Abstract:

Hepatocyte-like cells which express a Hnf polypeptide and a Foxa polypeptide, the method of generating the cells and the uses of the cells are provided.
**Hepatocyte-Like Cells and Uses Thereof**

**CROSS REFERENCE TO RELATED APPLICATION**

This application claims priority of Chinese Application No. 201010531420.4 filed on November 4, 2010. The content of the application is incorporated by reference in its entirety.

**FIELD OF THE INVENTION**

This invention relates to hepatocyte-like cells, related compositions, and related methods that are useful for improving liver function and treating various liver disorders.

**BACKGROUND OF THE INVENTION**

The liver is a vital organ in various vertebrates and some other animals. In the human body, the liver is the largest internal organ and provides many essential functions, including metabolic, exocrine and endocrine functions. The liver is necessary for survival. Without liver function, a human can only survive up to 24 hours. Currently, there is no way to compensate for long term absence of liver function, although liver dialysis can be used short term.

Disorders of the liver, including liver failure and end-stage liver diseases, are responsible for a huge number of deaths around the world and are a major burden on the health care system. Although liver transplantation has been successfully used for treat the disorders, its efficacy is limited and connected to many complications such as infection or rejection. Liver transplantation is also limited due to shortage of available donor organs and lifelong use of immunosuppression in recipients. Cell-based therapy, such as those based hepatocytes, on are believed to hold a great promise for treating these severe diseases.

Hepatocytes, the principal cell type in the liver, are responsible for function and regeneration of the adult liver. Along with biliary epithelial cells, hepatocytes are derived from the embryonic endoderm. Human hepatocytes can be used for modeling and understanding liver diseases, drug efficacy and toxicity testing, and cell replacement therapy. However, primary human hepatocytes are scarce and, despite their ability to efficiently proliferate in vivo, cannot be expanded in vitro.

Thus, there is a continuing unmet need for an unlimited source of human hepatocytes or hepatocyte-like cells.
SUMMARY OF INVENTION

This invention relates to a novel method for generating hepatocyte-like cells, related cells, and related methods.

One aspect of this invention features a method of generating hepatocyte-like cells. The method includes expressing in a starting cell a heterologous Hnf polypeptide and a heterologous Foxa polypeptide; and culturing the starting cell in a medium for a period of time to obtain one or more progeny cells thereof thereby generating hepatocyte-like cells. In one embodiment, the method further includes expressing in the starting cell a heterologous GATA4 polypeptide. The Hnf, Foxa, and GATA4 polypeptides can include the sequences of SEQ ID NOs: 1-3, respectively. In a preferred embodiment, the method further includes expressing in the starting cell one or more polypeptides that have sequences selected from the group consisting of SEQ ID NO: 4-14.

The starting cell can be a somatic cell. It can be a cell from an adult source, an embryonic source, or a fetal source. Examples of the cell include a fibroblast, an epithelium cell, a blood cell, a neuron, an embryonic cell, or a cell derived from a tissue or organ of a subject. Preferably, the starting cell is pl9^null or expresses the pl9^gene at a level lower than a predetermined level so that the cells can proliferate in vitro for a period of time and do not undergo cellular death or senescence as discussed below. The predetermined level can be one obtained from a control cell, e.g., a wildtype cell from a corresponding tissue or organ. To generate the hepatocyte-like cells, one can express the above-mentioned heterologous polypeptides in the starting cells and then culture the cells for a period of time, e.g., at least 2, 3, 4, 5, 6, 7, 10, 14 days. For example, the cell can be cultured for 2-30 days, e.g., 5-25 days, or 14-21 days.

In another aspect, this invention provides a cultured recombinant cell that contains, among others, (i) a first agent selected from a first group consisting of a heterologous Hnf polypeptide and a first nucleic acid encoding the Hnf polypeptide; and (ii) a second agent selected from a second group consisting of a heterologous Foxa polypeptide and a second nucleic acid encoding the Foxa polypeptide. The cell can further contain a third agent selected from a third group consisting of a heterologous GATA4 polypeptide and a third nucleic acid encoding the GATA4 polypeptide. The cell is positive for one or more hepatic functional genes as shown in Tables 2 and 3 below. The cell is capable of metabolizing one or more compounds selected from group consisting of phenacetin, testosterone, and diclofenac. In one embodiment, the cell is a hepatocyte-like cell that is obtained using the method described above.
In another aspect, this invention provides a pharmaceutical composition having the above-described cell and a pharmaceutically acceptable carrier. The invention also provides a bioartificial device having the cell. As discussed in detail below, the cell, pharmaceutical composition, and device can be used in a method for improving the liver function of a subject. To that end, one can administer to a subject in need thereof the cell, or implanting the device in the subject, thereby improving the liver function.

In yet another aspect, this invention provides a method of evaluating toxicity, carcinogenicity, or biotransformation activity of a test substance. The method includes contacting a test substance with the above-described cell, and examining a level of metabolic activity or viability of the cell. The value of the level indicates the toxicity, carcinogenicity, or biotransformation activity of the test substance.

This invention further provides a composition having (i) a first agent selected from a first group consisting of an isolated Hnf polypeptide and an isolated first nucleic acid encoding the Hnf polypeptide; and (ii) a second agent selected from a second group consisting of an isolated Foxa polypeptide and an isolated second nucleic acid encoding the Foxa polypeptide. In a preferred embodiment, the composition further contains a third agent selected from a third group consisting of an isolated GATA4 polypeptide and an isolated third nucleic acid encoding the GATA4 polypeptide. Also featured is a kit having the composition and a starting cell.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIGs. la-f are diagrams and photographs of an experimental design (la), results showing that three transcription factors induce hepatic conversion of tail-tip fibroblasts (lb-d), and effects of individual factor withdrawal from 3TF on epithelial colony formation (If), where each scale bar represents 100 μm and the data are presented as mean ± s.d.

FIGs. 2a-l are diagrams and photographs of characterization of iHep cells in vitro, showing that 3TF-induced iHep cells had typical epithelial morphology (2a); that epithelial conversion of TTFs was confirmed by immunofluorescent staining of Tjpl and E-cadherin (2b and c); that expression of indicated genes was analyzed by RT-PCR during the induction of iHep cells (2d); global gene expression by cDNA microarray assay (2e);
glycogen storage shown by PAS staining (2f); Dil-ac-LDL uptake in iHep cells (2g); ICG uptake in iHep cells (2h); secretory albumin protein levels as measured by ELISA during hepatic conversion (2i); and CYP metabolic activities of iHep cells (2j-l). *: P<0.05, t-test.

All scale bars: 50 mm. Data are presented as mean±s.d. in 2i-l.

FIGs. 3a-d are diagrams and photographs showing: (a) a schematic outline of iHep cell transplantation into livers of Fah−/−Rag2−/− mice; (b) Kaplan-Meier survival curves of primary-hepatocyte-transplanted Fah−/−Rag2−/− mice ("Hepa-F/R," n=10), iHep-cell-transplanted Fah−/−Rag2−/− mice ("iHep-F/R," n=12), TTF-transplanted Fah−/−Rag2−/− mice ("TTF-F/R," n=6), and control Fah−/−Rag2−/− mice ("F/R," n=10) after NTBC withdrawal (*, P<0.02, log-rank test); (c) repopulation of iHep cells in Fah−/−Rag2−/− livers as determined by Fah immunostaining; and (d) Fah immuno-staining and Y-chromosome FISH staining of serial liver sections from male Fah−/−Rag2−/− mice transplanted with female iHep cells, where the boundary of the Fah+ nodule is indicated by a dashed yellow line.

FIGs. 4a-g are diagrams and photographs showing that iHep cells restored liver functions of Fah−/−Rag2−/− mice, including (a) representative photographs of whole livers from Fah−/−Rag2−/− and iHep- Fah−/−Rag2−/− mice; (b-f) diagrams showing serum levels of tyrosine (4b), phenylalanine (4c), total bilirubin (4d), ALT (4e) and AST (4f) in wild-type (n=6), Hepa-Fah−/−Rag2−/− (n=5), iHep- Fah−/−Rag2−/− (n=5, sera collected 8 weeks after iHep transplantation) and control Fah−/−Rag2−/− mice (n=4, sera collected upon losing 20% of body weight). (*: P<0.05, t-test. Data are presented as mean±s.d.); and (g) representative photographs of iHep and PLC/PRF/5 cells (human hepatoma cell line) that were subcutaneously transplanted into the left and right flanks of NOD/SCID mice, respectively, where PLC/PRF/5-generated tumours are indicated by the dotted ovals.

FIGs. 5a-c are photographs showing that (a) hepatic marker genes Albumin and Tdo2 were induced by a combination of 14 transcription factors in 3T3 cells, wildtype MEFs and TTFs 5 days after infection; (b) wildtype TTFs underwent proliferation arrest and cell death 7 days after transduction of 14TF, while epithelial cells were formed in pl9Arf−/−TTFs after 14TF transduction; and (c) expressions of hepatic genes Albumin, Tdo2 and Ttr were analyzed by RT-PCR in 14TF-transduced p19Arf−/−TTFs.

FIGs. 6a and b are photographs showing mRNA levels of exogenous hepatic transcription factors (a) and of hepatic genes (b) in individual epithelial colonies derived from 14TF-transduced pl9Arf−/−TTFs.

FIGs. 7a-d are diagrams and photographs showing: (a) expression of indicated genes as analyzed by RT-PCR in pl9Arf−/−TTFs after transduction by different
transcription factors; (b) and (c) effects of individual factor withdrawal from 6TF and 5TF on epithelial colony formation (data are presented as mean±s.d.); and (d) stronger expression of hepatic genes [Albumin, Tdo2, Transferrin and E-cadherin] induced by the combination of Gata4, Hnfla and Foxa3 than that of Gata4, Hnfla and Foxa2, where endogenous Foxa2 and Foxa3 were induced by combination of Gata4, Hnfla and Foxa3.

FIG. 8 is a set of photographs showing hepatic conversion of MEFs by Gata4, Hnfla and Foxa3, where hepatic genes were determined by RT-PCR in pl9Arf-knockdown MEFs with overexpression of Gata4, Hnfla and Foxa3.

FIGs. 9a-c are diagram and a set of photographs showing that pl9Arf knockdown facilitates hepatic conversion of wildtype TTFs, where (a) efficient shRNA-mediated pl9Arf knockdown (“pl9Arf-shRNA”) was confirmed by qRT-PCR in TTFs. (*: t-test, P<0.05); (b) TTFs with pl9Arf knockdown were induced to show epithelial morphology after 3TF transduction; and (c) hepatic genes were up-regulated in pl9Arf-knockdown TTFs after 3TF transduction.

FIGs. 10a-f are diagrams and photographs of hepatic gene expression study in iHep cells, where (a) albumin positive cells were determined by flow cytometry analysis in 3TF-transduced TTFs; (b) mRNA levels of indicated genes were measured by qRT-PCR in TTF cells, primary hepatocytes cultured for 6 days (“Hepa”) and iHep cells (*: t-test, P<0.05); (c) and (d) albumin and Hnf4a proteins were detected by immunofluorescent staining; (e) expressions of exogenous 3TF were measured by qRT-PCR during hepatic conversion; (f) five 3TF-induced iHep cell colonies were picked up for mRNA expression analysis of hepatic genes (Albumin, Transferrin, Cpsi, CK8, CK18, E-cadherin, Tjpl, Cldnl, Foxal, Hif fα and Afp) and fibroblast-enriched genes (Collal, Pdgfr$, Postn, Thy1 and Csf1) by RT-PCR.

FIGs. 11a-f are diagrams and photographs of comparison of iHep cells with other cell lineages, where (a) expressions of hepatoblast marker genes were determined by PCR during hepatic conversion; (b) iHep cells were pretreated with 50µM 3-methylcholanthrene for 48 hours and levels of Cypal, Cyla2, Cyp3al1 and Cyp3al3 were measured by qRT-PCR; (c) bile duct marker genes were analyzed by PCR; (d) bile duct cells formed branching structures in a 3-dimension culture system (arrow heads), while iHep cells stopped proliferation under this condition; (e) marker genes for pancreatic exocrine cells (Prssl, Cela2a and Amy2a5) and endocrine cells (InsI, Ins2 and Glucagon) were analyzed in iHep cells, TTFs, primary hepatocytes and pancreatic cells; and (f) expressions of intestine marker genes were determined by PCR. *: t-test, P<0.05.
FIG. 12 is diagrams showing qRT-PCR results that confirmed up-regulated mRNA expression of several CYP enzymes in iHep cells after Phenolbarbital treatment.

FIG. 13 is a diagram showing Cyp2d22 activities of iHep cells as measured by the production of Bufuralol metabolite, 1'-OH-Bufuralol (P<0.05, t-test).

FIGs. 14a-e are a diagram and a set of photographs showing: (a) repopulation of primary hepatocytes in \textit{Fah}^+\textit{Rag}^{2+} \textit{livers} as shown by Fah staining of the liver; (b) body weight measured every week after NTBC removal (data are presented as mean+s.d. \(*: P<0.03, t\)-test); (c) repopulation of iHep cells in \textit{Fah}^+\textit{Rag}^{2+} \text{iHep} cells in F/R liver sections (brown staining; pictures of 4 areas were merged into one using ADOBE PHOTOSHOP (ADOBE SYSTEMS)); (d) \textit{Fah} wildtype allele and \textit{pl9Arf} wildtype and null alleles as analyzed by PCR using genomic DNA extracted from liver sections; and (e) female iHep donor cells that were transplanted into male F/R recipient mice, where serial liver sections of 8 Fah-positive (Faff) nodules were shown (nodule #2 - #9), Fah\textsuperscript{+} nodules are Y-FISH negative (Y-FISH-) (nodule #2 and #3). Note the Y-FISH positive endothelial cells (arrowhead) and inflammatory cells (arrows) from host in the Fah\textsuperscript{+} nodules (nodule #4 - #6). Yellow dash lines indicate the boundaries of Fah\textsuperscript{+} nodules (nodule #7 - #9).

FIGs. 15a-g are diagrams and photographs for study of restoring liver functions of \textit{Fah}^+\textit{Rag}^{2+} mice by iHep cell transplantation showing: (a) representative pictures of H&E stained liver sections from F/R and iHep-F/R mice, with arrowheads indicating dead hepatocytes in F/R livers; (b) serial liver sections stained by H&E and Fah immunostaining with H&E staining showing normal hepatic architecture formed by Fah\textsuperscript{+} cells (scale bars. 200\mu m); (c) immunostaining for Fah and Albumin of livers re-populated by iHep cells or primary hepatocytes; (d) Fah\textsuperscript{+} nodules isolated by laser-captured microdissection from serial liver sections and mRNA levels of indicated genes measured in repopulated iHep cells and repopulated primary hepatocytes in F/R recipient livers; and (e-g) serum levels of ornithine, alanine and glycine in WT (n=6), Hepa-F/R (n=5), iHep-F/R (n=5, sera collected 8 weeks after iHep transplantation) and control F/R mice (n=4, sera collected upon losing 20\% of body weight).\(*: P<0.05, \text{f-test.}\)

FIGs. 16a and b are photographs and a diagram showing that iHep cells are not tumorigenic after transplantation, where (a) serial sections of F/R livers 8 weeks after iHep cell transplantation were stained by Fah and Ki67 and Fah\textsuperscript{+} iHep cells were negatively stained for Ki67; (b) karyotypes of iHep cells were analyzed by measurement of chromosome numbers during mitosis.
FIG. 17 is a set of photographs showing expression of hepatic genes, Albumin, Afp, Transferrin, Ttr and Tat as analyzed by RT-PCR using mRNAs isolated from 293FT cells 6 days after Lentiviral infection.

FIGS. 18A-E are photographs showing that epithelial iHep cells formation was induced in human 293 FT cells (B-D) or in primary pI9Arf-null mouse TTF cultures (E) by overexpression of human FOXA2, HNFIA and GATA4 (B), human FOXA3, HNFIA and GATA4 (C), mouse Foxa3, Hnfia and Gata4 (D), and human FOXA3, HNFIA and GATA4 (E), where 293 FT cells expressing GFP was used as a control (A).

FIG. 19 is a photograph showing that overexpression of human FOXA3, HNFIA, and GATA4 induced the formation of epithelial human iHep cells from primary human fetal skin fibroblasts.

**DETAILED DESCRIPTION OF THE INVENTION**

This invention is based, at least in part, on unexpected discoveries that non-liver cells (e.g., adult fibroblast cells) can be converted to hepatocyte-like cells via (i) over-expressing as few as 2 (e.g., Hnf and Foxa) or 3 (Hnf, Foxa and GATA) heterologous transcription factors and (ii) decreasing expression of a cell cycle inhibitor (e.g., p19<sup>Irf</sup>) thereby increasing cell proliferation and by-passing proliferation arrest and associated cell death.

A hepatocyte-like cell (iHep cell) refers to a cell displaying one or more properties that are characteristic of mature, parenchymal hepatocytes as disclosed below. Preferably, an iHep cell may display at least one, two, three, four, five or more of the following properties: ability to use pyruvate as a sole carbon source; phase I biotransformation capacity (e.g. ethoxyresorufin, pentoxyresorufin, testosterone); phase II biotransformation capacity (e.g. 1-chloro-2,4 dinitrobenzene, 1,2-dichloro-4-nitrobenzene, 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole, estradiol, estrogen), the presence of cytochrome P450 protein and gene expression; inducibility of phase I and phase II biotransformation enzymes (e.g. beta-naphthoflavone, phenobarbital, methylcholanthrene); albumin secretion, urea production, glycogen storage, the presence of the expression of one or more of endogenous ALB, AFP, gamma-glutaryltransferase, hepatocyte nuclear factor (HNF) la, HNF 1β, HNF 3a, HNF 3β, HNF 4, HNF-6, anti-trypsin, CX32, MRP2, C/EBPa, tranthyretin, CK-18 and/or CFTR; polygonal morphology. In one embodiment, iHep cells of this invention showed an expression profile and hepatic function close to those of mature hepatocytes where some CYP genes were not induced, and CK19 and Afp were
upregulated. The iHep cells are not identical to hepatocytes. The iHep cells of this invention are genetically stable and not prone to tumor formation. They can be used for disease modeling, transplantation, and tissue engineering.

As mentioned above, there is an unmet need for an unlimited source of human hepatocytes or hepatocyte-like cells. Differentiating human embryonic stem cells (hESCs) into hepatocytes or the like has been recently developed. Although these hESCs derived cells show typical morphology and phenotypes of human hepatocytes, their uses as patient-compatible hepatocytes or the like are limited by the number of hESC lines available. The success in generating induced pluripotent stem cell (iPSC) makes it possible to produce hepatocytes from patient’s own cells, when iPSCs are differentiated to hepatic endoderm. Yet, cells derived from either hESC or iPSC pose the concern for contamination of undifferentiated pluripotent stem cells that could form teratoma in vivo. Multipotential mesenchymal stem cells (MSCs), which show in vitro proliferation and multiple lineage differentiations, can be differentiated in vitro into hepatocyte-like cells with appropriate hepatic gene expression and functional attributes. However, the application of MSC-derived hepatocyte-like cells is limited by the low efficiency and a mixture of differentiated cells derived.

As disclosed herein, conversion of mouse tail-tip fibroblasts to induce exogenous hepatocyte-like (iHep) cells were established by over-expression of transcription factor Hnfla, Foxa3, Gata4 and inactivation of p19arp. It was found that epithelial colony from fibroblasts was induced as early as 5 days after transduction of transcription factors, and iHep cells were obtained and readily expandable. iHep cells appeared to be epigenetically stable since exogenous transcription factors were silenced after lineage conversion. Remarkably, iHep cells with expression profile close to mature hepatocytes showed multiple hepatic functions in vitro, such as glycogen storage, Albumin secretion, low-density-lipoprotein transportation and metabolism of xenobiotics. By rigorous analysis of lineage markers, fibroblasts were only converted to mature hepatic cells, but not to hepatic progenitor cells or other cell lineages.

As disclosed herein, transcription factors Foxa3 and optionally, Gata4, can act as pioneer factors to trigger a global chromatin modification during hepatic conversion (Zaret et al. Cold Spring Harb. Symp. Quant. Biol. 73, 119-126 (2008) and Cirillo et al. Mol. Cell 9. 279-289 (2002)) and Hnfla can stabilize the hepatic gene expression, as Hnfla, Foxa2 and Hnf4a occupy each other's promoters and maintain the hepatic phenotype
(Kyrzmi et al. Genes Dev. 20, 2293-2305 (2006) and Odom et al. Science 303, 1378-1381 (2004)). Proliferative iHep cells can be obtained by inactivating p9^r^, a key component of the cellular senescence pathway that inhibits induced pluripotent stem cell reprogramming (Li et al. Nature 460, 1136-1139 (2009)). Inactivating other components of this pathway, such as p38 (Hui et al. Nature Genet. 39, 741-749 (2007)), can also be used to facilitate hepatic conversion as disclosed herein.

Transcription Factors Useful for the Invention

Various transcription factors can be used in this invention to generate iHep cells. Examples of them include those of the hepatocyte nuclear factor (Hnf) 1 or 4 subfamily (e.g., Hnf1α and Hnf4α), the forkhead box A protein (Foxa) family (e.g., Foxa1, Foxa2, and Foxa3), and the GATA family (e.g., GATA4). Other examples include members of the Hlf, Hhex, Jarid2, Coup-TFI, Lrhl, Fxr, and Pxr family or sub-family. Listed in Table 1 below are mouse genes encoding exemplary members of the transcription factors. Homologous from other species (e.g., human or other mammals) can also be used.

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<th>SEQ ID NO for corresponding polypeptides</th>
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Mouse Hnf1α (SEQ ID NO: 1):
WSKLSQLQTLLAALLESGLKREAIQNLGEGYVLMGEGPLDKGESCNGSGRDGLTELPLNGGETSGEDDTDDDEDFAPLPILKELENLSPHEAANQWAKWKLQWQLQNGGSLPSQNLQGSGYWETPDWRVAKMVKSLQHQQNHQPREDWDTQGNSHLSQHQNLKTPMKTKQRAALYTVRQKEPAQTFHQGGLILIEEPTGDELPTKGRRRNFKWGPAQQLILFPQ

Table 1

Mouse Foxa3 (SEQ ID NO: 2):
MLGSVMEHDLAESYWYYEAGEVYSPVNVFVTMAPLNSYTLNPLSSYPDDGPGLQQASLPFTGLTEPAPTA

P1·GPTFFSL·GGTSGSGAGASYAQPQPGL·VHGKE^4AKGYRPLAHAKPYPYISILTMIQAPGRMLTL·SE
IYQWMLDLFPYRENQQRQWQNSHRSLSFNCDFVKVARSFDKPKGKSYWALHPSGNNMFENCGYRQKRFK

KO2012058868 PCT/CN2011/001857
Mouse Gata4 (SEQ ID NO: 3):

LEEKAKKGNSATSASRNGTAGSATSATTTAATAVTSPAQPQPTPSEPEAQSDDVGLDCAEPPSSSTYPFGSLFEGELKLDAPYNNHPSINNLMSQETSTPSKDLVFGGYGAESGEGYVOSLYSRLNAS

Mouse Foxa1 (SEQ ID NO: 4):

LEELPGELKLDAPYNFNHPFSINNLMSEQTSTPSKLDVGFGGYGAESGEFGYVOSLYSRLNAS

Mouse Foxa2 (SEQ ID NO: 5):

LEELPGELKLDAPYNFNHPFSINNLMSEQTSTPSKLDVGFGGYGAESGEFGYVOSLYSRLNAS

Mouse Hnf4a (SEQ ID NO: 6):

LEELPGELKLDAPYNFNHPFSINNLMSEQTSTPSKLDVGFGGYGAESGEFGYVOSLYSRLNAS

Mouse Hnf6 (SEQ ID NO: 7):

LEELPGELKLDAPYNFNHPFSINNLMSEQTSTPSKLDVGFGGYGAESGEFGYVOSLYSRLNAS

Mouse Hlf (SEQ ID NO: 8):

LEELPGELKLDAPYNFNHPFSINNLMSEQTSTPSKLDVGFGGYGAESGEFGYVOSLYSRLNAS

Mouse Hhex (SEQ ID NO: 9):

LEELPGELKLDAPYNFNHPFSINNLMSEQTSTPSKLDVGFGGYGAESGEFGYVOSLYSRLNAS
Mouse Jarid2 (SEQ ID NO: 10):

MSKERPKRNI IQKKYDDSDGI PSSERWKVLKLYSLEKFNKQAQRKHQQGEGLASLKVNGGLNAFAQKALG

Mouse Coup-TF1 (SEQ ID NO: 11):

MAMWSSSRDDQRDDVAGGNPGGPAAQAARGGGGGEQQQAGSGAPHTPQTPGQPGAPATPGTAGDKGQGPP

Mouse Lrh (SEQ ID NO: 12):

MSASLDTGDFQFLKHGLTIAASAPGETRSHSPKREEQLREKARLPPHRRPAPAQRSRLLLLKFEVTEAPG

Mouse Fxr (SEQ ID NO: 13):

MVMQFGGLNPQ1Q1SLHSHSRLSGVFEPMGSMVFPKGLMLTEHAAFLQGQLQIDLELYSPNYNVFPFQQVPQGIS

Mouse Pxr (SEQ ID NO: 14):

MRPEESWSRVGLVQCEEADSALEEPINVEEEDGGLQICRVCGDKANGYHFNVMTCEGCKGFFRRAMKRNVRL

Mouse Pcx (SEQ ID NO: 15):

MVMQFGGLNPQ1Q1SLHSHSRLSGVFEPMGSMVFPKGLMLTEHAAFLQGQLQIDLELYSPNYNVFPFQQVPQGIS

Listed below are some of the cDNA sequences that can be used in this invention.

cDNA sequence for mouse Foxa3 gene, which encodes mouse Foxa3 protein:

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cDNA sequence for mouse Foxa3 gene, which encodes mouse Foxa3 protein:

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002  10  gtaagacactg  ggtctccccca  gggggccctc  agcctggtgt  tgtggggggc  cagggccccc
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015  75  ggcctgtaca  ttggaagagg  atggccaacc  ggtacccggg  gcaactctgcc  caaccacaacc
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100 100  ggccatggac  acctataacg  ttgccaaccg  gcgcaaggag  gaagccttcc  ggcacaagtt
101 105  ggcctgtaca  ttggaagagg  atggccaacc  ggtacccggg  gcaactctgcc  caaccacaacc
102 110  caccattcct  ctactacctc  ctctactacc  aagccttgct  aggctctgct  tattctcctc
103 115  gtaacctcgag  tggggaagtt  ccaccctgtc  ggtgtcttttt  gttcgtgatgt  gtgcgggctg
104 120  gctggtcagt  gggggactga  ggctagtgtg  gaccctatga  aggccagccc  ccagggccgg
105 125  ggtggctctg  ggtggctctg  ggtggctctg  ggtggctctg  ggtggctctg  ggtggctctg
106 130  ggccatggac  acctataacg  ttgccaaccg  gcgcaaggag  gaagccttcc  ggcacaagtt
107 135  ggcctgtaca  ttggaagagg  atggccaacc  ggtacccggg  gcaactctgcc  caaccacaacc
108 140  caccattcct  ctactacctc  ctctactacc  aagccttgct  aggctctgct  tattctcctc
109 145  gtaacctcgag  tggggaagtt  ccaccctgtc  ggtgtcttttt  gttcgtgatgt  gtgcgggctg
110 150  gctggtcagt  gggggactga  ggctagtgtg  gaccctatga  aggccagccc  ccagggccgg
111 155  ggtggctctg  ggtggctctg  ggtggctctg  ggtggctctg  ggtggctctg  ggtggctctg
112 160  ggccatggac  acctataacg  ttgccaaccg  gcgcaaggag  gaagccttcc  ggcacaagtt
113 165  ggcctgtaca  ttggaagagg  atggccaacc  ggtacccggg  gcaactctgcc  caaccacaacc
114 170  caccattcct  ctactacctc  ctctactacc  aagccttgct  aggctctgct  tattctcctc
115 175  gtaacctcgag  tggggaagtt  ccaccctgtc  ggtgtcttttt  gttcgtgatgt  gtgcgggctg
116 180  gctggtcagt  gggggactga  ggctagtgtg  gaccctatga  aggccagccc  ccagggccgg
117 185  ggtggctctg  ggtggctctg  ggtggctctg  ggtggctctg  ggtggctctg  ggtggctctg
118 190  ggccatggac  acctataacg  ttgccaaccg  gcgcaaggag  gaagccttcc  ggcacaagtt
119 195  ggcctgtaca  ttggaagagg  atggccaacc  ggtacccggg  gcaactctgcc  caaccacaacc
120 200  caccattcct  ctactacctc  ctctactacc  aagccttgct  aggctctgct  tattctcctc
121 205  gtaacctcgag  tggggaagtt  ccaccctgtc  ggtgtcttttt  gttcgtgatgt  gtgcgggctg
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ID NO: 19
cDNA sequence for mouse Gata4 gene, which encodes mouse Gata4 protein:

1 aggggacaag cccggagggc gcaggactgc cggtctggtc acaagtcttc cccggcctgc
2 tcagctccacc ggggccaggc ccgggccctg cgctgcctgc tcacagttcc cccggcctgc
3 ccacaaccac cctctctccc agtaaggtcc acggtgtacg cactctggag gcgagatggg acgggacact acctgtgcaa
4 tgcctgtggc ctctatcaca agatgaacgg catcaaccgg cccctcatta agcctcagcg
tgcctgtggc ctctatcaca agatgaacgg catcaaccgg cccctcatta agcctcagcg
tgcctgtggc ctctatcaca agatgaacgg catcaaccgg cccctcatta agcctcagcg
tgcctgtggc ctctatcaca agatgaacgg catcaaccgg cccctcatta agcctcagcg
tgcctgtggc ctctatcaca agatgaacgg catcaaccgg cccctcatta agcctcagcg
tgcctgtggc ctctatcaca agatgaacgg catcaaccgg cccctcatta agcctcagcg
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tgcctgtggc ctctatcaca agatgaacgg catcaaccgg cccctcatta agcctcagcg
cDNA sequence for human Foxa3 gene, which encodes human Foxa3 protein (NM_004497.2):

```
1  ggagcccggg gcgcccggag gcgcgggttg cacccgctata aagcgtggcc gcctccccgc
61 gcgcctggca gcagcttacc gcgggggtcg gcgggtgggt gcggggtttg gcggggtttg

121 cggtccccgt gcagcagctt gcagctgctt tcggggaagg cgggctggag cgttgccccac
gggggtggga gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg

181 ggaggggggg ctggggtctt tcggggcccct gcgggggttg gcgggggggg gcgggggggg
241 gcggcgtctt gcgggggtgc gcgggggttg gcgggggggg gcgggggggg gcgggggggg

301 cggccctctt ccggcgctgg ccgggggttg gcgggggggg gcgggggggg gcgggggggg
361 tccctgcacc cccctgtaca gcaggggggg gcgggggggg gcgggggggg gcgggggggg

421 ggcccacctg gcggggggtg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
481 ggggggcggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg

541 aacggggcct gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
601 ggcagctgg cagcagcagc gagcctggag ccgggggggg gcgggggggg gcgggggggg

661 gggaggaatc gcggggggtg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
721 tcggggtcat gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg

781 ccggccacgt gcagggggtc gcaggggggg gcgggggggg gcgggggggg gcgggggggg
841 ggcccgcccg gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
901 gtctgtgtgc gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
961 cctcccaggg cggcccaggag gcgggggggg gcgggggggg gcgggggggg gcgggggggg
```

```
55 tccctgcacc cccctgtaca gcaggggggg gcgggggggg gcgggggggg gcgggggggg
61 tccctgcacc cccctgtaca gcaggggggg gcgggggggg gcgggggggg gcgggggggg

121 cggtccccgt gcagcagctt gcagctgctt tcggggaagg cgggctggag cgttgccccac
gggggtggga gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg

181 ggaggggggg ctggggtctt tcggggcccct gcgggggttg gcgggggggg gcgggggggg
241 gcggcgtctt gcgggggtgc gcgggggttg gcgggggggg gcgggggggg gcgggggggg
```

```
301 cggccctctt ccggcgctgg ccgggggttg gcgggggggg gcgggggggg gcgggggggg
361 tccctgcacc cccctgtaca gcaggggggg gcgggggggg gcgggggggg gcgggggggg

421 ggcccacctg gcggggggtg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
481 ggggggcggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg

541 aacggggcct gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
601 ggcagctgg cagcagcagc gagcctggag ccgggggggg gcgggggggg gcgggggggg
```

```
661 gggaggaatc gcggggggtg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
721 tcggggtcat gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
```

```
781 ccggccacgt gcagggggtc gcaggggggg gcgggggggg gcgggggggg gcgggggggg
841 ggcccgcccg gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
```

```
901 gtctgtgtgc gcgggggggg gcgggggggg gcgggggggg gcgggggggg gcgggggggg
961 cctcccaggg cggcccaggag gcgggggggg gcgggggggg gcgggggggg gcgggggggg
```
1441 acattttggt ggcccactgg gtactgtgag gactgctaca ttgatggatg ttattggcta
1501 atccacctga tgtggtaagat gccacactct cgggtttccc tattttgggg tattaagatag
1561 cattttctgg atcgtcctgtag tggccccccc catttaagtg gccggccactc ttctttttggg
1621 tgtacctggc acagcttgggt cccacttctg ccccttggg gctgaacact tttttggggcc
1681 atgtgggtct tgtgagcaac tcatactgag tagtggcaaa cgtcagcttg ccaccaatgtg
1741 cccatggctgc tgtgctggcccc cgggtgtgctg cattgtgctt caacccctgtgc ttgctgggtt
1801 tgtgctggagtg ttcagaattgta gataactcct tgtgttcttc tgtaagccacc cctttttccc
1861 aactctgttgc ctcgagacca ccaagaaaaag tctgtaggat gtggaggtat tgtactaag
1921 tctgatctct ctcgagagct ctcgagactg tgtctgtttaa atcattaaag gctacgctgct
1981 cgcccttttaa aaaaaa aaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa
2041 aaaaaa (SEQ ID NO: 22)

cDNA sequence for human HNFla gene, which encodes human HNFla protein
(NM_000545.5):

1 cgtgagcgcc ctggagctgac gccacagtgtt ctaaaactag ccaatctgca cagggagctc
61 tgcctgcttg ggctagtca cggctggagc actggagccag tggggctgagt cggctggagc
120 gcagctgcag gtaaattcgc acacccagct gttcagcgcag ggttggatgc gccctgcttg
180 tgcctgcttg ggctagtca cggctggagc actggagccag tggggctgagt cggctggagc
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2220 aagcttggagct ggcttggagtgc ttcgagctgg cggctggagc actggagccag tggggctgagt cggctggagc
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2640 aagcttggagct ggcttggagtgc ttcgagctgg cggctggagc actggagccag tggggctgagt cggctggagc
2700 aagcttggagct ggcttggagtgc ttcgagctgg cggctggagc actggagccag tggggctgagt cggctggagc
2760 aagcttggagct ggcttggagtgc ttcgagctgg cggctggagc actggagccag tggggctgagt cggctggagc

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595
610
cDNA sequence for human *Gata4* gene, which encodes human *Gata4* protein (NM_002052.3):

1. ttggaggccg ccggcgcagg gcggcgagga ggcttccgct ccgctcagc tcgcggggtc
2. 61 ccacagggag cgctgccccg ccacccagac ccagccgccc gcgcggacg ggcagagaga
3. 121 agggacccag ctgcagcctt ctgcttcagc tcgcggggtc
4. 181 tcgtccgagt gggcgccgaa gcggcgcggc gcggcgcggc gcggcgcggc gcggcgcggc
5. 241 ctgggggcttc ccgctcggcc ccgctcggcc ccgctcggcc ccgctcggcc ccgctcggcc
6. 301 ctggggtctt ccgctcggcc ccgctcggcc ccgctcggcc ccgctcggcc ccgctcggcc
7. 361 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
8. 421 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
9. 481 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
10. 541 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
11. 601 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
12. 661 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
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15. 841 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
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19. 1081 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
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22. 1261 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
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33. 1921 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
34. 1981 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
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53. 3121 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
54. 3181 gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag gcagcagcag
55. 3241 a (SEQ ID NO: 23)
Members of the Hnf 1 subfamily are transcription factors that contain a POU-homeodomain and bind to DNA as homodimers. Among them, Hnf1a is highly expressed in the liver and is involved in the regulation of the expression of several liver-specific genes. Members of the Hnf4 subfamily are nuclear receptors and bind to DNA either as homodimers or as RXR heterodimers. Hnf4a, as a transcription factor, binds DNA as a homodimer, and controls the expression of several genes, including Hnf1a. This transcription factor plays a role in the development of the liver, kidney, and intestines.

Alternative splicing of this gene results in multiple transcript variants.

Forced box protein families are a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity. They are a subgroup of the helix-turn-helix class of proteins. The defining feature of these proteins is the forkhead box, a sequence of 80 to 100 amino acids forming a motif that
binds to DNA. This forkhead motif is also known as the winged helix due to the butterfly-like appearance of the loops in the protein structure of the domain. Foxa1, Foxa2, and Foxa3, also known as Hnfα, β, and γ, respectively, are members of the forkhead class of DNA-binding proteins. They are transcriptional activators for liver-specific transcripts such as albumin and transthyretin, and they also interact with chromatin.

GATA transcription factors are a family of zinc finger transcription factors. Members of this family recognize the GATA motif which is present in the promoters of many genes. Among them, GATA4 protein is known to regulate genes involved in embryogenesis and in myocardial differentiation and function. Mutations in this gene have been associated with cardiac septal defects.

As used herein, a particular transcription factor polypeptide(s) (e.g., a Hnf polypeptide, a Foxa polypeptide, a GATA4 polypeptide) refer a member(s) of a particular transcription factor family (e.g., one of the above-mentioned families), which include the corresponding transcription factor(s) described above, their homologous, polypeptide(s) having sequences thereof, and their mutant forms that retain substantial their transcription factor functions.

As disclosed herein, a forced expression of members of two or three of the above transcription factor families or subfamilies was sufficient to convert non-liver cells (such as adult fibroblast cells) to iHep cells. Accordingly, this invention provides agents that can convert non-liver cells to iHep cells, thereby supplying an unlimited cell source for modeling and understanding liver diseases, drug efficacy and toxicity testing, and cell replacement therapy.

Both polypeptides of the aforementioned transcription factors and nucleic acid encoding the polypeptides can be used to practice the invention. While many polypeptide preparations can be used, a highly purified or isolated polypeptide is preferred. The terms "peptide," "polypeptide," and "protein" are used herein interchangeably to describe the arrangement of amino acid residues in a polymer. A peptide, polypeptide, or protein can be composed of the standard 20 naturally occurring amino acid, in addition to rare amino acids and synthetic amino acid analogs. They can be any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

The peptid, polypeptide, or protein "of this invention" include recombinantly or synthetically produced fusion or chimeric versions of any of the aforementioned transcription factors, having the particular domains or portions that bind to the DNA site of the transcription factor and regulates the expression of a target gene of the transcription
factor. The term also encompasses polypeptides that have an added amino-terminal methionine (useful for expression in prokaryotic cells).

Within the scope of this invention are fusion proteins containing one or more of the afore-mentioned sequences and a heterologous sequence. A "chimeric" or "fusion" refers to the combination of amino acid sequences of different origin in one polypeptide chain by in-frame combination of their coding nucleotide sequences. The term explicitly encompasses internal fusions, i.e., insertion of sequences of different origin within a polypeptide chain, in addition to fusion to one of its termini. A heterologous polypeptide, nucleic acid, or gene is one that originates from a foreign species, or, if from the same species, is substantially modified from its original form. Two fused domains or sequences are heterologous to each other if they are not adjacent to each other in a naturally occurring protein or nucleic acid.

An "isolated" or "purified" peptide, polypeptide, or protein refers to a peptide, polypeptide, or protein that has been separated from other proteins, lipids, and nucleic acids with which it is naturally associated. The polypeptide/protein can constitute at least 10% (i.e., any percentage between 10% and 100%, e.g., 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, and 99%) by dry weight of the purified preparation. Purity can be measured by any appropriate standard method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. An isolated polypeptide/protein described in the invention can be purified from a natural source, produced by recombinant DNA techniques, or by chemical methods.

A "recombinant" peptide, polypeptide, or protein refers to a peptide, polypeptide, or protein produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired peptide. A "synthetic" peptide/polypeptide/protein refers to a peptide/polypeptide/protein prepared by chemical synthesis. The term "recombinant" when used with reference, e.g., to a cell, nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified.

"Overexpression" refers to the expression of a RNA or polypeptide or protein encoded by a DNA introduced into a host cell, wherein the RNA or polypeptide or protein is either not normally present in the host cell, or wherein the RNA or polypeptide or protein is present in said host cell at a higher level than that normally expressed from the endogenous gene encoding the RNA or polypeptide or protein.
The amino acid composition of each of the above-mentioned peptides/polypeptides/proteins may vary without disrupting their transcription factor functions - the ability to bind to a DNA site and enhance or inhibit the respective target gene expression. For example, it can contain one or more conservative amino acid substitutions. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), β-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in one of the above-described transcription factors (e.g., SEQ ID NOs: 1-14) is preferably replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of the sequences, such as by saturation mutagenesis, and the resultant mutants can be screened for the ability to bind to the respective DNA site(s) and trigger the respective cellular response to identify mutants that retain the activity as described below in the examples.

A functional equivalent of a peptide, polypeptide, or protein of this invention refers to a polypeptide derivative of the peptide, polypeptide, or protein, e.g., a protein having one or more point mutations, insertions, deletions, truncations, a fusion protein, or a combination thereof. It retains substantially the activity to of the above-mentioned transcription factors. The isolated polypeptide of this invention can contain one of SEQ ID NOs: 1-14, or a functional equivalent or fragment thereof. In general, the functional equivalent is at least 75% (e.g., any number between 75% and 100%, inclusive, e.g., 70 %, 80%, 85%, 90%, 95%, and 99%) identical to one of SEQ ID NOs. 1-14.

A polypeptide described in this invention can be obtained as a recombinant polypeptide. For example, to prepare a recombinant polypeptide, a nucleic acid encoding it can be linked to another nucleic acid encoding a fusion partner, e.g., glutathione-s-transferase (GST), 6x-His epitope tag, or M13 Gene 3 protein. The resultant fusion nucleic acid expresses in suitable host cells a fusion protein that can be isolated by methods known in the art. The isolated fusion protein can be further treated, e.g., by enzymatic digestion, to remove the fusion partner and obtain the recombinant polypeptide.

Due to their functions as transcription factors, the above-disclosed polypeptides can be associated with, e.g., conjugated or fused to, one or more of an amino acid sequence comprising a nuclear localization signal (NLS), a cell-penetrating peptide (CPP) sequence, and the like. In this manner, a composition of the invention as discussed below can include a transport enhancer. For example, the composition may include a penetration enhancing agent, such as MSM, for the delivery of the transcription factors or related therapeutic polypeptides to a cell and/or through the cell membrane and into the nucleus of the cell. The transcription factors then function to regulate transcription of target genes, thereby resulting in an induction of iHep cells. The transcription factors may be delivered by itself or as a fusion with one or more of an NLS, CPP, and/or other domains. See, e.g., Tachikawa et al. PNAS (2004) vol. 101, no. 42:15225-15230.

A cell-penetrating peptide (CPP) generally consists of less than 30 amino acids and has a net positive charge. CPPs internalize in living animal cells in vitro and in vivo in an endocytotic or receptor/energy-independent manner. There are several classes of CPPs with various origins, from totally protein-derived CPPs via chimeric CPPs to completely synthetic CPPs. Examples of CPPs are known in the art. See, e.g., U.S. Application Nos. 20090099066 and 20100279918. It is know that CPPs can delivery an exogenous protein to various cells.

Although the above-described transcription factors to be delivered to a cell may be fusion proteins including a NLS and/or CPP, in certain instances, the protein does not include an NLS and/or a CPP as the transport enhancer may serve the function of delivering the biologically active agent directly to the cell, and/or through the cell membrane into the cytoplasm of the cell and/or into the nucleus of the cell as desired. For instance, in certain instances, it may be desirable to deliver a biologically active protein to the cell wherein the protein is not conjugated or fused to another molecule. In such an instance, any biologically active protein may be delivered directly in conjunction with the transport enhancer.
All of naturally occurring versions, genetic engineered versions, and chemically synthesized versions of the above-mentioned transcription factors can be used to practice the invention disclosed therein. Polypeptides obtained by recombinant DNA technology may have the same amino acid sequence as a naturally occurring version (e.g., one of SEQ ID NOs: 1-14) or a functionally equivalent thereof. They also include chemically modified versions. Examples of chemically modified polypeptides include polypeptides subjected to conformational change, addition or deletion of a side chain, and those to which a compound such as polyethylene glycol has been bound. Once purified and tested by standard methods or according to the method described in the examples below or other methods known in the art, the polypeptides can be included in suitable composition.

For expressing the above-mentioned transcription factors, the invention provides a nucleic acid that encodes any of the polypeptides mentioned above. Preferably, the nucleotide sequences are isolated and/or purified. A nucleic acid refers to a DNA molecule (e.g., but not limited to, a cDNA or genomic DNA), an RNA molecule (e.g., but not limited to, an mRNA), or a DNA or RNA analog. A DNA or RNA analog can be synthesized from nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded. An "isolated nucleic acid" is a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid. The term therefore covers, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule but is not flanked by both of the coding sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein.

The present invention also provides recombinant constructs having one or more of the nucleotide sequences described herein. Example of the constructs include a vector, such as a plasmid or viral vector, into which a nucleic acid sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred embodiment, the construct further includes regulatory sequences, including a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. Appropriate cloning and expression vectors for
use with prokaryotic and eukaryotic hosts are also described in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press).

Examples of expression vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of or Simian virus 40 (SV40), bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, a nucleic acid sequence encoding one of the polypeptides described above can be inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and related sub-cloning procedures are within the scope of those skilled in the art.

The nucleic acid sequence in the aforementioned expression vector is preferably operatively linked to an appropriate transcription control sequence (promoter) to direct mRNA synthesis. Examples of such promoters include: the retroviral long terminal (LTR) or SV40 promoter, the E. coli lac or trp promoter, the phage lambda PL promoter, and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or viruses. The expression vector can also contain a ribosome binding site for translation initiation, and a transcription terminator. The vector may include appropriate sequences for amplifying expression. In addition, the expression vector preferably contains one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell cultures, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate nucleic acid sequences as described above, as well as an appropriate promoter or control sequence, can be employed to transform an appropriate host to permit the host to express the polypeptides described above (e.g., one of SEQ ID NOs: 1-14). Such vectors can be used in gene therapy. Examples of suitable expression hosts include bacterial cells (e.g., E. coli, Streptomyces, Salmonella typhimurium), fungal cells (yeast), insect cells (e.g., Drosophila and Spodoptera frugiperda (Sf9)), animal cells (e.g., CHO, COS, and HEK 293), adenoviruses, and plant cells. The selection of an appropriate host is within the scope of those skilled in the art. In some embodiments, the present invention provides methods for producing the above mentioned polypeptides by transfecting a host cell with an expression vector having a
nucleotide sequence that encodes one of the polypeptides. The host cells are then cultured under a suitable condition, which allows for the expression of the polypeptide.

As mentioned above, a nucleic acid sequence of this invention can be a DNA or RNA. The terms "RNA," "RNA molecule," and "ribonucleic acid molecule" are used interchangeably herein, and refer to a polymer of ribonucleotides. The term "DNA" or "DNA molecule" or "deoxyribonucleic acid molecule" refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally (e.g., by DNA replication or transcription of DNA, respectively). RNA can be post-transcriptionally modified. DNA and RNA also can be chemically synthesized. DNA and RNA can be single-stranded (i.e., ssRNA and ssDNA, respectively) or multi-stranded (e.g., double-stranded, i.e., dsRNA and dsDNA, respectively).

Starting Cells

As disclosed herein, the invention provides methods of generating iHep cells from non-liver cells (i.e., the starting cells). In one example, the methods involve introducing into starting cells heterologous transcription factors discussed above or nucleic acids encoding them so that the starting cells over-express the transcription factors. See, e.g., FIG. 1a. The modified starting cells are then cultured for a period of time, e.g., 14-21 days to generate iHep cells.

Various cells from a subject or animal can be used as the starting cells. In some embodiments, the starting cells are stem cells. The stem cells useful for the method described herein include but not limited to embryonic stem cell, mesenchymal stem cells, bone-marrow derived stem cells, hematopoietic stem cells, chondrocytes progenitor cells, epidermal stem cells, gastrointestinal stem cells, neural stem cells, hepatic stem cells, adipose-derived mesenchymal stem cells, pancreatic progenitor cells, hair follicular stem cells, endothelial progenitor cells, and smooth muscle progenitor cells. The stem cells can be pluripotent or multipotent. In some embodiments, the stem cell is an adult, fetal or embryonic stem cell. The stem cells can be isolated from umbilical, placenta, amniotic fluid, chorion villi, blastocysts, bone marrow, adipose tissue, brain, peripheral blood, blood vessels, skeletal muscle, and skin.

In some embodiments, the starting cells are differentiated cells. Examples include a fibroblast, an epithelium cell, a blood cell, a neuron, an embryonic cell, or a cell derived from a tissue or organ of a subject. These differentiated cells differ from stem cells in that differentiated cells generally do not undergo self-renewing proliferation while stem cells.
can undergo self-renewing cell division to give rise to phenotypically and genotypically identical daughters for an indefinite time and ultimately can differentiate into at least one final cell type.

The terms "proliferation" and "expansion" as used interchangeably herein refer to an increase in the number of cells of the same type by division. The term "differentiation" refers to a developmental process whereby cells become specialized for a particular function, for example, where cells acquire one or more morphological characteristics and/or functions different from that of the initial cell type. The term includes both lineage commitment and terminal differentiation processes. Differentiation may be assessed, for example, by monitoring the presence or absence of lineage markers, using immunohistochemistry or other procedures known to a skilled in the art. Differentiated progeny derived from progenitor cells may be, but are not necessarily, related to the same germ layer or tissue as the source tissue of the stem cells. For example, neural progenitor cells and muscle progenitor cells can differentiate into hematopoietic cell lineages.

To convert the differentiated cells to iHep cells, one needs to reprogram the differentiated cells so that they proliferate. This can be achieved by inactivating or down-regulating one or more components of the cellular senescence pathway that inhibits induced pluripotent stem cell reprogramming, such as pi9\(^{\beta\gamma}\) and p38 (Li, H. et al. Nature 460, 1136-1139 (2009); Hui, L. et al. Nature Genet. 39, 741-749 (2007)). Listed below are the polypeptide and cDNA sequences for one exemplary pi9\(^{\beta\gamma}\) (GenBank NM_009877):

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MGRRFLVTVVRQAGRPLQEDVFLKVFVRSSRPRPTASCATAFVNMLLRLERILRRGPHRNPGPDGDQGSR
SSSSAQLCRFLRPLYPHLYLGARRSAGRILPHAGGAAVRGGSACARCLGSPAARLGPAGTSHRAI
FRWLVFVYRNLRRPDRRA (SEQ ID NO: 17)
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1 tctcagaggtg cctcatactc gaaaggggcctt ggggggcggcgc ttctcacttc gcttgctca
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
121 tgttaggatcc agcgccgccc gcgccgctctg caagagaggggt ttcttcttttt gtatgatgg
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
181 ctatgcagagga ctctgcccaac gctctggcttc cttcttcttt ttcttcttttt ggcacgggacc
cgcggggggtt cttcgctgggt cttcgctgggt cttcgctgggt cttcgctgggt
241 cgttaggatcc agcgccgccc gcgccgctctg caagagaggggt ttcttcttttt gtatgatgg
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
301 caagctgttca gtagcagctct ttctgcgttact ttagaggatgc ggcacgggacc
cgcggggggtt cttcgctgggt cttcgctgggt cttcgctgggt cttcgctgggt
361 cagctgttca gtagcagctct ttctgcgttact ttagaggatgc ggcacgggacc
cgcggggggtt cttcgctgggt cttcgctgggt cttcgctgggt cttcgctgggt
421 cgttaggatcc agcgccgccc gcgccgctctg caagagaggggt ttcttcttttt gtatgatgg
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
481 cagctgttca gtagcagctct ttctgcgttact ttagaggatgc ggcacgggacc
cgcggggggtt cttcgctgggt cttcgctgggt cttcgctgggt cttcgctgggt
541 cgttaggatcc agcgccgccc gcgccgctctg caagagaggggt ttcttcttttt gtatgatgg
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
601 cagctgttca gtagcagctct ttctgcgttact ttagaggatgc ggcacgggacc
cgcggggggtt cttcgctgggt cttcgctgggt cttcgctgggt cttcgctgggt
661 cagctgttca gtagcagctct ttctgcgttact ttagaggatgc ggcacgggacc
cgcggggggtt cttcgctgggt cttcgctgggt cttcgctgggt cttcgctgggt
721 cgttaggatcc agcgccgccc gcgccgctctg caagagaggggt ttcttcttttt gtatgatgg
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
781 cagctgttca gtagcagctct ttctgcgttact ttagaggatgc ggcacgggacc
cgcggggggtt cttcgctgggt cttcgctgggt cttcgctgggt cttcgctgggt
841 cgttaggatcc agcgccgccc gcgccgctctg caagagaggggt ttcttcttttt gtatgatgg
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
ggagggcgtg cacggggggctt ggggggcggcgc ttctcacttc gcttgctca
901 cagctgttca gtagcagctct ttctgcgttact ttagaggatgc ggcacgggacc
cgcggggggtt cttcgctgggt cttcgctgggt cttcgctgggt cttcgctgggt
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(SEQ ID NO: 18)
Various means can be used for that purpose. In one embodiment, one can use the RNA interference (RNAi) technology or antisense technology. For example, one can generate a nucleic acid sequence that encode a small interference RNA (e.g., an RNAi agent) that targets one or more of genes encoding a component of the cellular senescence pathway and inhibits its expression or activity.

The term "RNAi agent" refers to an RNA, or analog thereof, having sufficient sequence complementarity to a target RNA to direct RNA interference. Examples also include a DNA that can be used to make the RNA. RNA interference (RNAi) refers to a sequence-specific or selective process by which a target molecule (e.g., a target gene, protein or RNA) is down-regulated. Generally, an interfering RNA ("iRNA") is a double stranded short-interfering RNA (siRNA), short hairpin RNA (shRNA), or single-stranded micro-RNA (miRNA) that results in catalytic degradation of specific mRNAs, and also can be used to lower or inhibit gene expression.

The term "short interfering RNA" or "siRNA" (also known as "small interfering RNAs") refers to an RNA agent, preferably a double-stranded agent, of about 10-50 nucleotides in length, preferably between about 15-25 nucleotides, more preferably about 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, the strands optionally having overhanging ends comprising, for example 1, 2 or 3 overhanging nucleotides (or nucleotide analogs), which is capable of directing or mediating RNA interference.

Naturally-occurring siRNAs are generated from longer dsRNA molecules (e.g., >25 nucleotides in length) by a cell’s RNAi machinery (e.g., Dicer or a homolog thereof).

The term "shRNA" refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region.

The term "miRNA" or "microRNA" refers to an RNA agent, preferably a single-stranded agent, of about 10-50 nucleotides in length, preferably between about 15-25 nucleotides in length, more preferably about 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, which is capable of directing or mediating RNA interference. Naturally-occurring miRNAs are generated from stem-loop precursor RNAs (i.e., premiRNAs) by Dicer. The term microRNA (or "miRNA") is used interchangeably with the term "small temporal RNA" (or "stRNA") based on the fact that naturally-occurring
microRNAs (or "miRNAs") have been found to be expressed in a temporal fashion (e.g., during development).

Thus, also within the scope of this invention is utilization of RNAi featuring degradation of RNA molecules (e.g., within a cell). Degradation is catalyzed by an enzymatic, RNA-induced silencing complex (RISC). A RNA agent having a sequence sufficiently complementary to a target RNA sequence (e.g., one or more of the above-mentioned genes of the cellular senescence pathway) to direct RNAi means that the RNA agent has a homology of at least 50%, (e.g., 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or 100% homology) to the target RNA sequence so that the two are sufficiently complementary to each other to hybridize and trigger the destruction of the target RNA by the RNAi machinery (e.g., the RISC complex) or process. A RNA agent having a "sequence sufficiently complementary to a target RNA sequence to direct RNAi" also means that the RNA agent has a sequence sufficient to trigger the translational inhibition of the target RNA by the RNAi machinery or process. A RNA agent also can have a sequence sufficiently complementary to a target RNA encoded by the target DNA sequence such that the target DNA sequence is chromatically silenced. In other words, the RNA agent has a sequence sufficient to induce transcriptional gene silencing, e.g., to down-modulate gene expression at or near the target DNA sequence, e.g., by inducing chromatin structural changes at or near the target DNA sequence.

The above-mentioned polynucleotides can be delivered to cells in vitro or in vivo using polymeric, biodegradable microparticle or microcapsule delivery devices known in the art. Another way to achieve uptake of the polynucleotides is using liposomes, prepared by standard methods. The polynucleotide can be incorporated alone into these delivery vehicles or co-incorporated with tissue-specific antibodies. Alternatively, one can prepare a molecular conjugate composed of a plasmid or other vector attached to poly-L-lysine by electrostatic or covalent forces. Poly-L-lysine binds to a ligand that can bind to a receptor on target cells (Cristiano, et al., 1995, J. Mol. Med. 73:479). Alternatively, tissue specific targeting can be achieved by the use of tissue-specific transcriptional regulatory elements that are known in the art. Delivery of naked DNA (i.e., without a delivery vehicle) to an intramuscular, intradermal, or subcutaneous site is another means to achieve in vivo expression.

siRNA, miRNA, and asRNA (antisense RNA) molecules can be designed by methods well known in the art. siRNA, miRNA, and asRNA molecules with homology sufficient to provide sequence specificity required to uniquely degrade any RNA can be
designed using programs known in the art, including, but not limited to, those maintained on websites for AMBION, Inc. and DHARMACON, Inc. Systematic testing of several designed species for optimization of the siRNA, miRNA, and asRNA sequences can be routinely performed by those skilled in the art. Considerations when designing short interfering nucleic acid molecules include, but are not limited to, biophysical, thermodynamic, and structural considerations, base preferences at specific positions in the sense strand, and homology. These considerations are well known in the art and provide guidelines for designing the above-mentioned RNA molecules.

An antisense polynucleotide (preferably DNA) of the present invention can be any antisense polynucleotide so long as it possesses a base sequence complementary or substantially complementary to that of the DNA encoding a key component of the cellular senescence pathway that inhibits induced pluripotent stem cell reprogramming and capable of suppressing expression of the component polypeptide. The base sequence can be at least about 70%, 80%, 90%, or 95% homology to the complement of the DNA encoding the polypeptide. These antisense DNAs can be synthesized using a DNA synthesizer.

The antisense DNA of the present invention may contain changed or modified sugars, bases or linkages. The antisense DNA, as well as the RNAi agent mentioned above, may also be provided in a specialized form such as liposomes, microspheres, or may be applied to gene therapy, or may be provided in combination with attached moieties. Such attached moieties include polycations such as polylysine that act as charge neutralizers of the phosphate backbone, or hydrophobic moieties such as lipids (e.g., phospholipids, cholesterol, etc.) that enhance the interaction with cell membranes or increase uptake of the nucleic acid. Preferred examples of the lipids to be attached are cholesterol or derivatives thereof (e.g., cholesteryl chloroformate, cholic acid, etc.). These moieties may be attached to the nucleic acid at the 3' or 5' ends thereof and may also be attached thereto through a base, sugar, or intramolecular nucleoside linkage. Other moieties may be capping groups specifically placed at the 3' or 5' ends of the nucleic acid to prevent degradation by nucleases such as exonuclease, RNase, etc. Such capping groups include, but are not limited to, hydroxyl protecting groups known in the art, including glycols such as polyethylene glycol, tetraethylene glycol and the like. The inhibitory action of the antisense DNA can be examined using a cell-line or animal based gene expression system of the present invention in vivo and in vitro.

The above-discussed nucleic acids encoding one or more of the polypeptides mentioned above or RNAi agents can be cloned in a vector for delivering to cells in vitro.
For in vivo uses, the delivery can target a specific tissue or organ (e.g., liver). Targeted delivery involves the use of vectors (e.g., organ-homing peptides) that are targeted to specific organs or tissues after systemic administration. For example, the vector can have a covalent conjugate of avidin and a monoclonal antibody to a liver specific protein.

In certain embodiments, the present invention provides methods for in vivo production of the above-mentioned iHep cells. Such method would achieve its therapeutic effect by introduction of the nucleic acid sequences into cells or tissues of a human or a non-human animal in need of an increase in liver function. Delivery of the nucleic acid sequences can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Preferred for therapeutic delivery of the nucleic acid sequences is the use of targeted liposomes.

Various viral vectors which can be utilized for gene therapy disclosed herein include, adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus and a lentivirus. Preferably, the retroviral vector is a lentivirus or a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes.

Recombinant lentivirus has the advantage of gene delivery into either dividing or non-dividing mammalian cells. The HIV-1 based lentivirus can effectively transduce a broader host range than the Moloney Leukemia Virus (MoMLV)-base retroviral systems. Preparation of the recombinant lentivirus can be achieved using the pLenti4/V5-DEST™, pLenti6/V5-DEST™ or pLenti vectors together with ViraPower™.

All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. Retroviral vectors can be made target-specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using a target-specific antibody or hormone that has a receptor in the target. Those of skill in the art will recognize that specific polynucleotide sequences can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the retroviral vector.

Another targeted system for delivery of nucleic acids is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules,
microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and delivered to cells in a biologically active form. Methods for efficient gene transfer using a liposome vehicle are known in the art. The composition of the liposome is usually a combination of phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylerine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Exemplary phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine. The targeting of liposomes is also possible based on, for example, organ-specificity, cell-specificity, and organelle-specificity and is known in the art.

When used *in vivo*, it is desirable to use a reversible delivery-expression system. To that end, the Cre-loxP or FLP/FRT system and other similar systems can be used for reversible delivery-expression of one or more of the above-described nucleic acids. See WO2005/112620, WO2005/039643, U.S. Applications 20050130919, 20030022375, 20020022018, 20030027335, and 20040216178. In particular, the reversible delivery-expression system described in US Application NO 20100284990 can be used to provide a selective or emergency shut-off

**Cell Conversion**

To covert the starting cells to iHep cells, the starting cells are cultured in culture medium, which is a nutrient-rich buffered aqueous solution capable of sustaining cell growth. Suitable culture media include but not limited to high glucose Dulbecco's Modified Eagle's Medium (DMEM), DMEM/F-15, Liebovitz L-15, RPMI 1640, Iscove's modified Dulbecco's media (IMDM), and Opti-MEM SFM. Chemically defined medium comprises a minimum essential medium such as Iscove's Modified Dulbecco's Medium (IMDM), supplemented with human serum albumin, human Ex Cyte lipoprotein, transferrin, insulin, vitamins, essential and non essential amino acids, sodium pyruvate, glutamine and a mitogen. A mitogen refers to an agent that stimulates cell division of a
cell. An agent can be a chemical, usually some form of a protein that encourages a cell to commence cell division, triggering mitosis. In one embodiment, serum-free media such as those described in WO96/39487, and the "complete media" as described in U.S. Pat. No. 5486359. In one preferred embodiment, one can use modified Block's medium supplemented with 0.1 nM dexamethasone, 20 µg l⁻¹ TGF-a, 10 µg l⁻¹ EGF, 4.2 mg l⁻¹ insulin, 3.8 mg l⁻¹ human transferrin and 5 µg l⁻¹ sodium selenite.

The starting cells are plated for culturing and differentiation onto an adherent substrate. In general, adherent substrates may be any substantially hydrophilic substrate. Adherent substrate surfaces may be generated via surface coating, e.g., coating of the polymeric or treated polymeric surfaces as above. In a non-limiting example, the coating may involve suitable poly-cations, such as, e.g., poly-ornithine or poly-lysine. For example, a coating can contain one or more components of extracellular matrix, e.g., the ECM proteins fibrin, laminin, collagen, preferably collagen type 1, glycosaminoglycans, e.g., heparin or heparan sulphate, fibronectin, gelatine, vitronectin, elastin, tenascin, aggrecan, agrin, bone sialoprotein, cartilage matrix protein, fibrinogen, fibulin, mucins, entactin, osteopontin, plasminogen, restrictin, serglycin, SPARC/osteonectin, versican, thrombo-spondin 1, or cell adhesion molecules including cadherins, connexins, selectins, by themselves or in various combinations.

In a preferred embodiment, the coating contains collagen, e.g., collagen type 1. Such coating may be particularly preferred during the differentiation protocol, since collagen, especially, collagen type 1, has been shown to aid maintenance of hepatocyte function, differentiation state and hepatic gene transcription.

After culturing for a period of time, the cultured cell population contains iHep cells. It shall be understood that the cultured cell population encompasses the progeny of a starting cell population obtainable as above, or the progeny of a fraction of the said cell population. Such progeny may be a non-clonal line, i.e., containing the offspring of multiple cells or cells from multiple colonies of a starting cell population obtainable as above; or such progeny may be a clonal sub-line, i.e., derived from a single cell or a single colony of the starting cell population.

Then, one can obtain a sample of the cultured cell population and confirm their status by examining one or more markers indicative of a hepatocyte-phenotype. The iHep cells generated according to the methods described herein should express characteristic markers indicative of liver function. For example, the cells are expected to express enzymes and other polypeptides associated with carbohydrate, protein, and lipid
metabolism. In one embodiment, they express a polypeptide associated with glycogen storage, glucose-6-phosphatase activity, decomposition of red blood cells, or plasma protein synthesis. In another, a cell of the invention expresses a polypeptide associated with urea production or synthesis of bile. In yet another embodiment, the cell expresses a polypeptide associated with cytochrome p450 (CYP3A4) activity, which is responsible for xenobiotic detoxification. In some other embodiments, the cell expresses arginase 1, which functions in physiologic detoxification and urea production.

The expression of a hepatocyte phenotype in a cell of the invention may be evaluated by analyzing mRNA. In some embodiments, the mRNAs of key enzymes and proteins expressed in the hepatocyte-like cell are evaluated by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Alternatively, iHep cells are characterized for a hepatocyte phenotype by analyzing the expression of hepatocyte markers (e.g., polypeptides characteristically expressed in hepatocytes by an immunoassay, such as an immunocytochemical assay or a Western blot. Examples of useful marks are described in Tables 2 and 3 and in the examples below.

One can also confirm the iHep cell status by evaluating their biological functions as shown in the examples below. More specifically, the cells can be evaluated for glycogen storage using Periodic Acid Schiff (PAS) functional staining for glycogen granules (Thompson S W, in Selected Histochemical and Histopathological Methods, C. C. Tomas, Sprungfield, 111, 1966; Sheehan D C and Hrapchak, B B. in Theory and Practice of Histotechnology, 2nd Ed., Battelle memorial Institute, Columbus, Ohio, 1987)), for urea production using colorimetrically (Miyoshi et al., 1998, J Biomater Sci Polym Ed 9: 227-237), for bile secretion by fluorescein diacetate time lapse assay (Gebhart et al. J. Cell Sci. 1982, 56233-244), for lipid synthesis by oil red O staining, and for glycogen synthesis (Passonneau et al. 1974, Anal. Biochem. 60:405-415).

Once the hepatocyte phenotype is confirmed, the iHep cells can be further purified or enriched according to the method described in the examples below or other methods known in the art. The resulting purified or enriched cell population contains at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% of iHep cells. The cells can be used in various ways as disclosed below.

Uses of iHep Cells

The above-described iHep cells, or a cell population containing them, or the progenies thereof, can be used in a variety of applications. One example is treating
diseases or liver metabolic deficiencies, e.g., liver metabolic deficiencies, liver degenerative diseases or fulminant liver failure, liver infections diseases, etc. via transplantation or implantation. Other examples include elucidating the mechanism of liver diseases and infections; screening cytotoxic compounds, carcinogens, mutagens growth/regulatory factors, pharma-ceutical compounds, etc., in vitro; evaluating metabolism, pharmacogenetics, or toxicity of an agent (e.g., a new or known drug); studying the pharmacological mechanism by which drugs and/or growth factors operate; diagnosing and monitoring cancer in a patient; gene therapy; and the production of biologically active products. Additional examples include uses in preparation of bio-
artificial liver devices and liver assist devices.

The cells of this invention as used herein refers to any of the staring cells to which one or more of the above-mentioned heterologous transcription factors have been introduced, as well as progenies of the cells such as the iHep cells and progeny thereof. Progenies as used herein includes cells derived from a parent, staring, or found cell via cell division or cell fusion with other cell(s).

Treatment of Liver Diseases

In an aspect, the invention provides methods for treating liver diseases or conditions. Also, the invention provides uses for the manufacture of a medicament for treating such liver diseases or conditions using the iHep cells disclosed herein (including iHep cells from humans and non-human animals) or the progeny thereof.

Such diseases may include disorders affecting liver tissue, and conditions affecting the hepatocyte viability and/or function (e.g., birth defects, the effect of a disease condition, the effect of trauma, toxic effects, viral infections, etc). Examples of the liver diseases or conditions include genetic liver diseases (e.g., Alagille syndrome), carbo-hydrate metabolism disorders (e.g., glycogen storage disease and galactosemia, fructosemia), amino acid metabolism disorders (e.g., tyrosinemia), glycolipid and lipid metabolism disorders (e.g., Niemann-Pick disease, Hunter's disease, Hurler's disease, and Wolman's disease), glycoprotein metabolism disorders (e.g., Gaucher's disease), metal storage disorders (e.g., Hemochromatosis and Wilson's Disease), peroxisomal disorders (e.g., Zellweger syndrome and mitochondrial cytopathies); hereditary disorders of bilirubin metabolism (e.g., Crigler-Najjar syndrome, Gilbert syndrome, and Dubin-Johnson syndrome), hereditary disorders of bile formation (e.g., progressive familial intrahepatic cholestasis), bile acid biosynthesis disorders, protein biosynthesis and targeting disorders (a)-
Antitrypsin deficiency and cystic fibrosis), acute liver failure arising from a combination of genetic and environmental factors.

The treatment methods include administering to the subject identified as in need of such treatment) an effective amount of a cell composition described herein, or a composition described herein to produce such a cell composition. Identifying a subject in need of such treatment can be in the judgment of a subject or a health care professional and can be subjective (e.g. opinion) or objective (e.g. measurable by a test or diagnostic method). Determination of those subjects "at risk" can also be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider (e.g., genetic test, enzyme or protein marker, family history, and the like). The compositions described herein may be also used in the treatment of any other disorders in which a reduction in liver function may be implicated.

The number of cells needed to restore liver function, fully or partially, varies depending on the degree of liver damage and the size, age and weight of the host. For example, the cells are administered in an amount effective to restore liver functions. Determination of effective amounts is well within the capability of those skilled in the art. The effective dose can be determined by using a variety of different assays designed to detect restoration of liver function. The progress of the transplant of the recipient can be determined using assays that include blood tests known as liver function tests. Such liver function tests include assays for alkaline phosphatase, alanine transaminase, aspartate transaminase and bilirubin. In addition, recipients can be examined for the presence or disappearance of features normally associated with liver disease such as, for example, jaundice, anemia, leukopenia, thrombocytopenia, increased heart rate, and high levels of insulin. Further, imaging tests such as ultrasound, computer assisted tomography (CAT) and magnetic resonance (MR) may be used to assay for liver function.

The iHep cells can be administered by conventional techniques such as injection of cells into the recipient host liver, injection into a site of liver lesion or at a site from which such cells can migrate to the site of the lesion (e.g. administration to spleen, portal vein, liver pulp, etc., e.g., by injection), or surgical transplantation of cells into the recipient host liver. In some instances it can be necessary to administer the iHep cells more than once to restore liver function. In addition, growth factors, such as G-CSF, or hormones, and TGFβ1 can be administered to the recipient prior to and following transplantation for the purpose of priming the recipient’s liver and blood to accept the transplanted cells and/or to generate an environment supportive of hepatic cell proliferation.
"Treating" or "treatment" refers to administration of a compound or agent to a subject who has a disorder with the purpose to cure, alleviate, relieve, remedy, delay the onset of, prevent, or ameliorate the disorder, the symptom of the disorder, the disease state secondary to the disorder, or the predisposition toward the disorder. The terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

A "subject" refers to a human and a non-human animal. In one embodiment, the subject is a human. In another, the subject is an experimental, non-human animal or animal suitable as a disease model. The term "animal" includes all vertebrate animals including humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages. In particular, the term "vertebrate animal" includes, but not limited to, humans, non-human primates (particularly higher primates), canines (e.g., dogs), felines (e.g., cats); equines (e.g., horses), bovines (e.g., cattle), porcine (e.g., pigs), rodent (e.g., mouse or rat), guinea pig, cat, rabbit, as well as in avians, such as birds, amphibians, reptiles, etc. The term "avian" refers to any species or subspecies of the taxonomic class avia, such as, but not limited to, chickens (breeders, broilers and layers), turkeys, ducks, a goose, a quail, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and cassowary. Examples of a non-human animal include all non-human vertebrates, e.g., non-human mammals and non-mammals mentioned above.

**Tissue-Engineering**

The invention also provides a tissue-engineered organ, or portion, or specific section thereof, as well as a tissue engineered device having the iHep cells of this invention or progenies thereof. A tissue engineered liver can provide a new therapy in which differentiated iHep cells are transplanted within three-dimensional polymer scaffolds to supplement or replace the function of a failing liver. Tissue-engineered organs can be used with a biocompatible scaffold to support cell growth in a three-dimensional configuration, which can be biodegradable.

The construction of a three-dimensional polymer-cell scaffold made of polymer and hepatocyte-like cell can be carried out according to WO/2003/076564 and U.S. Pat. Nos. 5624840 and 5759830. A tissue engineered liver can be made of iHep cells fabricated onto a matrix or a scaffold made of natural or manmade material. For example, the cells can be used to seed a decellularized liver scaffold as described in U.S. Patent
Application 20050249816. Manmade materials that can be used are often biodegradable polymers, such as the three-dimensional tissue culture system in which cells were laid over a polymer support system (See U.S. Pat. No. 5863531). Materials suitable for polymer scaffold fabrication include polylactic acid (PLA), poly-L-lactic acid (PLLA), poly-D-lactic acid (PDLA), polyglycolide, polyglycolic acid (PGA), polylactide-co-glycolide (PLGA), polydioxanone, polygluconate, polylactic acid-polyethylene oxide copolymers, modified cellulose, collagen, polyhydroxybutyrate, polyhydroxyprionionic acid, polyphosphoester, poly (alpha-hydroxy acid), polycaprolactone, polycarbonates, polyanides, polyethylene glycol, polyamides, polyethyleneglycols, polyamino acids, polyorthoesters, polyacetals, polycyanoacrylates, degradable urethanes, aliphatic polyesters polycrlylates, polymethylene, acyl substituted cellulose acetates, non-degradable polyurethanes, polystyrenes, polyvinyl chloride, polyvinyl fluoride, polyvinyl imidazole, chlorosulphonated polyolins, polyethylene oxide, polyvinyl alcohol, Teflon™, nylon silicon, and shape memory materials, such as poly (styrene-block-butadiene), polynorbornene, hydrogels, metallic alloys, and oligo (s-caprolactone) diol as switching segment/oligo (p-dioxanone) diol as physical crosslink. Other suitable polymers can be obtained by reference to The Polymer Handbook, 3rd edition (Wiley, N.Y., 1989). Such tissue engineered liver can be implanted into the patient to restore liver function.

This invention also provides use of the hepatocyte-like cells of the invention as part of a bioreactor, e.g. a liver assist device. Further, the iHep cells of this invention or their progenies can be used as biological components of detoxification devices such as liver perfusion or liver assist devices. Specifically, the cells of this invention can be used to construct extracorporeal liver assist device such as a bio-artificial liver for use by subjects having liver disorders that result in hepatic failure or insufficiency. The use of such bio-artificial livers involves the perfusion of the subject's blood through the bio-artificial liver. In the blood perfusion protocol, the subject's blood is withdrawn and passed into contact with the iHep cell cultures. During such passage, molecules dissolved in the patient's blood, such as bilirubin, are taken up and metabolized by the hepatocyte cultures. In addition, the hepatocyte-like cells provide factors normally supplied by liver tissue.

An exemplary liver assist device includes a rigid, plastic outer shell and hollow semi-permeable membrane fibers which are seeded with iHep cells of this invention or their progenies. The fibers can be coated with collagen, lectin, laminin, or fibronectin, for the attachment of cells. Body fluid from a subject can perfuse through the device for detoxification according to procedures known in the art and then returned to the subject.
**Drug Testing and Screening**

The iHep cells of this invention or their progenies can also be used as a tool for drug testing and development process. For example, one can use the cells to assess changes in gene expression patterns caused by drugs being considered for development. The changes in gene expression pattern from potential drugs can be compared with those caused by control drugs known to affect the liver. This allows one to screen compounds for their effects on the liver earlier in the development process without using animals, thereby saving time and money. In some embodiments, the iHep cells of this invention or their progenies are used in a high throughput drug screening, such as in the manner described in U.S. Pat. No. 7282366.

The iHep cells of this invention or their progenies can also be used to assess toxicity of various compounds or compositions of interest, e.g. chemical, pharmaceutical, cosmetic, biocidal or biological compounds, food additives or compositions, or biological agents. The use of differentiated cells may be preferred in such assays of toxicity, as the cells more closely resemble the cell types present in the liver of an organism. For example, a particular compound or composition is considered toxic or likely toxic, if it shows a detrimental effect on the viability of cells or on one or more aspect of cellular metabolism or function. The viability of cells *in vitro* may be measured using techniques known in the art, including colorimetric assays, such as the MTT (or MTT derivative) assays or LDH leakage assays, or using fluorescence-based assays, such as, e.g., the Live/Dead assay, CyQuant cell proliferation assay, or assays of apoptosis. Other useful assays include those that measure particular aspects of cellular metabolism or function.

**Carcinogenicity Evaluation**

It is known in the art that various compounds cause tumors in experimental animals such as mice even though they fail to act as mutagens in test organisms such as bacteria or fungi. One of the reasons for this phenomenon is metabolic activation; i.e., some chemicals are metabolically altered by enzymes in the liver (the P450 oxidase system and hydroxylation systems) or other tissues, creating new compounds that are both mutagenic and carcinogenic. In order to identify such carcinogens, people have used screening assays involving incubating a test chemical compound with liver extracts or liver tissues prior to exposure of the test organism to the metabolic product (Ames *et al.*, 1975, Mut. Res. 31:347-364; U.S. Patent No. 7026137). The iHep cells of this invention or their
progenies can be used as a substitute for the liver extracts or liver tissues described in the conventional assays.

Thus, the present invention also provides methods and assays to evaluate the carcinogenicity of a test compound or agent use the cells of this invention, which closely resemble the cell types present in the liver of an organism. These cells can be used in assays of both genotoxic and non-genotoxic (i.e., epigenetic) carcinogenicity. For example, one can contact the cells with a test agent and then examine neoplastic transformation or genetic stability of the cells. The agent is considered carcinogenic or likely carcinogenic, if it induces neoplastic transformation of the cells, or induces phenotypic changes in the cells that may be predictive of such neoplastic transformation, or induces genetic or metabolic changes that may potentially cause such neoplastic transformation.

Examples of phenotypic changes in the cells include, but are not limited to, morphological transformation, increased proliferation, dedifferentiation, independence of attachment, removal of contact inhibition of cells grown in monolayers, or expression of specific marker proteins. Such genetic changes in the cells may, but are not limited to, comprise DNA damage, chromosomal aberrations, e.g., chromosomal rearrangements, alterations in chromosome number (aneuploidy), or karyotype aberrations, gene mutations, e.g., point mutations, deletions or insertions. Agents that cause this kind of genetic changes are often referred to as mutagenic or mutagens. Accordingly, the cells provided by the present invention will be very useful in assays of mutagens, i.e., in assays of mutagenicity.

For the purposes of mutagenicity testing, the cells of the present invention can be genetically altered. For example, the cells may contain a transgene, encoding a polypeptide that increases the cells sensitivity to a particular proliferation-inhibiting agent. Consequently, genetic alterations in some cells removing the expression of such transgene would release these cells from this inhibition. Mutagenicity may then be assessed by methods of scoring such cells.

Other Uses

The cells of this invention can further used for various other uses. For example, they can be used in producing one or more proteins expressed in the liver.

One example is blood coagulation factors, which are useful for subjects with hemophilia and other blood clotting disorders. Currently, most of the preparations of blood coagulation factors are from donated blood and that presents the disadvantage that
the danger of transmitting hepatitis. Producing blood coagulation factors in vitro from the hepatocyte-like cell described herein greatly reduces the risk of transmitting hepatitis or other blood borne diseases. To produce coagulation factors, one can cultured the cells of this invention under suitable conditions. After the cultured hepatocyte-like cells have reached confluency, the supernatant culture media can be collected and purified according to methods known in the art, such as those described in U.S. Pat. No. 4789733 and Kane et al. J. Biol. Chem., 256:1002-1007, 1981.

Primary hepatocytes have versatile characteristics and functions. To use iHep cells for fully recapitulating primary hepatocytes, one can improve iHep cells in vitro for specialized purposes. For example, iHep cells as disclosed herein express several Cyp genes and acquire Cyp1a, Cyp3a and Cyp2c activities. By further optimization of iHep cells to express drug transporter genes and enhanced Cyp activities, one can obtain an alternative to primary hepatocytes for the early stages of drug discovery. Interestingly, preliminary data by the inventors implicate that mouse ESC-derived hepatocyte-like cells appeared to be more immature compared with iHep cells as disclosed herein. Nonetheless, a comprehensive comparison of iHep cells with other surrogate hepatocyte-like cells would be necessary, so that when a specialized hepatic function is desired one can decide which hepatocyte-like cells to choose.

Compositions

In a further aspect, the invention relates to a pharmaceutical composition comprising the human iHep cells, or iHep cells from other species including man, obtainable or directly obtained using the herein described methods, or a cell population comprising such as defined above, or the progeny thereof.

The term "pharmaceutical composition" refers to the combination of an active agent (e.g., cells or transcription factors disclosed herein) with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use in vivo or ex vivo. A "pharmaceutically acceptable carrier," after administered to or upon a subject, does not cause undesirable physiological effects. The carrier in the pharmaceutical composition must be "acceptable" also in the sense that it is compatible with the active ingredient and can be capable of stabilizing it (e.g., keeping iHep cells alive). One or more solubilizing agents can be utilized as pharmaceutical carriers for delivery of an active agent. Examples of a pharmaceutically acceptable carrier include, but are not limited to, biocompatible vehicles, adjuvants, additives, and diluents to achieve a composition usable
as a dosage form. Examples of other carriers include colloidal silicon oxide, magnesium stearate, cellulose, and sodium lauryl sulfate. Additional suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use, are described in Remington’s Pharmaceutical Sciences.

EXAMPLES

Materials and Methods

The following materials and methods apply to all examples, unless specifically noted otherwise.

Mice

p19\(^{A\beta}\) mice, Fah\(^{-/-}\) Rag2\(^{-/-}\) mice and NOD/SCID mice were maintained in specific pathogen-free husbandry. Fah\(^{-/-}\) Rag2\(^{-/-}\) mice were fed with drinking water containing 1.5 mg/l NTBC. The genetic background for p19\(^{A\beta}\) and Fah\(^{-/-}\) Rag2\(^{-/-}\) mice was C57B16/J 3 129Sv. Fah\(^{-/-}\) Rag2\(^{-/-}\) mice were used as the recipient to reduce immunological rejection of iHep cells after transplantation.

Molecular Cloning and Lentivirus Production

A multi-cloning site (CGGGATCCCAGCGCCGACTAGTCGACGGCGTCGAGGTT; SEQ ID NO: 15) was inserted into the Pmel restriction site of lentiviral vector pWPI (ADDGENE). cDNAs of candidate genes were cloned into the modified pWPI plasmid. For p19\(^{A\beta}\) shRNA expression, DNA oligonucleotides encoding p19\(^{A\beta}\) shRNA (CCGGTTAAGCTTTGAGCTAACCCTCAACAACATGTTCACTTTTTG; SEQ ID NO: 16) were inserted into the Agel and EcoRI restriction sites of the pLKO.1 plasmid. Constructed pWPI or pLKO.1 plasmids were then introduced to 293FT cells together with packaging plasmid psPAX2 (ADDGENE) and envelope plasmid pMD2.G (ADDGENE). After 48 h incubation, the medium containing lentiviruses was collected and passed through a 0.45 mm filter.

Fibroblast Culture and Bile Duct Induction

To isolate tail-tip fibroblasts, tails (each 5 cm in length) were cut from two-month-old mice. The dermis was peeled and the tails minced into 1-cm pieces. Two pieces were placed per 60-mm collagen-I-coated dish in 5 ml DMEM (SIGMA-ALDRICH) containing 10% FBS (SIGMA-ALDRICH). After 5 days incubation, fibroblasts that migrated out of the tails were transferred to new collagen-I-coated dishes. TTFs between passage 7 and 9 were used for iHep cell induction. Embryonic fibroblasts were isolated from E13.5

40
embryos. Head and visceral tissue were dissected and removed. The remaining tissues were minced and incubated with 0.25% trypsin (GIBCO) at 37 °C for 15 min. Isolated cells were plated onto a 60-mm collagen-I-coated dish in 5 ml DMEM containing 10% FBS. MEFs at passage 3 for were used lentiviral infection.

For bile duct differentiation, 1x10^4 cells were re-suspended in 1 ml DMEM/F12 medium with 1 ml freshly prepared collagen gel solution and poured into a 35-mm dish. After gel solidification, cells were cultured with 1.5 ml DMEM/F12 supplemented with 10% FBS, 1x ITS, 20 ng ml^-1 HGF for 3 days.

**Primary Hepatocyte Isolation and Culture**

Adult mice were subjected to standard two-step collagenase perfusion for isolation of primary hepatocytes. Briefly, the liver was pre-perfused through the portal vein with calcium-free buffer (0.5mM EGTA, 1x EBSS without Ca^{2+} and Mg^{2+}) and then perfused with collagenase (0.2 mgml^-1 collagenase type IV (SIGMA), 10mM HEPES, 1x EBSS with Ca^{2+} and Mg^{2+}). Parenchymal cells were purified by Percoll buffer (90% Percoll (SIGMA), 1x EBSS) at low-speed centrifugation (1,500 r.p.m., 10 min). Viability of isolated hepatocytes was around 90% as determined by Trypan blue. For microarray analysis, p19^drf/- primary hepatocytes were cultured in modified Block’s medium supplemented with 0.1 mM dexamethasone, 20 μg ml^-1 TGF-a, 10 μg ml^-1 EGF, 4.2 mgl^-1 insulin, S^mgl^-1 human transferrin and 5 μg ml^-1 sodiumselenite in collagen-I-coated dishes for 6 days before harvesting for RNA extraction. For other experiments, p19^drf/- primary hepatocytes were immediately lysated in TRIZOL for total RNA isolation.

**PCR**

For most experiments, total RNA was isolated from cells by TRIZOL (INVITROGEN). For RNA extraction from formalin-fixed-paraffin-embedded (FFPE) tissues, four serial sections mounted on polyethylene terephthalate (PET) membrane frame slides were deparaffinized and air dried. The first section was stained with anti-Fan antibody to identify the repopulated Fah^+ nodules. On the basis of the result of Fah immunostaining in the first section, Fah^+ tissues within the nodules were microdissected from the following three sections by a Leica LMD7000 Laser Microdissection Microscope (LEICA MICROSYSTEMS) with laser intensity of 45 and speed of 5. After microdissection, the remaining sections on the slides were further stained with anti-Fah antibody to confirm that only tissues inside Fah^+ nodules were separated. Microdissected
tissues from the same Fah⁺ nodule were pooled together for total RNA extraction using RNeasy FFPE Kit (QIAGEN).

A total of 1 µg RNA was reverse transcribed into cDNA with M-MLV Reverse Transcriptase (PROMEGA) according to the manufacturer's instructions. For DNA extraction from formalin-fixed-paraffin-embedded tissues, the QIAamp DNA FFPE Tissue Kit (QIAGEN) was applied according to the manufacturer's instructions. PCR was performed with HiFi Taq polymerase (TRANSGEN). Quantitative real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa) on an ABI 7500 fast real-time PCR system (APPLIED BIOSYSTEMS).

**Immunofluorescence**

For immunofluorescence staining, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and then incubated with PBS containing 0.2% Triton X-100 (SIGMA) for 15 min. Cells were then washed three times with PBS. After being blocked by 3% BSA in PBS for 60 min at room temperature, cells were incubated with primary antibodies at 4°C overnight, washed three times with PBS, and then incubated with appropriate fluorescence-conjugated secondary antibody for 60 min at room temperature in the dark. Nuclei were stained with DAPI (SIGMA). Primary and secondary antibodies were diluted in PBS containing 3% BSA. Antibodies used for immunofluorescence are as follows: mouse anti-Tjpl (INVITROGEN, 1:750), rabbit anti-E-cadherin (CELL SIGNALING, 1:500), mouse anti-albumin (R&D, 1:200), goat anti-Hnf4a (SANTA CRUZ, 1:200), Cy5-conjugated goat anti-mouse IgG (1:1,000), Cy3-conjugated goat anti-rabbit IgG (1:1,000), Cy3-conjugated donkey anti-goat IgG (JACKSON LAB-ORATORIES JACkSON LAB, 1:1,000). For Y-chromosome fluorescent in situ hybridization (FISH), liver samples of male Fah⁺Rag2⁻⁻ mice transplanted with female iHep cells were embedded in paraffin and hybridized with mouse Y-chromosome probe (ID LABS INC., Canada) according to manufacturer's instruction.

**FACS Analyses**

For intracellular staining of albumin, 10⁶ cells were harvested and fixed with 4% PFA for 30 min, and then permeabilized in staining buffer (PBS with 10% FBS and 0.5% saponin) for 10 min. Cells were then incubated with primary antibody (anti-albumin, R&D) for 30 min in staining buffer, followed with secondary antibody (Cy5-conjugated goat anti-mouse IgG, Jackson Laboratories) incubation for 30 min. Cells were analyzed
by the Calibur flow cytometer (BECTON DICKINSON). Data were analyzed with Windows Multiple Document Interface for Flow Cytometry (WinMDI, version 2.9).

**PAS Stain, Dil-Ac-LDL And ICG Uptake Assays, Alb ELISA And CYP Metabolism Assay**

Cells were stained by periodic acid-Schiff (PAS, SIGMA) and Dil-ac-LDL (INVITROGEN) following the manufacturer’s instructions. For the indocyanine green (ICG, SIGMA) uptake assay, cells were cultured in the medium supplemented with progesterone, pregnenolone-16a-carbonitrile and 8-bromo cAMP for 2 days. Cells had their medium changed with 1mg ml⁻¹ ICG and were incubated at 37 °C for 1 h, followed by washing with PBS three times.

To determine Alb secretion, TTFs transduced with three factors were cultured in the medium without phenol red. Culture supernatant was collected 24 h after medium change. The amount of Alb in the supernatant was determined by the mouse albumin ELISA kit (BETHYL LABORATORY) according to the manufacturer’s instructions. For the measurement of CYP enzyme activities, TTFs and iHep cells were cultured in the medium with 50 μM 3-methylcholanthrene for 48 h. Cells were dissociated and incubated with substrate in 200 ml incubation medium at different concentrations for 3 h at 37 °C. To stop the reaction, 800 μl cold methanol was added and centrifuged. The supernatants were collected for measurement of indicated productions by LC-MS/MS (AGILENT 1200 HPLC and ABI 4000 mass-spectrometer). Freshly isolated hepatocytes were used as a positive control. Total cell protein amount was used to normalize the data. Substrates and metabolic products for standard were purchased: phenacetin, diclofenac, bufuralol, acetaminophen, 4’-OH diclofenac (SIGMA), testosterone (FLUKA), 6β-OH-testosterone (CERILLIANT) and 1’-OH-bufuralol (TORONTO RESEARCH CHEMICALS).

**Microarray Analysis**

Total RNA extracted from p19⁰arf⁻/⁻ TTFs, p19⁰arf⁻/⁻ MEFs, pl¹⁻ hepatocytes cultured for 6 days, 3TF-transduced p19⁰arf⁻/⁻ TTFs without enrichment of epithelial cells, and iHep cells from different experiments was hybridized to whole mouse gene expression microarray (AGILENT) under the manufacturer’s instruction. Data were normalized by Gene-Spring (AGILENT). Microarray hybridization and analysis were carried out by ShanghaiBio Cooperation. Out of 29,153 annotated genes, 11,797 genes for which expression levels were at least twofold different between p19⁰arf⁻/⁻ TTFs and primary p19⁰arf⁻/⁻ hepatocytes were selected for analyses. Hierarchical clustering of samples was performed by Cluster 3.0 software. Average linkage with the uncentred correlation similarity metric...
was used for the clustering of samples. Original data were uploaded to the Gene Expression Omnibus database (accession number GSE23635).

**In vivo Function Analysis**

Fah\(^{-}\)Rag2\(^{-}\) mice were maintained with \(7.5\) mg\(^{-}\)l\(^{-1}\) NTBC in the drinking water. 8.33x10\(^{-5}\) iHep cells and 8.33x10\(^{-5}\) pl\(^{\wedge}\) TTFs were transplanted into the spleens of Fah\(^{-}\) Rag2\(^{-}\) mice at the age of 8-12 weeks, respectively. NTBC was withdrawn from the drinking water after cell transplantation. Ten Fah\(^{-}\)Rag2\(^{-}\) mice without any transplantation also had NTBC withdrawn as a control. A survival curve was generated by SPSS for windows using Kaplan-Meier method. Eight weeks after transplantation, the blood of surviving iHep-cell-transplanted Fah\(^{-}\)Rag2\(^{-}\) mice was collected from the retro-orbital sinus and centrifuged at 12,000 r.p.m. for 15 min. The serum was frozen at 280 °C until biochemical analyses. Total bilirubin, albumin, ALT, AST, blood urea nitrogen and creatinine were measured by 7600-020 clinical analyser (HITACHI). Amino acids were quantified by liquid chromatography-mass spectrometry ABI 3200 Q TRAP LC-MS/MS system (APPLIED BIOSYSTEM). After blood collection, mice were killed by cervical dislocation and livers were harvested, fixed and stained with Fah polyclonal antibody or haematoxylin and eosin as previously described. Blood and liver samples of control NTBC-offFah\(^{-}\)-Rag2\(^{-}\)- mice were collected after losing 20% body weight.

**Tumour Generation Assay**

The human hepatoma cell line PLC/PRF/5 was cultured in the same medium as iHep cells. iHep cells were induced and enriched as described above. After 21 days induction, cells were detached by trypsin and suspended in PBS. Seven NOD/SCID mice respectively were injected with 5x10\(^{6}\) iHep cells in the left subcutaneous flank and 5x10\(^{6}\) PLC/PRF/5 cells in the right subcutaneous flank. Tumour numbers were counted 8 weeks after injection.

**Statistics**

All data are presented as mean \(\pm\) s.d. For most statistical evaluation, an unpaired Student’s t-test was applied for calculating statistical probability in this study. For survival analysis, the Mantel-Cox log-rank test was applied. Statistical calculation was performed using STATISTICAL PROGRAM FOR SOCIAL SCIENCES SOFTWARE (SPSS, IBM). For all statistics, data from at least three independent samples or repeated experiments were used.
EXAMPLE 1

In this example, a group of transcription factors sufficient for inducing hepatocytes from fibroblasts were identified.

Fourteen mouse transcription factors ("14TF," Table 1) important for liver development and function (Kyrmizi et al. Genes Dev. 20, 2293-2305 (2006), Zaret., Nature Rev. Genet. 9, 329-340 (2008), Schrem et al, J. Pharmacol. Rev. 54, 129-158 (2002), and Schrem et al. Pharmacol. Rev. 56,291-330(2004)) were transduced into immortalized 3T3 fibroblasts, mouse embryonic fibroblasts (MEFs) and tail-tip fibroblasts (TTFs) via lentiviral infection. The hepatic genes albumin (Alb) and Tdo2 were induced in these cells at day 5 after infection (FIG. 5a), indicating that fibroblasts have the potential to be converted to hepatocytes.

To ensure that the process is independent of spontaneous immortalization and embryonic progenitors, TTFs were used to further study the 14 TFs. Wild-type TTFs showed proliferation arrest and cell death within 7 days after transduction (FIG. 1b), thereby inhibiting continuous hepatic conversion.

Because pi9cre (also called Cdkn2a)-nu\(^{pl^\text{Cre/}}\) hepatocytes proliferate in vitro without losing genetic stability (Mikula et al. Hepatology39,628-634(2004), pl^\text{Cre/} TTFs were used to overcome the proliferative limitation according to the design shown in FIG. 1a. Briefly, primary pi9cre TTFs were infected with lentiviruses expressing hepatic transcription factors. The cells were changed to modified Block's medium 2 days after infection and further cultured for 14-21 days.

Remarkably, proliferative cells with epithelial morphology were induced from mesenchymal pl9cre TTFs after transduction of 14TF (FIG. 5b). Moreover, these cells expressed Alb, Tdo2 and Ttr (FIG. 5c). Eleven epithelial colonies, picked up at day 21 after lentiviral transduction, expressed hepatic genes and the exogenous 14TF at different levels (FIG. 6). One epithelial colony, ET26, was further characterized (FIG. 1b). RT-PCR assays were carried out to examine expression of various genes in ET26, while primary hepatocytes and TTFs were used as controls.

The results show that ET26 cells expressed hepatic secretory protein genes, cytokeratin genes, epithelial cell adhesion genes and endogenous hepatic transcription factors (FIG. 1c). By contrast, expression of Colla1, Pdgfrb, Postn and Fspl (also called S100a4), genes typical for fibroblast, was down-regulated in ET26 cells (FIG. 1c).

Functionally, cytoplasmic accumulation of glycogen or low density lipoprotein was determined by periodic acid-Schiff (PAS) staining or Dil-ac-LDL intake. It was found that
ET26 cells showed glycogen storage as demonstrated in FIG. Id and uptake of Dil-labelled acetylated low density lipoprotein (Dil-ac-LDL, FIG. le).

These above results indicated that p19\(^{\text{Cre}}\). TTFs were converted into cells with significant hepatic gene expression and hepatic functions.

EXAMPLE 2

In this example, a number of key factors required for hepatic conversion were identified. More specifically, the following combinations were examined: (i) a combination of six factors ("6TF"), including Foxa2, Foxa3, Hnfla, Hnf4a, Hnf6 and Gata4, and (ii) a combination eight factors ("8TF"), including the just-mentioned 6TF plus Foxa1 and Hlf in the same manner described above.

It was found that either 6TF or 8TF converted TTFs to epithelial colonies with hepatic gene expression at comparable levels (FIGs. 7a and b). Upon withdrawal of Hnf6 from 6TF, it was found that there was significantly increased hepatic gene expression and epithelial colony formation (FIGs. 7a and b). For the remaining five factors ("5TF"), removal of Hnf4a further promoted the formation of epithelial colonies (FIG. 7c).

The remaining four factors were further grouped into two combinations: (1) Gata4, Hnfla and Foxa3 ("3TF") and (2) Gata4, Hnfla and Foxa2 ("3TF'"). It was found that 3TF showed a stronger effect than 3TF' on the induction of hepatic gene expression and epithelial colony formation (FIG. 7d). Remarkably, 3TF induced endogenous Foxa2 and Foxa3 expression (FIG. 7d), and removal of Foxa3 and Hnfla from 3TF failed to form epithelial colonies. On the other hand, combination of Foxa3 and Hnfla (i.e., removal of Gata4 from 3TF) were still able to induce formation of epithelial colonies, albeit at a lower degree (FIG. If), suggesting that GATA 4 is not absolutely required, but notably enhances the efficiency of hepatic conversion.

Intriguingly, it was found that 3TF triggered p19\(^{\text{Cre}}\). MEFs to express hepatic genes (FIG. 8), indicating the potential to induce hepatic conversion of embryonic fibroblasts. Furthermore, upon RNA-interference-mediated knockdown of p19\(^{\text{Cre}}\), it was found that 3TF also converted wild-type TTFs to epithelial cells with hepatic gene expression (FIG. 9).

EXAMPLE 3
In this example, assays were carried out to examine iHep cells induced by over-expression of Gata4, Hnfla and Foxa3 and the inactivation of p19Arf for their hepatic features.

It was found that, at day 6, the epithelial cells induced by 3TF were positively stained for tight junction protein 1 (Tjpl) and E-cadherin (FIGs. 2a-c). At day 14, 23% of epithelial cells were positive for Alb (FIG. 10a), indicating an efficient hepatic conversion. The increased expression of hepatic genes over time, for example, Alb, Trf, transferring (Trf) and CK18 (also called KrtlS), showed a progressively enhanced reprogramming (FIG. 2d and FIG. 10b, P<0.05).

Interestingly, it was found that iHep cells also expressed Afp and CK19 (also called Krtl9) (FIG. 2d). Protein expression of Alb and Hnf4a was confirmed by immunofluorescent staining in iHep cells (FIGs. 10c and d). Notably, expression levels of exogenous 3TF were markedly decreased during hepatic conversion, indicating that continuous expression of exogenous 3TF is not required (FIG. 10e).

Furthermore, individual iHep colonies showed similar expression patterns of hepatic genes and fibrotic genes (FIG. 10f), indicating a homogeneous conversion among individual TTFs. Although iHep cells expressed Afp and CK19 (FIG. 2d), other hepatoblast marker genes, such as Lin28b, Igfl2 and Dlkl (Li. et al., Gastroenterology 139, 2158-2169 (2010)), were undetectable during hepatic conversion (FIG. 11a).

Importantly, cytochrome P450 (CYP) enzymes specific to mature hepatocytes were detectable in iHep cells (FIG. 11b), suggesting that hepatic conversion undertakes a process without reversion to progenitors. Moreover, iHep cells neither expressed bile duct marker genes nor formed branching bile duct tubes in vitro (FIGs. 11c and d). The marker genes for pancreatic exocrine and endocrine cells and intestinal cells were also undetectable (FIGs. 11e and f). Therefore, the above results indicate that TTFs are not converted to lineages other than hepatocytes.

Microarray assays were carried out to compare the global expression profiles among iHep cells, TTFs, MEFs and hepatocytes cultured for 6 days. Pearson correlation analysis showed that iHep cells were clustered with cultured hepatocytes but separated from TTFs and MEFs (FIG. 2e).

Specifically, microarray data revealed that numerous hepatic functional genes were up-regulated in iHep cells compared to TTFs (FIG. 12 and Tables 2 and 3). When compared with cultured hepatocytes, 877 out of 29,153 annotated genes were found to be
up-regulated in iHep cells, including \textit{Afp}, \textit{CK19}, \textit{Fabp4} and \textit{S100a9}, whereas 817 genes were down-regulated, such as \textit{Cyp4bl}, \textit{Cyp2c40} and \textit{Apob} (fold change $>2$, $P<0.01$, t-test).

Shown in Table 3 are the results of genome-wide gene expression profile analysis of iHep cells. Global gene expression profiles of $pl9Arf$' TTFS, cultured $pl9Arf$' hepatocytes, and iHep cells were analyzed using Agilent whole genome oligo chips. Average expression levels of each listed gene in iHep cells were divided by the expression level of that gene in TTFS to calculate the ratio of iHep/TTF. The ratio of hepatocyte/TTF was calculated via dividing the expression level in cultured hepatocytes by the expression level in TTFS. Shown in Table 3 are microarray data of all CYP genes.

Notably, iHep cells established substantial hepatic functions. iHep cells accumulated PAS-positive glycogen aggregations and transported Dilac-LDL into the cytoplasm (FIG. 2f, g). Indocyanine green uptake was found in 20% of iHep cells (FIG. 2h). Furthermore, iHep cells secreted high amounts of Alb into medium (FIG. 2i, $P<0.05$).

Importantly, iHep cells metabolized phenacetin, testosterone and diclofenac (FIGs. 2j-l and Table 4, $P<0.05$), whereas metabolic activity for bufuralol was undetected (FIG. 13). More specifically, iHep cells were treated with Phenacetin, Testosterone, or Diclofenac at different concentrations. Metabolites of these chemicals were measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS) according to each standard curve. The results are shown in Table 4.
### Table 2

#### a) Glucose metabolism

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#### b) Fatty acid, cholesterol, bile acid metabolism

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#### c) Secretory protein

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#### d) Coagulation

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#### e) Drug metabolism

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Table 4

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<td>100</td>
<td>0.0</td>
<td>350.5±31.9</td>
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<td>1120.5±215.6</td>
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<th>6α-OH-Testosterone (µM)</th>
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<tr>
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<td>50</td>
<td>72.7±8.8</td>
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<th>Diclofenac (µM)</th>
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<th>4'-OH-Diclofenec (µM)</th>
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<tr>
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<td>50</td>
<td>32.5±13.6</td>
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<td>119.8±11.0</td>
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<td>200</td>
<td>131.7±26.5</td>
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</table>
EXAMPLE 4

In this example, the iHep cells prepared according to the method described above were transplanted into Fah<sup>−/−</sup> mice to examine whether the cells could proliferate in vivo and rescue the mice from death.

It was known that Fah<sup>−/−</sup> mice defective in tyrosine metabolism require 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC) supply for survival (Grompe et al. Genes Dev. 7 (12A), 2298-2307 (1993), Wang et al. Proc. Natl Acad. Sci. USA 100 (Suppl. 1), 11881-11888 (2003), Grompe et al. Nature Genet. 10, 453-460 (1995), and Overturfe al. Nature Genet. 12,266-273(1996)). After NTBC withdrawal ("NTBC-off "), Fah<sup>−/−</sup> mice undergo liver failure and death. They can be rescued by transplantation of wild-type primary hepatocytes, representing a useful model to characterize in vivo repopulation and functions of iHep cells. Immunodeficient Fah<sup>−/−Rag2<sup>−/−</sup></sup> mice were used for transplantation to reduce the likelihood of immunological rejection in the manner described above. The results are shown in FIGs. 3a and b and FIG. 14a.

It was found that ten Fah<sup>−/−Rag2<sup>−/−</sup></sup> mice without transplantation were all dead within 6.5 weeks after NTBC-off and showed continuous loss of body weight (FIG. 3b and FIG. 14b). Six Fah<sup>−/−Rag2<sup>−/−</sup></sup> mice transplanted with p<i>9<sup>ArF<sup>−/−</sup></sup></i>-TTFs were also dead after NTBC-off (FIG. 3b). In contrast, 5 out of 12 Fah<sup>−/−Rag2<sup>−/−</sup></sup> mice transplanted with iHep cells ("iHep-Fah<sup>−/−Rag2<sup>−/−</sup></sup>) were alive 8 weeks after NTBC-off and showed increased body weight (FIG. 3b and FIG. 14b, P<0.05).

Fah-positive (Fah<sup>+</sup>) iHep cells engrafting into liver sinusoid comprised 5% to 80% of total hepatocytes in iHep-Fah<sup>−/−Rag2<sup>−/−</sup></sup> livers (FIG. 3c and FIG. 14c). Moreover, Fah<sup>−/−</sup> wild-type and p<i>9<sup>ArF<sup>−/−</sup></sup></i>-nuH alleles were detected in iHep-Fah<sup>−/−Rag2<sup>−/−</sup></sup> livers by genomic PCR (FIG. 14d). To exclude the possibility of cell fusion between iHep and host cells, the Y chromosome in male livers transplanted with female iHep cells was stained. Twenty-five Fah<sup>+</sup> nodules in four male recipients were characterized and all of them were found to be negative for Y-chromosome staining, confirming that iHep cells do not fuse with host cells (FIG. 3d and FIG. 14e). These results indicate that transplanted iHep cells can repopulate and rescue Fah<sup>−/−Rag2<sup>−/−</sup></sup> recipients and that, without fusion with recipient liver cells, the iHep cell repopulation restored the normal liver architecture by replacing Fan<sup>−/−</sup> hepatocytes in death.

Macroscopically, iHep-Fah<sup>−/−Rag2<sup>−/−</sup></sup> livers were found to be normal and healthy, whereas livers from NTBC-off Fah<sup>−/−Rag2<sup>−/−</sup></sup> control mice were swelled with many necrotic lesions (FIG. 4a). The hexagonal hepatic lobule was destructed due to massive cell death.
in NTBC-off Fah"+"Rag2"−" livers (FIG. 15a). In contrast, iHep cell repopulation restored liver architecture without apparent cell death (FIGs. 15a and b).

Remarkably, both repopulated iHep cells and repopulated primary hepatocytes expressed Alb and other hepatic genes at comparable levels in Fah"+"Rag2"−" mice (FIGs. 12c and d). As shown in FIG. 15d, Fah" nodule were isolated by laser-captured microscopy from four serial liver sections. The first section was immunostained with anti-Fah antibody to locate the repopulated Fah" nodules in the recipient livers (Fah" nodules were brown stained and indicated by yellow dash lines). Fah" tissues with the nodules were microdissected from the other 3 sections. After microdissection, those leftover sections on the slides were further stained with anti-Fah antibody to confirm that only the Fah" nodules were microdissected. Tissues from the same Fah" nodule were pooled for RNA extraction. In total, 3 iHep cell-repopulated nodules and 3 primary hepatocyte-repopulated nodules were analyzed. mRNA levels of indicated genes were measured in repopulated iHep cells and repopulated primary hepatocytes in F/R recipient livers.

Moreover, serum levels of tyrosine, phenylalanine, ornithine, alanine and glycine were markedly reduced in iHep-Fah"+"Rag2"−" mice compared to NTBC-off Fah"+"Rag2"−" mice (FIGs. 4b and c, FIGs. 15e-g, and Table 5, P<0.05). iHep-Fah"+"Rag2"−" mice also showed decreased levels of total bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (FIGs. 4d-f and Table 6, P<0.05). These demonstrate that iHep cell transplantation substantially improves liver functions of NTBC-off Fah"+"Rag2"−" mice.

Thus, in contrast with other cell-type conversion via lineage-specific transcription factors (Vierbuchen et al. Nature 463, 1035-1041 (2010); Ieda et al. Cell 142, 375-386 (2010); Szabo et al. Nature 468, 521-526 (2010)), the in vivo function of iHep cells has been rigorously proven.

Assays were also carried out to examine whether the above-described iHep cells are tumorigenic. As shown in FIG. 16a, tumours were not found in iHep-Fah"+"Rag2"−" livers 2 months after transplantation. Indeed, Ki67 staining revealed that iHep cells ceased proliferation 8 weeks after transplantation. Moreover, it was found that iHep cells did not form tumours 8 weeks after subcutaneous xenograft in NOD/SCID mice (FIG. 4g). A total of 20 out of 25 analyzed iHep cells displayed 40 chromosomes after 17 passages, which was comparable with results from wild-type cells. These results indicate that iHep cells are genetic stable and not tumor prone.
Table 5

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<td>lle</td>
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<td>97.71±19.59</td>
<td>121.03±15.53*</td>
<td>109.86±27.12</td>
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</table>

Note: Serum levels of amino acids were measured in wildtype mice (WT), F/R mice 8 weeks after iHep cell transplantation (iHep-F/R), and F/R mice with 20% body weight losing after NTBC removal (F/R). Data are presented as means±s.d. Asterisks indicate the values are significantly reduced compared with those in F/R mice (P<0.05, f-test).
Table 6.

<table>
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<tr>
<th></th>
<th>Unit</th>
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<th>iHep-F/R</th>
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<td>μM</td>
<td>0.45±0.38</td>
<td>2.08±1.34*</td>
<td>45.68±30.70</td>
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<td>ALB</td>
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<td>38.30±1.89</td>
<td>25.46±2.78</td>
<td>26.24±5.47</td>
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<td>ALT</td>
<td>U/L</td>
<td>24.05±7.65</td>
<td>86.28±36.47*</td>
<td>153.92±45.92</td>
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<tr>
<td>AST</td>
<td>U/L</td>
<td>138.68±88.79</td>
<td>170.30±40.27*</td>
<td>308.82±87.75</td>
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<tr>
<td>BUN</td>
<td>mM</td>
<td>10.0±2.4</td>
<td>4.7±0.5*</td>
<td>9.4±4.9</td>
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<tr>
<td>Cr</td>
<td>μM</td>
<td>11.7±4.4</td>
<td>9.2±1.3*</td>
<td>13.4±3.0</td>
</tr>
</tbody>
</table>

Note: Serum levels of total bilirubin (TBIL), albumin (ALB), alanine transaminase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and Creatinine (Cr) were measured in wildtype mice (WT), F/R mice 8 weeks after iHep cell transplantation (iHep-F/R), and F/R mice with 20% body weight losing after NTBC removal (F/R). Data are presented as mean±s.d.. Asterisks indicate the values are significantly changed in iHep-F/R mice compared with those in F/R mice (P<0.05, t-test).

EXAMPLE 5

In this example, the above-described strategy for obtaining hepatocyte-like cells through direct lineage conversion was used to covert cells of human origin to human hepatocyte-like cells.

Briefly, human 293FT cells were forced to express human Foxa3 and Hnfla, as well as human Gata4, by infecting the cells with Lentiviruses expressing the transcription factors in the same manner described above. Expressions of hepatic genes, such as Albumin, Afp, Transferrin, Ttr and Tat were analyze by RT-PCR using mRNAs isolated from 293FT cells 6 days after Lentiviral infection in the same manner described above. As shown in FIG. 17, the expressions of these hepatic genes were induced and up-regulated.

The same assays were conducted using (i) Lentiviruses expressing human Foxa2, Hnfla, and human Gata4, or (ii) Lentiviruses expressing mouse Hnfla, Foxa3, and Gata4 in human 293FT cells. As shown in FIG. 17, similar results were obtained.

The human 293FT cells expressing the heterologous mouse or human transcription factors were examined under a microspore. It was found that, six days after Lentiviral infection, the 293FT cells showed a morphological similar to primary cultured hepatocytes. See FIGs. 18A-D. The similar morphology was also observed in primary p^null mouse TTFs that were infected with Lentiviruses expressing human FOXA3, HNF1A and GATA4. See FIG. 18E.

Furthermore, primary human fetal skin fibroblasts were infected with Lentiviruses expressing human FOXA3, HNF1A, and GATA4 in the same manner described above.
As shown in FIG. 19, overexpression of human FOXA3, HNFIA, and GATA4 induced the formation of epithelial human iHep cells from fetal skin fibroblasts.

The above results demonstrate that human, non-liver cells can also be converted to hepatocyte-like cells via over-expressing as few as two (e.g., Hnf and Foxa) or three (Hnf, Foxa, and GATA) heterologous transcription factors. The 293FT cell line is a fast-growing, highly transfectable clonal isolate derived from human embryonic kidney cells transformed with the SV40 large T antigen. The above results also suggest that presence of the SV40 large T antigen, like the p19ΔR′ knocking down, allowed the cells to by-pass proliferation arrest and associated cell death.

The foregoing examples and description of the preferred embodiments should be taken as illustrating, rather than as limiting the present invention as defined by the claims. As will be readily appreciated, numerous variations and combinations of the features set forth above can be utilized without departing from the present invention as set forth in the claims. Such variations are not regarded as a departure from the scope of the invention, and all such variations are intended to be included within the scope of the following claims.

All references cited herein are incorporated herein in their entireties.
CLAIMS

WHAT IS CLAIMED IS:

1. A method of generating hepatocyte-like cells, comprising
expressing in a starting cell a Hnf polypeptide and a Foxa polypeptide, and
culturing the starting cell in a medium for a period of time to obtain one or more
progeny cells thereof, thereby generating hepatocyte-like cells.

2. The method of claim 1, wherein the method further comprises expressing in
the starting cell a GATA4 polypeptide.

3. The method of claim 2, wherein the Hnf polypeptide comprises the sequence
of SEQ ID NO: 1; the Foxa polypeptide comprises the sequence of SEQ ED NO: 2, and the
GATA4 polypeptide comprises the sequence of SEQ ED NO: 3.

4. The method of claim 3, wherein the method further comprises expressing in
the starting cell one or more polypeptides have sequences selected from the group consisting
of SEQ ED NO: 4-14.

5. The method of claim 1, wherein the starting cell is a somatic cell.

6. The method of claim 1, wherein the starting cell is a fibroblast, an epithelium
cell, a blood cell, a neuron, an embryonic cell, or a cell derived from a tissue or organ of a
subject.

7. The method of claim 1, wherein the starting cell is p1^null or expresses the
p19^{ΔR} gene at a level lower than a predetermined level.

8. The method of claim 1, wherein the method further comprises introducing into
the starting cell an agent that inhibits expression or activity of the p19^{ΔR} gene.

9. The method of claim 8, wherein the agent is an antibody, a nucleic acid, a
polypeptide, or a small molecule compound.
10. The method of claim 9, wherein the agent is an RNAi agent.

11. The method of claim 10, wherein the RNAi agent comprises a double-stranded structure having a first strand and a second strand, said first and second strands each being between 19 and 30 nucleotides long, and wherein the first strand is encoded by SEQ ID NO: 16.

12. The method of claim 1, wherein the period of time is 2-30 days.

13. A cultured recombinant cell comprising (i) a first agent selected from a first group consisting of a heterologous Hnf polypeptide and a first nucleic acid encoding the Hnf polypeptide; and (ii) a second agent selected from a second group consisting of a heterologous Foxa polypeptide and a second nucleic acid encoding the Foxa polypeptide.

14. The cell of claim 13, wherein the cell further comprises a third agent selected from a third group consisting of a heterologous GATA4 polypeptide or a third nucleic acid encoding the GATA4 polypeptide.

15. The cell of claim 13, wherein the cell is positive for one or more of hepatic functional genes.

16. The cell of claim 13, wherein the cell is capable of metabolizing one or more compounds selected from group consisting of phenacetin, testosterone, and diclofenac.

17. The cell of claim 13, wherein the cell is a hepatocyte-like cell that is obtained using the method of claim 1.

18. The cell of claim 13, wherein the cell is pi9

19. The cell of claim 13, wherein the cell further comprises a fourth agent that inhibits expression or activity of the pi9

20. A pharmaceutical composition comprising the cell of claim 13 and a pharmaceutically acceptable carrier.

21. A bioartificial device comprising the cell of claim 13.

22. A method for improving the liver function of a subject, comprising (i) administering to a subject in need thereof the cell of claim 13 or (ii) implanting the device of claim 21 in the subject, thereby improving the liver function.

23. A method of evaluating toxicity, carcinogenicity, or biotransformation activity of a test substance, comprising contacting a test substance with the cell of claim 13, and examining a level of metabolic activity or viability of the cell, wherein the value of the level indicates the toxicity, carcinogenicity, or biotransformation activity of the test substance.

24. A composition comprising (i) a first agent selected from a first group consisting of an isolated Hnf polypeptide and a first nucleic acid encoding the Hnf polypeptide; and (ii) a second agent selected from a second group consisting of an isolated Foxa polypeptide and a second nucleic acid encoding the Foxa polypeptide.

25. The composition of claim 24, wherein the composition further comprises a third agent selected from a third group consisting of an isolated GATA4 polypeptide and a third nucleic acid encoding the GATA4 polypeptide.

26. A kit comprising the composition of claim 24 or 25, an agent that inhibits expression or activity of the pi94f gene, and a starting cell.

27. Use of the cultured recombinant cell of any of claims 11-15, the pharmaceutical composition of claim 20, or the bioartificial device of claim 21 in the manufacture of a medicament for improving the liver function of a subject.

28. The method of claim 12, wherein the period of time is 5-25 days.

29. The method of claim 28, wherein the period of time is 14-21 days.
FIGS. 1a-f
FIGs. 2a-l

a

No iHep cell injection
Liver failure and death

Fah<sup>−/−</sup>Rag2<sup>−/−</sup> mice maintained with NTBC water
Intrasplenic iHep cell injection
Repopulation and liver function restoration

b

Cumulative survival rate (%)

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c

Fah<sup>−/−</sup>Rag2<sup>−/−</sup>

iHep-Fah<sup>−/−</sup>Rag2<sup>−/−</sup>

Y-FISH

FIGs. 3a-d

2/12
FIGS. 4a-g

a. Different images depicting FAH and Rag2 knockout models.
b. Graphs showing tyrosine and phenylalanine levels.
c. Additional graphs likely related to enzyme activity or expression.
d. Bar charts representing total bilirubin levels.
e. Enzyme activity measures like ALT and AST.
f. Data on tumour-bearing mice and injected cell counts.

FIGS. 5a-c

a. Gel electrophoresis results for Albumin, Tdo2, and β-Actin.
b. Imaging results comparing WT TTF+14TF and p19Arf-null TTF+14TF.
c. Additional gel results for Albumin, Tdo2, Tr, and β-Actin.
FIG. 8

![Image of gel electrophoresis showing expression levels of Albumin, Tdo2, Transferrin, E-cadherin, and β-Actin across different conditions: H₂O, MEF+GFP; Primary hepatocyte; MEF+Gata4, Hnf1α, Foxa3.]

FIGS. 9a-c

![Graph showing relative mRNA levels for non-targeting vs. p19Arf-shRNA.](image)

![Image of TTF+p19Arf-shRNA+3TF condition showing cellular morphology.](image)

![Image of gel electrophoresis for TTF+GFP, Primary hepatocyte, TTF+p19Arf-shRNA+3TF conditions showing expression levels of Albumin, Aat, Ttr, Cldn2, and β-Actin.](image)
FIGs. 10a-f
FIGS. 11a-f
FIG. 12

Bufuralol metabolism

FIG. 13
FIGs. 14a-e
FIGS. 15a-g
FIGS. 16 a and b

FIG. 17
## INTERNATIONAL SEARCH REPORT

### A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

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)

IPC:C12N, A61M, A61K, A61P

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

Electronic database consulted during the international search (name of data base and, where practicable, search terms used)

Databases: DWPI, SipoABS, CPRSABS, CNABS, CNTXT, JPTXT, CNKI, ISI WEB OF KNOWLEDGE;

Search terms: hepatocyte, hepatic, fibroblast, hepatocyte nuclear factor, forkhead box a, hnf, foxa, hnf3, gata4, pI9, differentiation

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>Tao Liu, &quot;Effect of the Lentivirus-mediated Constitutive Expression of Foxa2 and Hnf4a upon the Hepatic Differentiation of Embryonic Stem Cells&quot;, Chinese Doctoral Dissertations, Full-text Database(Medicine and Health Sciences),No.50064-3,1 5May2011(15.05.2011),See p.50-69</td>
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* 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 filing of the international search report

09 Feb. 2012 (09.02.2012)

Name and mailing address of the ISA/CN

The State Intellectual Property Office, the P.R.China
6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088
Facsimile No. 86-10-62019451

Authorized officer

PANJUNYU

Telephone No. (86-10)62411086

Form PCT/ISA/210 (second sheet) (July 2009)
### INTERNATIONAL SEARCH REPORT

**International application No.**

PCT/CN2011/001857

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Form PCT/ISA/210 (continuation of second sheet) (July 2009)
INTERNATIONAL SEARCH REPORT

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 22
   because they relate to subject matter not required to be searched by this Authority, namely:
   
   claims 22 is directed to a method of treatment of the human/animal body

2. ☐ Claims Nos.:  
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on protest  ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.
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A. CLASSIFICATION OF SUBJECT MATTER

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C12N5/073 (2010.01) i
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C12N15/85 (2006.01) i
C12N15/113 (2010.01) i
A61M37/00(2006.01)i
A61K48/00(2006.01)i
A61P1/16(2006.01)n