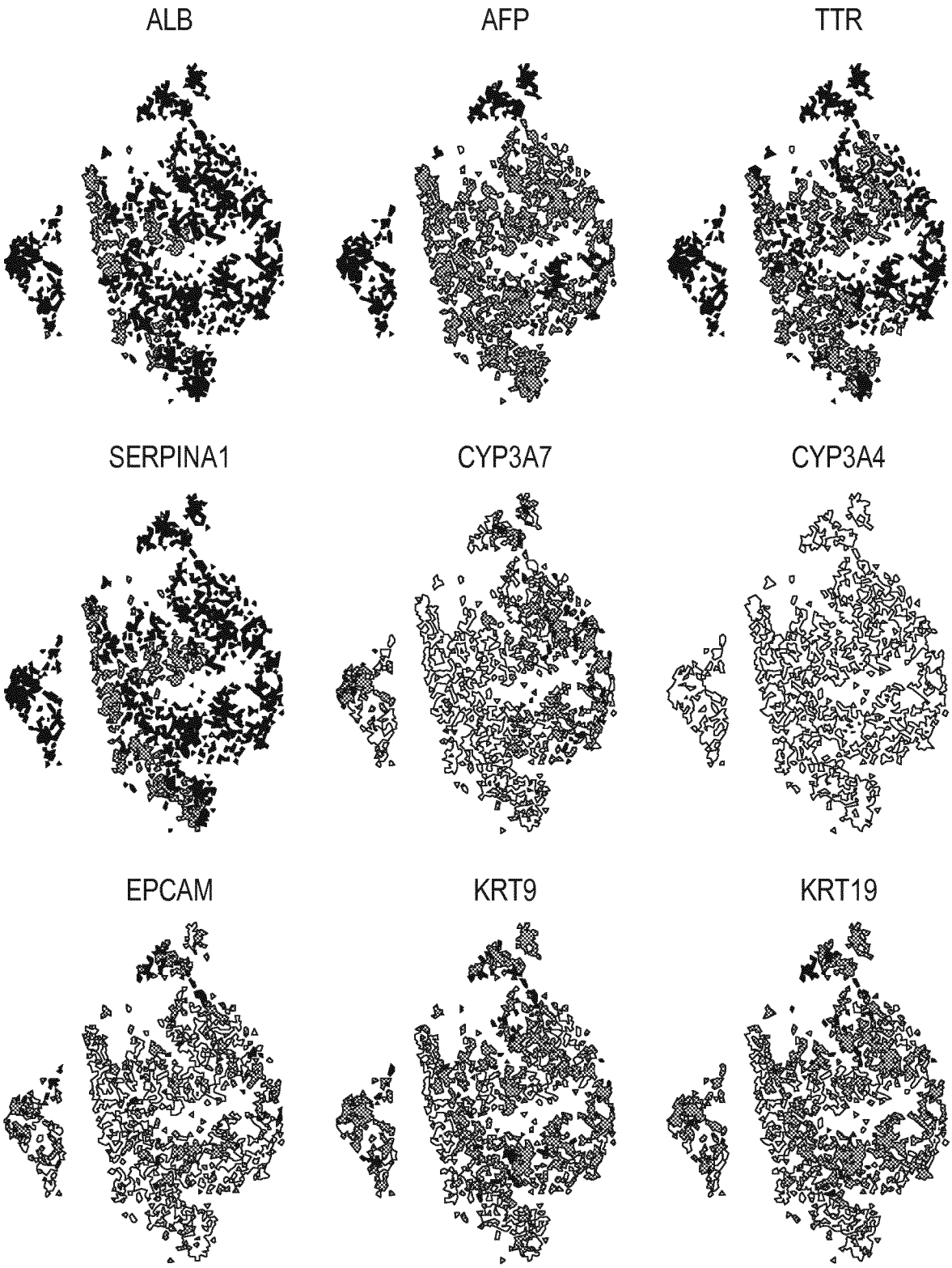

FIG. 4B

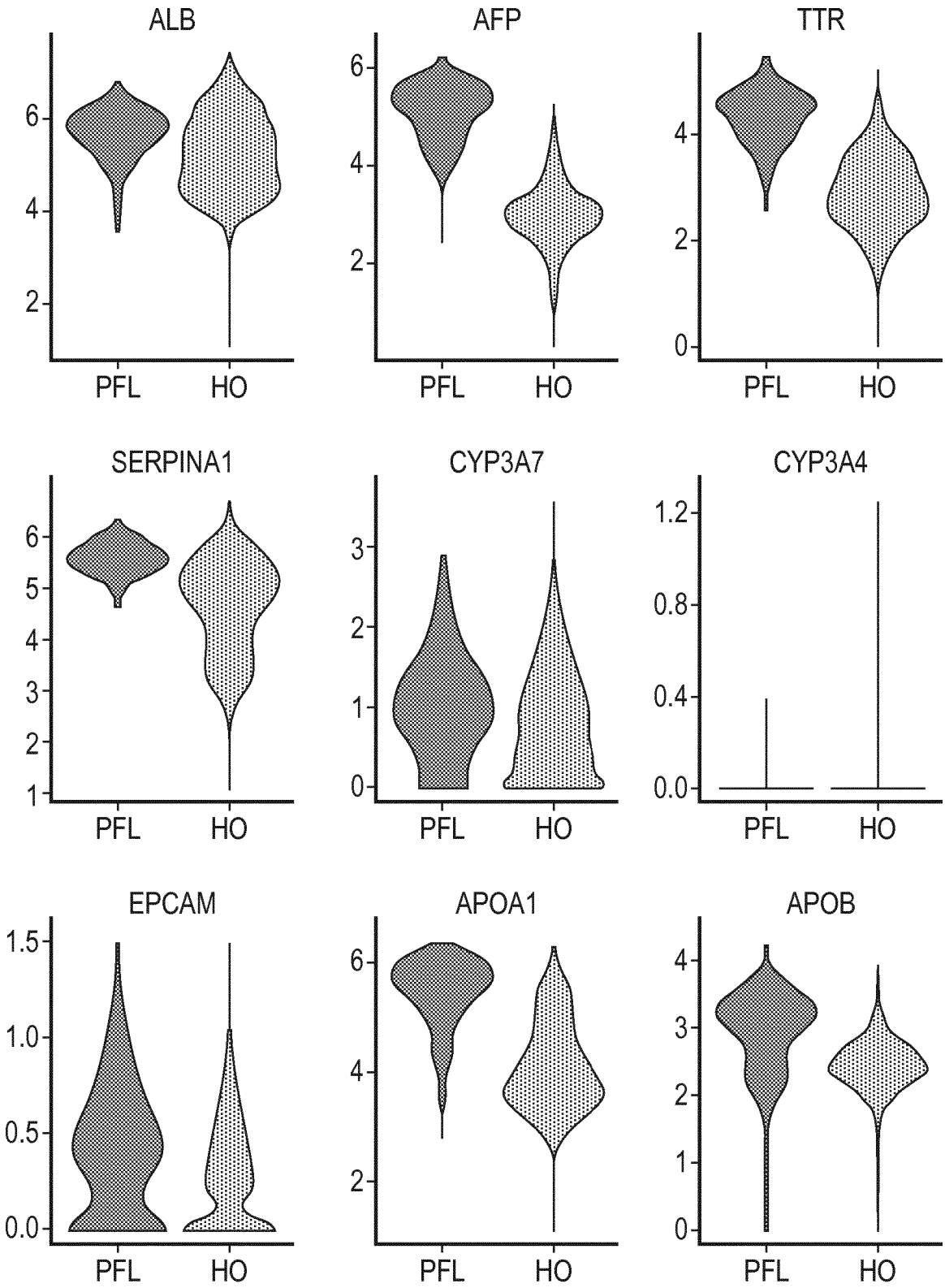

FIG. 4C

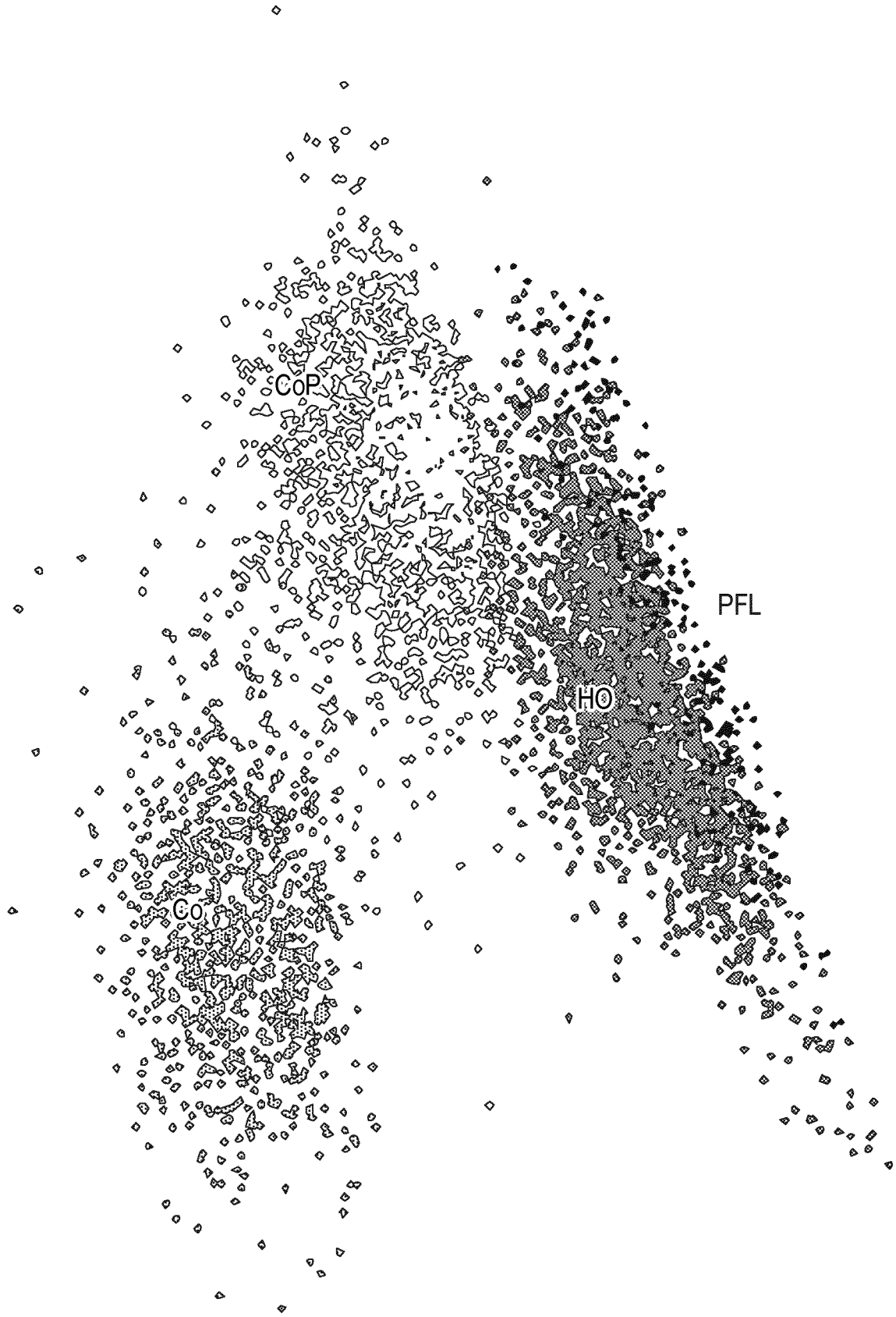

FIG. 4D

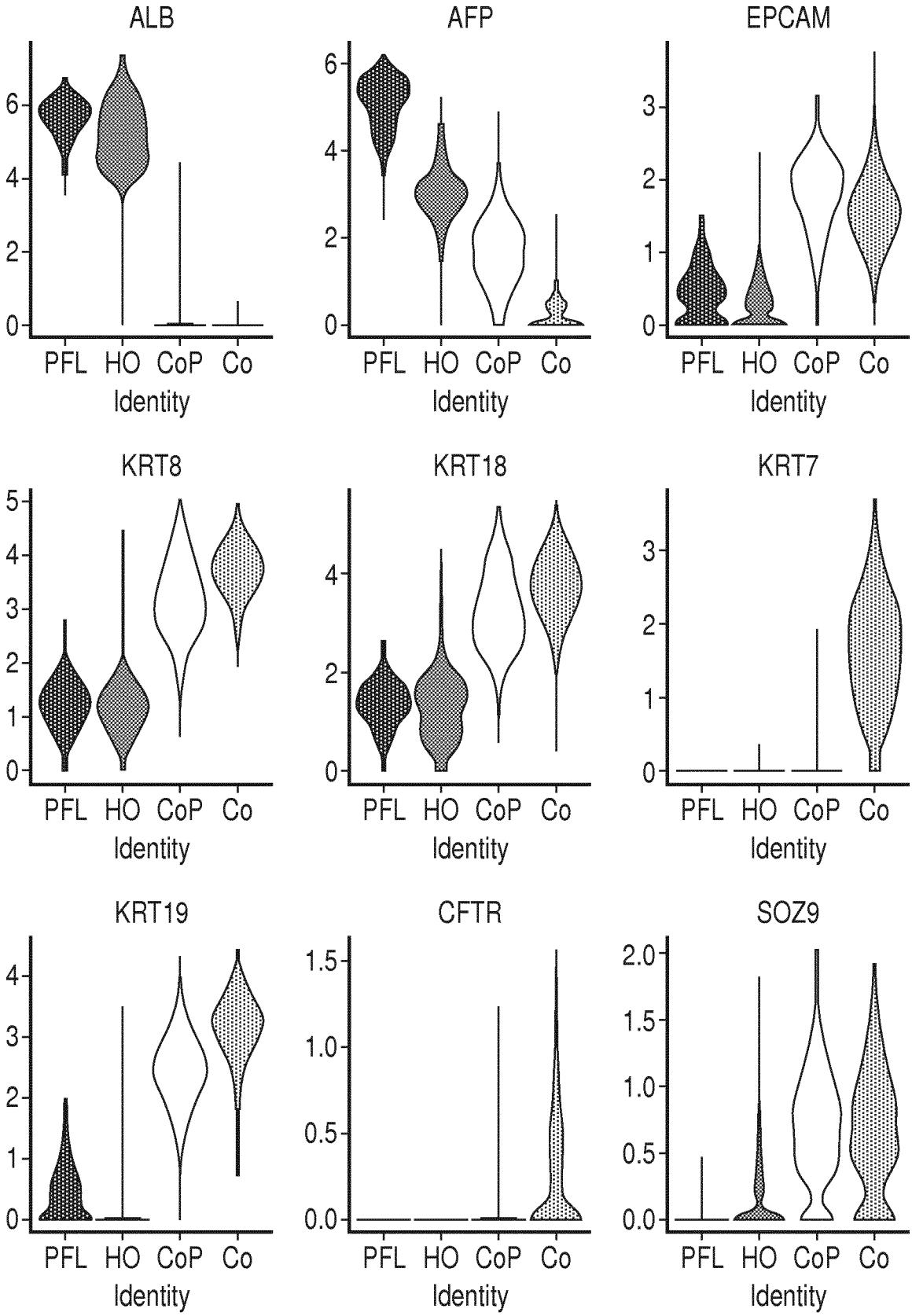
ALB
AFP
EPCAM
KRT8
KRT18
KRT7
KRT19
CFTR
SOZ9
PFL HO CoP Co
Identity

FIG. 4E

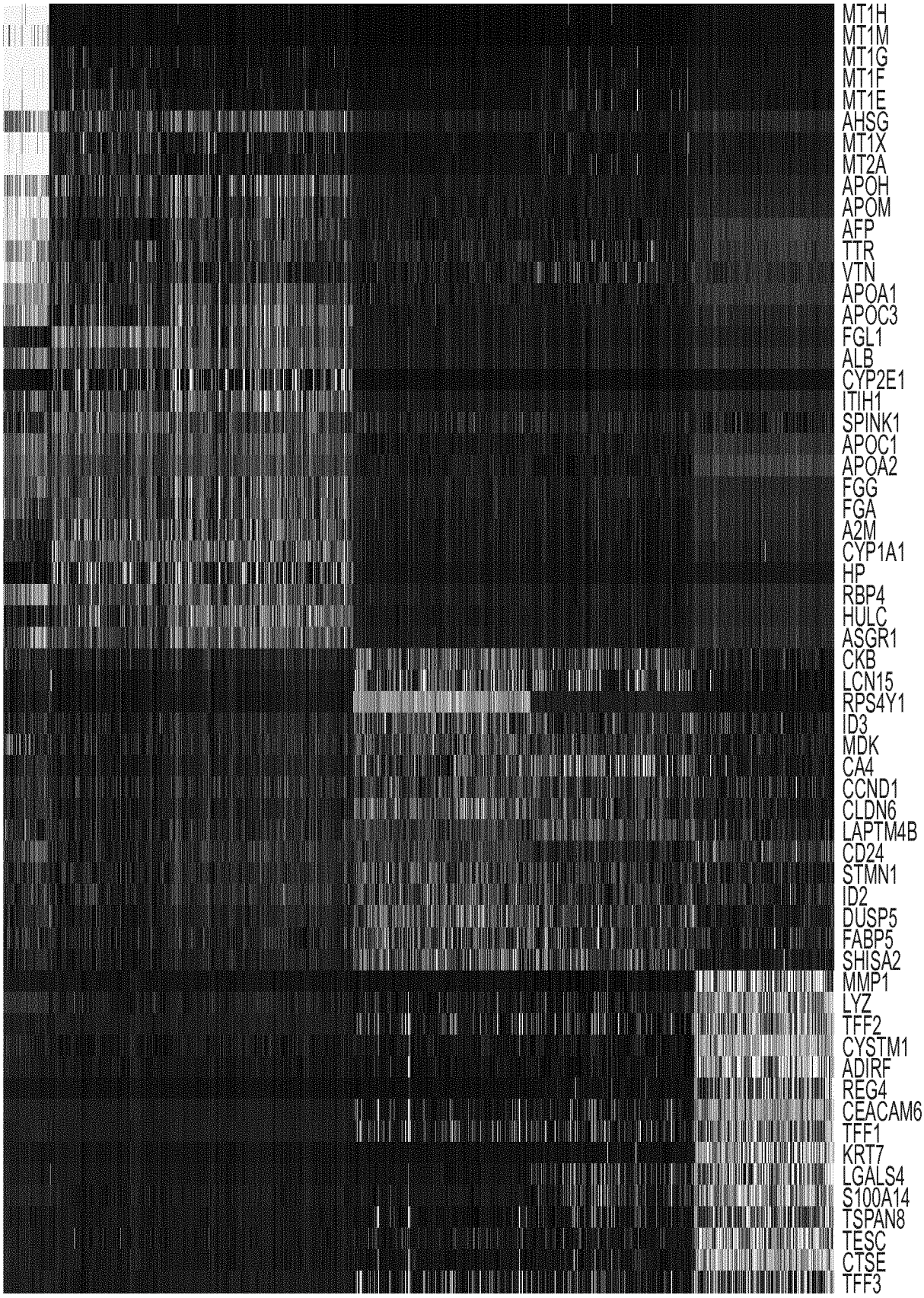
MT1H
MT1M
MT1G
MT1F
MT1E
AHSG
MT1X
MT2A
APOH
APOM
AFP
TTR
VTN
APOA1
APOC3
FGL1
ALB
CYP2E1
ITIH1
SPINK1
APOC1
APOA2
FGG
FGA
A2M
CYP1A1
HP
RBP4
HULC
ASGR1
CKB
LCN15
RPS4Y1
ID3
MDK
CA4
CCND1
CLDN6
LAPTM4B
CD24
STMN1
ID2
DUSP5
FABP5
SHISA2
MMP1
LYZ
TFF2
CYSTM1
ADIRF
REG4
CEACAM6
TFF1
KRT7
LGALS4
S100A14
TSPAN8
TESC
CTSE
TFF3
PFL
HO
CoP
Co

FIG. 4F

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/071574

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/071 C12Q1/68
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 C12Q

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, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAZUYA ANZAI ET AL: "Foetal hepatic progenitor cells assume a cholangiocytic cell phenotype during two-dimensional pre-culture", SCIENTIFIC REPORTS, vol. 6, no. 1, 23 June 2016 (2016-06-23), XP055534175, DOI: 10.1038/srep28283 abstract Methods - Three-dimensional (3D) culture of cells; page 10 - page 11 page 1, paragraph 2 -----	1-22
X	WO 2016/207621 A1 (CAMBRIDGE ENTPR LTD [GB]) 29 December 2016 (2016-12-29) claim 1 ----- -/--	14

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 October 2019	18/10/2019

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	Loubradou-Bourges, N

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	XINPING TAN ET AL: "[beta]-Catenin deletion in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development", HEPATOLOGY, vol. 47, no. 5, 7 April 2008 (2008-04-07), pages 1667-1679, XP055534724, US ISSN: 0270-9139, DOI: 10.1002/hep.22225 cited in the application abstract	14
X	MERITXELL HUCH ET AL: "In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration", NATURE, vol. 494, no. 7436, 1 January 2013 (2013-01-01), pages 247-250, XP055058163, ISSN: 0028-0836, DOI: 10.1038/nature11826 abstract	14
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/071574

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