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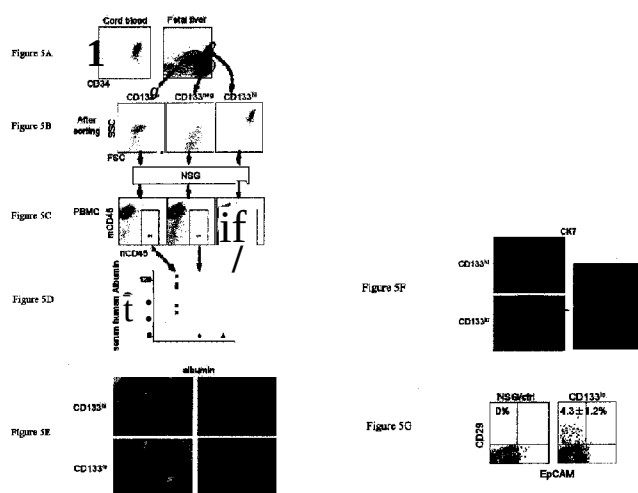
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(54) Title: METHODS OF RECONSTITUTING HUMAN HEPATOCYTES AND HUMAN HEMATOPOIETIC CELLS IN
NON-HUMAN MAMMALS



Figs. 5A-5G. *In vivo* transplantation of sorted cell subsets from human CD34⁺ fetal liver cells in NSG mice. (5A) Comparison of CD34⁺ cells purified from human cord blood (left) and fetal liver (right). Fluorescent CD133 and CD34 antibodies were used to analyze the cell phenotypes. Representative data from 5 cord blood and 3 fetal liver samples are shown. (5B) CD133^{int}, CD133^{lo}, CD133^{neg} cell subsets were isolated from CD34⁺ human fetal liver cells by cell sorting. The FACS plots show FSC versus SSC. (5C) These three populations were then injected separately into 3 groups of new born NSG pups (n=5). Ten weeks later, mononuclear cells from blood were prepared for staining of human CD45 and mouse CD45 antibodies. (5D) Sera were analyzed by human albumin specific ELISA. (5E) Livers from the mice injected with different cell subsets were harvested. Paraffin sections were prepared and stained with human specific albumin antibody to analyze the reconstitution of human hepatocytes. (5F) human specific CK7 antibody was used to stain human biliary epithelial cells in the liver sections. Black arrow indicates the positive biliary duct cells. (5G) Nine weeks after injection, single cell suspensions were prepared from the livers by perfusion and stained with CD29 and EpCAM antibodies (n=8). Liver from adult NSG mice served as negative control (n=2). Representative data are shown. See also Fig. 10.

(57) Abstract: The present invention is directed to mammalian hepatic progenitor/stem cells (HPSCs); methods of producing *in vivo* model containing livers repopulated with human hepatocytes, and optionally, a human hematopoietic system; the models produced by the methods; and uses of the models (e.g., to study the etiology and therapy of viral and nonviral human liver diseases, hepatocyte biology and human hepatomas). Also provided herein are abundant mammalian (e.g., human) hepatocyte progenitors which can be used in a variety of ways and methods of culturing HPSCs.

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METHODS OF RECONSTITUTING HUMAN HEPATOCYTES AND HUMAN HEMATOPOIETIC CELLS IN NON-HUMAN MAMMALS

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No.
5 61/362,168, filed on July 7, 2010. The entire teachings of the above application are
incorporated herein by reference.

BACKGROUND OF THE INVENTION

The human liver is an important organ for pharmacological studies aimed at
developing new human medicines. Mice containing livers repopulated with human
10 hepatocytes would provide excellent in vivo models for studies on human liver
functions, diseases and hepatotropic viruses such as chronic hepatitis virus (HBV and
HCV). Researchers have developed chimeric mice by partially repopulating the mouse
liver with human mature hepatocytes and used these mice for HBV and HCV studies
(Dandri, M., *et al*, *Hepatology*, 33:981-988 (2001); Tateno, C.Y., *et al*, *Am J Pathol*,
15 **165:901-912** (2004)). But the existing models require uPA transgenic mice which are
difficult to maintain as well as complicated surgical procedures. And furthermore, no
application has been shown to reconstitute human hematopoietic cells and human
hepatocytes in the same humanized mice, which would provide an opportunity to have
an insight into human in vivo immune responses to hepatotropic viruses. Therefore, a
20 small animal model that can meet both aspects would facilitate human liver disease
study and therapeutics development.

SUMMARY OF THE INVENTION

In severe injury, liver-cell progenitors may play a role in recovery, proliferating, and subsequently differentiating into mature liver cells. The proliferative potential that progenitor cells have is a major advantage for therapeutic exploitation compared to mature hepatocytes that have low proliferating activity. It has been reported that during rat fetal development, bipotential cells, known as hepatoblasts, are present in the liver (Shiojiri, N., *et al*, *Cancer Res*, 51:2611-2620 (1991)). Maturation of human fetal liver multi-potential cells along pathways into either hepatocytes or biliary epithelial cells has been demonstrated both in vivo and in vitro (Dan, Y.Y., *et al*, *Proc Natl Acad Sci USA*, 103:9912-9917 (2006)). Identifying these progenitors has major therapeutic potential for ex vivo pharmaceutical testing, bioartificial liver support, tissue engineering and gene therapy protocols.

Described herein is the investigation of whether the human fetal liver is an enriched stem cell pool and a resource for hematopoietic stem cells (HSC) as well as non-hematopoietic stem cells including human hepatocyte progenitors. A novel population of human hepatocyte progenitors were identified in fetal liver.

Human CD34⁺ stem cells were purified from total fetal liver cells by magnetic selection. Similar to cells from human cord blood, engraftment of these CD34⁺ cells into sub-lethally irradiated new born NSG mice led to the reconstitute human hematopoietic cells in the mice. However, human albumin⁺ hepatocytes were found only in the livers from fetal liver cell reconstituted mice. Furthermore, it is shown herein that within the CD34⁺ fetal liver cells CD34⁺CD133^{lo} cells are hepatocyte progenitors, referred to herein as hepatic progenitor/stem cells (HPSCs), that gave rise to hepatocytes following engraftment in NSG recipient mice, whereas CD34⁺CD133^{hi} cells are hematopoietic stem cells (HSCs) that gave rise to hematopoietic cells when engrafted in NSG recipient mice.

Accordingly, provided herein are isolated mammalian (*e.g.*, human) hepatic progenitor/stem cells (HPSCs), mammalian hepatic hematopoietic stem cells (HSCs), methods of reconstituting human hepatocytes, and optionally, human blood cell lineages

in a non-human mammal such as a mouse, uses of such non-human mammals (*e.g.*, humanized mammalian models) and methods of culturing human hepatocytes.

In one aspect, the invention is directed to an (one or more) isolated mammalian hepatic progenitor/stem cell (HPSC) which expresses CD34⁺CD133^{lo}. The HPSC can
5 further express CD117, CD44, CD45, CD73, vimentin, EpCAM, α -fetoprotein (AFP), albumin, CD166, CD24 or a combination thereof. In particular embodiments, the HPSC is a human HPSC. In other embodiments, the HPSC is a fetal HPSC. In a particular embodiment, the isolated mammalian HPSC is a human fetal HPSC.

Also described herein is an isolated mammalian hepatic hematopoietic stem cell
10 (HSC) which expresses CD34⁺CD133^{hi}. The HSC can further express CD45, CD44, CD117, CD166, CD90, CD105, CD97 or a combination thereof.

In another aspect, the invention is directed to a method of reconstituting human hepatocytes in a non-human mammal. The method comprises introducing human hepatic progenitor/stem cells (HPSCs) which express CD34⁺CD133^{lo} into an
15 immunodeficient non-human mammal, and maintaining the non-human mammal under conditions in which the non-human mammal's liver is reconstituted with human hepatocytes, thereby reconstituting human hepatocytes in a non-human mammal.

In another aspect, the invention is directed to a method of reconstituting human hepatocytes and human blood cell lineages in a non-human mammal. The method
20 comprises introducing human hepatic progenitor/stem cells (HPSCs) which express CD34⁺CD133^{lo} and human hematopoietic stem cells (HSCs) which express CD34⁺CD133^{hi} into an immunodeficient non-human mammal, and maintaining the non-human mammal under conditions in which the non-human mammal's liver is reconstituted with human hepatocytes and the non-human mammal's blood is
25 reconstituted with human blood cells, thereby reconstituting human hepatocytes and human blood cell lineages in a non-human mammal.

In another aspect, the invention is directed to method of producing a non-human mammal for use as a model of human liver disease. The method comprises introducing human hepatic progenitor/stem cells (HPSCs) which expresses CD34⁺CD133^{lo} into an

- immunodeficient non-human mammal; and maintaining the non-human mammal under conditions in which the non-human mammal's liver is reconstituted with human hepatocytes. One or more agents that produce a human liver disease are also introduced into the non-human mammal; and the non-human mammal is maintained under
- 5 conditions in which the human liver disease develops in the non-human mammal; thereby producing a non-human mammal for use as a model of human liver disease.

Non-human mammals produced by the methods described herein are also provided.

- In another aspect, the invention is directed to a method of identifying one or
- 10 more agents or treatment protocols that can be used to treat a human liver disease. The method comprises administering the one or more agents or treatment protocols to a non-human mammal that is a model of a human liver disease produced as described herein, and determining whether the human liver disease in the non-human mammal is alleviated. If the human liver disease is alleviated in the non-human mammal, then the
- 15 one or more agents or treatment protocols can be used to treat the human liver disease.

- In another aspect, the invention is directed to a method of identifying one or more effects of one or more agents on a human liver. The method comprises administering the one or more agents to a non-human mammal which comprises human hepatocytes produced as described herein and determining the effect of the agent on the
- 20 human liver cells of the non-human mammal, thereby identifying the effects of one or more agents on a human liver.

- In another aspect, the invention is directed to a method of identifying how one or more agents are metabolized by a human liver. The method comprises administering the one or more agents to a non-human mammal which comprises human hepatocytes
- 25 produced as described herein and maintaining the non-human mammal under conditions in which the one or more agents are metabolized by the human liver cells in the non-human mammal, thereby producing one or more metabolites in the non-human mammal. The one or more metabolites are detected and/or analyzed in the non-human mammal, thereby identifying how one or more agents are metabolized by a human liver.

In another aspect, the invention is directed to an *in vitro* method of obtaining mammalian (*e.g.*, human) hepatocytes. The method comprises contacting mammalian (*e.g.*, human) hepatic progenitor/stem cells (HPSCs) which express CD34⁺CD133^{lo} with hepatocyte differentiation medium, thereby producing a culture and maintaining the culture under conditions in which the HPSCs differentiate into mammalian (*e.g.*, human) hepatocytes, thereby obtaining mammalian (*e.g.*, human) hepatocytes.

The invention is also directed to mammalian (*e.g.*, human) hepatocytes (*e.g.*, cells, cell lines, cell cultures) produced by the methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows isolation of three CD34⁺ populations by fluorescence activated cell sorting (FACS).

Fig. 2 shows a comparison of albumin levels in mice injected with CD133^{lo} cells via intra-liver (i.l) and intra-cardiac (i.c) injection using ELISA.

Fig. 3 shows the results of cell surface phenotyping of CD133^{lo} cell population.

Figs. 4A-4B show CD34⁺ cells from human fetal liver gave rise to hematopoietic cells and hepatocytes in NSG recipient mice. CD34⁺ cells isolated from cord blood (n=3) (Fig. 4A) and fetal liver (n=3) (Fig. 4B) were engrafted separately into sublethally irradiated NSG newborn pups by intracardiac injection (n=20). Eight weeks later, peripheral blood mononuclear cells (PBMC) were stained for human CD45 (hCD45) and mouse CD45 (mCD45) and analyzed by flow cytometry. Representative plots are shown. The numbers indicate percentages of human and mouse CD45⁺ cells among total live cells. Paraffin sections of the livers were stained with antibody specific for human albumin. Representative stains are shown. Magnification, 100X.

Figs. 5A-5G show identification of HSCs and HPSCs among CD34⁺ fetal liver cells. (5A) Comparison of CD34⁺ cells from human cord blood (left) and fetal liver (right) for CD133 expression. Purified CD34⁺ cells were stained for CD133 and analyzed by flow cytometry. Representative CD34 versus CD133 staining profiles are shown from 5 cord blood and 3 fetal liver samples. (5B) CD133^{hi}, CD133^{lo} and CD133^{int} subsets were isolated from CD34⁺ fetal liver cells by cell sorting. Purified

cells were reanalyzed for purity. (5C) The three sorted cell populations were injected separately into 3 groups of NSG pups (n=5 each). Ten weeks later, PBMCs were stained for human CD45 and mouse CD45. Shown are representative staining profile from each group. (5D) Sera were analyzed for the level of human albumin by ELSIA. (5E and 5F)

- 5 Livers from the mice injected with CD34⁺CD133^{hi} and CD34⁺CD133^{lo} cells were harvested. Paraffin sections were stained with antibody specific for the human albumin (5E) or specific for human CK7 (5F). Arrow points to CK7 stained biliary duct cells. (8G) Nine weeks after reconstitution, single cell suspensions were prepared from the livers and stained for CD29 and EpCAM (n=8). Liver from non-reconstituted NSG
- 10 mice was used as negative controls (n=2). Representative EpCAM versus CD29 staining profiles are shown. Also see Figs. 10A-IOC.

- Figs. 6A-6E show in vitro differentiation of the three subpopulation of CD34⁺ fetal liver cells. (6A) Morphological changes of cultured CD34⁺CD133^{lo} cells over time. Shown are representative phase-contrast micrographs at 2, 14 and 28 days of
- 15 culture. (6B) Cells were trypsinized and counted at different time points during the culture. The total cell number on a given day were calculated by multiplying the cell number from manual counting with dilutions based on passage numbers. (6C) Representative images of immunofluorescent staining for albumin and DAPI on the culture cells 28 days after culture. (6D and 6E) Colony-forming assays. CD34⁺CD133^{hi},
- 20 CD34⁺CD133^{neg}, and CD34⁺CD133^{lo} cells were plated under culture conditions for colony formation. Shown are representative phase-contrast micrographs taken 9 days after culture (6D). Arrows point to the CFU-GEMM, CFU-GM, and BFU-E colonies. (6E) Comparison of the frequencies of the different colonies at day 9 after culture.

- Figs. 7A-7D show CD34⁺CD133^{lo} fetal liver cells exhibit long-term self-renewal
- 25 properties. (7A) Single cell suspensions of hepatocytes of primary recipient mice stained for human CD34 and CD133 followed by flow cytometry. Representative staining profile of CD34 versus CD133 is shown for one of eleven mice. The number indicates percentage of human cells within the gated region. (7B) Human CD34⁺ cells

were purified by magnetic cell sorting. The purified cells were stained for human CD34, CD133, CD44 and EpCAM. Staining profiles of CD34 versus CD133 and EpCAM versus CD44 are shown. (7C and 7D) The purified CD34⁺ cells were injected into sublethally irradiated newborn NSG pups (10⁵ cells per recipient). Eight weeks later, liver paraffin sections were stained for human albumin (7C) and the level of human albumin in the sera was quantified by ELISA (7D). Adult non-reconstituted NSG mice were used as negative control. One dot represents one mouse in 7D.

Figs. 8A-8F show transcription profiles of HPSCs are more closely related to those of HSCs from both fetal liver and cord blood than to mature hepatocytes. (8A) Comparison of surface phenotype of selected markers among HPSCs and HSCs from fetal liver and HSCs from cord blood. Staining profiles of human stem cell-related markers CD44, CD117, EpCAM, CD90, CD105, CD97, CD24, CD73, CD166 and CD45 are shown as histograms. Dark line, stained with specific antibodies; thin line, isotype controls. (8B) Comparison of relative transcript levels of selected genes among purified CD34⁺CD133^{hi}, CD34⁺CD133^{lo}, CD34⁺CD133^{neg} fetal liver cells and mature hepatocytes. Total RNA was isolated from purified cell subsets and assayed by quantitative RT-PCR. Data shown are obtained from two different fetal liver samples. (8C) Transcriptional profiling analysis. Shown is hierarchical cluster analysis of transcriptomes of the six cell types. Values at branches are AU p-values (left), BP values (right) and cluster labels (bottom). The height scale reflects a distance measure that is scaled between 0 and 1. CD34⁺CD133^{lo}, CD34⁺CD133^{hi} and CD34⁺CD133^{neg} cells were from three biological samples. (8D) Comparison of transcript levels for selected HSC-specific transcription factor between CD34⁺CD133^{lo} and CD34⁺CD133^{hi} cells by RT-PCR (n=2). (8E and 8F) Comparison of transcript levels for selected endoderm signature gene between CD34⁺CD133^{lo} and CD34⁺CD133^{hi} cells by RT-PCR (n=2). See also Figs. 12A-12C.

Fig. 9 shows a comparison of cell surface phenotype of human hepatoma cell lines with that of HPSCs and mature hepatocytes. Human CD34⁺CD133^{lo} fetal liver cells, human mature hepatocytes, Hep 3B cells, Hep G2 cells and SNU-423 cells were

stained for CD133, CD34, CD44, CD117, EpCAM, CD90, CD105, CD97, CD24, CD73, and CD166. Expression of each marker is shown as histogram. Dark line, stained with specific antibodies; thin line, isotype controls.

Figs. 10A-10C: (10A) Fetal liver cells were stained for CD34 and CD133 after CD34 selection. Shown are cytometry FSC and SSC data and phase-contrast micrograph of the sorted three cell populations. (10B) Human specific CK19 antibody was used to stain human biliary epithelial cells in the liver sections. Black arrows indicate the bile ducts positive for human CD19 while the white ones indicate the negative ducts. (10C) Cy3 fluorescence conjugated probe against human pan centromere and DAPI were used to stain human cells in the liver sections. Human fetal liver sections were used as positive control; NSG mouse liver sections were used as negative control. White arrows in the liver sections prepared from mice injected with CD133^{hi} cells indicate positive cells for human pan-centromere stainings.

Fig. 11: CD133^{hi} cells were sorted from CD34⁺ cells purified from human fetal liver. 1X10⁵ cells were injected intra-cardially into new born NSG mice (n= 10). 10 weeks after injection, splenocytes were isolated and stained for antibodies against human antigens: CD45, CD3, CD19, CD56, CD14, CD11c, BDCA-1, CD303, ILT7 and mouse CD45.

Figs. 12A-12C: (12A) CD133^{hi} and CD133^{lo} cells were sorted by FACS sorter and fixed on slides by Cytospin. Antibodies against human albumin, AFP, CK7 and CK19 were used to stain the cells. DAPI. Albumin, AFP, CK7 and CK19. (12B) Principal component analysis (PCA) of the microarray data. Shown is 3 dimensional plot of PCA. Each data point represents individual sample/array, with all 12 sample/arrays presented on the PCA plot. The percentage values indicate the proportion of total variance described by each PC. PC 1 principal component 1 (X-axis); PC 2 principal component 2 (Y-axis); PC 3 principal component 3 (Z-axis). CD133^{neg}, CD133^{hi} and CB HSC samples/arrays aggregate together which signify their similarity in expression profile. However, hepatocyte were separated from the other. (12C) Comparison of differentially expressed genes. Shown are number of common genes

shared by CD133^{lo} and **CD133^{hi}**. Compared to hepatocyte, CD133^{lo} and **CD133^{hi}** share 4636 differentially expressed genes but compared to CB HSC, CD133^{lo} and 133^{hi} shared only 468 genes.

DETAILED DESCRIPTION OF THE INVENTION

- 5** Hepatic progenitor/stem cells (HPSCs) are of great interest because of their potential applications in preclinical pharmaceutical testing and clinical transplantation. In rodents, hepatoblasts in fetal liver and oval cells in adult liver are known to exhibit hepatic progenitor cell properties as they can differentiate into both hepatocytes and biliary epithelial cells (cholangiocytes) *in vitro* as well as *in vivo* following adoptive
- 10** transfer into recipient mice or rats (Strick-Marchand, H., and Weiss, M. C. (2002), *Hepatology* 36, 794-804; Walkup, M. H., and Gerber, D. A. (2006), *Stem Cells* 24, 1833-1840). Although hepatoblasts and oval cells have also been reported in human fetal and adult livers, respectively, they are far less well characterized than their rodent counterparts.
- 15** Two major challenges have prevented the unequivocal identification and isolation of HPSCs from human fetal liver. First, there is a lack of a specific marker or a combination of markers for the identification and isolation of HPSCs from human fetal liver. Fetal liver is rich not only in HPSCs but also hematopoietic stem cells (HSCs). In rodents, some studies have shown that HPSCs share cell surface markers associated
- 20** with HSCs, such as CD34 (Blakolmer, *et al.* (1995) *Hepatology* 21, 1510-1516; Lemmer, E. R. *et al.* (1998), *J Hepatol* 29, 450-454), CD90 (Fiegel, H. C , *et al.* (2003) *Hepatology* 37, 148-154) and CD117 (Monga, S. P. *et al.* (2001), *Cell Transplant* 10, 81-89) while other studies have shown that HPSCs are distinct from HSCs by expressing hepatic and biliary cell markers such as α -fetoprotein (AFP), albumin and
- 25** cytokeratin (CK) (Kubota and Reid (2000), *Proc Natl Acad Sci USA*, 97, 12132-12137; Minguet *et al.* (2003) *J Clin Invest*, 112(8), 1152-1163; Nierhoff *et al.* (2005), *Hepatology* 42(1), 130-139; Tanimizu *et al.* (2003) *J Cell Sci*, 116 (PT 9), 1775-1786). Similar contradicting phenotypes have also been reported for HPSCs from human fetal

liver (Dan Y.Y. *et al*, *Proc Natl Acad Sci, USA*, 103, 9912-9917 (2006); Lemmer, E.R. *et al*. *J Hepatol*, 29, 450-454 (1998); Malhi, H. *et al*. *J Cell Sci*, 115, 2679-2688 (2002); Schmelzer, E. *et al*. (2007) *J Exp Med*, 204, 1973-1987). Furthermore, CD45⁺ CD34⁺ cells from mouse bone marrow were shown to give rise to hepatocytes following
5 adoptive transfer into recipient mice (Lagasse, E., *et al*. (2000), *Nat Med* 6, 1229-1234; Theise, N. D. *et al*. (2000a), *Hepatology* 31, 235-240). This kind of trans-differentiation was also observed in the patients receiving bone marrow transplantation (Theise, N. D. *et al*. (2000b), *Hepatology* 32, 11-16). Although some of these observed trans-differentiations were later shown to result from fusion between hematopoietic cells and
10 hepatocytes, confusion persists. Whether HPSCs and HSCs are separate lineages and can be uniquely isolated from human fetal liver remains unknown.

Second, there is a lack of small animal model to functionally evaluate the developmental potential of human HPSCs once isolated. While the progenitor and stem cell property of freshly isolated HPSCs from rodents can be readily evaluated by
15 adoptive transfer into syngeneic recipients, human HPSCs cannot be readily evaluated in immunocompetent mice or rats without inducing graft rejection. Even when immunodeficient mice, such as SCID (Malhi, H. *et al*. (2002), *J Cell Sci* 115, 2679-2688), NOD/SCID (Schmelzer, E., *et al*. (2007), *J Exp Med* 204, 1973-1987), Pfp/Rag2^{-/-} (Weiss, T. S. *et al*. (2008), **Gut 57, 1129-1138**) and Rag2^{-/-}γ^{-/-} (Dan, Y. Y., *et al*, (2006) *Proc Natl Acad Sci USA* 103, 9912-9917), were used, the widely used
20 protocol of surgical intra-splenic injection is cumbersome. Although human hepatocytes were detected in the recipient mice, the frequency of human hepatocytes in the mouse liver has not been accurately quantified due to generally poor reconstitution and the lack of quantitative assay. Instead, most studies of HPSCs from human fetal liver have
25 resorted to clonogenic and in vitro differentiation assays through long-term cultures of enriched progenitor cells. While the *in vitro* clonogenic assay could be very informative, it does not replace an *in vivo* assay under physiological conditions of the liver. Plus, the long-term in vitro culture could adversely affect the developmental potential of the isolated HPSCs. Thus, identification and characterization of human HPSCs urgently

needs an easy and efficient animal model to functionally evaluate the developmental potential of selected populations of fetal liver cells.

Construction of humanized mice by adoptive transfer of CD34⁺ cells from human fetal liver into sub-lethally irradiated NOD-SCID **n2rg**^{-/-} (NSG) mice is described herein. It was found that the recipient mice have not only human hematopoietic cells in the lymphoid systems but also human hepatocytes in the mouse liver. This technological breakthrough has enabled identification and isolation of HPSCs from human fetal liver that give rise to hepatocytes and biliary epithelial cells and HSCs that give rise to hematopoietic cells. Interestingly, the isolated HPSCs express many HSC markers and are transcriptionally more closely related to HSCs than to mature hepatocytes. In addition, shown herein is that some hepatoma cell lines exhibit the characteristic phenotype of HPSCs while others exhibit the surface phenotype of mature hepatocytes. Identification of human HPSCs and development of an efficient mouse model to evaluate these cells stimulates both basic and applied research of human HPSCs in health and diseases.

Shown herein is that simple adoptive transfer of CD34⁺ cells from human fetal liver into sublethally irradiated NOD-SCID **n2rg**^{-/-} (NSG) mice led to efficient development of both hematopoietic cells in the lymphoid organs and hepatocytes in the mouse liver. Also shown herein is that using this assay, CD34⁺CD133^{lo} fetal liver cells are HPSCs because they expanded and gave rise to both hepatocytes and biliary epithelial cells in primary recipients and were capable of self-renewal and secondary reconstitution. In contrast, CD34⁺CD133^{hi} fetal liver cells gave rise to hematopoietic cells in recipient mice. Besides expression of hematopoietic markers CD34 and CD133, HPSCs were transcriptionally more closely related to HSCs from both fetal liver and cord blood than to mature human hepatocytes. Nevertheless, HPSCs expressed mesenchymal markers CD73 and progenitor hepatocyte markers α -fetoprotein, albumin, vimentin, CK18, and CK19. Furthermore, some human hepatoma cell lines shared similar surface phenotype and transcriptional profiles of HPSCs. The results provided herein unequivocally identified HPSCs in human fetal liver and revealed a close

relationship between HPSCs and HSCs in the fetal liver. The simple and robust assay described herein facilitates both basic and applied research of human HPSCs in health and diseases.

Accordingly, the present invention is directed to mammalian HPSCs; methods
5 of producing *in vivo* models containing livers repopulated with human hepatocytes, and optionally, a human hematopoietic system; the models produced by the methods; and uses of the models (*e.g.*, to study the etiology and therapy of viral and nonviral human liver diseases, hepatocyte biology and human hepatomas). Also provided herein are abundant human hepatocyte progenitors which can be used in a variety of ways, as well
10 as methods of culturing HPSCs.

Specifically, in one aspect, the invention is directed to isolated HPSCs. In another aspect, the invention is directed to compositions of mammalian fetal liver cells comprising, consisting essentially of, or consisting of HPSCs which express CD34⁺ CD133^{lo}. In other aspects, the composition comprises, consists of, or consists
15 essentially of mammalian fetal HPSCs which express CD34⁺ CD133^{lo}. As described herein, the invention is also directed to uses of such compositions. The compositions can further comprise, consist essentially of, or consist of mammalian fetal hematopoietic stem cells (HSCs) which express CD34⁺ CD133^{hi}.

The invention is directed to an (one or more) isolated mammalian (*e.g.*, human)
20 fetal HPSC wherein the cell expresses CD34⁺CD133^{lo}. The fetal HPSC can further expresses CD1 17, CD44, CD45, CD73, vimentin, EpCAM, a-fetoprotein (AFP), albumin, CK7 and CK19 or a combination thereof. The invention is also directed to isolated mammalian (*e.g.*, human) fetal liver cells which comprise, consist essentially of, or consist of HPSC which express CD34⁺CD133^{lo} and HSCs which express
25 CD34⁺CD133^{hi}.

As used herein, "isolated" (*e.g.*, "isolated mammalian fetal HPSC"; "isolated mammalian fetal HSC") refers to substantially isolated with respect to the complex (*e.g.*, cellular) milieu in which it (*e.g.*, naturally) occurs (*e.g.*, organ, body, tissue, blood, or culture medium). In some instances, the isolated material will form part of a

composition (*e.g.*, a crude extract containing other substances), buffer system, culture system or reagent mix. In other circumstances, the material can be purified to essential homogeneity. Isolated mammalian fetal HPSCs can comprise at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% (on a total cell number basis) of all cells present. In one embodiment, the invention is directed to isolated, or substantially isolated (or purified, substantially purified) mammalian fetal HPSCs described herein.

The compositions of the invention can be used in a variety of ways. In one aspect, the invention is directed to a method of reconstituting human hepatocytes in a non-human mammal. The method comprises introducing human hepatic progenitor/stem cells (HPSCs) which express CD34⁺CD133^{lo} into an immunodeficient non-human mammal, and maintaining the non-human mammal under conditions in which the non-human mammal's liver is reconstituted with human hepatocytes, thereby reconstituting human hepatocytes in a non-human mammal.

In another aspect, the invention is directed to a method of reconstituting human hepatocytes and human blood cell lineages in a non-human mammal. The method comprises introducing human hepatic progenitor/stem cells (HPSCs) which express CD34⁺CD133^{lo} and human hematopoietic stem cells (HSCs) which express CD34⁺CD133^{hi} into an immunodeficient non-human mammal, and maintaining the non-human mammal under conditions in which the non-human mammal's liver is reconstituted with human hepatocytes and the non-human mammal's blood is reconstituted with human blood cells, thereby reconstituting human hepatocytes and human blood cell lineages in a non-human mammal.

In particular aspects, the human HPSCs expand at least 200 fold, 300 fold, or 400 fold during differentiation into human hepatocytes in the non-human mammal.

Mammalian (*e.g.*, human) HPSCs and/or HSCs can be obtained and purified as described herein, using routine methods known to those of skill in the art or from commercial sources. In one embodiment, HPSCs and/or HSCs are obtained from a fetal (*e.g.*, human) liver. In other embodiments, HPSCs and/or HSCs are obtained from

embryonic stem cells and/or cloned cells. For example, fetal liver (*e.g.*, single cell suspensions of human fetal liver) can be contacted with antibodies specific for the appropriate cell surface markers (*e.g.*, CD34⁺ CD133^{lo}; CD34⁺ CD133^{hi}; CD1 17; CD44; CD45; CD73; vimentin; EpCAM; α -fetoprotein (AFP); albumin) and separated
5 using fluorescence activated cell sorting (FACS) analysis. In yet other embodiments, the cells are further enriched for the HPSCs and/or HSCs prior to introduction into the non-human mammal. Methods for enriching an isolated cell population are routine and known to those of skill in the art.

The mammalian HPSCs and HSCs for use in the methods of the invention can
10 be introduced into the non-human mammal directly as obtained (*e.g.*, unexpanded) or manipulated (*e.g.*, expanded) prior to introduction into the non-human mammal. In one embodiment, the mammalian HPSCs and HSCs are not expanded prior to introducing the HSCs into the non-human mammal. In another embodiment, the mammalian HPSCs and HSCs are expanded prior to introducing the HSCs into the non-human mammal. As
15 will be appreciated by those of skill in the art there are a variety of methods that can be used to expand mammalian HPSCs and HSCs are expanded prior to introduction into the non-human mammal (see *e.g.*, Zhang, Y., *et al.*, *Tissue Engineering*, 12(8):2161-2170 (2006); Zhang CC, *et al.*, *Blood*, 111(7):3415-3423 (2008)). See for example, Maroun, K., *et al.*, *ISSCR, 7th Annual Meeting*, Abstract No. 1401 (July 8-11, 2009) and
20 PCT Application No. PCT/US2010/036664, filed May 28, 2010, published as WO 2011/002721 A1 which are incorporated herein by reference.

The mammalian HPSCs and HSCs for use in the methods can be obtained from a single donor or multiple donors. In addition, the mammalian HPSCs and HSCs used in the methods described herein can be freshly isolated, preserved (*e.g.*, cryopreserved) or
25 combinations thereof.

The amount of HPSCs, and optionally, HSCs that are introduced into the non-human mammal will vary depending on a variety of factors such as the cells, the non-human mammal, the application for which the cells are being introduced. In one embodiment, about 0.1×10^5 HPSCs to about 10×10^5 HPSCs are introduced into the

non-human mammal. In other embodiments, about 0.5×10^5 HPSCs, 1×10^5 , 1.5×10^5 , 2×10^5 , 2.5×10^5 , 3×10^5 , 3.5×10^5 , 4×10^5 , 4.5×10^5 , 5×10^5 , 5.5×10^5 , 6×10^5 , 6.5×10^5 , 7×10^5 , 7.5×10^5 , 8×10^5 , 8.5×10^5 , 9×10^5 , 9.5×10^5 , 10×10^5 , or 10.5×10^5 HPSCs re introduced into the non-human mammal. In a particular embodiment, about 1
5 $\times 10^5$ HPSCs are introduced into the non-human mammal. In another embodiment, about 2×10^5 HPSCs are introduced into the non-human mammal.

In the methods provided herein, HPSCs, and optionally HSCs, are introduced into a non-human mammal. As used herein, the terms "mammal" and "mammalian" refer to any vertebrate animal, including monotremes, marsupials and placental, that
10 suckle their young and either give birth to living young (eutharian or placental mammals) or are egg-laying (metatharian or nonplacental mammals). Examples of mammalian species that can be used to obtain HPSCs (*e.g.*, fetal) and/or HSCs (*e.g.*, fetal) include primates (*e.g.*, humans, monkeys, chimpanzees), rodents (*e.g.*, rats, mice, guinea pigs), canines, felines, and ruminants (*e.g.*, cows, pigs, horses). In some
15 embodiments, the HPSCs and/or HSCs are obtained from the fetus of a (one or more) mammal. In a particular embodiment, the fetal HPSCs and/or HSCs are human fetal HPSCs and/or HSCs.

Examples of non-human mammalian species that can be used to reconstitute human hepatocytes, and optionally human blood cell lineages, include non-human
20 primates (*e.g.*, monkeys, chimpanzees), rodents (*e.g.*, rats, mice, guinea pigs), canines, felines, and ruminants (*e.g.*, cows, pigs, horses). In one embodiment, the non-human mammal is a mouse. The non-human mammal used in the methods described herein can be adult, newborn (*e.g.*, < 48 hours old; pups) or *in utero*. In particular embodiment, the non-human mammal is a newborn or pup. In other embodiments, the non-human
25 mammal is an immunodeficient non-human mammal, that is, a non-human mammal that has one or more deficiencies in its immune system (*e.g.*, NSG or NOD scid gamma (NOD.Cg-Prkdcscid H2r^gtml Wjl/SzJ) mice) and, as a result, allows reconstitution of human hepatocytes and human blood cell lineages when fetal HPSCs and/or HSCs are introduced. For example, the non-human mammal lacks its own T cells, B cells, NK

cells or a combination thereof. In particular embodiments, the non-human mammal is an immunodeficient mouse, such as a non-obese diabetic mouse that carries a severe combined immunodeficiency mutation (NOD/scid mouse); a non-obese diabetic mouse that carries a severe combined immunodeficiency mutation and lacks a gene for the cytokine-receptor γ chain (NOD/scid IL2R $\gamma^{-/-}$ mouse); and a Balb/c rag $^{-/-}$ yc $^{-/-}$ mouse. Other specific examples of immunodeficient mice include, but are not limited to, severe combined immunodeficiency (*scid*) mice, non-obese diabetic (NOD)-*scid* mice, IL2rg $^{-/-}$ mice (e.g., NOD/LySz-*scid* IL2rg $^{-/-}$ mice, NOD/Shi-*scid* IL2rg $^{-/-}$ mice (NOG mice), BALB/c- *Rag $^{-/-}$ IL2rg $^{-/-}$* mice, *H2^d-Rag $^{-/-}$ IL2rg $^{-/-}$* mice), *NSD1Rag $^{-/-}$ IL2rg $^{-/-}$* mice.

In some embodiments, the non-human mammal is treated or manipulated prior to introduction of the cells. For example, the non-human mammal can be manipulated to further enhance engraftment and/or reconstitution of the cells. In one embodiment, the non-human mammal is irradiated prior to introduction of the cells. In another embodiment, one or more chemotherapeutics are administered to the non-human mammal prior to introduction of the cells. In other embodiments, the non-human mammal is not genetically engineered, and/or not treated to kill mouse liver cells and/or other mouse cells (e.g., immune cells) that would attack the human cells being introduced. In particular embodiments, the non-human mammal is sublethally irradiated, but not genetically engineered and/or not treated to kill mouse liver cells and/or other mouse cells (e.g., immune cells) that would attack the human cells being introduced, prior to introduction of the cells (e.g., HPSCs and/or HSCs).

As will also be appreciated by those of skill in the art, there are a variety of ways to introduce the cells into a non-human mammal. Examples of such methods include, but are not limited to, intradermal, intramuscular, intraperitoneal, intraocular, intrafemoral, intraventricular, intracranial, intrathecal, intravenous, intracardiac, intrahepatic, intra-bone marrow, subcutaneous, topical, oral and intranasal routes of administration. Other suitable methods of introduction can also include, *in utero* injection, hydrodynamic gene delivery, gene therapy, rechargeable or biodegradable

devices, particle acceleration devices ("gene guns") and slow release polymeric devices. The cells can be introduced in one or multiple injections. In a particular aspect, the cells are introduced in a single injection (*e.g.*, a single injection of cells, for example, on the first day the non-human mammal is born).

- 5 The amount of HPSCs introduced into the non-human mammal will vary depending upon a variety of factors such as the HPSCs being introduced, the non-human mammal, the application for which the non-human mammal is produced and the like. In particular embodiments about 50,000; 75,000; 100,000; 125,000; 150,000; 175,000; 200,000; 250,000; 275,000; 300,000; 350,000; 375,000; 400,000; 450,000; 10 475,000; or 500,000 HPSCs are introduced into the non-human mammal.

- In yet other embodiments, the HPSCs and/or HSCs are not treated (*e.g.*, not cultured) prior to introduction into the non-human mammal. That is, the HPSCs, and optionally the human HSCs, can be introduced directly (*e.g.*, immediately) after isolation, or the cells can be treated prior to introduction. In a particular embodiment, 15 the cells are introduced directly after isolation (*e.g.*, uncultured HPSCs are introduced). That is, in this embodiment, the cells are not treated with any agents and are not cultured for any length of time (0 days). In other embodiments, the cells are cultured about 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, 16 days 18 days, 20 days, 22 days, 24 days, 26 days, 28 days, 30 days, 32 days, 34 days, 36 days, 38 20 days, 40 days, 42 days, 44 days, 46 days, 48 days, 50 days, 52 days, 54 days, 56 days or 58 days. In other embodiments, the cells are cultured less than about one month or less than about two months.

- In particular aspects, the HPSCs introduced into the non-human mammal reconstitute the non-human mammal's liver with human hepatocytes, and the human 25 HSCs that are optionally introduced reconstitute the non-human mammal's blood with human blood cells. In particular embodiment, the human hepatocytes and human HSCs are "mature" (or "functional" or "biologically active") human hepatocytes and human blood cells. As will be appreciated by those of skill in the art, "mature" cells refer to the fact that the cells (whether human liver cells and/or human blood cells) express one or

more, and in some instances all, of the cell surface markers of the corresponding normal (wild type) cell found in humans, and as a result, function similarly in the non-human mammal as they function in a human.

5 The methods of producing the non-human mammals can further comprise assessing the reconstitution of human hepatocytes, the reconstitution of human blood cell lineages, or a combination thereof, in the non-human mammal. For example, reconstitution of human hepatocytes can be assessed by detecting human albumin, human CK7, human CK19 and combinations thereof in the non-human mammal (*e.g.*, in the serum or the liver of the non-human mammal). Assays for determining the
10 function of human liver cells and human blood lineage cells in the non-human mammal are known to those of skill in the art and are described herein. For example, as described herein and known in the art, detection of human albumin in the non-human mammal (*e.g.*, in the serum of the non-human mammal) indicates that the human liver cells are functional.

15 In addition, or in the alternative, reconstitution of human blood cell lineages can be assessed by detecting human CD45+ blood lineage cells (*e.g.*, in the blood, spleen, bone marrow, liver, or a combination thereof in the non-human mammal).

Non-human mammals produced by the methods described herein are also provided. The non-human mammals produced by the methods described herein can be
20 used in a variety of ways. For example, the non-human mammals can be used as models for pre-clinical testing of drugs, vaccines and human cell-based therapeutics prior to testing in the clinic.

Accordingly, in one aspect, the invention is directed to a method of identifying one or more agents or treatment protocols that can be used to treat a human liver
25 disease. The method comprises administering the one or more agents or treatment protocols to a non-human mammal that is a model of a human liver disease produced as described herein, and determining whether the human liver disease in the non-human mammal is alleviated. If the human liver disease is alleviated in the non-human

mammal, then the one or more agents or treatment protocols can be used to treat the human liver disease.

5 Examples of liver diseases include a primary liver cancer, a secondary liver cancer or cirrhosis of the liver. Specific examples of liver cancer include hepatocellular carcinoma, bile duct cancer, cholangiocarcinoma, angiosarcoma, or hepatoblastoma.

10 Examples of agents that produce the human liver disease are carcinogenic agents (*e.g.*, alphotoxin), infectious agents (*e.g.*, virus (*e.g.*, hepatitis virus (hepatitis A, hepatitis B, hepatitis C) or human immunodeficiency virus (HIV)), bacteria, parasite, environmental agents (*e.g.*, alcohol) or a combination thereof. The method can further
comprise reconstituting human blood cell lineages in the non-human mammal as described herein.

As one of skill in the art will appreciate, alleviation of a human liver disease includes removal of the disease, prolonging the life of the liver disease patient, or improving the quality of life of the liver disease patient or combinations thereof.

15 The method can further comprise determining whether the liver disease in the non-human mammal is alleviated as compared to a suitable control. As will be appreciated by those of skill in the art, a variety of suitable controls can be used. An example of a suitable control is a non-human mammal that is a model of human liver disease and that has not received the one or more agents or treatment protocols.

20 In another aspect, the invention is directed to a method of identifying one or more effects of one or more agents on a human liver. The method comprises administering the one or more agents to a non-human mammal which comprises human hepatocytes produced as described herein and determining the effect of the agent on the human liver cells of the non-human mammal, thereby identifying the effects of one or
25 more agents on a human liver. Methods for determining the effect of an agent on a human liver are apparent to those of skill in the art and include the agent's effect on albumin, alanine aminotransferase (ALT) (the ALT liver test), complement, hepatoglobin or a combination thereof. The agent can be, for example, a pharmaceutical composition, a drug, an environmental agent or a combination thereof. In addition, the

agent can have a therapeutic effect on the human liver cells, a toxic effect on the human liver cells, or no effect on the human liver cells.

In another aspect, the invention is directed to a method of identifying how one or more agents are metabolized by a human liver. The method comprises administering
5 the one or more agents to a non-human mammal which comprises human hepatocytes produced as described herein and maintaining the non-human mammal under conditions in which the one or more agents are metabolized by the human liver cells in the non-human mammal, thereby producing one or more metabolites in the non-human mammal. The one or more metabolites are detected and/or analyzed (*e.g.*, directly;
10 indirectly) in the non-human mammal, thereby identifying how one or more agents are metabolized by a human liver. One or more active metabolites, one or more toxic metabolites or a combination thereof can be detected.

In another aspect, the invention is directed to an *in vitro* method of obtaining mammalian hepatocytes (*e.g.*, mature human hepatocytes). The method comprises
15 contacting mammalian hepatic progenitor/stem cells (HPSCs) which express CD34⁺CD133^{lo} with hepatocyte differentiation medium, thereby producing a culture and maintaining the culture under conditions in which the HPSCs differentiate into mammalian hepatocytes, thereby obtaining mammalian hepatocytes.

In yet another aspect, the invention is directed to an *in vitro* method of obtaining
20 human hepatocytes (*e.g.*, mature human hepatocytes). The method comprises contacting human hepatic progenitor/stem cells (HPSCs) which express CD34⁺CD133^{lo} with hepatocyte differentiation medium, thereby producing a culture and maintaining the culture under conditions in which the HPSCs differentiate into human hepatocytes, thereby obtaining human hepatocytes.

25 Thus, the methods provided herein can be used to culture mammalian (*e.g.*, human) hepatocytes and establish mammalian (*e.g.*, human) hepatocytes (*e.g.*, hepatic cells, hepatic cell cultures and hepatic cell lines). In particular embodiments, the hepatocytes are cultured *in vitro* for days, months or years. For example the hepatocytes can be cultured for about 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 or more

days; or about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more months; or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more years. In particular embodiments, the hepatocytes (*e.g.*, hepatocytes, hepatic cell liners produced by the methods) are stable.

In particular embodiments, about 0.5×10^5 , 1×10^5 , 1.5×10^5 , 2×10^5 , 2.5×10^5 , 3×10^5 , 3.5×10^5 , 4×10^5 , 4.5×10^5 , 5×10^5 of the HPSCs are seeded on or in a suitable medium collagen and contacted with a hepatocyte differentiation medium. As will be appreciated by those of skill in the art a variety of suitable mediums are known and available. For example, the medium can comprise a collagen, a plastic, a synthetic scaffold and the like. As will also be appreciated by those of skill in the art, a variety of mediums that promote hepatocyte differentiation are known and available. In one embodiment, the hepatocyte differentiation medium comprises fetal bovine serum (FBS) (*e.g.*, about 5%), oncostatin, dexamethasone, insulin, transferring and selenous acid. The method can further comprise assessing differentiation of the HPSCs into hepatocytes, for example, by detecting albumin in the culture. The hepatocytes produced by the methods provided herein can be maintained as a fresh culture or can be preserved and/or stored (*e.g.*, cryopreserved) for days, months or years.

The invention is also directed to mammalian (*e.g.*, human) hepatocytes, cultures, and cell lines produced by the methods provided herein.

20

Exemplification

Example 1 Reconstitution of Human Hepatocytes and Human Hematopoietic Cells in Mice

Human fetal livers were minced and digested with collagenase. Single cell suspensions were prepared by grinding the digested liver and pressing it through a 100 μm cell strainer. The single cell suspensions were directly used for CD34⁺ cell purification. After staining with CD34 and CD133 antibodies, the FACS plot of purified cells showed there are three major cell populations: CD34⁺CD133^{high}, CD34⁺CD133^{low}, and CD34⁺CD133^{neg}. The CD34⁺ cells were injected into sub-lethally irradiated NSG

new born mice (200K/mouse). Eight weeks post injection, not only the reconstitution of human CD45⁺ blood lineage cells (blood), but also human albumin positive hepatocytes in liver were observed. CD34⁺ cells from human cord blood were used as a control. Most of cord blood CD34⁺ cells are CD133^{high} cells which are shown to be HSC herein. In the mice reconstituted with cord blood cells, human blood lineage cells were present but no human hepatocyte was detected.

To identify the role and the contribution of the three CD34⁺ cell populations in fetal liver, they were isolated by fluorescence activated cell sorting (FACS). The isolated cells showed >95% purity and >90% viability. See Fig. 1.

Cell populations were purified and injected into NSG mice separately by intra-cardiac injection. Each mouse received 200K cells. Eight weeks post injection, human CD45⁺ blood cells were only detected in mice injected with CD133^{hi*} cells, while CD133^{low} cells only contributed to the generation of human hepatocytes as indicated by the presence of human albumin in the serum. CD133^{neg} population didn't contribute to blood cells or hepatocytes.

Purified 200K CD133^{low} cells were injected into NSG mice either by i.l or i.c injection (5 mice in i.c group and 4 mice in i.l group). Eight weeks post injection, the human albumin levels in the serum were analyzed by ELISA. See Fig. 2.

CD34⁺ cells purified from fetal liver were stained with fluorescent antibodies. According to FSC and SSC, the CD133 low cells were much bigger than the other two populations. When gated on the CD34⁺CD133 low cell population, it showed that CD133 low cells expressed CD44, EpCAM, CD31 and CD117. See Fig. 3.

It was found that these CD133^{low} cells can only attach and grow on the collagen I coated plates. The sorted CD133^{low} cells were seeded in human hepatocyte differentiation medium. On day 2, they began attaching to the bottom of the collagen I coated wells shown as single cells. Pictures were taken every week to show the expansion and differentiation of these cells. On day 28, the cultured cells were stained with human albumin specific antibody and DAPI. It showed that a lot of cells differentiated from the sorted CD133^{low} cells were albumin positive. DAPI staining also showed that some of

the cells were multinucleated cells, which is a typical characteristic of human hepatocyte.

Example 2 Identification of Human Hepatic Progenitor/Stem Cells in Fetal Liver

5 Methods and Materials

Isolation of CD34⁺ cells from human fetal liver and cord blood

- Human fetal livers were obtained from aborted fetuses at 15-23 weeks of gestation in accordance with the institute ethical guidelines (Polkinhorne). All women have given written informed consent for the donation of fetal tissue for research. The
- 10 fetuses were collected under sterile condition within 2 h of the termination of pregnancy. The liver tissue from the fetus was initially cut into small pieces, followed by digestion with 2 mg/ml coUagenase VI prepared in DMEM for 15 min at 37°C with periodic mixing. Then, single cell suspension was prepared by passing the digested tissue through 100 μ m cell strainer (BD Biosciences). Umbilical cord blood was
- 15 obtained from the National Disease Research Interchange (NDRI) or the Singapore Cord Blood Bank. CD34⁺ cells from both fetal liver and cord blood were purified with the CD34 positive selection kit (Stem Cell Technologies, Vancouver, BC). The purity of CD34⁺ cells was 90 to 99%. Viable cells were counted by excluding dead cells with trypan blue. Cell isolation procedures were carried out under sterile condition in class
- 20 100-biosafety cabinet. To purify CD34⁺ CD133^{hi}, CD34⁺ CD133^{lo} and CD34⁺ CD133^{neg} cells, fetal liver cells were stained with anti-CD34 and anti-CD133 and the three cell populations were purified by sorting using Aria cell sorter (Beckton Dickinson). The purity of the sorted cells was between 90-99%.

25 Mice and cell injection

NSG mice were obtained from the Jackson Laboratory and bred in the animal facilities at Nanyang Technological University and National University of Singapore. Pups within 24 hrs of birth were sub-lethally irradiated (100 rads) and engrafted with HSCs by intra-cardiac or intra-hepatic injection. 2×10^5 CD34⁺ cells, or 1×10^5 CD34⁺

$CD133^{hi}$, $CD34^{+} CD133^{lo}$ or $CD34^{+} CD133^{neg}$ cells were injected per recipient. All research with human samples and mice was performed in compliance with the institutional guidelines.

5 Cells, cell culture and colony-forming assay

For in vitro hepatocyte differentiation, 2×10^5 $CD34^{+} CD133^{hi}$, $CD34^{+} CD133^{lo}$ and $CD34^{+} CD133^{neg}$ cells were seeded on collagen I (Sigma Chemical Co., St. Louis, MO)-coated tissue culture plates in IMDM medium supplemented with 10% fetal bovine serum (FBS), 20 ng/mL epidermal growth factor (Peprotech Inc, Rocky Hill, NJ), 20 ng/mL hepatocyte growth factor (HGF, Peprotech Inc), 10 ng/mL bFGF (Peprotech Inc) and 0.61 g/L nicotinamide (Sigma) for 14 days. Differentiation was induced by treating cells with maturation medium, consisting of IMDM supplemented with 5% FBS, 20 ng/mL HGF, 20 ng/mL oncostatin M (R&D Systems, Minneapolis, MN), 1 μ g/L dexamethasone (Sigma), and 50 mg/mL ITS⁺ premix (Sigma). Medium changes were performed twice weekly and hepatogenesis was assessed by immunofluorescence analysis for albumin production. All cultures were maintained at 37°C in a 5% CO₂ incubator.

For colony forming assays, 500 $CD34^{+} CD133^{hi}$, $CD34^{+} CD133^{lo}$ and $CD34^{+} CD133^{neg}$ cells were plated with complete MethoCult® methylcellulose medium (Stem

Cell Technologies) and cultured at 37°C in a 5% CO₂ incubator for 9 days. Mature

human hepatocytes were purchased from Invitrogen (Carlsbad, CA). Human hepatoma cell lines: Hep 3B, Hep G2, and SNU-423 were purchased from ATCC (Manassas, VA). All these cells were cultured according to the suppliers' instructions.

25 Secondary transfer

1 x 10⁵ purified CD34⁺CD133^{lo} cells were injected intra-hepatically into newborn NSG pups. After 9 weeks, single cell suspensions were prepared from recipient mouse livers by a two step perfusion. Mouse livers were first perfused with prewarmed liver perfusion medium (Invitrogen) for 10 min at 0.7 mlVmin and then
5 followed with prewarmed liver digestion medium (Invitrogen) for 10 min. Cells were washed with ice-cold DMEM medium. Cell viability after treatment exceeded 90% as assessed by Trypan blue dye exclusion. CD34⁺ cells were re-purified from the cell suspensions with the CD34 positive selection kit (Stem Cell Technologies). 1 x 10⁵ CD34⁺ cells were injected into sublethally irradiated newborn NSG pups. After another
10 8 weeks, the livers were harvested for histology and the sera were used for ELISA.

Flow Cytometry

Conjugated antibodies specific for EpCAM (9C4), CD44 (BJ18), CD34 (561), CD166 (3A6), CD105 (43A3), CD29 (TS2/16), CD24 (ML5), CD90 (5E10), CD73
15 (AD2), CD117 (104D2), CD97 (VIM3b), CD45 (HI30) were from BioLegend; and CD133 (EMK08) and mouse CD45.1 (A20) from eBioscience. Cells were stained with appropriate antibodies in 100 µl PBS containing 0.2% BSA and 0.05% sodium azide for 30 min on ice. Flow cytometry was performed on a LSRII flow cytometer using the FACSDiva software (BD, Franklin Lakes, NJ). 10,000 to 1,000,000 events were
20 collected per sample and analyzed using the Flowjo software.

Immunostainings

The livers were removed, embedded in paraffin and 5-micrometer-thick sections were prepared. After blocking, deparaffinized sections were stained with optimal
25 dilutions of rabbit anti-human albumin (AbCam), rabbit anti-human CK7 (Sigma) or rabbit anti-human CK19 (Sigma). Sections were developed with SuperPicture 3rd Gen IHC Detection Kit (Invitrogen). For immunofluorescence staining, cells were fixed with pre-chilled methanol and then blocked with 1% BSA/PBS. After blocking, cells were

stained with rabbit anti-human albumin antibody. Rhodamine conjugated goat anti-rabbit antibody (Santa Cruz) was served as the secondary antibody.

RT-PCR analysis

- 5 Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR) were used to analyzed the relative level of specific transcripts. Total RNAs were extracted from CD34⁺ CD133^{lo}, CD34⁺ CD133^{hi}, CD34⁺ CD133^{neg} cells and mature hepatocytes using RNeasy micro Kit (Qiagen, Valencia, CA) and transcribed into cDNA with iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, CA). The primers used for quantitative PCR are listed in the Table. For qPCR, SsoFast™ EvaGreen® Supermix (Bio-Rad) and CFX96 Real-Time PCR Detection System (Bio-Rad) were used according to the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay (ELISA)

- 15 Human albumin levels in the mouse sera were measured by a sandwich enzyme-linked immunosorbent assay. Human specific albumin ELISA kit was purchased from Bethyl Laboratories, Inc (Montgomery, TX).

Microarray analysis

- 20 Total RNAs from human hepatocytes, Hep 3B, cord blood HSCs, and CD34⁺ CD133^{lo}, CD34⁺ CD133^{hi}, CD34⁺ CD133^{neg} cells were isolated using the Qiagen RNeasy micro kit (Qiagen). RNA quality and quantity was determined using Bioanalyzer (Agilent Technologies) and nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific) respectively. Transcription analysis was conducted using
- 25 Agilent SurePrint G3 Human GE 8x60K Microarray (G4858A-028004; Agilent Technologies) following the Agilent 1-color microarray-based gene expression analysis protocol. Starting with 500 ng of total RNA, Cy3 labeled cRNA was produced according to manufacturer's protocol. For each sample, 1.65 ng of Cy3 labeled cRNAs were fragmented and hybridized for 17 hours in a rotating hybridization oven. Slides

were washed and then scanned with an Agilent Scanner. Data were obtained using the Agilent Feature Extraction software (version 9.5), with the 1-color defaults for all parameters. The Agilent Feature Extraction Software performed error modeling and adjusting for additive and multiplicative noise. The resulting data were processed using the RosettaResolver® system (version 7.2) (Rosetta Biosoftware, Kirkland, WA). Expression data from probes with annotated gene symbols were subjected to hierarchical clustering and a sample dendrogram was produced using the R (R version 2.11.1) package pvclust (pvclust_1.2-1), which is an add-on package for a statistical software R to assess the uncertainty in hierarchical cluster analysis. Probability values (p-values) for each cluster were calculated using bootstrap resampling techniques resulted in two types of p-values namely approximately unbiased (AU) p-value and bootstrap probability (BP) value. AU p-value is a better-unbiased p-value than BP value and clusters with p-value larger than 95% are strongly supported by data. Principal component analysis was performed using Genespring GX version 11 (Agilent technologies).

Results

CD34⁺ cells from human fetal liver give rise to both hematopoietic cells and hepatocytes following engraftment into NSG mice

To construct humanized mice, CD34⁺ cells isolated from both cord blood and fetal liver were used as sources of stem cells. Typically, 2×10^5 CD34⁺ cells (~95% purity) were engrafted into sublethally irradiated newborn pups of NSG mice by intracardiac injection. Eight weeks following reconstitution, as expected, human CD45⁺ leukocytes were detected in the peripheral blood of recipient mice regardless the source of stem cells (Figs. 4A-4B). When the liver sections of recipient mice were stained for human albumin, no positive signal was detected in the recipients that were engrafted with cord blood CD34⁺ cells (Fig. 4A). In contrast, a significant fraction of liver cells stained positive for human albumin in the recipient mice that were initially engrafted with fetal liver CD34⁺ cells (Fig. 4B). Generation of human albumin-expressing

hepatocytes was consistently observed using purified CD34⁺ cells from different fetal livers (>8), ranging from 15 to 23 weeks of gestation. The frequency of human albumin-positive hepatocytes was slightly higher when CD34⁺ fetal liver cells were engrafted into recipient pups by direct intra-hepatic injection. For this reason, intra-hepatic
5 injection was used in most of the experiments unless specified otherwise. These results indicate that in addition to HSCs, CD34⁺ cells from human fetal liver also contain HPSCs that can give rise to albumin-expressing human hepatocytes in the mouse liver.

CD34⁺CD133^{lo} cells give rise to human hepatocytes whereas CD34⁺CD133^{hi} cells give
10 rise to hematopoietic cells

CD34⁺ cells from fetal liver were separated into three distinct populations when co-stained with anti-CD133: high, low and negative (Fig. 5A), whereas CD34⁺ cells from cord blood were uniformly high for CD133. Based on forward scatter (FSC), CD34⁺CD133^{lo} cells were much larger in size than CD34⁺CD133^{hi} cells and
15 CD34⁺CD133^{neg} cells (Fig. 10A). Depending on the gestation stage of the fetal liver, the proportion of the CD34⁺CD133^{hi} cells ranged from 10% to 25% whereas the proportion of the CD34⁺CD133^{lo} cells ranged from 8% to 18% (data not shown). The proportion of both CD34⁺CD133^{hi} and CD34⁺CD133^{lo} cells decreased with increasing gestation time of the fetus.

20 To investigate the developmental potential of each subpopulation of CD34⁺ fetal liver cells, the three populations were purified by cell sorting and engrafted separately into sublethally irradiated newborn NSG pups. Ten weeks later, PBMCs from recipient mice were analyzed for the presence of human CD45⁺ leukocytes and sera were assayed for the level of human albumin by ELISA. When recipient pups were injected with
25 CD34⁺CD133^{hi} cells, human CD45⁺ cells were readily detected in PBMCs but no human albumin was detected in the sera (Fig. 5C and 5D). In contrast, when recipient pups were injected with CD34⁺CD133^{lo} cells, a significant level of human albumin was detected in the sera of recipient mice, but no human CD45⁺ cells were detected in PBMCs. When recipient pups were injected with CD34⁺CD133^{neg} cells, neither human

CD45⁺ cells nor human albumin was detected in the recipient mice. Consistently, only liver sections from mice engrafted with CD34⁺CD133^{lo} cells, but not those from mice engrafted with CD34⁺CD133^{hi} or CD34⁺CD133^{neg} cells, were stained positive for human albumin (Fig. SE) and for a human pan-centromere probe (Fig. IOC). In

- 5 addition, clusters of human biliary epithelial cells next to a venous channel were stained positive for human CK7 (Fig. 5F) and CK19 (Fig. 10B) in the liver sections from mice engrafted with CD34⁺CD133^{lo} cells, suggesting the development of human biliary epithelial cells in the mouse liver.

- To quantify the frequency and the number of human liver cells in the mouse
10 liver, nine weeks after injection of CD34⁺CD133^{lo} cells, recipient mice were carefully perfused and single cell suspensions of hepatocytes were prepared. The cells were stained for human hepatocyte marker CD29 (Aurich, H., *et al.* (2009) Gut 58, 570-581) and human hepatic progenitor cell marker EpCAM followed by flow cytometry. On average, approximately 4% of CD29⁺ EpCAM⁺ human hepatocytes was detected in the
15 liver of recipient mice that were engrafted with CD34⁺CD133^{lo} cells (Fig. 5G), whereas no CD29⁺ human hepatocytes were detected in the liver of non-reconstituted NSG mice. Based on the frequency of human hepatocytes and the total cells (5×10^8) in the liver of a 9-week-old mouse, the total number of human hepatocytes is about 2×10^7 per recipient mouse. Because only 1×10^5 CD34⁺CD133^{lo} cells were injected per recipient
20 mice initially, there is a 200-fold expansion during the differentiation from CD34⁺CD133^{lo} cells into human hepatocytes in the recipient mice.

- Together, these results show that based on CD133 expression, CD34⁺ human fetal liver cells can be separated into three subpopulations with different developmental potential: CD34⁺CD133^{hi} cells likely contain HSCs as they give rise to hematopoietic
25 cells in the recipient mice whereas CD34⁺CD133^{lo} cells likely contain HPSCs as they expand and give rise to both hepatocytes and biliary epithelial cells in recipient mice.

CD34⁺CD133^{lo} cells are capable of differentiating into human hepatocytes in vitro

When grown on collagen plates with hepatocyte differentiation medium (Lee et al., (2004) Hepatology 40, 1275-1284), CD34⁺CD133^{lo} cells adhered to the collagen substratum as a monolayer and gradually developed a typical polygonal morphology (Fig. 6A), with some of the cells becoming binucleated, a known characteristics of cultured hepatocytes (Fig. 9C). Development of hepatocytes were further confirmed by positive staining for human albumin on day 28. Cell numbers were counted on different days during the culture, which showed that the total cell numbers increased steadily (Fig. 6B), reaching a 50-fold increase in the total cell numbers by day 28 (Fig. 6B). In contrast, CD34⁺CD133^{hi} and CD34⁺CD133^{neg} cells did not proliferate in the hepatocyte differentiation medium. Thus, CD34⁺CD133^{lo} fetal liver cells are also capable of differentiating into hepatocytes in vitro.

To investigate the hematopoietic potential, CD34⁺CD133^{hi}, CD34⁺CD133^e and CD34⁺CD133^{neg} cells were analyzed by standard semi-solid colony forming assays. CD34⁺CD133^{hi} cells proliferated rapidly and gave rise to mixed hematopoietic colonies containing granulocytic, erythroid, monocyte-macrophage, and megakaryocyte elements (CFU-GEMM, >95%), while CD34⁺CD133^{neg} cells formed single colonies of granulocyte-macrophage progenitors (CFU-GM, ~25%) and erythroid precursor cells (BFU-E, >75%) (Fig. 6D and 6E). In contrast, CD34⁺CD133^{lo} cells did not generate any hematopoietic colonies but attached on the bottom of the plates as single cell layer. Consistent with in vitro differentiation, CD3⁺ T cells, CD19⁺ B cells, CD56⁺ NK cells, CD14⁺ monocyte/macrophages, CD11c⁺ BDCA-1⁺ myeloid dendritic cells (DCs), and ILT7⁺ CD303⁺ plasmacytoid DCs were detected in the spleen of recipient mice engrafted with CD34⁺CD133^{hi} cells (Fig. 11). These results further support the in vivo observation showing that CD34⁺CD133^{lo} cells contain HPSCs while CD34⁺CD133^{hi} cells contain the multipotent HSCs. CD34⁺CD133^{neg} cells are likely hematopoietic progenitors which do not stably reconstitute mice with human blood lineage cells.

CD34⁺CD133^{lo} fetal liver cells are capable of self-renewal

The stem cell property of CD34⁺CD133^{lo} fetal liver cells was further demonstrated by serial transfer experiments. In this experiment, purified CD34⁺CD133^{lo} fetal liver cells were first engrafted into sublethally irradiated newborn NSG pups. Nine weeks later, recipient mice were verified to have human hepatocyte reconstitution by assaying for serum level of human albumin. Livers from the primary recipient mice were perfused and single cell suspension was prepared. Although the frequency of human CD34⁺ cells in the mouse liver was extremely low (< 0.01%) (Fig. 7A), 6X10⁵ CD34⁺ cells from 11 nine-week old mice were purified. Flow cytometry analysis showed that the purified CD34⁺ human cells were also positive for CD133, CD44 and EpCAM (Fig. 7B). The purified cells were injected into six secondary newborn NSG pups (1X10⁵ cells per recipient). Eight weeks after secondary reconstitution, human albumin-expressing hepatocytes were detected in the liver sections and human albumin was detected in the sera of all six recipient mice (Fig. 7C and 7D). Thus, despite the expansion and differentiation in the primary recipient mice, some of the transferred CD34⁺CD133^{lo} cells can self-renew and maintain the ability to reconstitute human hepatocytes in the secondary recipient mice, indicating that CD34⁺CD133^{lo} cell population from the human fetal liver contains bona fide hepatic stem cells. For simplicity, the CD34⁺CD133^{lo} fetal liver cells are referred to as hepatic progenitor/stem cells (HPSCs) herein.

20

HPSCs are transcriptionally more similar to HSCs than to mature hepatocytes

Next, the cell surface phenotype of CD34⁺CD133^{hi} (HSCs) cells and CD34⁺CD133^{lo} (HPSCs) cells from fetal liver were characterized using CD34⁺CD133^{hi} HSCs from cord blood as a control. As shown in Figure 8A, CD34⁺CD133^{hi} cells from both fetal liver and cord blood shared a very similar staining pattern, including high levels of CD45, CD44, CD117 and CD116, medium levels of CD90, CD105 and CD97, and negative for EpCAM, CD24 and CD73. In general, CD34⁺CD133^{hi} cells from fetal liver stained more brightly than CD34⁺CD133^{hi} cells from cord blood, but the background staining of the former was also higher. In contrast, CD34⁺CD133^{lo} cells

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from fetal liver exhibited a very different staining pattern. They expressed significant levels of CD73, EpCAM, CD166 and CD45, low levels of CD44, CD1 17 and CD24, but negative for CD90, CD105 and CD97. The background staining of these cells was also much higher, consistent with their large size. Thus, HPSCs are not only positive for
 5 the mesenchymal stem cell markers such as EpCAM and CD73 but also some hematopoietic cell markers such as CD34, CD1 17 and even CD45.

Quantitative-PCR analyses showed that CD34⁺CD133^{lo} fetal liver cells expressed significant levels of hepatocyte and epithelial cell related transcripts, including CK18, AFP, albumin, CD31, c-met, vimentin, CK19, CK7 and CD29 (Fig.
 10 8B). CD34⁺CD133^{lo} HPSCs also expressed biliary cell markers such as CK19 and CK7. In contrast, mature human hepatocytes expressed a much higher level of albumin and were also positive for CK18, c-met and CD29 but not AFP, CD31 and vimentin. FL HSC expressed all the hematopoietic genes including CD45, CD1 17, CD34, and CD133, but the transcripts of albumin and AFP were also detected. Confirming the PCR
 15 results, immunofluorescence staining showed that all CD34⁺CD133^{lo} cells were positive for albumin, AFP, CK7 and CK19 while CD34⁺CD133^{hi} cells were negative (Fig. 12A). The uniform expression of both hepatic markers (albumin, AFP) and biliary markers (CK7, CK19) by CD34⁺CD133^{lo} cells indicates that they are a homogenous hepatic progenitor/stem cell population, capable of developing into both biliary cells and
 20 hepatocytes.

To further investigate the developmental relationship between HPSCs and HSCs from human fetal liver, transcriptional profiling of purified CD34⁺CD133^{lo}, CD34⁺CD133^{hi}, and CD34⁺CD133^{neg} fetal liver cells, as well as CD34⁺CD133^{hi} HSCs from cord blood, mature human hepatocytes, and HEP 3B, a hepatitis B virus (HBV)-
 25 transformed hepatoma cell line, was performed. Comparison of data from surface staining, PCR analysis and microarray showed good correlation among the three different assays. For example, CD34, CD45, CD133, EpCAM, AFP and CK19 were detected in CD34⁺CD133^{lo} HPSCs by all three methods. Hierarchical clustering and principal component analysis (Fig. 12B) showed that the transcriptional profile of

CD34⁺CD133⁺ HPSCs was much more similar to that of fetal liver and cord blood HSCs than to mature hepatocytes (Fig. 8C). HPSCs and HSCs from fetal liver shared 4636 genes that were up-regulated more than 5 fold as compared to hepatocytes, whereas they shared only 468 genes that were up-regulated 5 fold as compared to cord blood HSCs (Fig. 12C). Although HPSCs were transcriptionally closer to HSC, they did not express or had a much lower level of key HSC-specific transcriptional factors PBX1, SOX4, MEIS1 and HOXA9 (Fig. 8D). Instead, HPSCs expressed many endoderm specific genes (Seguin, C.A.*et al.* (2008), Cell Stem Cell 3, 182-195), such as transcription factors SOX-17, SOX-7, HNF1A, and HNF1B (Fig. 8E), as well as signature genes laminin, HSPG2 and collagen IV (Fig. 8F). These results showed that HPSCs are more closely related to HSCs in transcription than to mature hepatocytes, indicating a possibility of their common origin or trans-differentiation in fetal liver.

Some human hepatoma cells have similar surface marker expression pattern as HPSCs

15 Stem cells have been considered as potential origin of various human cancers. To explore the potential connection between the identified HPSCs and human hepatomas, three human hepatoma cell lines were analyzed for HPSC-related surface marker expression. Hep 3B is a HBV-transformed human hepatoma cell line (Knowles, B. B., *et al.* (1980) Science 209, 497-499); Hep G2 is a widely studied human

20 hepatoblastoma cell line and SNU-423 is a pleomorphic hepatocellular carcinoma cell line (Park, J. G. *et al.* (1995), Int J Cancer 62, 276-282). Hep 3B and Hep G2 expressed the surface markers of HPSCs except that Hep 3B expressed high levels of CD133 and CD34 while Hep G2 expressed only a modest level of CD133 and was negative for CD34 (Fig. 9). As shown by the microarray analysis (Fig. 8C), the transcriptional

25 profile of Hep 3B was more similar to HPSCs and HSCs from fetal liver than to mature hepatocytes. In contrast, the surface phenotype of SNU-423 was more similar to mature human hepatocytes, which were negative for all the progenitor/stem cell-related markers except for CD166 and CD73. In addition, SNU-423 was also positive for

CD44. These results indicate that the human HPSCs are targets for tumorigenesis and some human hepatoma cell lines may be directly derived from them.

Discussion

- 5 One major challenge for studying human HPSCs is lack of a simple and efficient in vivo assay for the developmental potential of cell populations from fetal liver, adult liver or other sources. As described herein, this challenge has been overcome by the development of a simple assay involving adoptive transfer of freshly isolated fetal liver cells into sublethally irradiated NSG pups. Development of human hepatocytes in the
- 10 recipient mouse liver was detected by assaying for human albumin in the serum, staining for human albumin-expressing cells in liver sections, and/or CD29⁺ human hepatocytes by flow cytometry. Development of human biliary epithelial cells in the mouse liver was assayed by staining of liver sections. Compared to the existing mouse models of human hepatocyte reconstitution, the present method is simple without
- 15 special genetic engineering, chemical treatment or surgical procedure of recipient mice. A much higher degree of human liver chimerism can be achieved by transplanting mature human hepatocytes into immunodeficient mice, such as urokinasetype plasminogen activator (uPA) transgenic (Mercer, D. F. *et al.* (2001), *Nat Med* 7, 927-933; Meuleman, ? *et al.* (2005), *Hepatology* 41, 847-856) and fumarylacetoacetate
- 20 hydrolase (FAH) knockout (Azuma, H., *et al.* (2007) *Nat Biotechnol* 25, 903-910) mice. However, these recipient mice are specially engineered and the endogenous mouse hepatocytes have to be ablated when human hepatocytes are transferred. Despite their potential utility in a variety of applications, these recipient mice have not been used to assay developmental potential of human HPSCs. Recently, human hepatic progenitor
- 25 cells were assayed for their developmental potential in adult immunodeficient mice by intra-splenic injection (Dan, Y. Y., *et al.*, (2006) *Proc Natl Acad Sci USA* 103, 9912-9917; Malhi, H. *et al.* (2002), *J Cell Sci* 115, 2679-2688; Schmelzer, E., *et al.* (2007), *J Exp Med* 204, 1973-1987; Weiss, T. S. *et al.* (2008), *Gut* 57, 1129-1138). In addition to the requirement of advanced surgical procedure and chemical treatment of recipient

mice, reconstitution of human hepatocytes in the liver of recipient mice is extremely low (on the order of 0.01 %). In contrast, the simple method of direct injection of human HPSCs into the sublethally irradiated NSG pups described herein led to approximately 4% of human hepatocytes in the mouse liver. The robust reconstitution is probably due to irradiation, which likely kills some of mouse HPSCs, and the use of neonates as recipients, where human HPSCs can incorporate into the mouse liver and expand as the mouse grows. Another advantage of the methods provided herein is simultaneous reconstitution of both hematopoietic lineage cells and hepatocytes in the same recipient when CD34⁺ fetal liver cells are injected. Such a humanized mouse could be especially useful for studying biological processes and disease mechanisms that requires interactions between human hepatocytes and immune system in the same recipient mice.

Development of the simple and robust in vivo assay has enabled identification of HPSCs from human fetal liver. Because CD34⁺ fetal liver cells give rise to both hepatocytes and hematopoietic cells in NSG recipient mice, identification was based on the use of CD34 in combination with other markers. By testing a series of surface markers, it was found that anti-CD 133 staining can further separate CD34⁺ fetal liver cells into three distinct populations: CD133^{hi}, CD133^{lo}, and **CD133^{neg}**. By testing the developmental potential of each subpopulation in NSG recipients, it was shown that CD34⁺ CD133^{hi} cells contain HSCs as they gave rise to T, B, NK, DC, monocyte/macrophages in NSG recipient mice and CFU-GEMM in vitro. Consistently, HSCs from cord blood are uniformly high for CD133. In contrast, CD34⁺ CD133^{lo} cells did not give rise to any hematopoietic cells but hepatocytes in the recipient mouse liver and hepatocytes in vitro. Based on the number of CD34⁺ CD133^{lo} cells transferred and the number of human hepatocytes recovered per recipient mouse, HPSCs expand approximately 200 fold during their differentiation in vivo. Despite the tremendous expansion, some human cells can apparently self-renew, remain CD34⁺ CD133^{lo} phenotype in the recipient liver, and give rise to hepatocyte reconstitution in secondary recipient mice. Thus, these findings showed that the combination of CD34 and CD133

staining separated HSCs and HPSCs in human fetal liver and that CD34⁺ CD133^{lo} fraction of human fetal liver cells contained bona fide hepatic stem cells.

Detailed analysis of CD34⁺ CD133^{hi} human HPSCs reveals that these cells express additional hematopoietic markers including CD117, CD44 and CD45, mesenchymal cell markers such as CD73 and vimentin, and progenitor hepatocyte markers, such as EpCAM, AFP and albumin. Importantly, HPSCs uniformly expressed these different lineage markers when assayed by either flow cytometry or immunofluorescence, suggesting that the CD34⁺ CD133^{hi} HPSC population is rather uniform. The phenotype of HPSCs that we identified here share similarities as well as significant differences from those reported previously. Schmelzer et al divided EpCAM⁺ human fetal liver cells into hepatoblasts and HPSCs. Hepatoblasts expressed AFP, albumin, CK19, CD133, and CD44 whereas HPSCs were positive for CK19, CD44, CD133, neural cell adhesion molecule (NCAM), and weakly positive for albumin, but negative for AFP. Moreover, their HPSCs and hepatoblasts are negative for hematopoietic markers CD34, CD45 and CD90. Weiss et al reported the use of CD90 to isolate human hepatic progenitor cells which are positive for hematopoietic markers CD34 and c-kit, biliary cell marker CK19 and hepatic marker HepParl. They further showed that the CD90⁺ fetal liver cells gave a low level (%) reconstitution of human hepatocytes when transplanted into adult immunodeficient mice. While the precise basis underlying the slightly different phenotype of HPSCs from different studies is not known, one possibility is that the isolated cell populations are still mixed populations and that different stages of progenitor cells were isolated by different groups.

The physical and functional separation of HPSCs from HSCs in human fetal liver has prompted investigation of the controversial relationship between HPSCs and HSCs. Based on the expression of hematopoietic markers by HPSCs (Blakolmer, *et al.* (1995) *Hepatology* 21, 1510-1516; Lemmer, E. R. *et al.* (1998), *J Hepatol* 29, 450-454) and the controversial result of trans-differentiation from HSCs to hepatocytes in mice, it was hypothesized that HSCs can trans-differentiate into the hepatic lineage. By

- performing genome-wide transcriptional analysis, the relationship between HSCs and HPSCs in human fetal liver has been systematically investigated as described herein. Compared to mature human hepatocytes, HPSCs are transcriptionally much closer to HSCs from both fetal liver and cord blood. While HPSCs transcribe many genes that
- 5 are traditionally associated with hematopoiesis, HSCs from fetal liver, but not cord blood, also had a significant level of albumin and AFP transcript but not protein (Fig. 9A and 11B). Nevertheless, HPSCs lack the core transcriptional networks, such as PBX1 and HOXA9, that define HSCs. Because virtually all purified HPSCs from fetal liver expressed AFP, albumin, CK7 and CK19 by intracellular staining, the HPSCs
- 10 described herein are unlikely to have significant contamination of HSCs. Thus, the sharing of transcriptional profiles between HPSCs and HSCs indicate that the likelihood of trans-differentiation from HSCs to HPSCs during fetal development or more provocatively both HSCs and HPSCs originate from the same precursor in the fetal liver.
- 15 Identification of human HPSCs has also allowed investigation of possible connections between HPSCs and human liver cancers. Although it has been long thought that the mature hepatocyte represents the cell of origin of all hepatocellular carcinomas (HCCs), a growing body of evidence indicates that some HCCs in human and animals originate from HPSCs. Consistent with this notion, it was found, as
- 20 described herein, that some human hepatomas share similar cell surface phenotype and transcriptional profile to HPSCs, indicating that they are likely derived from transformation of HPSCs. In contrast, some other human hepatomas share similar surface phenotype as mature hepatocytes, indicating that they are likely derived from transformation of mature hepatocytes.
- 25 In summary, by developing a simple and robust assay of human HPSCs, human HPSCs from HSCs in fetal liver have been identified. However, both types of fetal cells are highly related transcriptionally, indicating their common origin in the fetal liver.

Gene Name	Primers	Gene Name	Primers
Alpha-fetoprotein	Forward: CTTTGGGCTGCTCGCTA TGA (SEQ ID NO: 1) Reverse: ATGGCTTGGAAGTTCCG GGTC (SEQ ID NO: 2)	SOX17	Forward: GTGGACCGCACGGAAT TTG (SEQ ID NO: 29) Reverse: GAGGCCCATCTCAGGC TTG (SEQ ID NO: 30)
Keratin 7 (CK 7)	Forward: AGACGGAGTTGACAGA GCTG (SEQ ID NO: 3) Reverse: GGATGGCCCGGTTCATC TC (SEQ ID NO: 4)	SOX7	Forward: AGCCGGAGCAGACCTT CTT (SEQ ID NO: 31) Reverse: CCGGGGAGTAATAGGC AGG (SEQ ID NO: 32)
Keratin 19 (CK 19)	Forward: ACCAAGTTTGAGACGGA ACAG (SEQ ID NO: 5) Reverse: CCCTCAGCGTACTGATT TCCT (SEQ ID NO: 6)	Laminin	Forward: ACTTTCAAGACATTCC GTCCAG (SEQ ID NO: 33) Reverse: CCTCACAGTCATAGGC GAAGTAT (SEQ ID NO: 34)
Keratin 18 (CK 18)	Forward: TGATGACACCAATATCA CACGAC (SEQ ID NO: 7) Reverse: TACCTCCACGGTCAACC CA (SEQ ID NO: 8)	HSPG2	Forward: CAGATGCCCCATTGGC TATTC (SEQ ID NO: 35) Reverse: GCTGGCAATTCAGGCA GTG (SEQ ID NO: 36)

Albumin	Forward: TTTATGCCCCGGAAGCTC CTTT (SEQ ID NO: 9) Reverse: TGTTTGGCAGACGAAGC CT (SEQ ID NO: 10)	Collagen IV	Forward: GGATGCTGTTGAAAGG TGAAAGA (SEQ ID NO: 37) Reverse: GGTGGTCCGGTAAATC CTGG (SEQ ID NO: 38)
GAPDH	Forward: CATGAGAAGTATGACAA CAGCCT (SEQ ID NO: 11) Reverse: AGTCCTTCCACGATACC AAAGT (SEQ ID NO: 12)	GATA4	Forward: ACACCCCAATCTCGAT ATGTTTG (SEQ ID NO: 39) Reverse: GTTGCACAGATAGTGA CCCGT (SEQ ID NO: 40)
CD133	Forward: CATCCACAGATGCTCCT AAGGC (SEQ ID NO: 13) Reverse: GCTTTATGGGAGTCTTG GGTC (SEQ ID NO: 14)	HNF1A	Forward: CGGAGGAACCGTTTCA AGTG (SEQ ID NO: 41) Reverse: GCATTCCGCCCTATTG CAC (SEQ ID NO: 42)
CD34	Forward: CAACACCTAGTACCCTT GGAAGT (SEQ ID NO: 15) Reverse: ACTGTCGTTTCTGTGAT GTTTGT (SEQ ID NO: 16)	HNF1B	Forward: TGTACGCACACAAGCA GGAA (SEQ ID NO: 43) Reverse: GTTGGTGAGTGTACTG ATGCTG (SEQ ID NO: 44)
CD45	Forward: ACAGCCAGCACCTTTCC	CD31	Forward: AACAGTGTTGACATGA

	TAC (SEQ ID NO: 17) Reverse: GTGCAGGTAAGGCAGC AGA (SEQ ID NO: 18)		AGAGCC (SEQ ID NO: 45) Reverse: TGTAACACAGCACGTC ATCCTT (SEQ ID NO: 46)
EpCAM	Forward: TGTCTGTGAAAACACTACA AGCTGG (SEQ ID NO: 19) Reverse: AGCCATTCATTTCTGCC TTCATC (SEQ ID NO: 20)	c-met	Forward: TGGTGCAGAGGAGCAA TGG (SEQ ID NO: 47) Reverse: CATTCTGGATGGGTGT TTCCG (SEQ ID NO: 48)
CD29	Forward: CAAGCAGGGCCAAATTG TGG (SEQ ID NO: 21) Reverse: CCTTTGCTACGGTTGGT TACATT (SEQ ID NO: 22)	Vimentin	Forward: GAACGCCAGATGCGTG AAATG (SEQ ID NO: 49) Reverse: CCAGAGGGAGTGAATC CAGATTA (SEQ ID NO: 50)
CD117	Forward: GGCGACGAGATTAGGCT GTTA (SEQ ID NO: 23) Reverse: GCCTTTTCCGTGATCCA TTCA (SEQ ID NO: 24)	SOX4	Forward: GACCTGCTCGACCTGA ACC (SEQ ID NO: 51) Reverse: CCGGGCTCGAAGTTAA AATCC (SEQ ID NO: 52)
PBX1	Forward: TGAGCGTGCAGTCACTC AATG (SEQ ID NO: 25) Reverse: TGAAGGGGTAGTAGCAT	HOXA9	Forward: TACGTGGACTCGTTCC TGCT (SEQ ID NO: 53) Reverse: CGTCGCCTTGGACTGG

	CCTG (SEQ ID NO: 26)		AAG (SEQ ID NO: 54)
MEIS1	Forward: GATATAGCCGTGTTCGC CAAA (SEQ ID NO: 27) Reverse: CGGTGGCAGAAATTGTC ACAT (SEQ ID NO: 28)		

The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

- 5** While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. An isolated mammalian fetal hepatic progenitor/stem cell (HPSC) wherein the cell expresses CD34⁺CD133^{lo}.
- 5 2. The HPSC of Claim 1 which further expresses CD1 17, CD44, CD45, CD73, vimentin, EpCAM, α -fetoprotein (AFP), albumin, CK19, CK7 or a combination thereof.
3. The HPSC of Claim 1 wherein the cell is a human HPSC.
4. A method of reconstituting human hepatocytes in a non-human mammal
10 comprising
 - a) introducing human hepatic progenitor/stem cells (HPSCs) which express CD34⁺CD133^{lo} into an immunodeficient non-human mammal, and
 - b) maintaining the non-human mammal under conditions in which the non-human mammal's liver is reconstituted with human hepatocytes,
15 thereby reconstituting human hepatocytes in a non-human mammal.
5. The method of Claim 4 wherein the HPSCs are obtained from a human fetal liver.
6. The method of Claim 4 wherein the HPSCs further express CD1 17, CD44, CD45, CD73, vimentin, EpCAM, α -fetoprotein (AFP), albumin, CK19, CK7 or
20 a combination thereof.
7. The method of Claim 4 wherein the immunodeficient non-human mammal is an immunodeficient mouse.

8. The method of Claim 7 wherein the immunodeficient mouse is selected from the group consisting of: a non-obese diabetic mouse that carries a severe combined immunodeficiency mutation (NOD/scid mouse); a non-obese diabetic mouse that carries a severe combined immunodeficiency mutation and lacks a gene for the cytokine-receptor γ chain (NOD/scid IL2R $\gamma^{T'}$ mouse) and a Balb/c rag^{-/-} yc^{-/-} mouse.
9. The method of Claim 7 wherein the mouse is a new born mouse.
10. The method of Claim 7 wherein the mouse is sublethally irradiated prior to introduction of the HPSCs into the mouse.
- 10 11. The method of Claim 4 wherein the human HPSCs expand at least about 200 fold during differentiation into human hepatocytes in the non-human mammal.
12. The method of Claim 4 wherein about 1×10^5 HPSCs are introduced into the non-human mammal.
13. The method of Claim 4 wherein the HPSCs are about 95% pure.
- 15 14. The method of Claim 4 wherein the HPSCs are introduced via intracardiac injection, intrahepatic injection or a combination thereof.
15. The method of Claim 14 wherein the cells are introduced in a single injection.
16. The method of Claim 4 further comprising assessing the reconstitution of human hepatocytes in the non-human mammal.
- 20 17. The method of Claim 16 wherein reconstitution of human hepatocytes is assessed by detecting human albumin, human CK7, human CK19 or a combination thereof in the non-human mammal.

18. The method of Claim 17 wherein the human albumin, human CK7, human CK19 or the combination thereof is detected in the serum of the non-human mammal, the liver of the non-human mammal or a combination thereof.
19. The method of Claim 4 further comprising introducing human hepatocytes from the non-human mammal into a second non-human mammal, and maintaining the second non-human mammal under conditions in which the second the non-human mammal's liver is reconstituted with human hepatocytes, thereby reconstituting human hepatocytes in a non-human mammal.
20. The method of Claim 4 further comprising reconstituting human blood cell lineages in the non-human mammal comprising
- c) introducing human hematopoietic stem cells (HSCs) which express CD34⁺CD133^{hi} into the immunodeficient non-human mammal, and
- d) maintaining the non-human mammal under conditions in which the non-human mammal's blood is reconstituted with human blood cells,
- thereby further reconstituting human blood cell lineages in a non-human mammal.
21. The method of Claim 20 wherein the HSCs are obtained from a human fetal liver.
22. The method of Claim 20 further comprising assessing the reconstitution of human blood cell lineages in the non-human mammal.
23. The method of Claim 20 wherein the reconstitution of human blood cell lineages is assessed by detecting human CD45⁺ blood cells.
24. The method of Claim 23 wherein the human CD45⁺ blood cells are detected in the blood, spleen, bone marrow, liver, or a combination thereof in the non-human mammal.

25. A method of producing a non-human mammal for use as a model of human liver disease comprising
- a) introducing human hepatic progenitor/stem cells (HPSCs) which express CD34⁺CD133¹⁰ into an immunodeficient non-human mammal,
- 5 b) maintaining the non-human mammal under conditions in which the non-human mammal's liver is reconstituted with human hepatocytes,
- c) introducing one or more agents that produce a human liver disease into the non-human mammal, and
- d) maintaining the non-human mammal under conditions in which the human
- 10 liver disease develops in the non-human mammal,
- thereby producing a non-human mammal for use as a model of human liver disease.
26. The method of Claim 25 wherein the liver disease is a primary liver cancer, a secondary liver cancer or cirrhosis of the liver.
- 15 27. The method of Claim 26 wherein the liver cancer is hepatocellular carcinoma, bile duct cancer, cholangiocarcinoma, angiosarcoma, or hepatoblastoma.
28. The method of Claim 25 wherein the one or more agents that produce the human liver disease are one or more carcinogenic agents, one or more infectious agents, one or more environmental agents or a combination thereof.
- 20 29. The method of Claim 28 wherein the one or more carcinogenic agents is alphatoxin; the one or more infectious agents is a virus, a bacteria, or a parasite; and the one or more environmental agents is alcohol.
30. The method of Claim 29 wherein the virus is hepatitis virus or human immunodeficiency virus (HIV).

31. The method of Claim 25 wherein the HPSCs are obtained from a human fetal liver.
32. The method of Claim 25 wherein the HPSCs further express CD117, CD44, CD45, CD73, vimentin EpCAM, α -fetoprotein (AFP), albumin, CK19, CK7 or a combination thereof.
- 5
33. The method of Claim 25 wherein the immunodeficient non-human mammal is an immunodeficient mouse.
34. The method of Claim 33 wherein the immunodeficient mouse is selected from the group consisting of: a non-obese diabetic mouse that carries a severe combined immunodeficiency mutation (NOD/scid mouse); a non-obese diabetic mouse that carries a severe combined immunodeficiency mutation and lacks a gene for the cytokine-receptor γ chain (NOD/scid IL2R $\gamma^{-/-}$ mouse) and a Balb/c rag^{7-/-} γ c^{-/-} mouse.
- 10
35. The method of Claim 33 wherein the mouse is a new born mouse.
- 15
36. The method of Claim 33 wherein the mouse is sublethally irradiated prior to introduction of the HPSCs into the mouse.
37. The method of Claim 25 wherein the human HPSCs expand at least about 200 fold during differentiation into human hepatocytes in the non-human mammal.
38. The method of Claim 25 wherein about 1×10^5 HPSCs are introduced into the non-human mammal,
- 20
39. The method of Claim 25 wherein the HPSCs are about 95% pure.
40. The method of Claim 25 wherein the HPSCs are introduced via intracardiac injection, intrahepatic injection or a combination thereof.
41. The method of Claim 40 wherein the cells are introduced in a single injection.

42. The method of Claim 25 further comprising assessing the reconstitution of human hepatocytes, biliary cells or a combination thereof in the non-human mammal.
- 5 43. The method of Claim 42 wherein reconstitution of human hepatocytes and biliary cells is assessed by detecting human albumin, human CK7, human CK19 or a combination thereof in the non-human mammal.
44. The method of Claim 43 wherein the human albumin is detected in the serum of the non-human mammal, the liver of the non-human mammal or a combination thereof.
- 10 45. The method of Claim 25 further comprising reconstituting human blood cell lineages in the non-human mammal.
46. The method of Claim 45 wherein human blood cell lineages are reconstituted in the non-human mammal comprising introducing human hematopoietic stem cells (HSCs) that express a CD34⁺CD133^{hi} into the non-human mammal.
- 15 47. A non-human mammal produced by the method of Claim 4.
48. A non-human mammal produced by the method of Claim 20.
49. A non-human mammal produced by the method of Claim 25.
50. A method of identifying one or more agents or treatment protocols that can be used to treat a human liver disease comprising
- 20 a) administering the one or more agents or treatment protocols to a non-human mammal of Claim 49; and
- b) determining whether the human liver disease in the non-human mammal is alleviated,

wherein if the human liver disease is alleviated in the non-human mammal, then the one or more agents or treatment protocols can be used to treat the human liver disease.

- 5 **51.** The method of Claim 50 further comprising comparing whether the liver disease in the non-human mammal is alleviated compared to a control.
- 10 **52.** The method of Claim 51 wherein the control is a non-human mammal that is a model of human liver disease and that has not received the one or more agents or treatment protocols.
- 15 **53.** A method of identifying one or more effects of one or more agents on a human liver comprising
- 15 a) administering the one or more agents to a non-human mammal of Claim 47; and
- 20 b) determining the effect of the agent on the human liver cells of the non-human mammal;
- 20 thereby identifying the effects of one or more agents on a human liver.
- 25 **54.** The method of Claim 53 wherein the agent is a pharmaceutical composition, a drug, an environmental agent or a combination thereof.
- 25 **55.** The method of Claim 54 wherein the agent has a therapeutic effect on the human liver cells, a toxic effect on the human liver cells, or no effect on the human liver cells.

56. A method of identifying how one or more agents are metabolized by a human liver comprising
- a) administering the one or more agents to a non-human mammal of Claim 47;
- 5 c) maintaining the non-human mammal under conditions in which the one or more agents are metabolized by the human liver cells in the non-human mammal, thereby producing one or more metabolites in the non-human mammal; and
- 10 b) detecting the one or more metabolites in the non-human mammal;
- thereby identifying how one or more agents are metabolized by a human liver.
- 15 57. The method of Claim 56 wherein one or more active metabolites, one or more toxic metabolites or a combination thereof are detected.
58. An in vitro method of obtaining mammalian hepatocytes comprising:
- a) contacting mammalian hepatic progenitor/stem cells (HPSCs) which express
- 20 CD34⁺CD133⁺ with hepatocyte differentiation medium, thereby producing a culture; and
- b) maintaining the culture under conditions in which the HPSCs differentiate into mammalian hepatocytes,
- thereby obtaining mammalian hepatocytes.
- 25 59. The method of Claim 58 wherein the mammalian hepatocytes are human hepatocytes and human hepatocytes are obtained.

- 50 -

60. The method of Claim 58 wherein about 2×10^5 of the HPSCs are seeded on collagen.
- 5 61. The method of Claim 60 wherein the hepatocyte differentiation medium comprises 5% fetal bovine serum (FBS), oncostatin, dexamethasone, insulin, transferrin and selenous acid.
62. The method of Claim 58 further comprising assessing differentiation of the
10 HPSCs into mammalian hepatocytes.
63. The method of Claim 62 wherein differentiation of the HPSCs into mammalian hepatocytes is assessed by detecting mammalian albumin in the culture.
- 15 64. Mammalian hepatocytes produced by the method of Claim 58.
65. Human hepatocytes produced by the method of Claim 59.

Figure 1

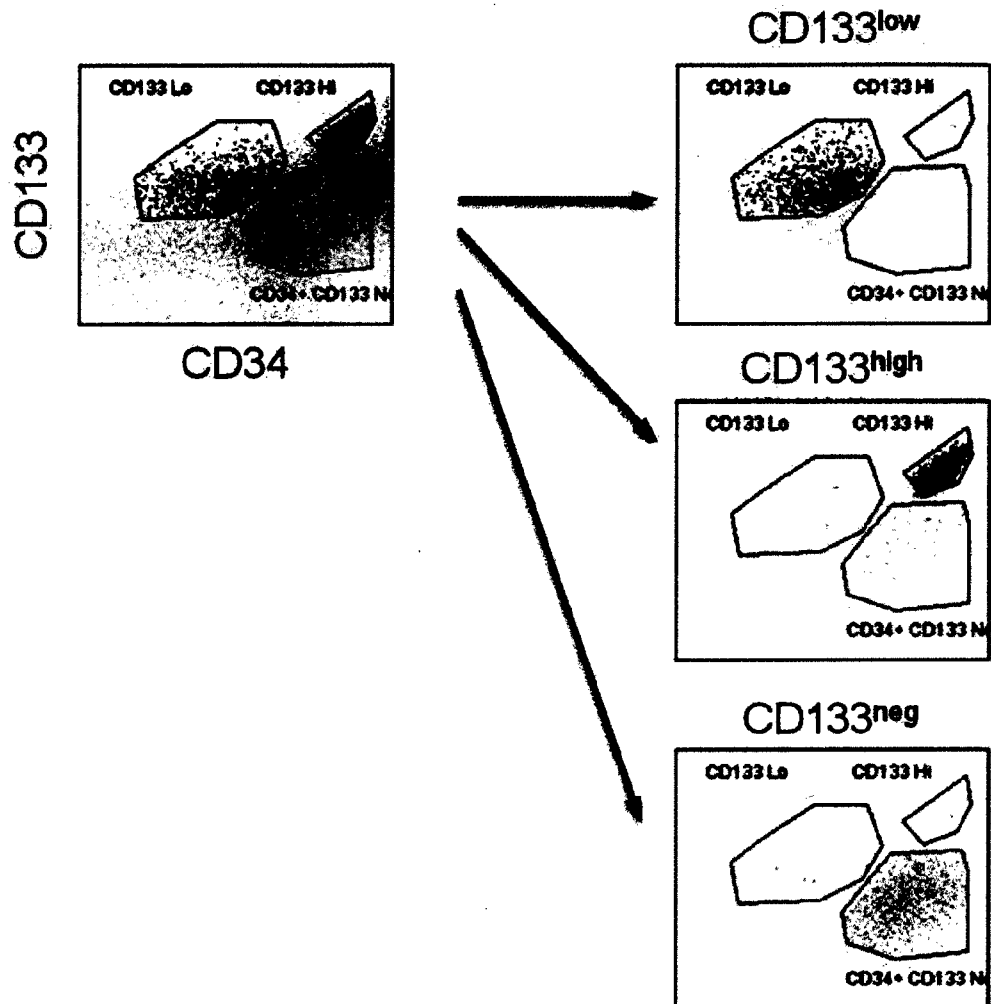


Figure 2

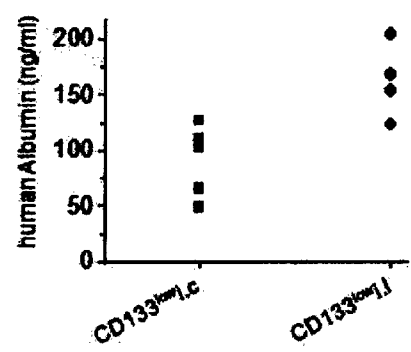
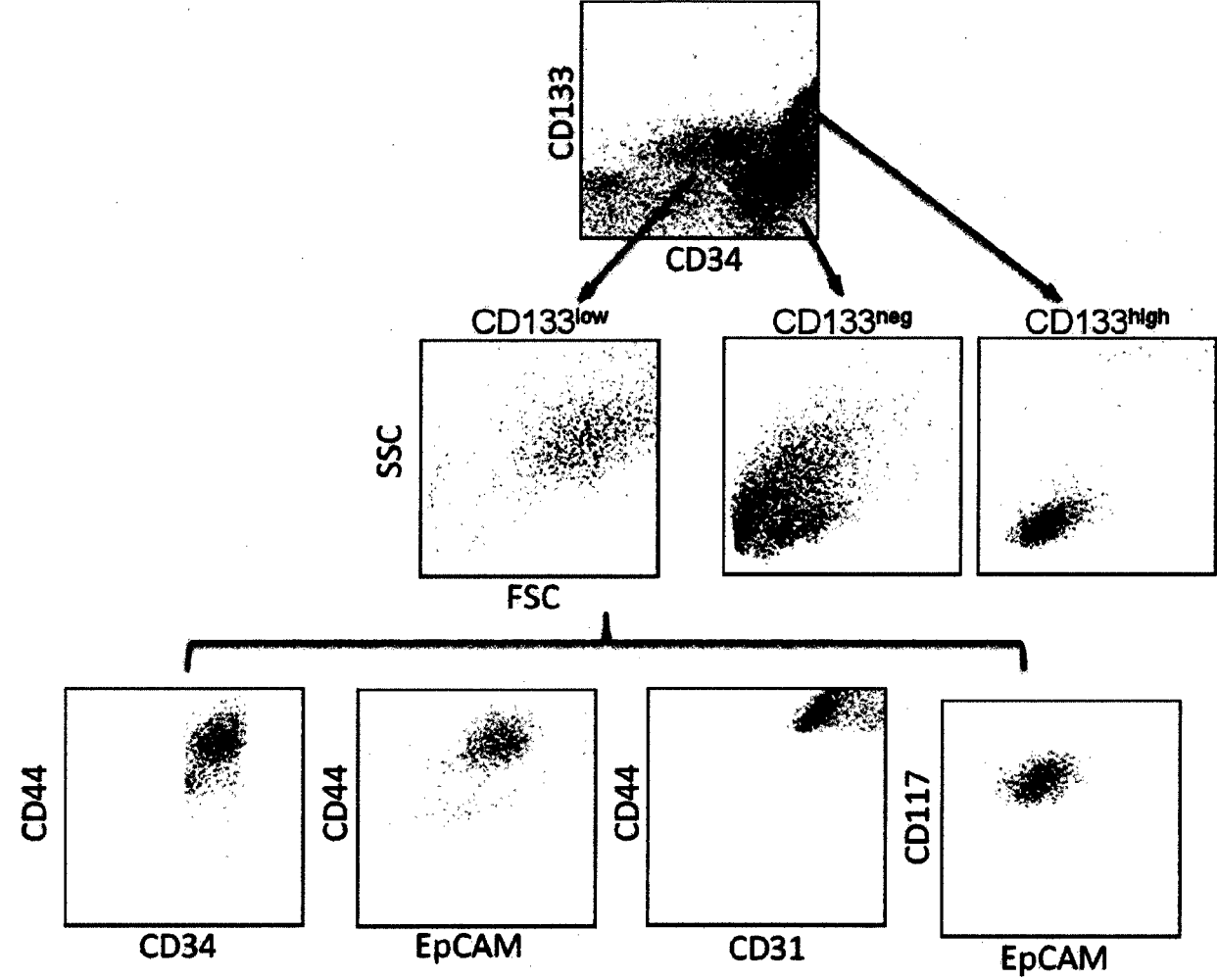


Figure 3



Figures 4A-4B

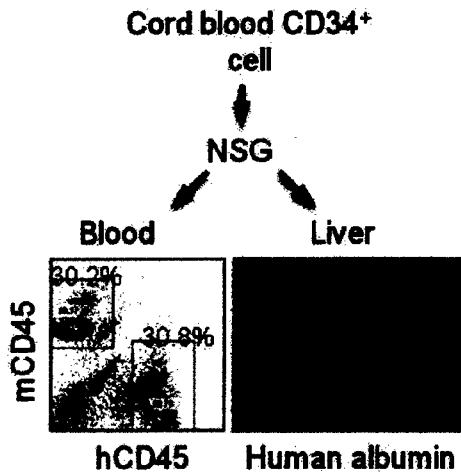


Figure 4A

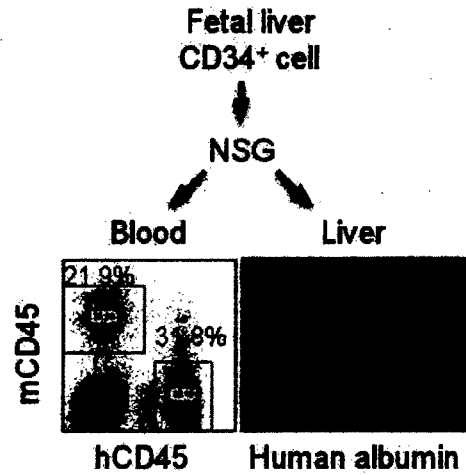


Figure 4B

Figs. 4A-4B. CD34⁺ cells from human fetal liver can give rise to human blood cells and hepatocytes *in vivo*. Cord blood (n=3) and fetal liver cells (n=3) selected by CD34 were transplanted into sub-lethally irradiated NSG new born pups by intra-cardiac injection separately (n=20). Eight weeks later, mononuclear cells were prepared from blood and stained with human CD45 and mouse CD45 antibodies. Representative plots are shown. Paraffin sections of the livers were also prepared and stained with human albumin specific antibodies (Magnification 100X).

Figures 5A-5E

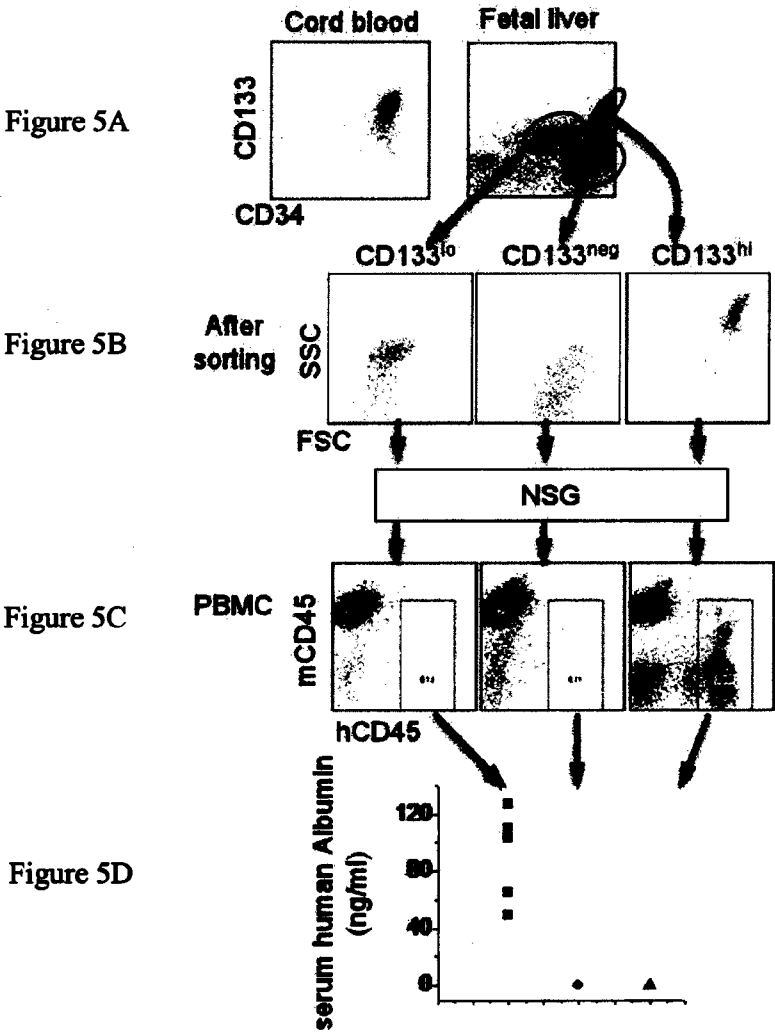
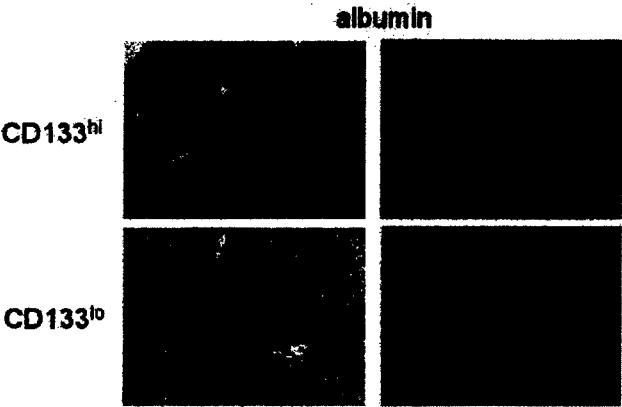
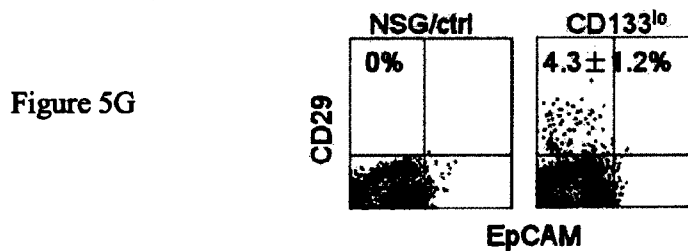
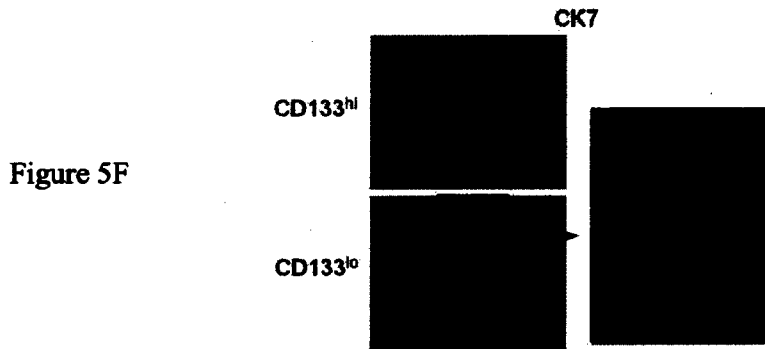


Figure 5E



Figures 5F-5G



Figs. 5A-5G. *In vivo* transplantation of sorted cell subsets from human CD34⁺ fetal liver cells in NSG mice. (5A) Comparison of CD34⁺ Cells purified from human cord blood (left) and fetal liver (right). Fluorescent CD133 and CD34 antibodies were used to analyze the cell phenotypes. Representative data from 5 cord blood and 3 fetal liver samples are shown. (5B) CD133^{hi}, CD133^{lo}, CD133^{neg} cell subsets were isolated from CD34⁺ human fetal liver cells by cell sorting. The FACS plots show FSC versus SSC. (5C) These three populations were then injected separately into 3 groups of new born NSG pups (n=5). Ten weeks later, mononuclear cells from blood were prepared for staining of human CD45 and mouse CD45 antibodies. (5D) Sera were analyzed by human albumin specific ELSIA. (5E) Livers from the mice injected with different cell subsets were harvested. Paraffin sections were prepared and stained with human specific albumin antibody to analyze the reconstitution of human hepatocytes. (5F) human specific CK7 antibody was used to stain human biliary epithelial cells in the liver sections. Black arrow indicates the positive biliary duct cells. (5G) Nine weeks after injection, single cell suspensions were prepared from the livers by perfusion and stained with CD29 and EpCAM antibodies (n=8). Liver from adult NSG mice served as negative control (n=2). Representative data are shown. See also Fig. 10.

Figures 6A-6C

Figure 6A

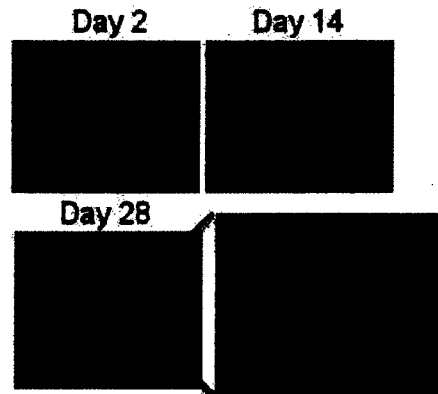


Figure 6B

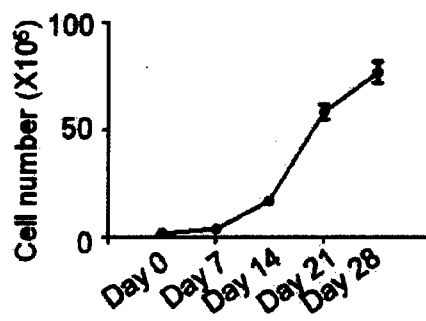
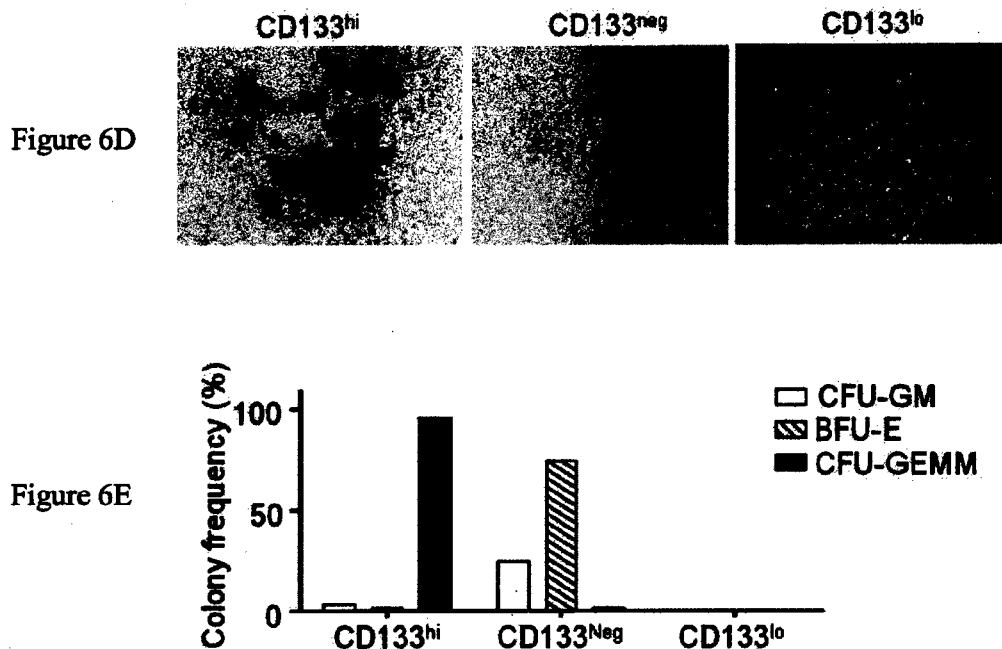


Figure 6C

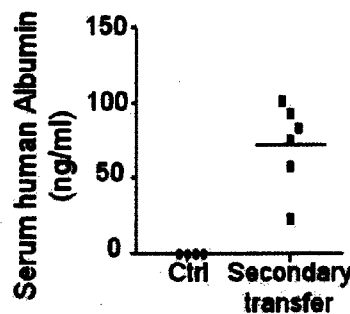
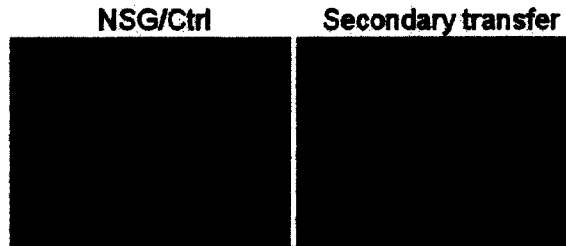
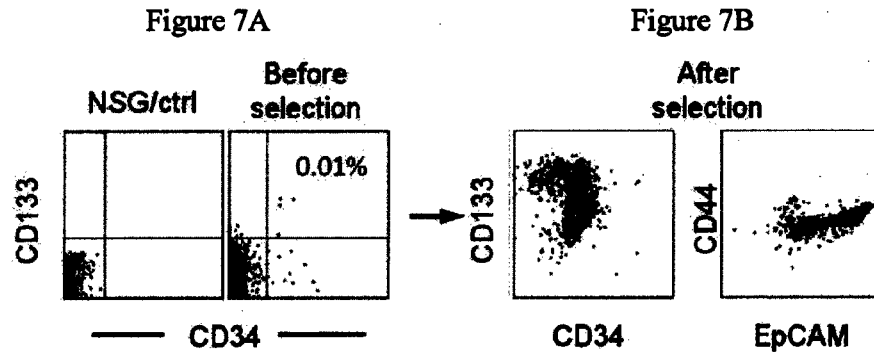


Figures 6D-6E



Figs. 6A-6E. *In vitro* differentiation of the isolated cell populations. (6A) CD133^{lo} cells differentiate into hepatocytes *in vitro*. Phase-contrast micrograph data show the growing and morphology change of cells at different days. (6B) Cells were trypsinized and absolute number were counted at different time points during the *in vitro* differentiation. (6C) illustrates immunofluorescence for albumin and DAPI staining on the differentiated cells on Day 28. (6D) Colony-forming assay. Nine days after plate the cells, phase-contrast micrograph pictures were taken. Black arrows indicate the CFU-GEMM, CFU-GM, and BFU-E colonies. (6E) Colonies were counted on day 9. Shown are the frequencies of the different colonies. See also Fig. 11.

Figures 7A-7D



Figs. 7A-7D. The CD133^{lo} cells have self-renewal properties in vivo. (7A-7B) Re-purification of hepatic progenitor cells by CD34 selection. Liver single cell suspensions of the mice that were injected with CD133^{lo} cells were prepared by perfusion and then stained with CD34 and CD133 antibodies to analyze the frequency of HPC (n=11). CD34⁺ cells were purified from the single cell suspensions by magnetic particles. After selection, the recovered cells were stained with CD34, CD133, CD44 and EpCAM antibodies. Adult NSG mice were used as negative control. (7C) The re-purified 600K CD34⁺ cells were injected into 6 new born NSG pups. Eight weeks later, liver paraffin sections were prepared and stained for human albumin. (7D) Sera were collected for albumin test by ELISA (n=6). Adult NSG mice were used as control.

Figures 8A-8B

Figure 8A

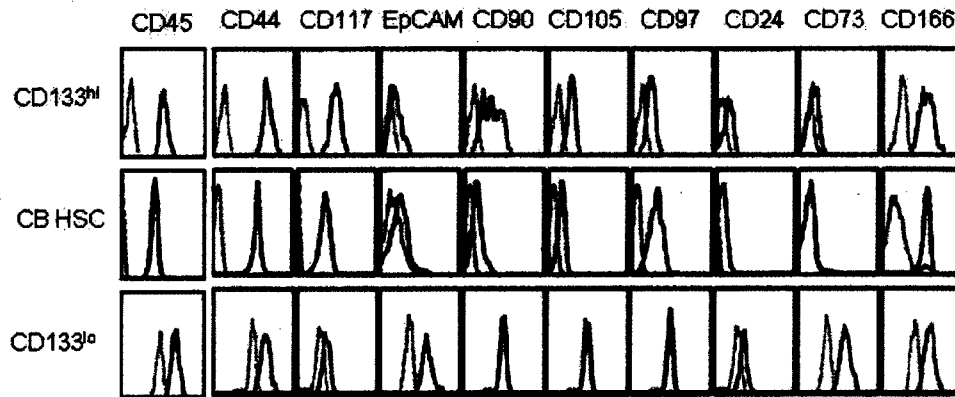
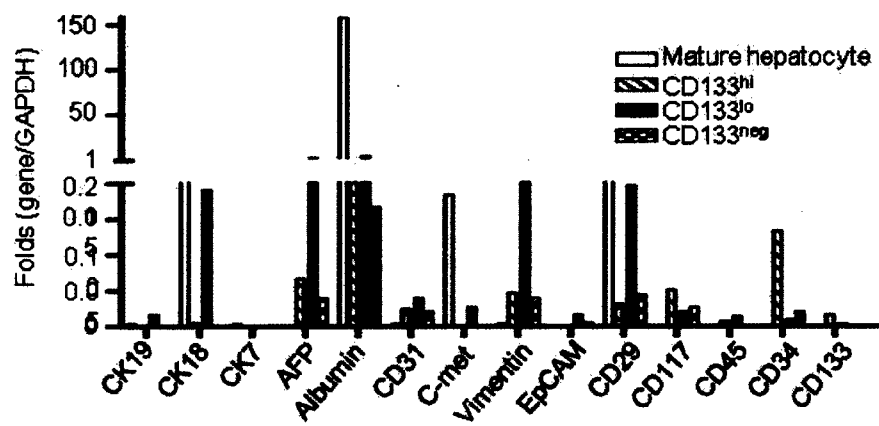


Figure 8B



Figures 8C-8F

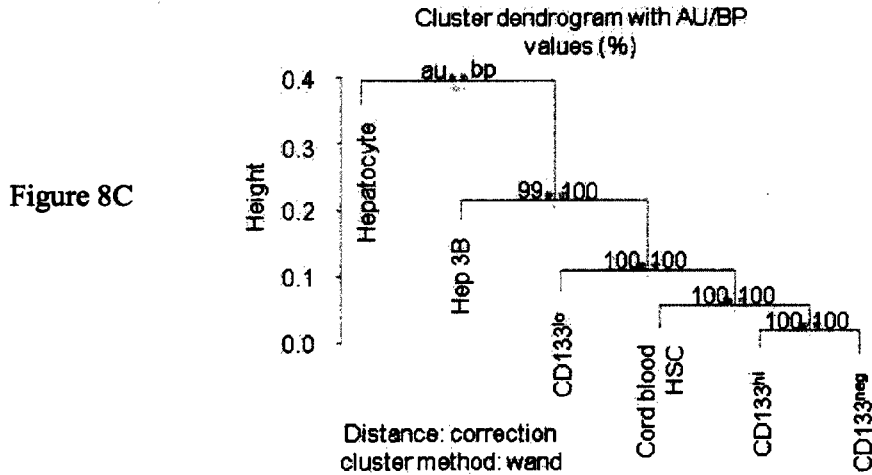


Figure 8D

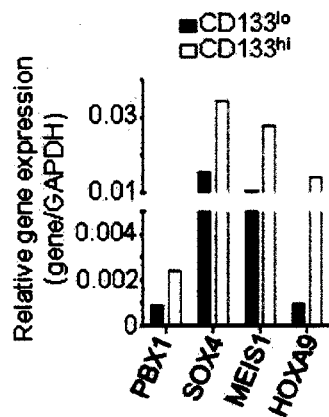


Figure 8E

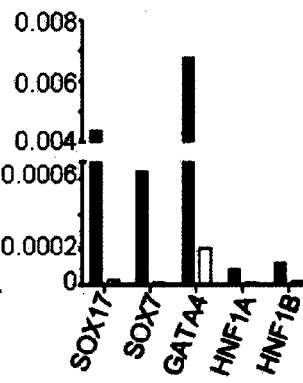
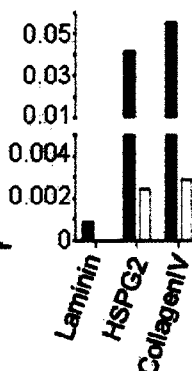


Figure 8F



Figs. 8A-8F. Gene expression profile of HpSC and HSC. (8A) HSC and HpSC surface phenotyping. CD133^{hi} and CD133^{lo} cells were stained for human stem cell related markers: CD44, CD117, EpCAM, CD90, CD105, CD97, CD24, CD73, CD166 and CD45. Cord blood HSC was used as a parallel control. (8B) Total RNA of CD133^{hi}, CD133^{lo}, CD133^{neg} and mature hepatocytes were prepared and analyzed by real-time PCR. The results are obtained from two different fetal liver samples. (8C) Transcriptional profiling of cell populations. Shown is hierarchical cluster analysis of transcriptomes of the six cell types. Values at branches are AU *p*-values (left), BP values (right) and cluster labels (bottom). The height scale reflects a distance measure that is scaled between 0 and 1. CD133^{lo}, CD133^{hi} and CD133^{neg} cells were from three biological samples. (8D) Comparison of HSC specific transcription factor gene expressions in CD133^{lo} and CD133^{hi} cells by RT-PCR (n=2). (8E and 8F) Comparison of endoderm signature gene expressions in CD133^{lo} and CD133^{hi} cells by RT-PCR (n=2). See also Figs. 12A-12C.

Figure 9

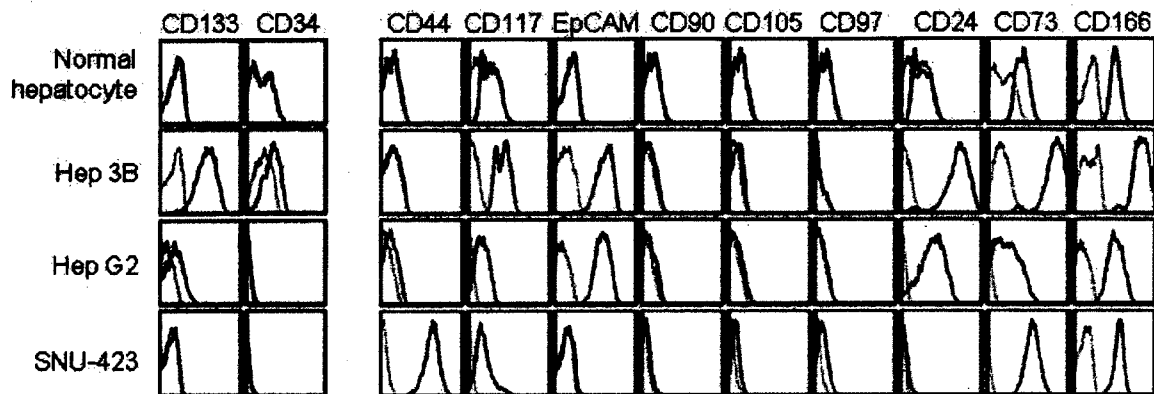


Fig. 9. human hepatoma cell phenotyping. Human mature hepatocytes, Hep 3B cells, Hep G2 cells and SNU-423 cells were stained for CD133, CD34, CD44, CD117, EpCAM, CD90, CD105, CD97, CD24, CD73, and CD166.

Figures 10A-10B

Figure 10A

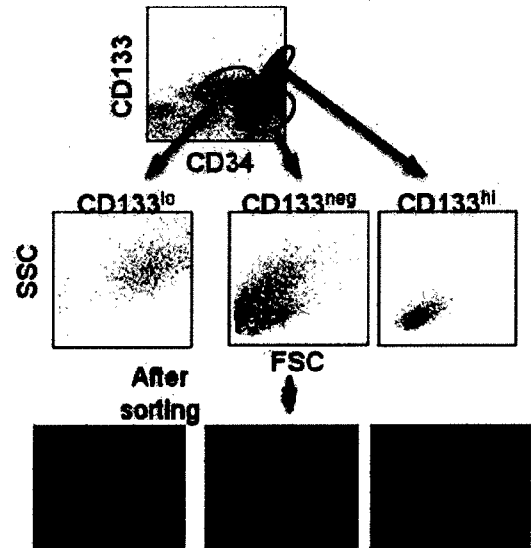


Figure 10B

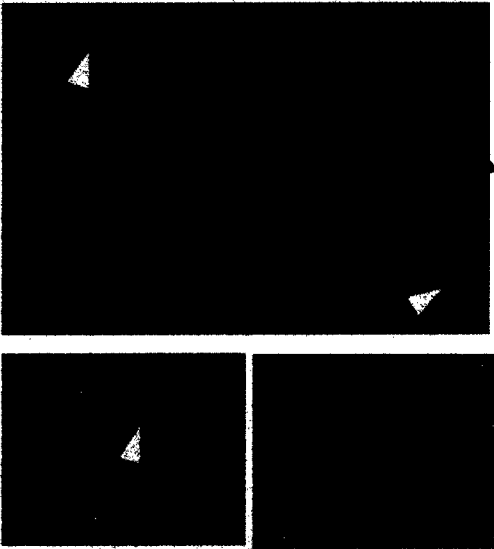
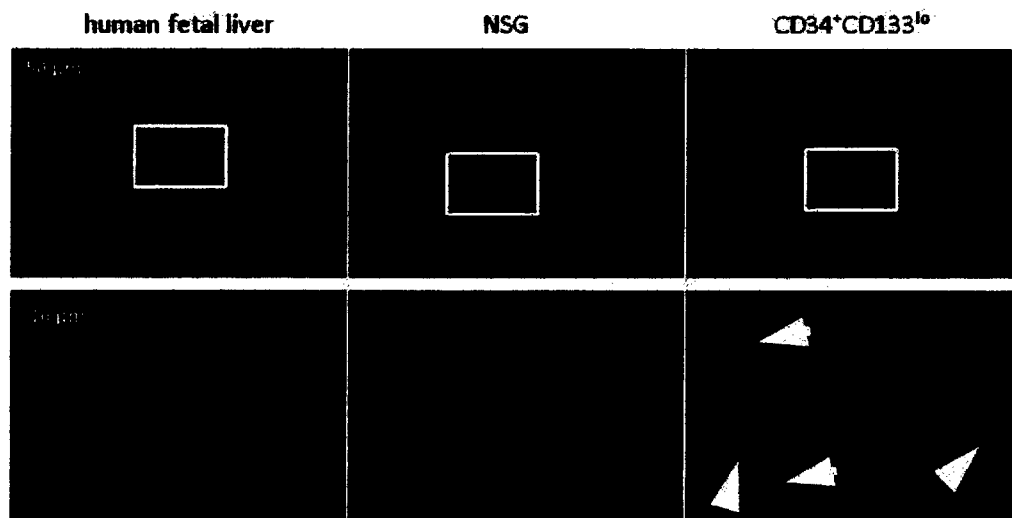


Figure 10C



Figs. 10A-10C. (10A) Fetal liver cells were stained for CD34 and CD133 after CD34 selection. Shown are cytometry FSC and SSC data and phase-contrast micrograph of the sorted three cell populations. (10B) Human specific CK19 antibody was used to stain human biliary epithelial cells in the liver sections. Black arrows indicate the bile ducts positive for human CD19 while the white ones indicate the negative ducts. (10C) Cy3 fluorescence conjugated probe against human pan centromere (red) and DAPI (blue) were used to stain human cells in the liver sections. Human fetal liver sections were used as positive control; NSG mouse liver sections were used as negative control. White arrows in the liver sections prepared from mice injected with CD133^{lo} cells indicate positive cells for human pan-centromere stainings.

Figure 11

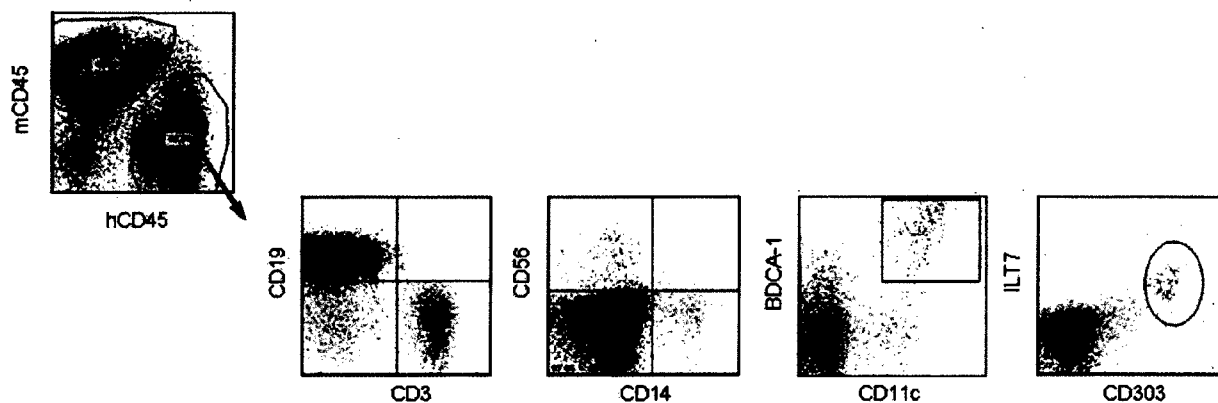
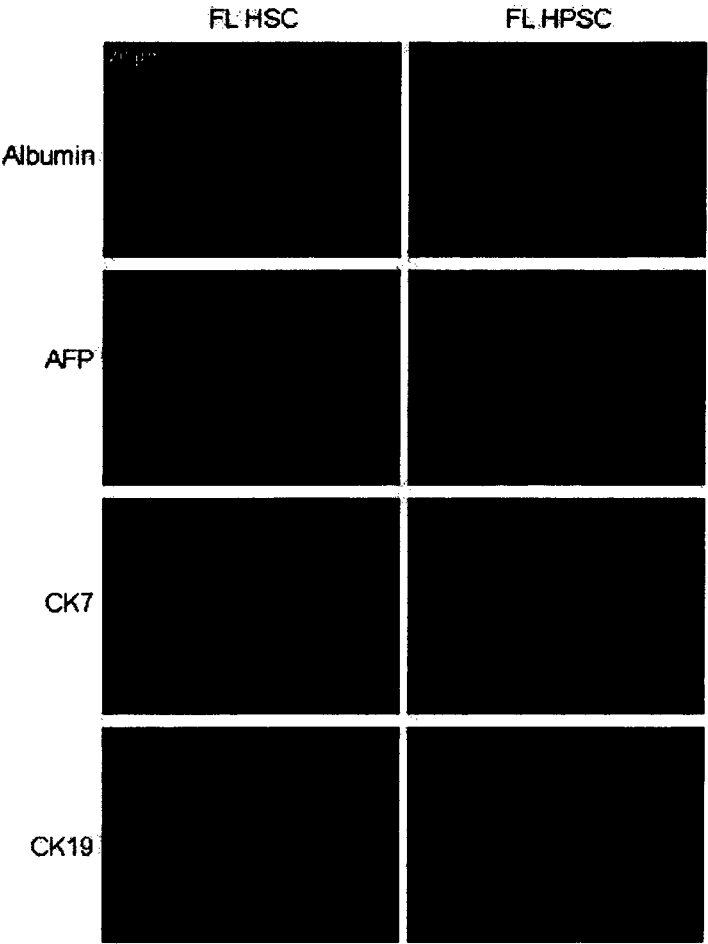


Fig. 11. CD133^{hi} cells were sorted from CD34⁺ cells purified from human fetal liver. 1×10^5 cells were injected intra-cardically into new born NSG mice (n=10). 10 weeks after injection, splenocytes were isolated and stained for antibodies against human antigens: CD45, CD3, CD19, CD56, CD14, CD11c, BDCA-1, CD303, ILT7 and mouse CD45.

Figure 12A



Figures 12B-12C

Figure 12B

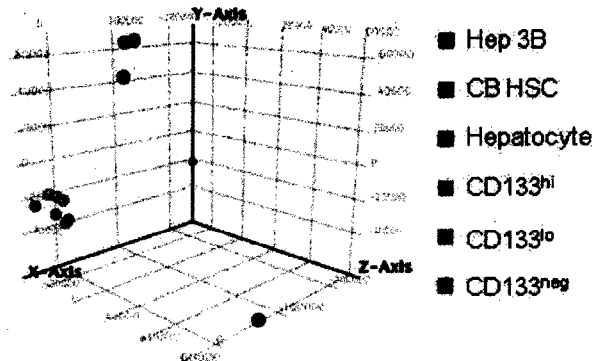
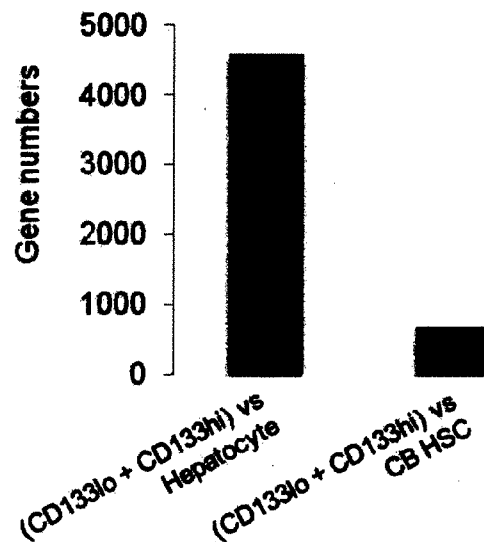


Figure 12C



Figs. 12A-12C. (12A) CD133^{hi} and CD133^{lo} cells were sorted by FACS sorter and fixed on slides by Cytospin. Antibodies against human albumin, AFP, CK7 and CK19 were used to stain the cells. Blue: DAPI. Green: Albumin, AFP, CK7 and CK19. (12B) Principal component analysis (PCA) of the microarray data. Shown is 3 dimensional plot of PCA. Each data point represents individual sample/array, with all 12 sample/arrays presented on the PCA plot. The percentage values indicate the proportion of total variance described by each PC. PC 1 principal component 1 (X-axis); PC 2 principal component 2 (Y-axis); PC 3 principal component 3 (Z-axis). Arrays are distinguished by colour and replicates represent by the same colour. CD133^{Neg}, CD133^{high} and CB HSC samples/arrays aggregate together which signify their similarity in expression profile. However, hepatocyte were separated from the other. (12C) Comparison of differentially expressed genes. Shown are number of common genes shared by CD133^{low} and CD133^{hi}. Compared to hepatocyte, CD133^{low} and CD133^{hi} share 4636 differentially expressed genes but compared to CB HSC, CD133^{low} and 133^{hi} shared only 468 genes.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG20 11/000234

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12N 5/02 (2006.0 1)

C12N 5/0735 (20 10.0 1)

C12N 5/073 (20 10.0 1)

C12N 5/22 (2006.0 1)

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)

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

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

WPI, EPODOC, MEDLINE, CAPLUS, BIOSIS. Keywords: hepatic, liver, progenitor cells, CD133, CD34 and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAO, M. S. <i>et al</i> , 'Characterization of hepatic progenitors from human fetal liver during second trimester', World Journal of Gastroenterology, 2008, vol. 14, no. 37, pages 5730-5737 The whole document, but particularly the abstract	1-3
X	WO 2007/07 1339 A 1 (UNIVERSITE CATHOLIQUE DE LOU VAIN) 28 June 2007 The whole document, but particularly page 7, lines 9-23; page 10, lines 9-15 and 20-21; page 41, lines 13-25; page 51, lines 19-30; page 53, lines 7-13 and 18; page 60, line 22 - page 61, line 4; Figure 3; Example 1	4-18, 25-44, 47-65



Further documents are listed in the continuation of Box C



See patent family annex

♦	Special categories of cited documents:		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier application or patent but published on or after the international filing date	"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
"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)	"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
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

26 August 2011

Date of mailing of the international search report

26.08.2011

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INTERNATIONAL SEARCH REPORT

International application No.

PCT /SG201 1/000234

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHMELZER, E. <i>et al</i> , 'Human hepatic stem cells from fetal and postnatal donors', Journal of Experimental Medicine, 2007, vol. 204, no. 8, pages 1973-1987 The whole document, but particularly the abstract; page 1981, column 1, paragraph 2- page 1982, column 2, paragraph 1; Table 1	4-18, 47-49, 58-65

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG20 1 1/000234

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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.:
because they relate to subject matter not required to be searched by this Authority, namely:
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:

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are 6 different inventions based on the following features that separate the claims into distinct groups:

- Claims 1-3. The feature of an isolated mammalian fetal hepatic progenitor/stem cell (HPSC) that expresses CD34⁺CD133⁺ is specific to this group of claims.
- Claims 4-46. The feature of producing non-human mammals which contain human hepatocytes by introducing human HPSCs that express CD34⁺CD133⁺ is specific to this group of claims.
- Claims 47-49. The feature of non-human mammals which contain human hepatocytes is specific to this group of claims.
- Claims 50-57. The feature of identifying and testing agents that affect the human liver by administering them to non-human mammals which contain human hepatocytes is specific to this group of claims.
- Claims 58-62. The feature of obtaining mammalian hepatocytes *in vitro* by contacting mammalian HPSCs that express CD34⁺CD133⁺ with hepatocytes differentiation medium is specific to this group of claims.
- Claims 63 and 64. The feature of mammalian hepatocytes is specific to this group of claims.

See Supplemental Box I

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 additional fees, this Authority did not invite payment of additional fees.
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.

Supplemental Box I

(To be used when the space in any of Boxes I to IV is not sufficient)

Continuation of Box No: III

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claims and therefore cannot provide the required technical relationship. Therefore there is no special technical feature present in the claims and the requirements for unity of invention are consequently not satisfied *apriori*.

INTERNATIONAL SEARCH REPORT**Information on patent family members****International application No.****PCT/SG201 1/000234**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	200707 1339	AU	2006328988	CA	26345 10	CN	10 1356264
		EP	1969 118	EP	228 1875	JP	2009520474
		US	20083 11094				
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001 .							
END OF ANNEX							