METHODS AND COMPOSITIONS FOR PRODUCING HEPATOCYTE-LIKE CELLS

GOVERNMENT RIGHTS

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BACKGROUND

A major goal for regenerative medicine is to facilitate human tissue replacement through transplantation of stem cells that can be harvested from readily accessible tissues. For example, orthotopic liver transplantation is the only effective treatment for end-stage liver disease or severe liver injury, but its utility is severely limited by the lack of donor liver tissue and by the requirement for lifelong immunosuppression.

A large number of adipocyte-derived stem cells (ASCs) can be easily obtained using a commonly performed procedure, liposuction. Additionally, methods for inducing ASC differentiation into hepatocyte-like cells (iHeps) have been developed. Liver regeneration via transplantation of iHeps (e.g., autologous iHeps) is a highly attractive possibility for regenerative medicine. By this method, ASC obtained by liposuction are induced to differentiate into iHeps *in vitro,* and then iHeps are transplanted into the donor's liver. The abundance and accessibility of adipose tissue ensures that there is a source of readily available ASCs. Moreover, liver regeneration using autologous iHeps would not require immunosuppression.

However, since a patient can die rapidly after acute liver failure (e.g., caused by acetaminophen toxicity), the prolonged culture period associated with the currently known method to produce iHeps from ASCs severely limits the clinical utility for producing iHeps. Furthermore, the known method to produce iHeps from ASCs is also characterized by low efficiency and low yield.

The production of hepatocyte-like cells from adipocyte-derived stem cells for use in therapy and/or research will benefit from reduced cost, reduced culture time, increased efficiency, and increased yield.

Publications

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SUMMARY

Methods are provided for producing a population of hepatocyte-like cells (iHeps) from a population of adipocyte-derived stem cells (ASCs). Aspects of the methods include placing a population of ASCs into a three dimensional culture (e.g., hanging drop suspension culture, high density culture, spinner flask culture, microcarrier culture, etc.) and contacting the cells with a first and second culture medium. Also provided are methods of treating an individual, which include producing a population of iHeps from a population of ASCs, and administering an effective number of iHeps into the individual.

The presence of iHeps may be confirmed by various methods, including for example, contacting the induced cell population with an antibody specific for a hepatocyte marker protein, and determining the percentage of cells positive for expression. In some embodiments, 15% or more of cells of the induced cell population are hepatocyte-like cells. In some cases, 37% or more of the cells of the induced cell population are hepatocyte-like cells. In some embodiments, the elapsed time to generate iHeps from ASCs is less than 13 days (e.g., less than 10 days). The high efficiency of iHep production and the short time frame over which iHeps can be produced by the subject methods facilitate treatment methods because liver damage (e.g., liver failure, e.g., due to acetaminophen toxicity) can rapidly cause death (e.g., in 2 weeks or less). Kits for practicing the methods of the disclosure are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

Figures 1A-E provide a comparison of SCi-Heps to Chi-Heps, and a comparison of the methods of generating SCi-Heps and Chi-Heps. (**A**) The two different methods for

inducing the differentiation of ASC into iHeps are shown. The top panel shows the method for chemically differentiating ASC into Chi-Heps. The cells isolated from lipo-aspirates are cultured for 3-15 days ASC cells. These cells are then differentiated from mesoderm into endodermal cells over a 3-day period (stage 1), and then further differentiated into Chi-Heps (stage 2) using defined media containing growth factors. The bottom panel shows one embodiment of a subject method for producing SCi-Heps. (**B**) Bright field images (100x) showing the change in the morphology of the spindle-shaped ASC as they differentiate into Chi- Heps (via chemical differentiation) or SCi-Heps using a subject method for producing SCi-Heps. Relative to Chi-Heps, SCi-Heps have a greater cellular density and colony-like morphology that more closely resembles hepatocytes. (**C**) The percentage of ASC, Chi-Heps, SCi-Heps and iPS-Heps expressing the CK8/18 marker found on mature hepatocytes. Unstained ASC were used to characterize the background level of staining (control), and 100% of human hepatocytes express this marker. (**D**) Immunofluorescence staining images (at 100x) of control ASC, Chi- Heps, SCi-Heps or hepatocytes for human CK8/18 expression, LDL uptake or PAS staining. Chi-Heps and SCi-Heps, but not ASC, can endocytose LDL and can synthesize glycogen (PAS stain). (**E**) The amount of human albumin or urea secreted into the supernatant by Chi-Heps, SCi-Heps and ASC cultured for the indicated time period. SCi-Heps produced albumin and urea well before Chi-Heps produced detectable amounts of these analytes, while ASC did not produce albumin or urea. In panels C and E, each bar represents the average (+SEM) of 4 biologically independent samples analyzed.

Figure 2 A-C depict gene expression in Chi-Heps, SCi-Heps and adipocytes. (**A**) iHeps have increased levels of hepatocyte-specific mRNAs and decreased levels of adipocyte-specific mRNAs. RT-PCR analysis was used to measure the level of hepatocyte-specific (FoxA2, AFP, ALB, AAT, TDO2) or adipocyte-specific (CD37) mRNA expression in Chi-Heps, SCi-Heps, iPS-Heps or ASCs. For each gene, the mRNA levels in Chi-Heps (after 15 days of differentiation), SCi-Heps (after 9 days of differentiation) and iPS-Heps (after 30 days of differentiation) are normalized relative to that in ASCs. (**B**) RT-PCR analysis of the gene expression in ASCs, and in SCi-Heps after 3, 6 and 12 days of induced differentiation. The level of CD105 mRNA expression (which is an ASC marker) was decreased by 35-fold ($p = 0.001$); while the level of hepatocyte (FoxA2 and ALB) mRNA expression was increased by 25- and 51-fold, respectively ($p=0.0007$ and $9x10^{-5}$, respectively), in SCi-Heps after 12 days of hepatocyte differentiation. The mRNA levels in SCi-Heps were normalized relative to that in ASCs. Each data point in panels A-B represents the average ± SEM for 3 independent determinations. As shown in **Figure 11**, there were significant changes in the level of expression of each gene in SCi-Heps after 3, 6

and 12 days of differentiation. (**C**) An illustration of the spatial relationship between the gene expression profiles obtained from ASCs, Chi-Heps, SCi-Heps, iPS-Heps and hepatocytes. Microarray-based global gene expression data was analyzed as described in the methods section. The lengths of each connecting edge (and the number shown) indicate the distance between the expression profiles for the cell types at each of the corresponding vertices. This distance is determined by summing the squares of the differences in the level of expression for each gene on the array for the two cell types at the vertices of each line. This diagram indicates that the gene expression pattern in Chi- and SCi-Heps is closer to that of hepatocytes than is that of iPS-Heps. Also, the deviation of the iPS-Heps pattern from the ASC-hepatocyte axis is larger than that of iHeps, which indicates that iPS-Heps have a larger number of gene expression changes that are external to both ASCs and hepatocytes than are found in Chi- or SCi-Heps.

Figures 3 A-D demonstrate the implantation (via injection) of SCi-Heps into the mouse liver. **(A)** An ultrasound generated image (in transverse view) of SCi-Heps being injected through a 30G needle directly into the right lobe of the liver of a TK-NOG mouse. (**B**) Chimeric mice produced human albumin after SCi-Hep transplantation. The amount of human albumin in plasma was serially measured over an 8-week period after transplantation of SCi-Heps into 4 ganciclovir-conditioned mice. Each dashed line shows the amount of human albumin measured in a chimeric mouse at the indicated time. In all 4 chimeric mice, human serum albumin was detectable 4 weeks after transplantation, and the amount increased with time after transplantation. In contrast, albumin was not produced by any of the 4 mice that were recipients of control, undifferentiated ASCs (red triangles and solid line). (**C**) Immunofluorescent staining of liver sections (at 100x magnification) obtained from TK-NOG mice 4 weeks after implantation of SCi-Heps, (a-c) or undifferentiated ASCs (d-f). The liver sections were stained with human specific anti-albumin (a, d), anti-CK8/18 (b, e), or anti ASGR1 antibodies, and counter-strained with DAPI to show the cell nuclei. (**D**) Immunofluorescent staining of liver sections (200x magnification) obtained from TK-NOG mice 4 weeks after transplantation with SCi-Heps or undifferentiated ASCs. The liver sections were stained with anti-CK8/18, anti-Ki67 or anti-ZO-1 antibodies; and were then counter-strained with DAPI to show the cell nuclei.

Figures 4 A-B demonstrate that SCi-Heps do not form tumors in immunocompromised mice, but iPS-Heps do. (**A**) As early as three weeks after $5x10^4$ iPS-Heps were implanted under the kidney capsule of NOG mice, palpable tumors were formed in the area of implantation. In contrast, no tumors were detected 2-months after the same number of Chi- or SCi-Heps were implanted (top row). The tumors were visible in tissue sections obtained from the area of iPS-Hep implantation, while only normal tissue was

present in the area of Chi-Hep implantation (middle row). The magnification of images in the top and middle rows are 10x. The images in the bottom rows were obtained from the boxed regions of the corresponding image and are shown at 200x magnification. (**B**) Tumor formation after iPS-Heps implantation. Top row: Images of the tumors formed 3 weeks after iPS-Hep cells were implanted under the kidney capsule. The 300x and 100x images were obtained from the indicated region (dashed box) of the corresponding 40x and 100x images. Bottom row: Two months after the iPS-iHep cells were implanted, the tumors contained cells from all three germ cell layers. These images are at 200x magnification. The scale bars shown are 50 μm.

Figures 5 A-C provide FACS analysis performed using an anti-Sox 17 antibody, which recognizes to a marker for endodermal cells. ASC were isolated from three different donors and induced to differentiate using the standard (Chi-Hep) or spherical culture (SCi-Heps) methods. After 4 days of differentiation, FACS was performed using an anti-Sox 17 antibody, which recognizes to a marker for endodermal cells. The percentage of ASC differentiating into endodermal cells after spherical culture (61.2 ± 2.4) more than doubled that obtained using the standard method (25.7 ± 1.3).

Figures 6 A-B provides FACS analysis performed using an anti-CD105 antibody, which binds to a marker for ASC. ASC were isolated from three different donors and induced to differentiate into SCi- Heps using the spherical culture method. After 9 days of differentiation, FACS was performed using an anti-CD105 antibody, which binds to a marker for ASC. While 98.6 ±0.5% of ASC expressed CD105, the percentage of SCi-Heps expressing this ASC marker was only 9.0 ± 0.5%.

Figure 7 demonstrates that iPS-Heps can synthesize glycogen (**a,b**) and endocytose low-density lipoproteins (LDL) (**c,d**). The LDL immunofluorescence and PAS stained images are shown at 100x (a, c) and 400x magnification (b, d). In contrast, ASC could not endocytose LDL (e, 100x), nor did they stain with PAS (f, 100x).

Figures 8 A-D demonstrate that iPS-Heps have hepatocyte properties. The amount of human albumin (**A**) or urea nitrogen (**B**) secreted into the supernatant by Chi-Heps, iPS-Heps and ASCs are shown at the indicated time points. Chi-Heps produced albumin and urea well before iPS-Heps produced detectable amounts of these analytes, while control ASCs did not produce albumin or urea. (**C**) Chi-Heps (after 15 days) and iPS-Heps (after 30 days of differentiation), but not ASCs, can mediate a CYP3A4-dependent drug biotransformation reaction. (**D**) iPS-Heps have increased levels of hepatocyte-specific and decreased levels of adipocyte-specific mRNAs. RT-PCR analysis was used to measure the level of hepatocyte-specific (FoxA2, AFP, ALB, AAT, A1AT, TDO2) or adipocyte-specific (CD37, CD29) mRNA expression in s, iPS-Heps or ASCs. For each gene, the mRNA levels

in in Chi-Heps (after 15 days of differentiation) and iPS-Heps (after 30 days of differentiation) are normalized relative to that of the corresponding mRNA level in ASCs. Each bar or data point in panels A-D represents the average (±SEM) of 4 biologically independent samples that were analyzed.

Figure 9 provides principal component analysis for the gene expression data obtained from ASC, Chi- Hep, SCi-Hep, iPS-Hep and hepatocytes. The first (x-axis) and the second (y-axis) principle components (PC) explained 46.4% and 26.5%, respectively, of the total variance in the expression profile. Of note, iPS-Heps had a large amount of deviation along PC2 relative to ASCs and hepatocytes, while Chi-Heps and SCi-heps were right in between the ASCs and hepatocytes. Consistent with other analyses (including the analysis shown in **Figure 2C**), this graph indicates that iPS-Heps had a large number of gene expression changes that were not present in hepatocytes (relative to ASCs).

Figure 10 demonstrates that SCi-Heps do not express ASGR-1 *in vitro*. Permeabilized human hepatocytes (**a-c**) or SCi-Heps (**d-f**), which were analyzed after 9 days of *in vitro* differentiation, were stained with an anti-human ASGR-1 antibody and counter-stained with DAPI. The immunofluorescence images (200x magnification) show that human hepatocytes, but not SCi-Heps expressed ASGR-1. The scale bars shown are 50 µm.

Figure 11 presents a table depicting the numbers of differentially expressed genes in hepatocytes, Chi-Heps, SCi-Heps, and iPS-heps relative to ASCs. From analysis of microarray data, the indicated number of genes whose expression was significantly increased, decreased or unchanged in hepatocytes relative to ASCs were selected. (A gene's expression was considered as altered in hepatocytes if the absolute value of the fold change was >5 and the adjusted p value was <0.01.) Each of the 3 panels indicates the number (and percentage) of the selected genes whose expression was increased, decreased or not significantly changed (NS) when the expression profiles in Chi- Heps, SCi-Heps or iPS-Heps (relative to ASCs). For each of these comparisons, the expression of a gene was considered altered in iHeps if the absolute value of the fold change was >2 and the adjusted p value was <0.01. It is noteworthy that 18% of the genes whose expression was not altered in hepatocytes had an altered level of expression in iPS-Heps.

Figure 12 presents a table depicting changes in the level of expression of genes (*Foxa2, Albumin*) expressed in hepatocytes and in adipocyte-specific (*CD105*) mRNAs during induced SCi-Hep differentiation. RT-PCR was used to measure the level of expression of 3 genes (*Foxa2, Albumin* and *CD105*) in ASC, and in SCi-Heps after 3, 6 and 12 days of induced differentiation. The measured average expression levels for each gene in SCi-Heps on the indicated day (relative to its level of expression in ASC) and the

corresponding p-values for the expression differences are shown. Each value is the average fold-change for 3 independent measurements; a positive value indicates that its expression was increased in SCi-Heps relative to ASC, while a negative value indicates that it was decreased. A one-sample t-test was applied using the log-transformed expression level to assess the statistical significance (P-value) of the measured expression differences.

Figures 13 A-B demonstrate that ASCs cultured by stirred suspension culture (spinner flask culture in this case) form a high density of cellular aggregates that resemble the spheres formed during hanging drop suspension culture. Images shown are at 10x magnification. (A) The morphology of ASC cellular aggregates formed after spinner flask culture of ASCs for 24 hours. The morphology of the aggregates is very similar to that of the 'spheres' formed after culturing ASCs by the hanging drop method. The increased cell density (hence the term "high density culture") in the spinner flask culture is readily apparent when comparing panel (A) to panel (B). (B) 'spheres' formed after culturing ASCs by the hanging drop method for 48 hours.

Figures 14 A-B demonstrate that ASCs cultured by stirred suspension culture (spinner flask culture in this case) form a greater percentage (A) of CD34+ cells (adipose stem cells) and a roughly equal percentage (B) of SOX17+ cells (endodermal precursor cells) compared to ASCs cultured by the hanging-drop method. X-axis is the intensity of signal detected.

Figures 15 A-B demonstrate that iHeps produced using spinner flask culture (SS-Hep) had the specific cytochrome CYP enzymes CYP3A4 and CYP1A1, and the activity of CYP3A4 was strongly induced by dexamethasone (Dex) treatment. YJM is a human hepatocyte cell line. CYP activity was normalized to cell viability. Results are presented with (+) and without (-) Dex induction.

Figure 16 demonstrates that iHeps produced using spinner flask culture (SS-Hep) secreted an increased level of human albumin (hAlb) compared to Chi-Heps and SCi-Heps (approximately 16-fold relative to SCi-Heps and approximately 96-fold relative to Chi-Heps).

DETAILED DESCRIPTION OF THE EMBODIMENTS

Methods are provided for producing a population of hepatocyte-like cells (iHeps) from a population of adipocyte-derived stem cells (ASCs). Aspects of the methods include placing a population of ASCs into a three dimensional culture (e.g., hanging drop suspension culture, high density culture, spinner flask culture, microcarrier culture, etc.), and contacting the cells with a first and second culture medium. Also provided are methods of treating an individual, which include producing a population of iHeps from a population of

ASCs, and administering an effective number of iHeps into the individual. Kits for practicing the methods are also described herein.

Before the present methods and compositions are described, it is to be understood that this invention is not limited to particular method or composition described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supercedes any disclosure of an incorporated publication to the extent there is a contradiction.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the peptide" includes reference to one or more peptides and equivalents thereof, e.g.

polypeptides, known to those skilled in the art, and so forth.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed

Definitions

The terms "specific binding," "specifically binds," and the like, refer to non-covalent or covalent preferential binding to a molecule relative to other molecules or moieties in a solution or reaction mixture (e.g., an antibody specifically binds to a particular polypeptide or epitope relative to other available polypeptides). In some embodiments, the affinity of one molecule for another molecule to which it specifically binds is characterized by a K_D (dissociation constant) of 10^{-5} M or less (e.g., 10^{-6} M or less, 10^{-7} M or less, 10^{-8} M or less, 10^{-9} M or less, 10^{-10} M or less, 10^{-11} M or less, 10^{-12} M or less, 10^{-13} M or less, 10^{-14} M or less, 10^{-15} M or less, or 10^{-16} M or less). "Affinity" refers to the strength of binding, increased binding affinity being correlated with a lower K_D.

The term "specific binding member" as used herein refers to a member of a specific binding pair (i.e., two molecules, usually two different molecules, where one of the molecules, e.g., a first specific binding member, through non-covalent means specifically binds to the other molecule, e.g., a second specific binding member).

The term "specific binding agent" as used herein refers to any agent that specifically binds a biomolecule (e.g., a marker such as a nucleic acid marker molecule, a protein marker molecule, etc.). In some cases, a "specific binding agent" for a marker molecule (e.g., a hepatocyte marker molecule) is used. Specific binding agents can be any type of molecule. In some cases, a specific binding agent is an antibody or a fragment thereof. In some cases, a specific binding agent is nucleic acid probe (e.g., an RNA probe; a DNA probe; an RNA/DNA probe; a modified nucleic acid probe, e.g., a locked nucleic acid (LNA) probe, a morpholino probe, etc.; and the like)

As used herein, a "marker molecule" does not have to be definitive (i.e., the marker does not have to definitely mark the cell as being of a particular type. For example, the expression of a marker molecule by a cell can be indicative (i.e., suggestive) that the cell is of a particular cell type. For example, if 3 cell types (type A, type B, and type C) express a particular marker molecule (e.g., a particular mRNA, a particular protein, etc.), expression of that marker molecule by a cell cannot necessarily be used by itself to definitively determine that the cell is a type A cell. However, expression of such a marker can *suggest* that the cell

is a type A cell. In some cases, expression of such a marker, combined with other evidence, can definitively show that the cell is a type A cell. As another illustrative example, if a particular cell type is known to express two or more particular marker molecules (e.g., mRNAs, proteins, a combination thereof, etc.) then the expression by a cell of one of the two or more particular marker molecules can be suggestive, but not definitive, that the cell is of the particular type in question. In such a case, the marker is still considered a marker molecule.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity. "Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Antibody fragment", and all grammatical variants thereof, as used herein are defined as a portion of an intact antibody comprising the antigen binding site or variable region of the intact antibody, wherein the portion is free of the constant heavy chain domains (i.e. CH2, CH3, and CH4, depending on antibody isotype) of the Fc region of the intact antibody. Examples of antibody fragments include Fab, Fab', Fab'-SH, $F(ab')_2$, and Fv fragments; diabodies; any antibody fragment that is a polypeptide having a primary structure consisting of one uninterrupted sequence of contiguous amino acid residues (referred to herein as a "single-chain antibody fragment" or "single chain polypeptide"), including without limitation (1) single-chain Fv (scFv) molecules (2) single chain polypeptides containing only one light chain variable domain, or a fragment thereof that contains the three CDRs of the light chain variable domain, without an associated heavy chain moiety (3) single chain polypeptides containing only one heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety and (4) nanobodies comprising single Ig domains from non-human species or other specific single-domain binding modules; and multispecific or multivalent structures formed from antibody fragments. In an antibody fragment comprising one or more heavy chains, the heavy chain(s) can contain any constant domain sequence (e.g. CH1 in the IgG isotype) found in a non-Fc region of an intact antibody, and/or can contain any hinge region sequence found in an intact antibody, and/or can contain a leucine zipper sequence fused to or situated in the hinge region sequence or the constant domain sequence of the heavy chain(s).

As used in this disclosure, the term "epitope" means any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms also apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

A “TK-NOG mouse”, as used herein refers to a mouse in which a herpes simplex virus type 1 thymidine kinase (HSVtk) transgene is expressed within the liver of a highly immunodeficient NOG mouse. Mouse liver cells expressing the HSVtk transgene can be ablated after a brief exposure to a non-toxic dose of ganciclovir (GCV). In some embodiments, an individual receiving treatment can be a mouse. For example, a TK-NOG mouse with ablated liver cells can be considered an individual with reduced liver function (i.e., an individual with liver damage) and the mouse can therefore be considered to be an individual receiving treatment when subject iHEPS (describe in detail below) are transplanted into the mouse. Transplanted human liver cells can be stably maintained within the liver of TK-NOG mice, and TK-NOG mice with transplanted human liver cells are referred herein as humanized TK-NOG mice. The reconstituted liver of humanized TK-NOG mice can be a mature and functioning human organ, and can generate a human-specific profile of drug metabolism. The 'humanized liver' can be stably maintained in humanized TK-NOG mice with a high level of function for a prolonged period (e.g., at least 8 months). For more information about TK-NOG mice, refer to: (i) Hasegawa et al, Biochem Biophys Res Commun. 2011 Feb 18;405(3):405-10; (ii) Yamazaki et al, Chem Res Toxicol. 2012 Feb 20;25(2):274-6; (iii) Hu et al., Pharmacogenet Genomics. 2013 Feb;23(2):78-83; and (iv) Yamazaki et al, Chem Res Toxicol. 2013 Mar 18;26(3):486-9; which are hereby incorporated by reference in their entirety.

The term “stem cell” is used herein to refer to a mammalian cell that has the ability both to self-renew and to generate a differentiated cell type (see Morrison et al. (1997) Cell 88:287-298). In the context of cell ontogeny, the adjective "differentiated", or “differentiating” is a relative term. A "differentiated cell" is a cell that has progressed further down the developmental pathway than the cell it is being compared with. Thus, pluripotent stem cells can differentiate into lineage-restricted progenitor cells (e.g., adipocyte-derived stem cells), which in turn can differentiate into end-stage cells (e.g., adipocytes, osteoblasts,

chondrocytes, etc.), which play a characteristic role in a certain tissue type, and may or may not retain the capacity to proliferate further. Stem cells (and differentiated progeny) may be characterized by both the presence of specific markers (e.g., proteins, RNAs, etc.) and the absence of specific markers. Stem cells may also be identified by functional assays both *in vitro* and *in vivo*, particularly assays relating to the ability of stem cells to give rise to multiple differentiated progeny.

The stem cells of interest are mammalian, where the term refers to cells isolated from any animal classified as a mammal, including humans, domestic and farm animals, and zoo, laboratory, sports, or pet animals, such as dogs, horses, cats, cows, mice, rats, rabbits, *etc*. In some embodiments, the mammal is a human and the mammalian cells (e.g., a population of adipocyte-derived stem cells) are therefore human cells (e.g., a population of human adipocyte-derived stem cells).

The terms "passaging" or "passage" (i.e., splitting or split) in the context of cell culture are known in the art and refer to the transferring of a small number of cells into a new vessel. Cells can be cultured if they are split regularly because it avoids the senescence associated with high cell density. For adherent cells, cells are detached from the growth surface as part of the passaging protocol. Detachment is commonly performed with the enzyme trypsin and/or other commercially available reagents (e.g., TrypLE, EDTA (Ethylenediaminetetraacetic acid), a policemen (e.g., a rubber policemen) for physically scrapping the cells from the surface, etc.). A small number of detached cells (e.g., as few as one cell) can then be used to seed a new cell population, e.g., after dilution with additional media. Therefore, to passage a cell population means to dissociate at least a portion of the cells of the cell population, dilute the dissociated cells, and to plate the diluted dissociated cells (i.e., to seed a new cell population).

The terms "media" and "medium" are herein used interchangeably. Cell culture media is the liquid mixture that baths cells during in vitro culture.

The term "population", e.g., "cell population" or "population of cells", as used herein means a grouping (i.e., a population) of two or more cells that are separated (i.e., isolated) from other cells and/or cell groupings. For example, a 6-well culture dish can contain 6 cell populations, each population residing in an individual well. The cells of a cell population can be, but need not be, clonal derivatives of one another. A cell population can be derived from one individual cell. For example, if individual cells are each placed in a single well of a 6-well culture dish and each cell divides one time, then the dish will contain 6 cell populations. A cell population can be any desired size and contain any number of cells greater than one cell. For example, a cell population can be 2 or more, 10 or more, 100 or more, 1,000 or more, 5,000 or more, 10^4 or more, 10^5 or more, 10^6 or more, 10^7 or more, 10^8 or more, 10^9

or more, 10^{10} or more, 10^{11} or more, 10^{12} or more, 10^{13} or more, 10^{14} or more, 10^{15} or more, 10^{16} or more, 10^{17} or more, 10^{18} or more, 10^{19} or more, or 10^{20} or more cells.

METHODS

Aspects of the disclosure include methods of producing a population of hepatocyte-like cells from a population of adipocyte-derived stem cells (ASCs). The methods generally involve placing a population of ASCs into a three dimensional culture (e.g., hanging drop suspension culture, high density culture, spinner flask culture, microcarrier culture, etc.) to produce an ASC-derived cellular aggregate (e.g., sphere); contacting cells of the ASC-derived cellular aggregate with a first culture medium comprising Activin A and a fibroblast growth factor (FGF) to produce a precursor cell population; and contacting cells of the precursor cell population with a second culture medium comprising hepatocyte growth factor (HGF) to produce an induced cell population that comprises induced hepatocyte-like cells (iHeps).

Adipose derived stem cells (ASCs). The term "adipose-derived stem cell" refers to a population of adipose cells found in post-natal mammals that are pluripotent and have the potential to differentiate into a variety of cell types including but not limited to cells of osteogenic, adipogenic, and chondrogenic lineages. ASCs can also differentiate into hepatocyte-like cells. ASCs have been referred to in the literature as adipose-derived adult stem (ADAS) cells, adipose-derived adult stromal cells, adipose-derived stromal cells (ADSCs), adipose stromal cells (ASCs), adipose mesenchymal stem cells (AdMSCs), lipoblast, pericyte, preadipocyte, and processed lipoaspirate (PLA) cells. The International Fat Applied Technology Society (IFATS) reached a consensus to adopt the term "adipose-derived stem cells" (ASCs) to identify the isolated, plastic-adherent, multipotent cell population. Thus, the term "Adipose-derived stem cell" (ASC) is used herein in accordance with the IFATS consensus, which will be known to one of ordinary skill in the art. In contrast to cell lines, ASCs have not undergone immortalization.

A number of scientific publications have described the underlying biology of ASCs, preclinical studies for the use of ASCs in regenerative medicine in various fields have been performed, and the efficacy of ASCs has been determined in several clinical trials.

ASCs for use in the subject methods can be isolated by any convenient methods, which will be known to one of ordinary skill in the art. Subcutaneous adipose tissue samples can generally be obtained under local anesthesia. Current methods used for isolating ASCs generally include collagenase digestion followed by centrifugal separation to isolate the Stromal Vascular Fraction from primary adipocytes.

As a non-limiting example, to isolate ASCs, adipose tissue obtained as excised

surgical specimens (e.g., a biopsy) or as lipoaspirates can be digested with a collagenase enzyme (e.g., a bacterially-derived collagenase) in the presence of calcium to release the individual cell components. Subsequently, mature adipocytes can be separated (e.g., via differential centrifugation), from the remaining cells, which form a Stromal Vascular Fraction (SVF) pellet. The SVF cell population includes endothelial cells, fibroblasts, Band T-lymphocytes, macrophages, myeloid cells, pericytes, pre-adipocytes, smooth muscle cells, and the culture adherent ASCs. After culture (e.g., 4 to 6 days) with medium containing about 10% fetal bovine serum (any convenient culture medium can be used), a single milliliter of human lipoaspirate will yield between 0.25 to 0.375 × 10^6 ASCs capable of differentiating along the adipogenic (adipocyte), chondrogenic (chondrocyte), and osteogenic (osteoblast) lineages in vitro. ASCs display a fibroblast-like morphology and lack the intercellular lipid droplets seen in adipocytes. Isolated ASCs are typically expanded in monolayer culture on standard tissue culture plastics with a basal medium containing 10% fetal bovine serum. Since liposuction from a single patient often results in > 1 L of tissue, it is feasible to generate hundreds of millions of ASCs from a single donor within a single in vitro cell culture passage In contrast to the SVF cells, ASCs are relatively homogeneous based on their expression profile of surface antigens.

In general, the isolation of ASCs from a lipoaspirate can include: (**1**) wash the lipoaspriate in buffered saline solution; (**2**) subject the lipoaspirate to collagenase digestion; (**3**) centrifuge and isolate the stromal vascular fraction (SVF) pellet; (**4**) culture the heterogeneous SVF cells on an adherent surface; and (**5**) isolate adherent ASCs. Culture of SVF cells under standard conditions eventually (within the first few passages) results in the appearance of ASCs, which is a relatively homogeneous population of mesodermal or mesenchymal cells. ASCs can be verified, for example, by demonstrating that the cells can differentiate into multiple different lineages (e.g., adipocytes can be identified using, for example, Oil Red O stain; osteoblasts can be identified using, for example, Alizarin Red stain; and chondrocytes can be identified using, for example, Alcian Blue stain). Protein and nucleic acid markers can also be used to verify differentiation into multiple lineages.

The International Society for Cellular Therapy (ISCT) and IFATS have established minimal criteria defining SVF cells and ASC based on functional and quantitative criteria. The four criteria used herein are: (1) ASCs are plastic-adherent when maintained under standard culture conditions; (2) ASCs have the capacity for osteogenic, adipogenic, and chondrogenic differentiation; (3) ASCs express the markers (i.e., molecular markers) CD29, CD34, CD36, CD49f, CD73, CD90 (Thy-1), CD105, CD133, c-kit, and c-met; and (4) ASCs are negative for CD45, CD106, and CD31. The adipocytic, chondroblastic and osteoblastic differentiation assays (e.g.., Oil Red O stain, Alcian blue stain, and Alizarin red stain,

respectively) can be used to assess potency and differentiation capacity, and can be used in conjunction with a quantitative evaluation of differentiation either biochemically or by reverse transcription polymerase chain reaction. The colony-forming unit - fibroblast (CFU-F) assay is recommended by the IFATS to calculate population doublings capacity of ASCs.

For more information regarding the nature of ASCs, including the isolation and culture of ASCs, see (i) Gimble et al., Circ Res. 2007 May 11;100(9):1249-60: "Adipose-derived stem cells for regenerative medicine"; (ii) Gimble et al., Organogenesis. 2013 Jan 1;9(1): "Adipose-derived stromal/stem cells: A primer"; (iii) Bourin et al, Cytotherapy. 2013 Jun;15(6):641-8: "Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT)"; (iv) Gentile et al, Stem Cells Transl Med. 2012 Mar;1(3):230-6: "Concise review: adipose-derived stromal vascular fraction cells and platelet-rich plasma: basic and clinical implications for tissue engineering therapies in regenerative surgery"; and (v) Mizuno et al., Stem Cells. 2012 May;30(5):804-10: "Concise review: Adipose-derived stem cells as a novel tool for future regenerative medicine"; all of which are hereby incorporated by reference in their entirety.

As used herein, the term "adipose tissue" refers to fat including the connective tissue that stores fat. Adipose tissue contains multiple regenerative cell types, including ASCs and endothelial progenitor and precursor cells.

Three dimensional culture. Methods of the disclosure include placing a population of ASCs into a three dimensional culture to produce an ASC-derived cellular aggregate (e.g., sphere). The term "three dimensional culture" as used herein refers to the culture of cells in a way that includes low shear force (and consequently low turbulence), and high mass transfer of nutrients. One non-limiting example of "three dimensional culture" is the culture of cells as aggregates (e.g., spheres, i.e., spheroid formation). Placing a population of cells (e.g., ASCs) into a three dimensional culture can cause the cells to form cellular aggregates (e.g., cellular spheres). Thus, subject cells (e.g., a population of ASCs) can be placed into a three dimensional culture to produce an ASC-derived cellular aggregate (e.g, sphere). Cellular aggregates (e.g, spheres) can be produced by a number of three dimensional culture techniques, including but not limited to hanging drop suspension culture, high density cell culture (e.g., stirred suspension culture such as spinner flask culture, e.g., with or without microcarriers; bioreactor culture, e.g., with or without microcarriers; etc.), and the like (see examples section).

In some embodiments, cells can be placed into a three dimensional culture at any

convenient chosen density (e.g., via dilution or concentration). For example, in some cases, cells are placed into a three dimensional culture at a cell density in a range of from $1x10^2$ cells/ml to $1x10^7$ cells/ml (e.g., from $5x10^2$ cells/ml to $5x10^6$ cells/ml, from $1x10^3$ cells/ml to $5x10^6$ cells/ml, from $5x10^3$ cells/ml to $5x10^6$ cells/ml, from $5x10^3$ cells/ml to $1x10^5$ cells/ml, from $5x10^3$ cells/ml to $5x10^4$ cells/ml, from $7x10^3$ cells/ml to $3x10^4$ cells/ml, from $8x10^3$ cells/ml to $3x10^4$ cells/ml, $1x10^4$ cells/ml, from $1x10^4$ cells/ml to $1x10^6$ cells/ml, from $5x10^4$ cells/ml to $1x10^6$ cells/ml, from $7x10^4$ cells/ml to $7x10^5$, from $1x10^5$ cells/ml to $7x10^5$, from $3x10^5$ cells/ml to $7x10^5$, from $4x10^5$ cells/ml to $6x10^5$, or $5x10^5$).

In practicing the methods of the disclosure, ASCs can be cultured in three dimensional culture for a period of time sufficient for the formation of cellular aggregates (e.g., spheres, which can resemble embryoid bodies). In some embodiments, ASCs are cultured using three dimensional culture (i.e., ASCs are placed into a three dimensional culture) for 3 days or less (e.g., 2.5 days or less, 2 days or less, 1.5 days or less, 1 day or less, 12 hours or less, 8 hours or less, 6 hours or less, or 4 hours or less). In some embodiments, ASCs are cultured using three dimensional culture for a period of time in a range of from 2 hours to 3 days (e.g., from 2 hours to 3 days, from 2 hours to 2.5 days, from 2 hours to 2 days, from 2 hours to 1.5 days, from 2 hours to 1 day, from 6 hours to 3 days, from 6 hours to 2.5 days, from 6 hours to 2 days, from 6 hours to 1.5 days, from 6 hours to 1 day, from 6 hours to 12 hours, from 8 hours to 3 days, from 8 hours to 2.5 days, from 8 hours to 2 days, from 8 hours to 1.5 days, from 8 hours to 1 day, from 8 hours to 12 hours, from 12 hours to 2.5 days, from 12 hours to 2 days, from 12 hours to 1.5 days, from 12 hours to 1 day, from 1 day to 3 days, from 1 day to 2.5 days, from 1 day to 2 days, from 1 day to 1.5 days, from 1.5 days to 3 days, from 1.5 days to 2.5 days, from 1.5 days to 2 days, from 2 days to 3 days, from 1 day, 1.5 days, from 2 days, from 2.5 days, or 3 days).

Hanging drop suspension culture. In some embodiments, the three dimensional culture is a hanging drop suspension culture. Thus, in some embodiments, aggregates of subject cells (e.g., ASCs) are formed by placing the cells in a hanging drop suspension culture. Hanging drop suspension culture is a technique often used to form embryoid bodies from embryonic stem cells (ESCs). Cells in fluid are placed in droplets (usually in the range of 5-50 µl in size) onto a surface (e.g., surface of a Petri dish, a coverslip, glass, plastic etc.) and the surface is inverted such that the cells are suspended from the surface. The cells are thus cultured under the surface in a suspended droplet instead of being cultured on top of the surface. The suspended droplets are sometimes referred to as hanging drops. Some cells form aggregates, (referred to as 'spheres' and/or 'spheroids') when cultured using the hanging drop suspension culture.

In some embodiments, cells can be placed into hanging drop suspension culture at a chosen density (e.g., via dilution or concentration). For example, in some cases, cells are placed into hanging drop suspension culture at a cell density in a range of from $1x10^2$ cells/ml to $1x10^7$ cells/ml (e.g., from $5x10^2$ cells/ml to $5x10^6$ cells/ml, from $1x10^3$ cells/ml to $5x10^6$ cells/ml, from $5x10^3$ cells/ml to $5x10^6$ cells/ml, from $5x10^3$ cells/ml to $1x10^5$ cells/ml, from $5x10^3$ cells/ml to $5x10^4$ cells/ml, from $7x10^3$ cells/ml to $3x10^4$ cells/ml, from $8x10^3$ cells/ml to $3x10^4$ cells/ml, $1x10^4$ cells/ml, from $1x10^4$ cells/ml to $1x10^6$ cells/ml, from $5x10^4$ cells/ml to $1x10^6$ cells/ml, from $7x10^4$ cells/ml to $7x10^5$, from $1x10^5$ cells/ml to $7x10^5$, from $3x10^5$ cells/ml to $7x10^5$, from $4x10^5$ cells/ml to $6x10^5$, or $5x10^5$).

In practicing the methods of the disclosure, ASCs can be cultured by the hanging drop method (i.e., cultured using hanging drop suspension culture) for a period of time sufficient for the formation of cellular aggregates (e.g., spheres, which can resemble embryoid bodies). In some embodiments, ASCs are cultured using hanging drop suspension culture (i.e., ASCs are placed into a hanging drop suspension culture) for 3 days or less (e.g., 2.5 days or less, 2 days or less, 1.5 days or less, 1 day or less, 12 hours or less, 8 hours or less, 6 hours or less, or 4 hours or less). In some embodiments, ASCs are cultured using hanging drop suspension culture for a period of time in a range of from 2 hours to 3 days (e.g., from 2 hours to 3 days, from 2 hours to 2.5 days, from 2 hours to 2 days, from 2 hours to 1.5 days, from 2 hours to 1 day, from 6 hours to 3 days, from 6 hours to 2.5 days, from 6 hours to 2 days, from 6 hours to 1.5 days, from 6 hours to 1 day, from 6 hours to 12 hours, from 8 hours to 3 days, from 8 hours to 2.5 days, from 8 hours to 2 days, from 8 hours to 1.5 days, from 8 hours to 1 day, from 8 hours to 12 hours, from 12 hours to 2.5 days, from 12 hours to 2 days, from 12 hours to 1.5 days, from 12 hours to 1 day, from 1 day to 3 days, from 1 day to 2.5 days, from 1 day to 2 days, from 1 day to 1.5 days, from 1.5 days to 3 days, from 1.5 days to 2.5 days, from 1.5 days to 2 days, from 2 days to 3 days, from 1 day, 1.5 days, from 2 days, from 2.5 days, or 3 days).

In some embodiments, an ASC-derived cellular aggregate (e.g., sphere) is removed from three dimensional culture (e.g., hanging drop suspension culture, high density culture, spinner flask culture, microcarrier culture, etc.). For example, a cellular aggregate (e.g., sphere) can be suspended in fluid and plated onto a two-dimensional surfaced that is not considered a three dimensional culture. In some cases, aggregates (e.g., spheres) are collected (e.g., by centrifugation) and suspended. In some cases, the aggregates (e.g., spheres) are collected and suspended at a density in a range of from 15 aggregates/ml to 45 aggregates/ml (e.g., from 20 aggregates/ml to 40 aggregates/ml, from 25 aggregates/ml to 35 aggregates/ml, or 30 aggregates/ml). The aggregates can be suspended in any convenient media (e.g., stage 1 media, described below). In some cases, the collected

(suspended) aggregates (e.g., spheres) are seeded (i.e., plated, e.g., onto matrigel-coated dishes). In some embodiments, an ASC-derived cellular aggregate (e.g., sphere) is removed from three dimensional culture (e.g., hanging drop suspension culture, high density culture, spinner flask culture, microcarrier culture, etc.) simultaneous with, or prior to, contacting cells of the ASC-derived cellular aggregate (e.g., sphere) with a stage 1 medium. Thus, in some cases, cells are cultured (e.g., contacted with) a stage 1 medium after being removed from three dimensional culture. In some embodiments, cells of the ASC-derived cellular aggregate (e.g., sphere) are contacted with a stage 1 medium while still in three dimensional culture (e.g., in order to transfer the cells to non-inverted culture conditions).

High density culture - microcarrier culture. In some embodiments, the three dimensional culture is a high density culture. Thus, aggregates (e.g., spheres) of subject cells (e.g., ASCs) are formed by placing the cells in a high density culture. In some embodiments, a high density culture is a microcarrier culture. In some embodiments, the three dimensional culture is a microcarrier culture. Thus, aggregates (e.g., spheres) of subject cells (e.g., ASCs) are formed by placing the cells in a microcarrier culture. The term "microcarrier culture" is used herein to refer to the culture of cells on a support matrix (e.g., a spherical support matrix). In this system, cells are propagated on the surface of small solid particles suspended in the growth medium by slow agitation. The cells attach and grow to confluence on the surface of the microcarriers.

Microcarriers can be produced in various shapes and sizes, spherical being the most common, and their density allows them to be maintained in suspension with gentle stirring. Each microcarrier should have dimensions that can facilitate cell growth for several doublings. In this way at the end of the cell growth, each microcarrier will support several hundred cells on its surface. Spherical microcarriers usually have diameters of 100–250μm. In some embodiments, microcarriers are spheres with a diameter in a range of from 100μm to 250μm.The size distribution of the spherical microcarriers should be low (e.g., ±25 μm) in order to prevent uneven distribution of cells on the microcarriers. Density of the microcarriers should be slightly above 1 (e.g., 1.02–1.05 g/ml) in order to maintain the microcarriers in suspension at minimal agitation speed.

Microcarriers can be made from a number of different materials including diethylaminoethanol (DEAE)-dextran, dextran, glass, polystyrene plastic, acrylamide (e.g., polyacrylamide), collagen, etc. In some cases, biodegradable microcarriers can be used as the scaffold for in vivo transplantation of cells. The MC surface is available for cell growth while the mobility of MCs in the medium generates a homogeneity that is similar to the

suspension environment used in traditional mammalian and microbial submerged cultures.

The surface of a microcarrier can be derivatized with functional groups such as recombinant proteins, positively charged tertiary quaternary or primary amines, gelatin, collagen, other extracellular matrix (ECM) proteins and peptides (e.g. RGD peptide). The positively charged MCs attract the cells (which are negatively charged), by electrostatic forces. The optimal amount of positive charge is generally found to be between 1 and 2 milliequivalents/g dry materials (for cross-linked dextran or polyacrylamide beads derivatized with tertiary amines). At this level, cells attach to the microcarriers efficiently (about 90% within 1 h) without negative effect on cell growth. Coating with collagen or ECM protein results in lower cell attachment but usually supports better growth of cells at low inoculation levels.

Several types of microcarriers are available commercially including dextran-based (Cytodex, GE Healthcare), collagen-based (Cultispher, Percell), and polystyrene-based (SoloHill Engineering) microcarriers. They differ in their porosity, specific gravity, optical properties, presence of animal components, and surface chemistries. It is possible to categorize the microcarriers into six groups:

Group 1: Non-porous smooth (e.g. polystyrene microcarriers) or microporous microcarriers (e.g. Cytodex 1) with positive charges. These microcarriers are suitable for culturing adherent cells that form a continuous monolayer of cells on the surface of the microcarriers in stirred cultures. This group includes also Whatman's anion exchange celluloses (DE-53), cylindrical shaped microcarriers that have been used successfully in culturing cell lines (BHK and MDCK).

Group 2: Collagen coated microcarriers (e.g. Cytodex 3 and FACT 102-L). These microcarriers are chemically coupled with collagen and are suitable for culturing sensitive cells with low plating efficiency. The collagen coating is also designed to facilitate cell harvesting.

Group 3: ECM coated microcarriers (Pro-F 102-L). Pro-F 102-L is coated with recombinant fibronectin which is designed for culturing of sensitive cells in serum free conditions.

Group 4: Non-charged microcarriers (e.g. Glass beads and tissue culture Polystyrene MC P 102-L). These microcarriers have similar surface properties as classical 2D tissue culture surfaces.

Group 5: Macroporous microcarriers (e.g. Cytopore and Cultispher).Macroporous microcarriers with pore sizes in the range of 10–70 µm on the surface. They provide higher cell surface areas for growth and offer better mechanical protection to the cells from shear stress generated by stirrers, spargers or spin filters.

Group 6: Weighted microcarriers (Cytoline). These microcarriers are designed for use in fluidized bed perfusion cultures. These commercial microcarriers have been designed according to the needs for propagating anchorage dependent cell lines used in production of vaccine and biopharmaceuticals.

In practicing the methods of the disclosure, ASCs can be cultured by microcarrier culture (i.e., cultured using microcarrier culture) for a period of time sufficient for the formation of cellular aggregates (e.g., spheres, which can resemble embryoid bodies). In some embodiments, ASCs are cultured using microcarrier culture (i.e., ASCs are placed into a microcarrier culture) for 3 days or less (e.g., 2.5 days or less, 2 days or less, 1.5 days or less, 1 day or less, 12 hours or less, 8 hours or less, 6 hours or less, or 4 hours or less). In some embodiments, ASCs are cultured using microcarrier culture for a period of time in a range of from 2 hours to 3 days (e.g., from 2 hours to 3 days, from 2 hours to 2.5 days, from 2 hours to 2 days, from 2 hours to 1.5 days, from 2 hours to 1 day, from 6 hours to 3 days, from 6 hours to 2.5 days, from 6 hours to 2 days, from 6 hours to 1.5 days, from 6 hours to 1 day, from 6 hours to 12 hours, from 8 hours to 3 days, from 8 hours to 2.5 days, from 8 hours to 2 days, from 8 hours to 1.5 days, from 8 hours to 1 day, from 8 hours to 12 hours, from 12 hours to 2.5 days, from 12 hours to 2 days, from 12 hours to 1.5 days, from 12 hours to 1 day, from 1 day to 3 days, from 1 day to 2.5 days, from 1 day to 2 days, from 1 day to 1.5 days, from 1.5 days to 3 days, from 1.5 days to 2.5 days, from 1.5 days to 2 days, from 2 days to 3 days, from 1 day, 1.5 days, from 2 days, from 2.5 days, or 3 days).

For examples of the use of microcarriers (e.g., using spinner flasks, bioreactors, etc.) for various purposes and with various types of cells, refer to scientific literature such as: (i) Chen et al, Biotechnol Adv. 2013 Mar 24. pii: S0734-9750(13)00065-7: Application of human mesenchymal and pluripotent stem cell microcarrier cultures in cellular therapy: Achievements and future direction; (ii) Pacak et al, PLoS One. 2013;8(1):e55187: Microcarrier-based expansion of adult murine side population stem cells; (iii) Torgan et al, Med Biol Eng Comput. 2000 Sep;38(5):583-90: Differentiation of mammalian skeletal muscle cells cultured on microcarrier beads in a rotating cell culture system; and (iv) Park et al, Tissue Eng Part B Rev. 2013 Apr;19(2):172-90: Microcarriers designed for cell culture and tissue engineering of bone; as well as patent literature such as U.S. patents: 8524492, 8426176, 7947471, 7670839, 7361493, 6214618, and 5153133, 4910142, and 4824946; all of which are hereby incorporated by reference in their entirety.

Microcarrier culture generally requires agitation of the microcarriers (e.g., the cell-loaded microcarriers). Agitation can include simple agitation or shaking, agitation using a spinner flask, and/or agitation using a bioreactor (also known as a rotating cell culture system (RCCS), a rotary cell culture system (RCCS), or a rotating chamber system).

High density culture - Spinner flask culture. In some embodiments, the three dimensional culture is a high density culture. Thus, aggregates (e.g., spheres) of subject cells (e.g., ASCs) are formed by placing the cells in a high density culture. In some embodiments, a high density culture is a spinner flask culture. Spinner flask culture is an example of a stirred suspension culture. Stirred suspension culture (i.e., mass suspension culture)(e.g., spinner flask culture, bioreactor culture, etc.) can be used to culture cells in the absence of microcarriers or in the presence of microcarriers. Thus, in some embodiments, a microcarrier culture is agitated using a spinner flask. In some embodiments, a three dimensional culture (e.g., a high density culture) is a spinner flask culture without (i.e., in the absence of) microcarriers. In the absence of microcarriers, ASCs placed in stirred suspension culture (e.g., spinner flask culture, bioreactor culture) still form cellular aggregates (e.g., spheres)(Figure 13). Thus, in some embodiments, a three dimensional culture (e.g., a high density culture) is a spinner flask culture without (i.e., in the absence of) microcarriers.

Spinner flasks (i.e., stirrer bottles) are generally designed to facilitate the culture of high volumes of cells by providing homogenous circulation of cells (e.g., cells in the absence of microcarriers, cells on microcarriers, etc.) and medium (i.e., nutrients), and superior gas exchange (e.g., oxygenation). Spinner flasks can be made of any convenient material (e.g., borosilicate glass, tissue culture grade plastics, etc.) and are usually a flat-bottom flask with a stirring device (e.g., usually a vertical impeller or a hanging stir bar) that can be controlled using a magnetic stir plate. A spinner flask can also include two angled side arms to allow for the introduction of pipettes. Any convenient spinner flask that provides for high density culture can be used in practicing the subject methods.

In some embodiments, cells can be placed into a spinner flask culture at any convenient chosen density (e.g., via dilution or concentration). For example, in some cases, cells are placed into a spinner flask culture at a cell density in a range of from $1x10^2$ cells/ml to $1x10^7$ cells/ml (e.g., from $5x10^2$ cells/ml to $5x10^6$ cells/ml, from $1x10^3$ cells/ml to $5x10^6$ cells/ml, from $5x10^3$ cells/ml to $5x10^6$ cells/ml, from $5x10^3$ cells/ml to $1x10^5$ cells/ml, from $5x10^3$ cells/ml to $5x10^4$ cells/ml, from $7x10^3$ cells/ml to $3x10^4$ cells/ml, from $8x10^3$ cells/ml to $3x10^4$ cells/ml, $1x10^4$ cells/ml, from $1x10^4$ cells/ml to $1x10^6$ cells/ml, from $5x10^4$ cells/ml to $1x10^6$ cells/ml, from $7x10^4$ cells/ml to $7x10^5$, from $1x10^5$ cells/ml to $7x10^5$, from $3x10^5$ cells/ml to $7x10^5$, from $4x10^5$ cells/ml to $6x10^5$, or $5x10^5$).

In practicing the methods of the disclosure, ASCs can be cultured by spinner flask culture (i.e., cultured using spinner flask culture) for a period of time sufficient for the formation of cellular aggregates (e.g., spheres, which can resemble embryoid bodies). In some embodiments, ASCs are cultured using spinner flask culture (i.e., ASCs are placed

into a spinner flask culture) for 3 days or less (e.g., 2.5 days or less, 2 days or less, 1.5 days or less, 1 day or less, 12 hours or less, 8 hours or less, 6 hours or less, or 4 hours or less). In some embodiments, ASCs are cultured using spinner flask culture for a period of time in a range of from 2 hours to 3 days (e.g., from 2 hours to 3 days, from 2 hours to 2.5 days, from 2 hours to 2 days, from 2 hours to 1.5 days, from 2 hours to 1 day, from 6 hours to 3 days, from 6 hours to 2.5 days, from 6 hours to 2 days, from 6 hours to 1.5 days, from 6 hours to 1 day, from 6 hours to 12 hours, from 8 hours to 3 days, from 8 hours to 2.5 days, from 8 hours to 2 days, from 8 hours to 1.5 days, from 8 hours to 1 day, from 8 hours to 12 hours, from 12 hours to 2.5 days, from 12 hours to 2 days, from 12 hours to 1.5 days, from 12 hours to 1 day, from 1 day to 3 days, from 1 day to 2.5 days, from 1 day to 2 days, from 1 day to 1.5 days, from 1.5 days to 3 days, from 1.5 days to 2.5 days, from 1.5 days to 2 days, from 2 days to 3 days, from 1 day, 1.5 days, from 2 days, from 2.5 days, or 3 days).

High density culture - Bioreactor culture. In some embodiments, the three dimensional culture is a high density culture. Thus, aggregates (e.g., spheres) of subject cells (e.g., ASCs) are formed by placing the cells in a high density culture. In some embodiments, a high density culture is a bioreactor culture. Bioreactor culture is an example of a stirred suspension culture. Stirred suspension culture (i.e., mass suspension culture)(e.g., spinner flask culture, bioreactor culture, etc.) can be used to culture cells in the absence of microcarriers or in the presence of microcarriers. Thus, in some embodiments, a microcarrier culture is agitated using a bioreactor. In some embodiments, a three dimensional culture (e.g., a high density culture) is a bioreactor culture without (i.e., in the absence of) microcarriers. In the absence of microcarriers, ASCs placed in stirred suspension culture (e.g., spinner flask culture, bioreactor culture) still form cellular aggregates (e.g., spheres). Thus, in some embodiments, a three dimensional culture (e.g., a high density culture) is a bioreactor culture without (i.e., in the absence of) microcarriers.

Bioreactors (also known as a rotating cell culture system (RCCS), a rotary cell culture system (RCCS), or a rotating chamber system) can control environmental conditions such as gas (e.g., air, oxygen, nitrogen, carbon dioxide) flow rates, temperature, pH, dissolved oxygen levels, agitation speed/circulation rate, and the like. Like spinner flasks, bioreactors are designed to facilitate the culture of high volumes of cells by providing homogenous circulation of cells (e.g., cells in the absence of microcarriers, cells on microcarriers, etc.) and medium (i.e., nutrients), and superior gas exchange (e.g., oxygenation). Any convenient bioreactor that provides for high density cell culture can be used with the disclosed methods and various suitable bioreactors are known in the art. Examples of suitable biorecactors include, but are not limited to: rotating wall microgravity

bioreactors, rotating wall vessel bioreactors (RWVB), stirred tank bioreactors, perfused bioreactors, fluidized bed bioreactors. Bioreactors can be made from a large variety of different materials, including, but not limited to: stainless steel, polytetraflouroethylene (PTFE), glass, and the like.

Bioreactors can allow for the scaling up of culture in, for example, conventional stainless steel or disposable bioreactors that are used for propagation of suspended mammalian cells. Bioreactors range in size. In some cases a bioreactor culture is a culture in the range of from 5 liters to 105 liters (e.g., from 5 liters to 15 liters, from 8 liters to 12 liters, from 15 liters to 25 liters, from 25 liters to 40 liters, from 40 liters to 50 liters, from 45 liters to 55 liters, from 55 liters to 65 liters, from 65 liters to 75 liters, from 75 liters to 85 liters, from 85 liters to 95 liters, or from 95 liters to 105 liters).

In some embodiments, cells can be placed into a bioreactor culture at any convenient chosen density (e.g., via dilution or concentration). For example, in some cases, cells are placed into a bioreactor culture at a cell density in a range of from $1x10^2$ cells/ml to $1x10^7$ cells/ml (e.g., from $5x10^2$ cells/ml to $5x10^6$ cells/ml, from $1x10^3$ cells/ml to $5x10^6$ cells/ml, from $5x10^3$ cells/ml to $5x10^6$ cells/ml, from $5x10^3$ cells/ml to $1x10^5$ cells/ml, from $5x10^3$ cells/ml to $5x10^4$ cells/ml, from $7x10^3$ cells/ml to $3x10^4$ cells/ml, from $8x10^3$ cells/ml to $3x10^4$ cells/ml, $1x10^4$ cells/ml, from $1x10^4$ cells/ml to $1x10^6$ cells/ml, from $5x10^4$ cells/ml to $1x10^6$ cells/ml, from $7x10^4$ cells/ml to $7x10^5$, from $1x10^5$ cells/ml to $7x10^5$, from $3x10^5$ cells/ml to $7x10^5$, from $4x10^5$ cells/ml to $6x10^5$, or $5x10^5$).

In practicing the methods of the disclosure, ASCs can be cultured in bioreactor culture for a period of time sufficient for the formation of cellular aggregates (e.g., spheres, which can resemble embryoid bodies). In some embodiments, ASCs are cultured using bioreactor culture (i.e., ASCs are placed into a bioreactor culture) for 3 days or less (e.g., 2.5 days or less, 2 days or less, 1.5 days or less, 1 day or less, 12 hours or less, 8 hours or less, 6 hours or less, or 4 hours or less). In some embodiments, ASCs are cultured using bioreactor culture for a period of time in a range of from 2 hours to 3 days (e.g., from 2 hours to 3 days, from 2 hours to 2.5 days, from 2 hours to 2 days, from 2 hours to 1.5 days, from 2 hours to 1 day, from 6 hours to 3 days, from 6 hours to 2.5 days, from 6 hours to 2 days, from 6 hours to 1.5 days, from 6 hours to 1 day, from 6 hours to 12 hours, from 8 hours to 3 days, from 8 hours to 2.5 days, from 8 hours to 2 days, from 8 hours to 1.5 days, from 8 hours to 1 day, from 8 hours to 12 hours, from 12 hours to 2.5 days, from 12 hours to 2 days, from 12 hours to 1.5 days, from 12 hours to 1 day, from 1 day to 3 days, from 1 day to 2.5 days, from 1 day to 2 days, from 1 day to 1.5 days, from 1.5 days to 3 days, from 1.5 days to 2.5 days, from 1.5 days to 2 days, from 2 days to 3 days, from 1 day, 1.5 days, from 2 days, from 2.5 days, or 3 days).

Culturing cells in differentiation media. The term "differentiation media" as used herein refers to media can be used to induce cells to differentiate. For example, stage 1 and stage 2 media (described below) are two types of differentiation media used to induce ASCs to differentiate into hepatocyte-like cells. In some embodiments, the methods include removing the ASC-derived cellular aggregate (e.g., sphere) from three dimensional culture (e.g., hanging drop suspension culture, high density culture, spinner flask culture, bioreactor culture, stirred suspension culture, microcarrier culture, etc.) and contacting cells of the ASC-derived cellular aggregate (e.g., for 3 days or less) with a first culture medium (e.g., stage 1 media) comprising Activin A and a fibroblast growth factor (FGF) to produce a precursor cell population. In some embodiments, cells of the precursor cell population are contacted with a second culture medium (e.g., stage 2 media) comprising hepatocyte growth factor (HGF) (e.g., for 7 days or less) to produce an induced cell population that comprises induced hepatocyte-like cells (iHeps). All proteins listed below that can be included in a suitable differentiation media, can be provided from any convenient source (e.g., purified from tissue, recombinant, etc.).

In some embodiments, cells of an ASC-derived cellular aggregate (e.g., sphere) are contacted with stage 1 media (as defined below, e.g., a culture medium having Activin A and an FGF) for 3 days or less (e.g., 2.5 days or less, 2 days or less, 1.5 days or less, 1 day or less, or 12 hours or less) to produce a precursor cell population. In some embodiments, cells of an ASC-derived cellular aggregate (e.g., sphere) are contacted with stage 1 media for a period of time in a range of from 12 hours to 3 days (e.g., 12 hours to 2.5 days, 12 hours to 2 days, 12 hours to 1.5 days, 12 hours to 1 day, 1 day to 3 days, 1 day to 2.5 days, 1 day to 2 days, 1 day to 1.5 days, 1.5 days to 3 days, 1.5 days to 2.5 days, 1.5 days to 2 days, 2 days to 3 days, 1 day, 1.5 days, 2 days, 2.5 days, or 3 days) to produce a precursor cell population.

In some embodiments, cells of the precursor cell population are contacted with stage 2 media (as defined below, e.g., a culture medium having hepatocyte growth factor (HGF)) for 8 days or less (e.g., 7.5 days or less, 7 days or less, 6.5 days or less, 6 days or less, 5.5 days or less, 5 days or less, 4.5 days or less, 4 days or less, 3.5 days or less, 3 days or less, 2.5 days or less, 2 days or less, 1.5 days or less, 1 day or less, or 12 hours or less) to produce an induced cell population that comprises induced hepatocyte-like cells. In some embodiments, cells of the precursor cell population are contacted with stage 2 media for a period of time in a range of from 12 hours to 8 days (e.g., 12 hours to 8 days, 1 day to 8 days, 2 days to 8 days, 3 days to 8 days, 4 days to 8 days, 5 days to 8 days, 6 days to 8 days, 2 days to 7 days, 3 days to 7 days, 4 days to 7 days, 5 days to 7 days, 6 days to 7 days, 5.5 days to 8 days, 5.5 days to 7.5 days, 5.5 days to 6.5 days, 6 days to 7.5 days, 6.5

days to 7.5 days, 1 day, 2 days, 3 days, or 3 days) to produce an induced cell population that comprises induced hepatocyte-like cells.

In some embodiments, the total time elapsed from (i) placing a population of ASCs into a three dimensional culture, to (ii) producing an induced cell population that comprises induced hepatocyte-like cells (iHeps), is less than 13 days (e.g., less than 12.5 days, less than 12 days, less than 11.5 days, less than 11 days, less than 10.5 days, less than 10 days, less than 9.5 days, less than 9 days, less than 8.5 days, less than 8 days, less than 7.5 days, less than 7 days, less than 6.5 days, less than 6 days, less than 5.5 days, less than 5 days, less than 4.5 days, or less than 4 days).

In some embodiments, the total time elapsed from (i) placing a population of ASCs into a three dimensional culture, to (ii) producing an induced cell population that comprises induced hepatocyte-like cells (iHeps), is 13 days or less (e.g., 12.5 days or less, 12 days or less, 11.5 days or less, 11 days or less, 10.5 days or less, 10 days or less, 9.5 days or less, 9 days or less, 8.5 days or less, 8 days or less, 7.5 days or less, 7 days or less, 6.5 days or less, 6 days or less, 5.5 days or less, 5 days or less, 4.5 days or less, or 4 days or less).

In some embodiments, the total time elapsed from (i) placing a population of ASCs into a three dimensional culture, to (ii) producing an induced cell population that comprises induced hepatocyte-like cells (iHeps), is in a range of from 12 hours to 13 days (e.g., from 1 day to 13 days, from 2 days to 13 days, from 3 days to 13 days, from 4 days to 13 days, from 4.5 days to 13 days, from 5 days to 13 days, from 5.5 days to 13 days, from 6 days to 13 days, from 6.5 days to 13 days, from 7 days to 13 days, from 7.5 days to 13 days, from 8 days to 13 days, from 8.5 days to 13 days, from 9 days to 13 days, from 9.5 days to 13 days, from 10 days to 13 days, from 10.5 days to 13 days, from 11 days to 13 days, from 11.5 days to 13 days, from 12 hours to 12.5 days, from 1 day to 12.5 days, from 2 days to 12.5 days, from 3 days to 12.5 days, from 4 days to 12.5 days, from 4.5 days to 12.5 days, from 5 days to 12.5 days, from 5.5 days to 12.5 days, from 6 days to 12.5 days, from 6.5 days to 12.5 days, from 7 days to 12.5 days, from 7.5 days to 12.5 days, from 8 days to 12.5 days, from 8.5 days to 12.5 days, from 9 days to 12.5 days, from 9.5 days to 12.5 days, from 10 days to 12.5 days, from 10.5 days to 12.5 days, from 11 days to 12.5 days, from 11.5 days to 12.5 days, from 12 hours to 12 days, from 1 day to 12 days, from 2 days to 12 days, from 3 days to 12 days, from 4 days to 12 days, from 4.5 days to 12 days, from 5 days to 12 days, from 5.5 days to 12 days, from 6 days to 12 days, from 6.5 days to 12 days, from 7 days to 12 days, from 7.5 days to 12 days, from 8 days to 12 days, from 8.5 days to 12 days, from 9 days to 12 days, from 9.5 days to 12 days, from 10 days to 12 days, from 10.5 days to 12 days, from 11 days to 12 days, from 3 days to 11 days, from 3 days to

10 days, from 3 days to 9.5 days, from 3 days to 9 days, from 6 days 10 days, or from 4 days to 10 days).

Basal cell culture media. Subject cells (e.g., ASCs, iHeps, differentiating ASCs, etc.) are cultured in an appropriate liquid nutrient medium, referred to herein as "basal cell culture media.". The stage 1 and stage 2 media are basal culture media supplemented with at least one additional component, as described below. Various basal media formulations are available (e.g., Dulbecco's Modified Eagle Medium (DMEM), RPMI, Iscove's medium, hepatocyte culture medium (HCM) (Lonza, Cat: cc-3198) *etc.*).

Any convenient culture media can serve as a basal culture medium. For example, a suitable tissue culture medium can contain components such as a vitamin; an amino acid (e.g., an essential amino acid, a non-essential amino acid, etc.); a pH buffering agent; a salt; an antimicrobial agent (e.g., an antibacterial agent, an antimycotic agent, etc.); serum (e.g., fetal bovine serum, human serum, calf serum, horse serum, goat serum etc.); an energy source (e.g., a sugar); a nucleoside; a lipid; trace metals; a cytokine; a growth factor; a stimulatory factor; additives (e.g., pyruvate (0.1-5 mM), glutamine (0.5-5 mM), etc.) and the like. Any convenient cell culture media can be used, and as is known in the art, various cell types grow better in particular media preparations. For example, in some cases, particular media formulations have been optimized to culture specific types of cells (e.g., ASCs, hepatocytes (hepatocyte culture medium (HCM) (Lonza, Cat: cc-3198)), neural progenitors, embryonic stem cells, etc.). Accordingly, any convenient cell culture media can be used as a basal culture media and may be tailored to the culture of ASCs and/or their differentiating progeny.

One exemplary, non-limiting, suitable basal medium is RPMI (Roswell Park Memorial Institute) 1640, which is well known in the art as a basal medium that allows the culture of mammalian cells with serum (Fetal Bovine Serum (FBS)) supplementation. Another suitable basal culture media is Advanced RPMI 1640, which is similar to classic RPMI 1640, but allows for serum supplementation to be reduced by 50-90% with no change in growth rate or morphology. Advanced RPMI 1640 can contain, for example, glucose, non-essential amino acids, sodium pyruvate, and phenol red. The complete formulation is readily available (e.g., online) to one of ordinary skill in the art for RPMI 1640, for Advanced RPMI 1640, and for many suitable commercially available basal culture media. Another suitable basal culture media is CLONETICS™ hepatocyte culture medium (HCM™) (available, ofr example, from Lonza – Catalog number 3198).

Stage 1 medium. "Stage 1 medium" is also referred to herein as a "first culture medium." A suitable stage 1 medium is a basal culture medium (e.g., advanced RPMI 1640, RPMI 1640, etc.) suitable for the culture of cells (e.g., ASCs, differentiating ASCs, etc.) supplemented with at least activin A (e.g.,100 ng/ml) and a fibroblast growth factor (e.g., FGF4, e.g., 20 ng/ml). In some cases, stage 1 medium includes a Wnt signaling agonist (e.g., Wnt3a, e.g., 50 ng/ml). In some cases, stage 1 medium includes a supplement such as B27 (e.g., 20%), which is known in the art. In some cases, a stage 1 medium includes a Wnt signaling agonist and a supplement (e.g., B27). In some cases, the basal culture media used for stage 1 media is advanced RPMI 1640 and it is supplemented with activin A (e.g.,100 ng/ml), FGF4 (e.g., 20 ng/ml), Wnt3a (e.g., 50 ng/ml), and 20% B27. In general, Stage 1 medium drives ASC differentiation toward the endoderm lineage.

Activins are dimeric proteins consisting of β subunits, which are connected by disulfide bonds. There are three different forms of activin: (i) homodimeric activin A (2 β_A subunits); (ii) homodimeric activin B (2 β_B subunits); and (iii) heterodimeric activin AB (1 β_A and 1 β_B subunit). The term "Activin A" as used herein refers to a homodimer of β_A subunits. A β_A subunit is the polypeptide also referred to as" Inhibin beta A chain" or "INHBA." The amino acid sequence of Inhibin beta A chain is:

MPLLWLRGFLLASCWIIVRSSPTPGSEGHSAAPDCPSCALAALPKDVPNSQPEMVEAVKK
HILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVEIEDDIGRRAEMNELMEQTS
EIITFAESGTARKTLHFEISKEGSDLSVVERAEVWLFLKVPKANRTRTKVTIRLFQQQKHPQ
GSLDTGEEAEEVGLKGERSELLLSEKVVDARKSTWHVFPVSSSIQRLLDQGKSSLDVRIAC
EQCQESGASLVLLGKKKKKEEEGEGKKKGGGEGGAGADEEKEQSHRPFLMLQARQSED
HPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPSHIAGTS
GSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDIQNMIVEEC
GCS (SEQ ID NO: 1)

In some embodiments, a subject stage 1 medium comprises Activin A at a concentration in a range of from about 50 ng/ml to about 150 ng/ml (e.g., from about 60 ng/ml to about 140 ng/ml, from about 70 ng/ml to about 130 ng/ml, from about 80 ng/ml to about 120 ng/ml, from about 85 ng/ml to about 115 ng/ml, from about 90 ng/ml to about 110 ng/ml, from about 95 ng/ml to about 105 ng/ml, from about 97.5 ng/ml to about 102.5 ng/ml, or about 100ng/ml).

Fibroblast growth factors (FGFs) are a well described family of proteins (related by sequence, structure, and function), with 18 mammalian members. The FGFs are grouped into 6 subfamilies based on differences in sequence homology and phylogeny: FGFs 1 and 2; FGFs 3, 7, 10, and 22; FGFs 4-6; FGFs 8, 17, and 18; FGFs 9, 16 and 20; and FGFs 19,

21 and 23. In some cases, a suitable FGF is FGF 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 16, 17, 18, 20, and/or 22. In some cases, a suitable FGF is FGF4, FGF5, and/or FGF6. In some cases, a suitable FGF is FGF4.

The amino acid sequences of exemplary human FGFs are:

FGF1:

MAEGEITTFTALTEKFNLPPGNYKKPKLLYCSNGGHFLRILPDGTVDGTRDRSDQHIQLQ
LSAESVGEVYIKSTETGQYLAMDTDGLLYGSQTPNEECLFLERLEENHYNTYISKKHAEK
NWFVGLKKNGSCKRGPRTHYGQKAILFLPLPVSSD (SEQ ID NO: 2)

FGF2:

MVGVGGGDVEDVTPRPGGCQISGRGARGCNGIPGAAAWEAALPRRRPRRHPSVNPRSR
AAGSPRTRGRRTEERPSGSRLGDRGRGRALPGGRLGGRGRGRAPERVGGRGRGRGTA
APRAAPAARGSRPGPAGTMAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLR
IHPDGRVDGVREKSDPHIKLQLQAEERGVVSIKGVCANRYLAMKEDGRLLASKCVTDECFF
FERLESNNYNTYRSRKYTSWYVALKRTGQYKLGSKTGPGQKAILFLPMSAKS
(SEQ ID NO: 3)

FGF3:

MGLIWLLLLSLLEPGWPAAGPGARLRRDAGGRGGVYEHLGGAPRRRKLYCATKYHLQLH
PSGRVNGSLENSAYSILEITAVEVGIVAIRGLFSGRYLAMNKRGRLYASEHYSAECEFVER
IHELGYNTYASRLYRTVSSTPGARRQPSAERLWYVSVNGKGRPRRGFKTRRTQKSSLFLP
RVLDHRDHEMVRQLQSGLPRPPGKGVQPRRRRQKQSPDNLEPSHVQASRLGSQLEASA
H (SEQ ID NO: 4)

FGF4:

MSGPGTAAVALLPAVLLALLAPWAGRGGAAAPTAPNGTLEAELERRWESLVALSLARLPV
AAQPKEAAVQSGAGDYLLGIKRLRRLYCNVGIGFHLQALPDGRIGGAHADTRDSLLELSPV
ERGVVSIFGVASRFFVAMSSKGKLYGSPFFTDECTFKEILLPNNYNAYESYKYPGMFIALSK
NGKTKKGNRVSPTMKVTHFLPRL (SEQ ID NO: 5)

FGF5:

MSLSFLLLLFFSHLILSAWAHGEKRLAPKGQPGPAATDRNPRGSSSRQSSSSAMSSSSAS
SSPAASLGSQGSGLEQSSFQWSPSGRRTGSLYCRVGIGFHLQIYPDGKVNGSHEANMLS
VLEIFAVSQGIVGIRGVFSNKFLAMSKKGKLHASAKFTDDCKFRERFQENSYNTYASAIHR
TEKTGREWYVALNKRGKAKRGCSPRVKPQHISTHFLPRFKQSEQPELSFTVTVPEKKKPP

SPIKPKIPLSAPRKNTNSVKYRLKFRFG (SEQ ID NO: 6)

FGF6:

MALGQKLFITMSRGAGRLQGTLWALVFLGILVGMVVPSPAGTRANNTLLDSRGWGTLLSR
SRAGLAGEIAGVNWESGYLVGIKRQRRLYCNVGIGFHLQVLPDGRISGTHEENPYSLLEIST
VERGVVSLFGVRSALFVAMNSKGRLYATPSFQEECKFRETLLPNNYNAYESDLYQGTYIAL
SKYGRVKRGSKVSPIMTVTHFLPRI (SEQ ID NO: 7)

FGF7:

MHKWILTWILPTLLYRSCFHIICLVGTISLACNDMTPEQMATNVNCSSPERHTRSYDYMEG
GDIRVRRLFCRTQWYLRIDKRGKVKGTQEMKNNYNIMEIRTVAVGIVAIKGVESEFYLAMN
KEGKLYAKKECNEDCNFKELILENHYNTYASAKWTHNGGEMFVALNQKGIPVRGKKTKKE
QKTAHFLPMAIT (SEQ ID NO: 8)

FGF8:

MGSPRSALSCLLLHLLVLCLQAAQEGPGRGPALGRELASLFRAGREPQGVSQQHVREQSL
VTDQLSRRLIRTYQLYSRTSGKHVQVLANKRINAMAEDGDPFAKLIVETDTFGSRVRVRGA
ETGLYICMNKKGKLIAKSNGKGKDCVFTEIVLENNYTALQNAKYEGWYMAFTRKGRPRKG
SKTRQHQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFTRSLRGSQRTWAPEPR
(SEQ ID NO: 9)

FGF9:

MAPLGEVGNYFGVQDAVPFGNVPVLPVDSPVLLSDHLGQSEAGGLPRGPAVTDLDHLKGI
LRRRQLYCRTGFHLEIFPNGTIQGTRKDHSRFGILEFISIAVGLVSIRGVDSGLYLGMNE
KGELYGSEKLTQECVFREQFEENWYNTYSSNLYKHVDTGRRYYVALNKDGTPREGTRTK
RHQKFTHFLPRPVDPDKVPELYKDILSQS (SEQ ID NO: 10)

FGF10:

MWKWILTHCASAFPHLPGCCCCCFLLLFLVSSVPVTCQALGQDMVSPEATNSSSSSFSSP
SSAGRHVRSYNHLQGDVRWRKLFSFTKYFLKIEKNGKVSGTKKENCPYSILEITSVEIGV
VAVKAINSNYYLAMNKKGKLYGSKEFNNDCKLKERIEENGYNTYASFNWQHNGRQMYVAL
NGKGAPRRGQKTRRKNTSAHFLPMVVHS (SEQ ID NO: 11)

FGF16:

MAEVGGVFASLDWDLHGFSSSLGNVPLADSPGFLNERLGQIEGKLQRGSPTDFAHLKGIL
RRRQLYCRTGFHLEIFPNGTVHGTRHDHSRFGILEFISLAVGLISIRGVDSGLYLGMNER

GELYGSKKLTRECVFREQFEENWYNTYASTLYKHSDSERQYYVALNKDGSPREGYRTKR
HQKFTHFLPRPVDPSKLPSMSRDLFHYR (SEQ ID NO: 12)

FGF17:
MGAARLLPNLTLCLQLLILCCQTQGENHPSPNFNQYVRDQGAMTDQLSRRQIREYQLYSR
TSGKHVQVTGRRISATAEDGNKFAKLIVETDTFGSRVRIKGAESEKYICMNKRGKLIGKPSG
KSKDCVFTEIVLENNYTAFQNARHEGWFMAFTRQGRPRQASRSRQNQREAHFIKRLYQG
QLPFPNHAEKQKQFEFVGSAPTRRTKRTRRPQPLT (SEQ ID NO: 13)

FGF18:
MYSAPSACTCLCLHFLLLCFQVQVLVAEENVDFRIHVENQTRARDDVSRKQLRLYQLYSRT
SGKHIQVLGRRISARGEDGDKYAQLLVETDTFGSQVRIKGKETEFYLCMNRKGKLVGKPD
GTSKECVFIEKVLENNYTALMSAKYSGWYVGFTKKGRPRKGPKTRENQQDVHFMKRYPK
GQPELQKPFKYTTVTKRSRRIRPTHPA (SEQ ID NO: 14)

FGF20:
MAPLAEVGGFLGGLEGLGQQVGSHFLLPPAGERPPLLGERRSAAERSARGGPGAAQLAH
LHGILRRRQLYCRTGFHLQILPDGSVQGTRQDHSLFGILEFISVAVGLVSIRGVDSGLYLG
MNDKGELYGSEKLTSECIFREQFEENWYNTYSSNIYKHGDTGRRYFVALNKDGTPRDGAR
SKRHQKFTHFLPRPVDPERVPELYKDLLMYT (SEQ ID NO: 15)

FGF22:
MRRRLWLGLAWLLLARAPDAAGTPSASRGPRSYPHLEGDVRWRRLFSSTHFFLRVDPGG
RVQGTRWRHGQDSILEIRSVHVGVVVIKAVSSGFYVAMNRRGRLYGSRLYTVDCRFRERI
EENGHNTYASQRWRRRGQPMFLALDRRGGPRPGGRTRRYHLSAHFLPVLVS
(SEQ ID NO: 16)

In some embodiments, a subject stage 1 medium comprises an FGF (e.g., FGF4, FGF5, FGF6, etc.) at a concentration in a range of from 5 ng/ml to 50 ng/ml (e.g., from 5 ng/ml to 40 ng/ml, from 10 ng/ml to 40 ng/ml, from 10 ng/ml to 30 ng/ml, from 15 ng/ml to 25 ng/ml, from 17.5 ng/ml to 22.5 ng/ml, 15 ng/ml, 17.5 ng/ml, 20 ng/ml, 22.5 ng/ml, 25 ng/ml or 30 ng/ml).

Wnt signaling agonist - In some embodiments, a suitable "Stage 1 medium" includes a Wnt signaling agonist. A Wnt signaling agonist triggers the nuclear localization of β-Catenin and results in the activation of canonical Wnt signaling. There are two main

branches of the Wnt signaling pathway: (1) the canonical β-Catenin dependent Wnt signaling pathway and (2) the non-canonical β-Catenin independent pathways, which include planar cell polarity (PCP) signaling as well as Calcium signaling (Gao, et. al, Cell Signal. 2010 May;22(5):717-27. Epub 2009 Dec 13). As used herein, the terms "Wnt signaling" and "Wnt/β-Catenin signaling" are used interchangeably to refer to the canonical β-Catenin dependent Wnt signaling pathway. As such, a "Wnt signaling agonist" increases output from the β-Catenin dependent Wnt signaling pathway. Activation of the Wnt pathway culminates when the protein β-Catenin enters the cell nucleus (for recent review of the canonical β-Catenin dependent Wnt signaling pathway see Clevers et. al., Cell. 2012 Jun 8;149(6):1192-205: Wnt/β-catenin signaling and disease). However, in the absence of Wnt signaling, free cytosolic β-Catenin is incorporated into a complex, known in the art as the β-Catenin destruction complex, which includes the proteins Axin, Adenomatous Polyposis Coli (APC), and glycogen synthase kinase (GSK-3β). Phosphorylation of β-Catenin by GSK-3β designates β-Catenin for the ubiquitin pathway and degradation by proteasomes (via βTRCP).

The binding of a canonical Wnt to its receptor (a Frizzled protein) leads to the activation of Dishevelled (Dvl) proteins, which inhibit glycogen synthase kinase-3β (GSK-3β) activity (i.e., phosphorylation of β-Catenin), leading to the cytosolic stabilization of β-Catenin. Stabilized β-Catenin then enters the nucleus and associates with the TCF/LEF (T Cell-specific transcription Factor / Lymphoid Enhancer Factor) family of transcription factors to induce transcription of important downstream target genes. Thus, in the absence of Wnt signaling, cytosolic (and therefore nuclear) levels of β-Catenin are kept low by negative regulatory components of the pathway while in the presence of Wnt signaling, cytosolic (and therefore nuclear) levels of β-Catenin are stabilized by positive regulatory components of the pathway.

By "negative regulatory components" of the Wnt pathway, it is meant proteins that function by antagonizing the Wnt pathway, thus resulting in decreased pathway output (i.e., decreased target gene expression). Examples of known negative regulatory components of the Wnt pathway include, but are in no way limited to: WIF, sFRP, Dkk, APCDD1, Notum, SOST, Axin, APC, GSK-3β, CK1γ, WTX, and βTrCP.

By "positive regulatory components" of the Wnt pathway, it is meant proteins that function by enhancing the Wnt pathway, thus resulting in increased pathway output (i.e., increased target gene expression). Examples of known positive regulatory components of the Wnt pathway include, but are in no way limited to: Wnt (e.g., Wnt1, Wnt3, Wnt3a, Wnt7a, and/or Wnt8), Norrin, R-spondin, PORCN, Wls, Frizzled, LRP5 and LRP6, Tspan12, Lgr4, Lgr5, Lgr6, Dvl, β-Catenin, and TCF/LEF. In some embodiments, a subject Wnt

signaling agonist is a positive regulatory component of the canonical Wnt signaling pathway (e.g., Wnt1, Wnt3, Wnt3a, Wnt7a, Wnt8, etc.).

In some embodiments, a subject Wnt signaling agonist is "specific for" or "specifically binds to" a component of the Wnt signaling pathway such that binding results in increased pathway output (i.e., transcription of target genes). The binding of an agonist can be mediated by covalent or non-covalent interactions or a combination of covalent and non-covalent interactions. When the interaction of the agonist with the component to which it specifically binds produces a non-covalently bound complex, the binding which occurs is typically electrostatic, hydrogen-bonding, or the result of lipophilic interactions. In particular, specific binding is characterized by the preferential binding of one member of a pair to a particular species relative to other species within the family of compounds to which the corresponding member of the binding member belongs. Thus, for example, a Wnt signaling agonist that is specific for the negative regulatory component GSK-3 preferably binds to GSK-3 relative to other proteins in the cell.

A subject Wnt signaling agonist is any molecule (e.g., a chemical compound; a non-coding nucleic acid, e.g., a non-coding RNA; a polypeptide; a nucleic acid encoding a polypeptide, etc.) that results in increased output (i.e., increased target gene expression) from the Wnt signaling pathway. For example, a Wnt signaling agonist can function by stabilizing, enhancing the expression of, or enhancing the function of a positive regulatory component of the pathway or by destabilizing, decreasing the expression of, or inhibiting the function of a negative regulatory component of the pathway. Thus, a Wnt signaling agonist can be a polypeptide positive regulatory component (e.g., Wnt3, wnt3a, Wnt1, and the like), and/or a nucleic acid encoding one or more positive regulatory components of the pathway. A Wnt signaling agonist can also be a small molecule or nucleic acid that stabilizes a positive regulatory component of the pathway either at the level of mRNA or protein.

In some embodiments, a Wnt signaling agonist functions by stabilizing β-Catenin, thus allowing nuclear levels of β-Catenin to rise. β-Catenin can be stabilized in multiple different ways. As multiple different negative regulatory components of the Wnt signaling pathway function by facilitating the degradation of β-Catenin, a subject Wnt signaling agonist can be a small molecule or nucleic acid inhibitor (e.g., microRNA, shRNA, etc.)(functioning at the level of mRNA or protein) of a negative regulatory component of the pathway. For example, in some embodiments, the Wnt signaling agonist is an inhibitor of GSK-3β. In some such embodiments, the inhibitor of GSK-3β is a small molecule chemical compound (e.g., TWS119, BIO, CHIR-99021, SB 216763, SB 415286, CHIR-98014 and the like).

TWS119: 3-(6-(3-aminophenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yloxy)phenol
(Ding et. al, Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7632-7. Epub 2003 Jun 6)
BIO: 6-bromo-3-[(3E)-1,3-dihydro-3-(hydroxyimino)-2H-indol-2-ylidene]-1,3-dihydro-(3Z)-2H-indol-2-one
or (2'*Z*,3'*E*)-6-Bromoindirubin-3'-oxime
(Meijer et. al, Chem Biol. 2003 Dec;10(12):1255-66)
CHIR-99021: 6-[[2-[[4-(2,4-dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile
(Bennett et al., J Biol Chem. 2002 Aug 23;277(34):30998-1004. Epub 2002 Jun 7)
SB 216763: 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione
(Cross et al., J Neurochem. 2001 Apr;77(1):94-102)
SB 415286: 3-(3-chloro-4-hydroxyphenylamino)-4-(2-nitrophenyl)-1H-pyrrole-2,5-dione
(Cross et al., J Neurochem. 2001 Apr;77(1):94-102)
CHIR-98014: N2-(2-(4-(2,4-dichlorophenyl)-5-(1H-imidazol-1-yl)pyrimidin-2-ylamino)ethyl)-5 nitropyridine-2,6-diamine
(Ring et al., Diabetes. 2003 Mar;52(3):588-95)

Any convenient Wnt agonist may be used. In some embodiments, the Wnt agonist is Wnt3a. The amino acid sequence of human Wnt3a is:

MAPLGYFLLLCSLKQALGSYPIWWSLAVGPQYSSLGSQPILCASIPGLVPKQLRFCRNYVEI
MPSVAEGIKIGIQECQHQFRGRRWNCTTVHDSLAIFGPVLDKATRESAFVHAIASAGVAFA
VTRSCAEGTAAICGCSSRHQGSPGKGWKWGGCSEDIEFGGMVSREFADARENRPDARS
AMNRHNNEAGRQAIASHMHLKCKCHGLSGSCEVKTCWWSQPDFRAIGDFLKDKYDSASE
MVVEKHRESRGWVETLRPRYTYFKVPTERDLVYYEASPNFCEPNPETGSFGTRDRTCNV
SSHGIDGCDLLCCGRGHNARAERRREKCRCVFHWCCYVSCQECTRVYDVHTCK
(SEQ ID NO: 17).

In some cases, a subject stage 1 medium comprises Wnt3a at a concentration in a range of from about 20 ng/ml to about 80 ng/ml (e.g., from about 20 ng/ml to about 80 ng/ml, from about 25 ng/ml to about 75 ng/ml, from about 30 ng/ml to about 70 ng/ml, from about 35 ng/ml to about 65 ng/ml, from about 40 ng/ml to about 60 ng/ml, from about 45 ng/ml to about 55 ng/ml, from about 47.5 ng/ml to about 52.5 ng/ml, or about 50ng/ml).

Stage 2 medium. "Stage 2 medium" is also referred to herein as a "second culture medium." A suitable stage 2 medium is a basal culture medium (e.g., hepatocyte culture

medium (HCM™), advanced RPMI 1640, RPMI 1640, etc.) suitable for the culture of cells (e.g., differentiating ASCs, hepatocytes, etc.) supplemented with at least hepatocyte growth factor (HGF). In some cases, stage 2 medium includes a fibroblast growth factor (e.g., FGF4, e.g., 25 ng/ml).

In some embodiments, a stage 2 media further includes a component selected from: an FGF (e.g., FGF4, e.g., 25 ng/ml), oncostatinM (OSM, e.g., 30 ng/ml), dexamethasone (Dex, e.g., $2x10^{-5}$ M), dimethyl sulfoxide (DMSO, e.g., 0.1%), and a combination thereof. In some cases, the basal culture media used for stage 2 media is CLONETICS™ (available, for example, from Lonza – Catalog number 3198) and it is supplemented with HGF (e.g., 150 ng/ml), an FGF (e.g., FGF4, e.g., 25 ng/ml), oncostatinM (e.g., 30 ng/ml), dexamethasone (e.g., $2x10^{-5}$ M), and DMSO (e.g., 0.1%).

Hepatocyte growth factor (HGF) is a protein that functions as a potent mitogen, hepatotrophic factor, and/or a growth factor. The amino acid sequence of human HGF is:

MWVTKLLPALLLQHVLLHLLLLPIAIPYAEGQRKRRNTIHEFKKSAKTTLIKIDPALKIKTKKVN
TADQCANRCTRNKGLPFTCKAFVFDKARKQCLWFPFNSMSSGVKKEFGHEFDLYENKDYI
RNCIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHSFLPSSYRGKDLQENYCRNPRGEEGG
PWCFTSNPEVRYEVCDIPQCSEVECMTCNGESYRGLMDHTESGKICQRWDHQTPHRHK
FLPERYPDKGFDDNYCRNPDGQPRPWCYTLDPHTRWEYCAIKTCADNTMNDTDVPLETT
ECIQGQGEGYRGTVNTIWNGIPCQRWDSQYPHEHDMTPENFKCKDLRENYCRNPDGSE
SPWCFTTDPNIRVGYCSQIPNCDMSHGQDCYRGNGKNYMGNLSQTRSGLTCSMWDKNM
EDLHRHIFWEPDASKLNENYCRNPDDDAHGPWCYTGNPLIPWDYCPISRCEGDTTPTIVN
LDHPVISCAKTKQLRVVNGIPTRTNIGWMVSLRYRNKHICGGSLIKESWVLTARQCFPSRD
LKDYEAWLGIHDVHGRGDEKCKQVLNVSQLVYGPEGSDLVLMKLARPAVLDDFVSTIDLP
NYGCTIPEKTSCSVYGWGYTGLINYDGLLRVAHLYIMGNEKCSQHHRGKVTLNESEICAGA
EKIGSGPCEGDYGGPLVCEQHKMRMVLGVIVPGRGCAIPNRPGIFVRVAYYAKWIHKIILTY
KVPQS (SEQ ID NO: 18).

In some embodiments, a subject stage 2 medium includes HGF at a concentration in a range of from 75 ng/ml to 250 ng/ml (e.g., from 100 ng/ml to 200 ng/ml, from 120 ng/ml to 180 ng/ml, from 125 ng/ml to 175 ng/ml, from 130 ng/ml to 170 ng/ml, from 135 ng/ml to 165 ng/ml, from 140 ng/ml to 160 ng/ml, from 145 ng/ml to 155 ng/ml, 120 ng/ml, 125 ng/ml, 130 ng/ml, 135 ng/ml, 140 ng/ml, 145 ng/ml, 150 ng/ml, 155 ng/ml, 160 ng/ml or 175 ng/ml).

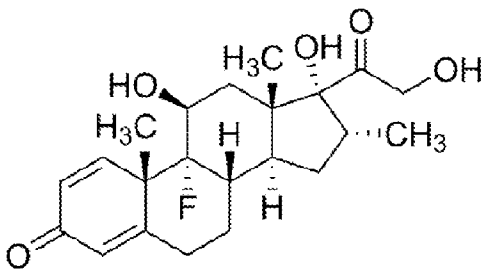
In some embodiments, a subject stage 2 medium includes an FGF (e.g., FGF4) at a concentration in a range of from 5 ng/ml to 55 ng/ml (e.g., from 5 ng/ml to 45 ng/ml, from 10 ng/ml to 45 ng/ml, from 10 ng/ml to 35 ng/ml, from 15 ng/ml to 35 ng/ml, from 20 ng/ml to 30 ng/ml, from 22.5 ng/ml to 27.5 ng/ml, 15 ng/ml, 17.5 ng/ml, 20 ng/ml, 22.5 ng/ml, 25 ng/ml, 27.5 ng/ml, or 30 ng/ml).

Oncostatin M (OSM) is a polypeptide that functions as a cytokine and belongs to the interleukin 6 group of cytokines. The amino acid sequence of human OSM is:

MGVLLTQRTLLSLVLALLFPSMASMAAIGSCSKEYRVLLGQLQKQTDLMQDTSRLLDPYIRI QGLDVPKLREHCRERPGAFPSEETLRGLGRRGFLQTLNATLGCVLHRLADLEQRLPKAQD LERSGLNIEDLEKLQMARPNILGLRNNIYCMAQLLDNSDTAEPTKAGRGASQPPTPTPASD AFQRKLEGCRFLHGYHRFMHSVGRVFSKWGESPNRSRRHSPHQALRKGVRRTRPSRKG KRLMTRGQLPR (SEQ ID NO: 19)

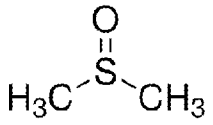
In some embodiments, a subject stage 2 medium includes oncostatinM (OSM) at a concentration in a range of from 10 ng/ml to 50 ng/ml (e.g., from 15 ng/ml to 45 ng/ml, from 20 ng/ml to 40 ng/ml, from 22.5 ng/ml to 37.5 ng/ml, from 25 ng/ml to 35 ng/ml, from 27.5 ng/ml to 32.5 ng/ml, 25 ng/ml, 27.5 ng/ml, 30 ng/ml, 32.5 ng/ml, or 35 ng/ml).

Dexamethasone (Dex) is a synthetic member of the glucocorticoid class of steroid drugs that has anti-inflammatory and immunosuppressant properties. Dex is also known as (11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione, 9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione, 9α-Fluoro-16α-methylprednisolone, and Prednisolone F. Dex has the CAS number 50-02-2 and the chemical structure:



In some embodiments, a subject stage 2 medium comprises dexamethasone (Dex) at a concentration in a range of from $1x10^{-6}$ M to $1x10^{-4}$ M (e.g., from $5x10^{-6}$ to $5x10^{-5}$ M, from $1x10^{-5}$ M to $3x10^{-5}$ M, from $1.5x10^{-5}$ M to $2.5x10^{-5}$ M, or $2X10^{-5}$ M).

Dimethylsulfoxide (DMSO) is an organosulfur compound with the formula $(CH_3)_2SO$ that is a colorless liquid and is a polar aprotic solvent that dissolves both polar and nonpolar compounds. DMSO is also known as Methyl sulfoxide, has the CAS number 67-68-5, and has the chemical structure:



In some embodiments, a subject stage 2 medium comprises DMSO at a concentration in a range of from 0.01% to 1% (e.g., from 0.025% to 0.5%, from 0.05% to 0.2%, or 0.1%).

Hepatocyte-like cells. The term "hepatocyte-like cell" as used herein refers to a cell (e.g., of a cell population, e.g., a cell that is induced according to the subject methods) that is positive for (i.e., exhibits) one or more hepatocyte characters. Hepatocyte characters (i.e., characteristics of hepatocyte-like cells) include, but are not limited to:

(i) expression of (i.e., positive for) one or more hepatocyte markers (e.g., glucose-6-phosphatase, albumin (ALB), alpha-1-antitrypsin (AAT, also known as SERPINA1), cytokeratin 8 (CK8), cytokeratin 18 (CK18), cytokeratin 8/18 (CK8/18), asialoglycoprotein receptor 1 (ASGR1), alcohol dehydrogenase 1, arginase Type I, cytochrome p450 3A4 (CYP3A4), liver-specific organic anion transporter (LST-1), forkhead box protein A2 (FoxA2), alpha-fetoprotein (AFP), tryptophan 2,3-dioxygenase (TDO2), or a combination thereof);

(ii) activity of liver enzymes such as glucose-6-phosphatase, CYP3A4, and/or CYP1A1;

(iii) production and/or secretion of liver products (e.g., as measured in bodily fluids such as blood, serum, plasma, etc.)(e.g., bile, urea, and/or albumin);

(iv) exhibition of a hepatocyte metabolic properties (e.g., ability to detoxify xenobiotics; endocytosis of LDL, synthesis of glycogen, cytochrome P450 1A2 detoxification activity, and the like);

(v) exhibition of hepatocyte morphological features;

(vi) ability to engraft into the liver of an immunodeficient indidual (e.g., a mouse, a human, etc.); and

(vii) lack of expression of (negative for) one or more non-hepatocyte markers (e.g., adipocyte markers, e.g., CD37, CD29, etc.; ASC markers, e.g., CD105; and the like).

A subject hepatocyte-like cell does not have to be positive for all tested hepatocyte characters in order for the cell to be considered a hepatocyte-like cell. For example, in some cases, a subject hepatocyte-like cell is positive for all tested hepatocyte characters. However, in some cases, a subject hepatocyte-like cell is positive for one or more

hepatocyte character; and is also negative for one or more hepatocyte characters. In some cases (described below in more detail), a hepatocyte-like cell is transplanted into an individual. In such cases, the hepatocyte-like cell can be (i) positive for all tested hepatocyte characters; or (ii) positive for one or more hepatocyte character(s); and negative for one or more hepatocyte character(s).

Thus, in some cases, hepatocyte-like cells do not express all known hepatocyte markers and/or test positive for all hepatocyte functional tests. For example, in some cases, hepatocyte-like cells that are induced according to the subject methods do not express ASGR1. However, as demonstrated in the examples section below (e.g., **Figure 3C**), in some cases, hepatocyte-like cells exhibit a hepatocyte character (e.g., express a hepatocyte marker such as ASGR1) after transplantation (e.g., into an individual such as a mouse and/or a human) that they did not exhibit prior to transplantation. For example, in some cases, hepatocyte-like cells that are induced according to the subject methods do not express the hepatocyte marker ASGR1 prior to transplantation into an individual, but do express ASGR1 after transplantation. Thus, in some cases, it is not required that hepatocyte-like cells induced according to the subject methods exhibit all known hepatocyte characters (e.g., production of a liver product, expression of a hepatocyte marker, etc.) or even exhibit all tested hepatocyte characters prior to the use of the cells for transplantation. For example, in some cases, hepatocyte-like cells that are induced according to the subject methods are positive for one or more hepatocyte characters, and are negative for one or more hepatocyte characters, but are still considered hepatocyte-like cells and can still be transplanted into an individual.

In some embodiments, the subject methods include the step of verifying the presence of induced hepatocyte-like cells (iHeps) in the induced cell population. Verifying can rely on cellular phenotypes (e.g., gene or protein expression, drug metabolism profile, responsiveness to particular drugs, etc.) known in the art to be characteristic of hepatocytes. For example, verifying can be performed by testing for any one or more of the hepatocyte characters listed above (e.g., expression of a hepatocyte marker, lack of a expression of a non-hepatocyte marker, production and/or secretion of a liver product, activity of a liver enzyme, exhibition of ta hepatocyte metabolic property, etc.). In some cases, verifying comprises determining the percentage of cells of the induced cell population that are iHeps.

In some embodiments, verifying includes contacting cells of the induced cell population with a specific binding agent (e.g., a nucleic acid probe, an antibody, etc.) specific for a hepatocyte marker (e.g., mRNA, protein, etc.), and determining the percentage of cells positive for expression, wherein cells positive for expression are iHeps.

Suitable markers are listed above. In some embodiments, verifying includes contacting the induced cell population with a binding agent (e.g., a nucleic acid probe, an antibody, etc.) specific for a non-hepatocyte marker (e.g., mRNA, protein, etc.), and determining the percentage of cells negative for expression, wherein cells negative for expression are iHeps. Suitable markers are listed above. In some cases, 10% or more of the cells of the induced cell population are determined to be iHeps (e.g., 10.5% or more, 11% or more, 12.5% or more, 15% or more, 17.5% or more, 20% or more, 22.5% or more, 25% or more, 27.5% or more, 30% or more, 32.5% or more, 35% or more, 37% or more, 40% or more, 45% or more, 50% or more, 55% or more, 60% or more, 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 98% or more, 99% or more, or 100%). In some embodiments, the percent of cells of the induced cell population that are determined to be iHeps is in a range of from 10% to 100% (e.g., from 10% to 90%, from 10% to 80%, from 10% to 70%, from 10% to 60%, from 10% to 50%, from 10% to 45%, from 10% to 100%, from 40% to 100%, from 10% to 37.5%, from 10% to 37%, from 15% to 90%, from 15% to 80%, from 15% to 70%, from 15% to 60%, from 15% to 50%, from 15% to 45%, from 15% to 100%, from 40% to 100%, from 15% to 37.5%, from 15% to 37%, from 20% to 90%, from 20% to 80%, from 20% to 70%, from 20% to 60%, from 20% to 50%, from 20% to 45%, from 20% to 100%, from 40% to 100%, from 20% to 37.5%, from 20% to 37%, from 25% to 90%, from 25% to 80%, from 25% to 70%, from 25% to 60%, from 25% to 50%, from 25% to 45%, from 25% to 100%, from 40% to 100%, from 25% to 37.5%, from 25% to 37%, from 30% to 90%, from 30% to 80%, from 30% to 70%, from 30% to 60%, from 30% to 50%, from 30% to 45%, from 30% to 100%, from 40% to 100%, from 30% to 37.5%, or from 30% to 37%).

It will be understood by those of skill in the art that expression levels reflect detectable amounts of the marker (e.g., nucleic acid or protein) on and/or in the cell. A cell that is negative for staining (e.g., the level of binding of a marker specific reagent is not detectably different from a matched control) may still express minor amounts of the marker. And while it is commonplace in the art to refer to cells as "positive" or "negative" for a particular marker, actual expression levels are quantitative traits. The number of detected molecules can vary by several logs, yet still be characterized as "positive".

When a protein marker is used, the staining intensity (e.g., of a marker-specific antibody) can be monitored by flow cytometry, where lasers detect the quantitative levels of fluorochrome (which is proportional to the amount of cell marker bound by specific reagents, e.g. antibodies). Flow cytometry, or FACS, can also be used to separate cell populations based on the intensity of binding to a specific reagent, as well as other parameters such as cell size and light scatter. Although the absolute level of staining may differ with a particular

fluorochrome and reagent preparation, the data can be normalized to a control.

In order to normalize the distribution to a control, each cell is recorded as a data point having a particular intensity of staining. These data points may be displayed according to a log scale, where the unit of measure is arbitrary staining intensity. In one example, the brightest stained cells in a sample can be as much as 4 logs more intense than unstained cells. When displayed in this manner, it is clear that the cells falling in the highest log of staining intensity are bright, while those in the lowest intensity are negative. The "low" positively stained cells have a level of staining brighter than that of an isotype matched control, but is not as intense as the most brightly staining cells normally found in the population. An alternative control may utilize a substrate having a defined density of marker on its surface, for example a fabricated bead or cell line, which provides the positive control for intensity.

Enrichment of hepatocyte-like cells. To increase the fraction of obtained cells that are iHeps, it is sometimes advantageous to enrich for (i.e., purify) the produced iHeps. In particular aspects, the hepatocyte-like cells provided herein may be selected or enriched by using a screenable or selectable reporter expression cassette comprising a mature hepatocyte-specific transcriptional regulatory element operably linked to a reporter gene, or by cell sorting (e.g., magnetic cell sorting, Fluorescence Activated Cell Sorting (FACS), and the like) using an antibody against a hepatocyte-specific marker (e.g., a cell surface antigen such as ASGR1).

To aid selection or enrichment, the ASCs, may comprise a selectable or screenable reporter expression cassette comprising a reporter gene. The reporter expression cassette may comprise a hepatocyte-specific transcriptional regulatory element operably linked to a reporter gene (e.g., any of the fluorescent proteins, e.g., blue, yellow, red, green, etc.). Non-limiting examples of hepatocyte-specific transcriptional regulatory element include a promoter of glucose-6-phosphatase, albumin (ALB), alpha-1-antitrypsin (AAT, also known as SERPINA1), cytokeratin 8 (CK8), cytokeratin 18 (CK18), asialoglycoprotein receptor 1 (ASGR1), alcohol dehydrogenase 1, arginase Type I, cytochrome p450 3A4 (CYP3A4), liver-specific organic anion transporter (LST-1), forkhead box protein A2 (FoxA2), alpha-fetoprotein (AFP), and tryptophan 2,3-dioxygenase (TDO2).

Enrichment using antibodies (e.g., magnetic cell sorting, FACS, and the like) specific for cell surface markers of ASCs (e.g., CD29, CD34, CD36, CD49f, CD73, CD90 (Thy-1), CD105, CD133, c-kit, c-met, and the like), endodermal precursor cells (e.g., SOX17), hepatocyte precursor/progenitor cells (e.g., N-cadherin, E-cadherin, EpCAM, and NCAM), and/or hepatocytes (e.g., ASGR1) have the advantage of not requiring genetic modification

of the cells to be enriched. Magnetic cell sorting and FACS have the ability to analyze multiple surface markers simultaneously, and they can be used to sort ASCs, endodermal precursor cells, hepatocyte precursor/progenitor cells, and/or hepatocytes (e.g., iHeps) based on the expression of a cell surface marker. In some cases, iHeps do not express ASGR1. However, in some cases, e.g, if they are cultured longer and/or are further differentiated, iHeps do express ASGR1. In cases where cells that (i) do not express ASGR1; and/or (ii) express markers of hepatocyte precursor cells and/or endodermal precursor cells, are administered to an individual, the cells can further differentiate after administration into the individual (as demonstrated in the examples section below).

In some cases, cells that are produced by the subject methods can be enriched (e.g., sorted) by using an expressed cell surface marker. For example, cells of the precursor cell population (e.g., ASCs that have been contacted by a stage 1 medium) and/or cells of the induced cell population (e.g., cells of the precursor cell population that have been contacted with stage 2 medium) can be enriched using any marker that is expressed by hepatocyte precursor/progenitor cells (e.g., N-cadherin, E-cadherin, EpCAM, and NCAM) and/or endodermal precursor cells (e.g., SOX17). Cells of the induced cell population (e.g., cells of the precursor cell population that have been contacted with stage 2 medium) can be enriched using any marker that is expressed by hepatocyte precursor/progenitor cells (e.g., N-cadherin, E-cadherin, EpCAM, NCAM, etc.), endodermal precursor cells (e.g., SOX17), and/or hepatocytes (e.g., ASGR1) prior to further culture (e.g., to facilitate for further differentiation) and/or prior to administration to an individual. A cell population that includes ASCs (e.g., a population of cells from liposuction) can be enriched for ASCs using ASC cell surface markers (e.g., CD29, CD34, CD36, CD49f, CD73, CD90 (Thy-1), CD105, CD133, c-kit, c-met, and the like).

Cryopreservation. In some embodiments, subject cells (e.g., iHeps) are preserved for future use. Specifically, iHeps can be cryopreserved by performing the following steps: (i) Detach cells from growth cell surface, (ii) rinse cells (e.g., wash with PBS via centrifugation), (iii) resuspend cells (e.g., cell pellet) in media with 10% DMSO, and (vi) freeze cells and store in liquid nitrogen using standard tissue culture techniques commonly used in the art to preserve cells. Cells can be frozen at 1-50 million cells per vial. When ready for use, iHeps should be thawed in a manner as commonly known in the art for thawing frozen cultured cells.

Methods of treatment

Aspects of the disclosure include methods of treating an individual. The subject methods of treatment generally include producing a population of hepatocyte-like cells from

a population of adipose-derived stem cells (e.g., using the methods described above), and administering (e.g., injecting, transplanting, etc.) an effective number of hepatocyte-like cells into an individual. The ASCs can be from any source and can be derived by any convenient method. Exemplary methods of isolating/extracting/obtaining ASCs are described above. In some cases the ASCs are autologous (i.e., from the same individual into which the iHeps will be administered) (e.g., to reduce the possibility and/or severity of an immune response). In some cases, the ASCs are from a related individual (e.g., to reduce the possibility and/or severity of an immune response). In some cases, the ASCs are from an unrelated individual. In some cases, the ASCs are from an individual of another species (e.g., human ASCs administered to a mouse).

The terms "treatment", "treating", "treat" and the like are used herein to generally refer to obtaining a desired pharmacologic and/or physiologic effect. The effect can be prophylactic in terms of completely or partially preventing a disease or symptom(s) thereof and/or may be therapeutic in terms of a partial or complete stabilization or cure for a disease and/or adverse effect attributable to the disease. The term "treatment" encompasses any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing the disease and/or symptom(s) from occurring in a subject who may be predisposed to the disease or symptom but has not yet been diagnosed as having it; (b) inhibiting the disease and/or symptom(s), i.e., arresting development of a disease and/or the associated symptoms; or (c) relieving the disease and the associated symptom(s), i.e., causing regression of the disease and/or symptom(s). Those in need of treatment can include those already inflicted (e.g., those with liver malfunction, liver disease, liver damage, etc.) as well as those in which prevention is desired.

The terms "recipient", "individual", "subject", "host", and "patient", are used interchangeably herein and refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired, particularly humans. "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, sheep, goats, pigs, camels, etc. In some embodiments, the mammal is human.

A therapeutic treatment is one in which the subject is inflicted prior to administration and a prophylactic treatment is one in which the subject is not inflicted prior to administration. In some embodiments, the subject has an increased likelihood of becoming inflicted or is suspected of being inflicted prior to treatment. In some embodiments, the subject is suspected of having an increased likelihood of becoming inflicted.

In some embodiments, the individual to be treated is an individual with reduced liver function (e.g., an individual with liver damage). The term "liver damage" as used herein

refers to any damage (e.g., caused by disease, trauma, unexplained, etc.) resulting in reduced liver function. An individual with reduced liver function (e.g., reduced relative to the individual's basal level of function; reduced relative to a healthy individual of comparable age, weight, sex, etc.; and the like) is referred to herein as an individual with a damaged liver (i.e., an individual with liver damage). Thus, the terms "liver damage" and "reduced liver function" are used synonymously herein. In some cases, an individual with "liver damage" is an individual with liver disease.

The cause for reduced liver function may be known or unknown. How to determine whether a given individual has reduced liver function (and therefore also how to determine whether an individual receiving treatment is recovering or has recovered liver function) will be known to one of ordinary skill in the art. Liver function tests can include liver enzyme tests, also known as liver function tests (LFTs), a group of blood tests that detect inflammation and damage to the liver. Blood protein markers of liver damage may include, for example: increased aspartate aminotransferase (AST), also known as SGOT; increased alanine aminotransferase (ALT), also known as SGPT; increased alkaline phosphatase; increased 5' nucleotidase; decreased albumin; decreased globulin; and increased gamma-glutamyl transpeptidase (GGT). If, for example, elevated amounts (and/or activity) of the above protein markers (or a decreased level for albumin and/or globulin) are detected in the blood, liver damage may be present. Additional liver function tests include: (i) prothrombin time (PT), a test of the time it takes for a blood sample to clot (e.g., if low levels of clotting factors are present, the prothrombin time is longer), which can be reported as international normalized ratio (INR) (a standardized way for laboratories to report PT so that results can be compared accurately across laboratories); and (ii) a bilirubin test: bilirubin blood levels may be elevated in people with impaired bile flow, which can occur in severe liver disease, gallbladder disease, or other bile system conditions.

Examples of symptoms, illnesses, and/or diseases that can be considered to indicate "liver damage" (and that can be treated by transplanting subject induced hepatocyte-like cells into an individual having the symptoms, illness, and/or disease), include, but are not limited to: acute liver failure, alcohol-related liver disease, Alagille syndrome, alpha 1-antitrypsin deficiency, alveolar hydatid disease, autoimmune hepatitis, bacillary peliosis, biliary atresia , Budd–Chiari syndrome, chronic liver disease, cirrhosis, congenital hepatic fibrosis, congestive hepatopathy, fatty liver, galactosemia, gastric antral vascular ectasia, Gilbert syndrome, hemochromatosis, hepatitis (A, B, and/or C), hepatic encephalopathy, hepato-biliary diseases, hepatolithiasis, hepatopulmonary syndrome, hepatorenal syndrome, hepatosplenomegaly, hepatotoxicity, hepatocellular carcinoma, hepatic encephalopathy, jaundice, Laennec's cirrhosis, liver abscess, liver cysts, liver

cancer, liver failure, Lyngstadaas syndrome, non-alcoholic fatty liver disease, pediatric end-stage liver disease, peliosis hepatis, polycystic liver disease, primary biliary cirrhosis, primary sclerosing cholangitis (PSC), progressive familial intrahepatic cholestasis, Reye syndrome, type I glycogen storage disease, viral hepatitis, Wilson disease, Zahn infarct, and Zieve's syndrome.

In some cases, iHeps are cultured for a period of time prior to transplantation (e.g., in HCM™ for 2 days). Cells (e.g., iHeps) can be provided to the individual (i.e., administered into the individual) alone or with a suitable substrate or matrix, e.g. to support their growth and/or organization in the tissue to which they are being transplanted (e.g., liver). In some embodiments, the matrix is a scaffold (e.g., an organ scaffold). In some embodiments, $1x10^3$ or more cells will be administered (e.g., transplanted), for example $5x10^3$ or more cells, $1x10^4$ or more cells, $5x10^4$ or more cells, $1x10^5$ or more cells, $5x10^5$ or more cells, 1 x 10^6 or more cells, $5x10^6$ or more cells, $1x10^7$ or more cells, $5x10^7$ or more cells, $1x10^8$ or more cells, $5x10^8$ or more cells, 1 x 10^9 or more cells, $5x10^9$ or more cells, or $1x10^{10}$ or more cells. In some embodiments, subject cells are administered into the individual on microcarriers (e.g., cells grown on biodegradable microcarriers).

The cells induced by the subject methods (iHeps) may be administered in any physiologically acceptable excipient (e.g., William's E medium), where the cells may find an appropriate site for survival and function (e.g., organ reconstitution). The cells may be introduced by any convenient method (e.g., injection, catheter, or the like).

The cells may be introduced to the subject (i.e., administered into the individual) via any of the following routes: parenteral, subcutaneous, intravenous, intracranial, intraspinal, intraocular, or into spinal fluid. The cells may be introduced by injection (e.g., direct local injection), catheter, or the like. Examples of methods for local delivery (e.g., delivery to the liver) include, e.g., by bolus injection, e.g. by a syringe, e.g. into a joint or organ; e.g., by continuous infusion, e.g. by cannulation, e.g. with convection (see e.g. US Application No. 20070254842, incorporated here by reference); or by implanting a device upon which the cells have been reversably affixed (see e.g. US Application Nos. 20080081064 and 20090196903, incorporated herein by reference).

In some cases, iHeps are administered into an individual by ultrasound-guided liver injection. In this way, cells can be placed directly into a liver lobe (e.g., in humans, or even in mice using a small animal ultrasound system). Brightness mode (B-mode) can be used to acquire two-dimensional images for an area of interest with a transducer and cells can be injected in solution (e.g., 100µl to 300µl, e.g., 200 µl of, for example, William's E medium) into one site or many sites (e.g., 1-30 sites) in the liver using, for example, a 30G needle.

The number of administrations of treatment to a subject may vary. Introducing cells into an individual may be a one-time event; but in certain situations, such treatment may elicit improvement for a limited period of time and require an on-going series of repeated treatments. In other situations, multiple administrations of iHeps may be required before an effect is observed. As will be readily understood by one of ordinary skill in the art, the exact protocols depend upon the disease or condition, the stage of the disease and parameters of the individual being treated.

A "therapeutically effective dose" or "therapeutic dose" is an amount sufficient to effect desired clinical results (i.e., achieve therapeutic efficacy). A therapeutically effective dose can be administered in one or more administrations. For purposes of this disclosure, a therapeutically effective dose of iHeps is an amount that is sufficient, when administered to (e.g., transplanted into) the individual, to palliate, ameliorate, stabilize, reverse, prevent, slow or delay the progression of the disease state (e.g., liver damage) by, for example, providing functions normally provided by a healthy liver. In some cases, transplanted iHeps integrate into the individual's liver and become part of the liver. In some cases, transplanted iHeps do not integrate into the individual's liver, but can still provide functions normally provided by a healthy liver.

In some embodiments, a therapeutically effective dose of iHeps is about $1x10^3$ or more cells (e.g., $5x10^3$ or more, $1x10^4$ cells, $5x10^4$ or more, $1x10^5$ or more, $5x10^5$ or more, 1 x 10^6 or more, $5x10^6$ or more, $1x10^7$ cells, $5x10^7$ or more, $1x10^8$ or more, $5x10^8$ or more, 1 x 10^9 or more, $5x10^9$ or more, or $1x10^{10}$ or more). In some embodiments, a therapeutically effective dose of iHeps is in a range of from about $1x10^3$ cells to about $1x10^{10}$ cells (e.g, from about $5x10^3$ cells to about $1x10^{10}$ cells, from about $1x10^4$ cells to about $1x10^{10}$ cells, from about $5x10^4$ cells to about $1x10^{10}$ cells, from about $1x10^5$ cells to about $1x10^{10}$ cells, from about $5x10^5$ cells to about $1x10^{10}$ cells, from about $1x10^6$ cells to about $1x10^{10}$ cells, from about $5x10^6$ cells to about $1x10^{10}$ cells, from about $1x10^7$ cells to about $1x10^{10}$ cells, from about $5x10^7$ cells to about $1x10^{10}$ cells, from about $1x10^8$ cells to about $1x10^{10}$ cells, from about $5x10^8$ cells to about $1x10^{10}$, from about $5x10^3$ cells to about $5x10^9$ cells, from about $1x10^4$ cells to about $5x10^9$ cells, from about $5x10^4$ cells to about $5x10^9$ cells, from about $1x10^5$ cells to about $5x10^9$ cells, from about $5x10^5$ cells to about $5x10^9$ cells, from about $1x10^6$ cells to about $5x10^9$ cells, from about $5x10^6$ cells to about $5x10^9$ cells, from about $1x10^7$ cells to about $5x10^9$ cells, from about $5x10^7$ cells to about $5x10^9$ cells, from about $1x10^8$ cells to about $5x10^9$ cells, from about $5x10^8$ cells to about $5x10^9$, from about $5x10^3$ cells to about $1x10^9$ cells, from about $1x10^4$ cells to about $1x10^9$ cells, from about $5x10^4$ cells to about $1x10^9$ cells, from about $1x10^5$ cells to about $1x10^9$ cells, from about $5x10^5$ cells to about $1x10^9$ cells, from about $1x10^6$ cells to about $1x10^9$ cells, from about

$5x10^6$ cells to about $1x10^9$ cells, from about $1x10^7$ cells to about $1x10^9$ cells, from about $5x10^7$ cells to about $1x10^9$ cells, from about $1x10^8$ cells to about $1x10^9$ cells, from about $5x10^8$ cells to about $1x10^9$, from about $5x10^3$ cells to about $5x10^8$ cells, from about $1x10^4$ cells to about $5x10^8$ cells, from about $5x10^4$ cells to about $5x10^8$ cells, from about $1x10^5$ cells to about $5x10^8$ cells, from about $5x10^5$ cells to about $5x10^8$ cells, from about $1x10^6$ cells to about $5x10^8$ cells, from about $5x10^6$ cells to about $5x10^8$ cells, from about $1x10^7$ cells to about $5x10^8$ cells, from about $5x10^7$ cells to about $5x10^8$ cells, or from about $1x10^8$ cells to about $5x10^8$ cells).

The cells of this disclosure can be supplied in the form of a pharmaceutical composition, comprising an isotonic excipient prepared under sufficiently sterile conditions for human administration. For general principles in medicinal formulation, the reader is referred to Cell Therapy: Stem Cell Transplantation, Gene Therapy, and Cellular Immunotherapy, by G. Morstyn & W. Sheridan eds, Cambridge University Press, 1996; and Hematopoietic Stem Cell Therapy, E. D. Ball, J. Lister & P. Law, Churchill Livingstone, 2000. Choice of the cellular excipient and any accompanying elements of the composition will be adapted in accordance with the route and device used for administration. The composition may also comprise or be accompanied with one or more other ingredients that facilitate the engraftment or functional mobilization of the cells. Suitable ingredients include matrix proteins that support or promote adhesion of the cells, or complementary cell types.

Cells of the subject methods may be genetically altered in order to introduce genes useful in the differentiated hepatocytes, *e.g.* repair of a genetic defect in an individual, selectable marker, etc. Cells may also be genetically modified to enhance survival, control proliferation, and the like. Cells may be genetically altered by transfection or transduction with a suitable vector, homologous recombination, or other appropriate technique, so that they express a gene of interest. In some embodiments, a selectable marker is introduced, to provide for greater purity of the desired differentiating cell.

The cells of this disclosure can also be genetically altered in order to enhance their ability to be involved in tissue regeneration, or to deliver a therapeutic gene to a site of administration. A vector is designed using the known encoding sequence for the desired gene, operatively linked to a promoter that is either pan-specific or specifically active in hepatocytes.

Many vectors useful for transferring exogenous genes into target mammalian cells are available. The vectors may be episomal, *e.g.* plasmids, virus derived vectors such cytomegalovirus, adenovirus, *etc.*, or may be integrated into the target cell genome, through homologous recombination or random integration, *e.g.* retrovirus derived vectors such MMLV, HIV-1, ALV, *etc.* For modification of stem cells, lentiviral vectors are preferred.

Lentiviral vectors such as those based on HIV or FIV gag sequences can be used to transfect non-dividing cells, such as the resting phase of human stem cells (see Uchida *et al.* (1998) P.N.A.S. **95**(20):11939-44).

Combinations of retroviruses and an appropriate packaging line may also find use, where the capsid proteins will be functional for infecting the target cells. Usually, the cells and virus will be incubated for at least about 24 hours in the culture medium. The cells are then allowed to grow in the culture medium for short intervals in some applications, *e.g.* 24-73 hours, or for at least two weeks, and may be allowed to grow for five weeks or more, before analysis. Commonly used retroviral vectors are "defective", *i.e.* unable to produce viral proteins required for productive infection. Replication of the vector requires growth in the packaging cell line.

The host cell specificity of the retrovirus is determined by the envelope protein, *env* (p120). The envelope protein is provided by the packaging cell line. Envelope proteins are of at least three types, ecotropic, amphotropic and xenotropic. Retroviruses packaged with ecotropic envelope protein, *e.g.* MMLV, are capable of infecting most murine and rat cell types. Ecotropic packaging cell lines include BOSC23 (Pear *et al.* (1993) P.N.A.S. 90:8392-8396). Retroviruses bearing amphotropic envelope protein, *e.g.* 4070A (Danos *et al, supra.*), are capable of infecting most mammalian cell types, including human, dog and mouse. Amphotropic packaging cell lines include PA12 (Miller *et al.* (1985) Mol. Cell. Biol. **5**:431-437); PA317 (Miller *et al.* (1986) Mol. Cell. Biol. **6**:2895-2902) GRIP (Danos *et al.* (1988) PNAS **85**:6460-6464). Retroviruses packaged with xenotropic envelope protein, *e.g.* AKR *env*, are capable of infecting most mammalian cell types, except murine cells.

The vectors may include genes that must later be removed, *e.g.* using a recombinase system such as Cre/Lox, or the cells that express them destroyed, *e.g.* by including genes that allow selective toxicity such as herpesvirus TK, bcl-xs, *etc.* Suitable inducible promoters are activated in a desired target cell type, either the transfected cell, or progeny thereof. By transcriptional activation, it is intended that transcription will be increased above basal levels in the target cell by at least about 100 fold, more usually by at least about 1000 fold.

UTILITY

The results described in the Examples below demonstrate that functioning human liver tissue can be generated *in vivo* after direct implantation of hepatocyte-like cells, derived from ASCs using the subject methods, into the liver. The SCi-Heps (iHeps induced using the subject methods) were produced from ASCs by *in vitro* differentiation for a short period in chemically defined media. SCi-Heps exhibited many of the *in vitro* functional

properties of mature hepatocytes, and they were able to stably reconstitute functioning human liver *in vivo* in a murine model system, and implantation studies demonstrated that SCi-Heps have a very low malignant potential. Thus, readily-accessible stem cells were used to rapidly build functioning human liver tissue *in vivo*. The time to generate iHeps from ASCs is greatly reduced by the subject methods to under 13 days (e.g., 9 days). In addition, the efficiency of iHep production is greatly increased by the subject methods to greater that 10% (e.g., 37%). Both of these features facilitate treatment methods because liver damage (e.g., liver failure, e.g., due to acetaminophen toxicity) can rapidly cause death (e.g., in 2 weeks or less).

These procedures can be scaled to enable autologous liver regeneration in humans. Since there are ~140 x 10^6 hepatocytes per gram of human or mouse liver tissue; a 1500 gram adult human liver has ~2 x10^{11} hepatocytes, while a 1.8 gram mouse liver has 2.5 x 10^8 cells. After 1 million human hepatocytes are transplanted into a TK-NOG mouse, ~50% replacement of the mouse hepatocytes is routinely obtained, which amounts to ~1.2 x 10^8 functioning human hepatocytes in the mouse liver. This represents a 120-fold increase (or 7 population doublings) in the number of human hepatocytes generated *in vivo* relative to the number of transplanted cells. If a similar regeneration efficiency is achieved in humans, 800 million SCi-Heps (10^{11}/120) would be transplanted to replace 50% of the cells in a human liver. Of relevance to this question, 1 liter (1000 g) of lipo-aspirate can easily be obtained from a single liposuction procedure. This volume is estimated to contain 2-10 x 10^9 cells, of which 1-10% are estimated to be ASCs. Thus, one liter of lipo-aspirate contains between 2 x 10^7 and 10^9 ASCs, which can provide a sufficient number of cells to enable autologous human liver replacement. Human liver regenerative procedures, including ultrasound-guided direct liver Implantation (used in the examples below), are scalable and appropriate for human clinical use.

The examples below demonstrate that spherical culture coupled with a chemical differentiation process can increase the yield of SCi-Heps by 7-fold compared to other known methods. Thus, the spherical culture method helps to ensure that a sufficient number of SCi-Heps can be obtained to enable liver regeneration in a human subject. The spherical culture method also reduces the culture volume by 12-fold and decreases the time in culture by 40%; these factors dramatically reduce the cost of differentiating ASC into iHeps. The estimated cost of the medium and growth factors required for producing Chi-Heps is ~$49,000 per liter of lipo-aspirate processed, but the subject method reduces these costs by over 20-fold (to ~$2400 per liter of lipo-aspirate processed).

The fact that implanted SCi-Heps did not form tumors (see the Examples below) indicates that these cells have a markedly reduced risk of causing tumors. In contrast to the

results obtained with SCi-Heps, readily palpable tumors were noted within 3 weeks after implantation of iPS-Heps. The rapid tumor onset should not be surprising, since this iPS-derived cell population has a large percentage of un-differentiated cells. However, ~10^9 cells must be transplanted to regenerate a human liver, but even as few as 100-1000 cells with malignant potential in this population could cause tumor formation. These numbers create a very significant hurdle for any selective method used to prepare iPS-derived cells for liver regeneration. The fact that implanted SCi-Heps did not form tumors indicates that these cells have a markedly reduced risk of causing tumors to form. Since gancyclovir-induced liver damage in genetically engineered TK-NOG mice resembles the acute liver failure in humans that is caused by exogenous toxicants or drug overdose, methods disclosed here can provide cells that can be used to treat a damaged liver (e.g., a patient with acute liver failure).

Hepatocyte-like cells produced by the subject methods provide a source of donor cells for cell replacement in damaged livers. As such, the hepatocyte-like cells may be used for tissue reconstitution or regeneration in a human patient, an individual in need of such treatment, and/or for the reconstitution of human liver tissue in a non-human animal (e.g., a mouse, which can then be used, for example, for research purposes including drug screening, drug testing, etc.). The cells are administered in a manner that permits them to graft or migrate to the intended tissue site and reconstitute or regenerate the functionally deficient area.

The differentiated cells of this disclosure can also be used to prepare antibodies that are specific for markers of hepatocytes. Polyclonal antibodies can be prepared by injecting a vertebrate animal with cells of this disclosure in an immunogenic form. Production of monoclonal antibodies is described in such standard references as U.S. Pat. Nos. 4,491,632, 4,472,500 and 4,444,887, and Methods in Enzymology 73B:3 (1981). Specific antibody molecules can also be produced by contacting a library of immunocompetent cells or viral particles with the target antigen, and growing out positively selected clones. See Marks et al., New Eng. J. Med. 335:730, 1996, and McGuiness et al., Nature Biotechnol. 14:1449, 1996. A further alternative is reassembly of random DNA fragments into antibody encoding regions, as described in EP patent application 1,094,108 A.

Gene expression may be examined before, during, and/or after the production of hepatocyte-like cells by the subject methods. The expressed set of genes may be compared against other subsets of cells, against progentior cells, against terminally differentiated hepatocytes, against ASCs, and the like, as known in the art. Any suitable qualitative or quantitative methods known in the art for detecting specific mRNAs can be used. mRNA can be detected by, for example, hybridization to a microarray, next-

generation sequencing, *in situ* hybridization, by reverse transcriptase-polymerase chain reaction (rtPCR), or in Northern blots containing poly A mRNA. One of skill in the art can readily use these methods to determine differences in the size or amount of mRNA transcripts between two samples.

Any suitable method for detecting and comparing mRNA expression levels in a sample can be used in connection with the methods of the disclosure. For example, the mRNA from a sample can be sequenced via next-generation sequencing methods known in the art such as nanopore sequencing (e.g. as described in Soni et al Clin Chem 53: 1996-2001 2007, or as described by Oxford Nanopore Technologies), Illumina's reversible terminator method, Roche's pyrosequencing method (454), Life Technologies' sequencing by ligation (the SOLiD platform) or Life Technologies' Ion Torrent platform. Examples of such methods are described in the following references: Margulies et al (Nature 2005 437: 376–80); Ronaghi et al (Analytical Biochemistry 1996 242: 84–9); Shendure (Science 2005 309: 1728); Imelfort et al (Brief Bioinform. 2009 10:609-18); Fox et al (Methods Mol Biol. 2009;553:79-108); Appleby et al (Methods Mol Biol. 2009;513:19-39) and Morozova (Genomics. 2008 92:255-64), which are incorporated by reference for the general descriptions of the methods and the particular steps of the methods, including all starting products, reagents, and final products for each of the steps.

Alternatively, gene expression in a sample can be detected using hybridization analysis, which is based on the specificity of nucleotide interactions. Oligonucleotides or cDNA can be used to selectively identify or capture DNA or RNA of specific sequence composition, and the amount of RNA or cDNA hybridized to a known capture sequence determined qualitatively or quantitatively, to provide information about the relative representation of a particular message within the pool of cellular messages in a sample. Hybridization analysis can be designed to allow for concurrent screening of the relative expression of hundreds to thousands of genes by using, for example, array-based technologies having high density formats, including filters, microscope slides, or microchips, or solution-based technologies that use spectroscopic analysis (e.g., mass spectrometry).

Hybridization to arrays may be performed, where the arrays can be produced according to any suitable methods known in the art. For example, methods of producing large arrays of oligonucleotides are described in U.S. 5,134,854, and U.S. 5,445,934 using light-directed synthesis techniques. Using a computer controlled system, a heterogeneous array of monomers is converted, through simultaneous coupling at a number of reaction sites, into a heterogeneous array of polymers. Alternatively, microarrays are generated by deposition of pre-synthesized oligonucleotides onto a solid substrate, for example as described in PCT published application no. WO 95/35505.

Methods for collection of data from hybridization of samples with an array are also well known in the art. For example, the polynucleotides of the cell samples can be generated using a detectable fluorescent label, and hybridization of the polynucleotides in the samples detected by scanning the microarrays for the presence of the detectable label. Methods and devices for detecting fluorescently marked targets on devices are known in the art. Generally, such detection devices include a microscope and light source for directing light at a substrate. A photon counter detects fluorescence from the substrate, while an x-y translation stage varies the location of the substrate. A confocal detection device that can be used in the subject methods is described in U.S. Patent no. 5,631,734. A scanning laser microscope is described in Shalon *et al.*, *Genome Res.* (1996) 6:639. A scan, using the appropriate excitation line, is performed for each fluorophore used. The digital images generated from the scan are then combined for subsequent analysis. For any particular array element, the ratio of the fluorescent signal from one sample is compared to the fluorescent signal from another sample, and the relative signal intensity determined.

Methods for analyzing the data collected from hybridization to arrays are well known in the art. For example, where detection of hybridization involves a fluorescent label, data analysis can include the steps of determining fluorescent intensity as a function of substrate position from the data collected, removing outliers, i.e. data deviating from a predetermined statistical distribution, and calculating the relative binding affinity of the targets from the remaining data. The resulting data can be displayed as an image with the intensity in each region varying according to the binding affinity between targets and probes.

Pattern matching can be performed manually, or can be performed using a computer program. Methods for preparation of substrate matrices (e.g., arrays), design of oligonucleotides for use with such matrices, labeling of probes, hybridization conditions, scanning of hybridized matrices, and analysis of patterns generated, including comparison analysis, are described in, for example, U.S. 5,800,992.

In vitro induced hepatocyte-like cells (iHeps) produced by the subject methods provide also provide a source of cells for novel hepatic drug discovery, development, and safety testing. The use of in vitro iHeps produced by the subject methods offers the pharmaceutical industry an invaluable tool for preclinical screening of candidate drugs to treat cardiomyopathy, arrhythmia, and heart failure, as well as therapeutics to combat secondary cardiac toxicities. The development of new screens using In vitro iHeps produced by the subject methods should reduce the time and cost of bringing new drugs to market.

In screening assays for biologically active agents (e.g., small molecule compounds, peptides, viruses, *etc.*) of the subject hepatocyte-like cells, usually a culture comprising the subject hepatocyte-like cells, is contacted with the agent of interest, and the effect of the agent assessed by monitoring output parameters, such as expression of markers, cell viability, drug metabolism, and the like.

Agents of interest for screening include known and unknown compounds that encompass numerous chemical classes, primarily organic molecules, which may include organometallic molecules, inorganic molecules, genetic sequences, etc. An important aspect of the disclosure is to evaluate candidate drugs, including toxicity testing; and the like.

In addition to complex biological agents, such as viruses, candidate agents include organic molecules comprising functional groups necessary for structural interactions, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, frequently at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules, including peptides, polynucleotides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Included are pharmacologically active drugs, genetically active molecules, etc. Compounds of interest include chemotherapeutic agents, hormones or hormone antagonists, etc. Exemplary of pharmaceutical agents suitable for this disclosure are those described in, "The Pharmacological Basis of Therapeutics," Goodman and Gilman, McGraw-Hill, New York, New York, (1996), Ninth edition, under the sections: Water, Salts and Ions; Drugs Affecting Renal Function and Electrolyte Metabolism; Drugs Affecting Gastrointestinal Function; Chemotherapy of Microbial Diseases; Chemotherapy of Neoplastic Diseases; Drugs Acting on Blood-Forming organs; Hormones and Hormone Antagonists; Vitamins, Dermatology; and Toxicology, all incorporated herein by reference.

Test compounds include all of the classes of molecules described above, and may further comprise samples of unknown content. Of interest are complex mixtures of naturally occurring compounds derived from natural sources such as plants. While many samples will comprise compounds in solution, solid samples that can be dissolved in a suitable solvent may also be assayed. Samples of interest include manufacturing samples, pharmaceuticals, libraries of compounds prepared for analysis, and the like. Samples of interest include compounds being assessed for potential therapeutic value, i.e. drug candidates.

Compounds, including candidate agents, are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds, including biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

Agents are screened for biological activity by adding the agent to at least one and usually a plurality of cell samples, usually in conjunction with cells lacking the agent. The change in parameters in response to the agent is measured, and the result evaluated by comparison to reference cultures, e.g. in the presence and absence of the agent, obtained with other agents, etc.

The agents are conveniently added in solution, or readily soluble form, to the medium of cells in culture. The agents may be added in a flow-through system, as a stream, intermittent or continuous, or alternatively, adding a bolus of the compound, singly or incrementally, to an otherwise static solution. In a flow-through system, two fluids are used, where one is a physiologically neutral solution, and the other is the same solution with the test compound added. The first fluid is passed over the cells, followed by the second. In a single solution method, a bolus of the test compound is added to the volume of medium surrounding the cells. The overall concentrations of the components of the culture medium should not change significantly with the addition of the bolus, or between the two solutions in a flow through method.

Preferred agent formulations do not include additional components, such as preservatives, that may have a significant effect on the overall formulation. Thus preferred formulations consist essentially of a biologically active compound and a physiologically acceptable carrier, e.g. water, ethanol, DMSO, etc. However, if a compound is liquid without a solvent, the formulation may consist essentially of the compound itself.

A plurality of assays may be run in parallel with different agent concentrations to obtain a differential response to the various concentrations. As known in the art, determining the effective concentration of an agent typically uses a range of concentrations resulting from 1:10, or other log scale, dilutions. The concentrations may be further refined with a second series of dilutions, if necessary. Typically, one of these concentrations

serves as a negative control, i.e. at zero concentration or below the level of detection of the agent or at or below the concentration of agent that does not give a detectable change in the phenotype.

The cells may be freshly isolated, cultured, genetically altered as described above, or the like. The cells may be environmentally induced variants of clonal cultures: *e.g.* split into independent cultures and grown under distinct conditions, for example with or without virus; in the presence or absence of other biological agents. The manner in which cells respond to an agent, particularly a pharmacologic agent, including the timing of responses, is an important reflection of the physiologic state of the cell.

Parameters are quantifiable components of cells, particularly components that can be accurately measured, desirably in a high throughput system. A parameter can be any cell component or cell product including cell surface determinant, receptor, protein or conformational or posttranslational modification thereof, lipid, carbohydrate, organic or inorganic molecule, nucleic acid, *e.g.* mRNA, DNA, *etc.* or a portion derived from such a cell component or combinations thereof. While most parameters will provide a quantitative readout, in some instances a semi-quantitative or qualitative result will be acceptable. Readouts may include a single determined value, or may include mean, median value or the variance, *etc.* Characteristically a range of parameter readout values will be obtained for each parameter from a multiplicity of the same assays. Variability is expected and a range of values for each of the set of test parameters will be obtained using standard statistical methods with a common statistical method used to provide single values.

For further elaboration of general techniques useful in the practice of this disclosure, the practitioner can refer to standard textbooks and reviews in cell biology, tissue culture, and embryology. With respect to tissue culture and stem cells, the reader may wish to refer to Teratocarcinomas and embryonic stem cells: A practical approach (E. J. Robertson, ed., IRL Press Ltd. 1987); Guide to Techniques in Mouse Development (P. M. Wasserman et al. eds., Academic Press 1993); Embryonic Stem Cell Differentiation in Vitro (M. V. Wiles, Meth. Enzymol. 225:900, 1993); Properties and uses of Embryonic Stem Cells: Prospects for Application to Human Biology and Gene Therapy (P. D. Rathjen et al., Reprod. Fertil. Dev. 10:31, 1998).

KITS

Also provided are kits for use in the subject methods. The subject kits include any combination of components for used in stage 1 and/or stage 2 media. For example, in some cases, a kit includes a Wnt signaling agonist (e.g., Wnt3a), activin A, an FGF (e.g., FGF4,

FGF5, FGF6, etc.), and HGF. In some embodiments, a kit can further include oncostatinM, dexamethasone, and/or dimethyl sulfoxide. In some embodiments, a subject kit includes a basal media (e.g., RPMI 1640). In some embodiments, a subject kit includes an antibody specific for a hepatocyte marker protein for use in a verifying step (or an enrichment step). In some such embodiments, the hepatocyte marker protein is selected from the group consisting of: glucose-6-phosphatase, albumin (ALB), alpha-1-antitrypsin (AAT, also known as SERPINA1), cytokeratin 8 (CK8), cytokeratin 18 (CK18), cytokeratin 8/18 (CK8/18), asialoglycoprotein receptor 1 (ASGR1), alcohol dehydrogenase 1, arginase Type I, cytochrome p450 3A4 (CYP3A4), liver-specific organic anion transporter (LST-1), forkhead box protein A2 (FoxA2), alpha-fetoprotein (AFP), tryptophan 2,3-dioxygenase (TDO2), and a combination thereof. In some embodiments, a subject kit includes assay reagents (e.g, for measuring bile, albumin, and/or urea; for performing hepatocyte assays such as LDL endocytosis assays, glycogen synthesis assays, cytochrome P450 1A2 detoxification activity assays; and the like) for use in a verifying step.

In addition to the above components, the subject kits may further include (in certain embodiments) instructions for practicing the subject methods. These instructions may be present in the subject kits in a variety of forms, one or more of which may be present in the kit. One form in which these instructions may be present is as printed information on a suitable medium or substrate, e.g., a piece or pieces of paper on which the information is printed, in the packaging of the kit, in a package insert, and the like. Yet another form of these instructions is a computer readable medium, e.g., diskette, compact disk (CD), flash drive, and the like, on which the information has been recorded. Yet another form of these instructions that may be present is a website address which may be used via the internet to access the information at a removed site.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., room temperature (RT); base pairs (bp); kilobases (kb); picoliters (pl); seconds (s or sec); minutes (m or min); hours (h or

hr); days (d); weeks (wk or wks); nanoliters (nl); microliters (ul); milliliters (ml); liters (L); nanograms (ng); micrograms (ug); milligrams (mg); grams ((g), in the context of mass); kilograms (kg); equivalents of the force of gravity ((g), in the context of centrifugation); nanomolar (nM); micromolar (uM), millimolar (mM); molar (M); amino acids (aa); kilobases (kb); base pairs (bp); nucleotides (nt); intramuscular (i.m.); intraperitoneal (i.p.); subcutaneous (s.c.); and the like.

Disclosed here is an efficient, high-yield, cost-effective method for producing hepatocyte-like cells from adipose-derived stem cells. The hepatocyte-like cells produced by this method have many of the functional properties of mature hepatocytes *in vitro*, and can stably reconstitute a functioning human liver *in vivo* in a murine model system. The following materials and methods were used for Examples 1 and 2 below.

Materials and Methods

ASC preparation and differentiation. Lipo-aspirates were obtained as de-identified samples from human donors undergoing liposuction at Stanford University Medical Center according to protocol that was approved by the Stanford University Medical Center IRB. ASC were prepared from these samples as described (Banas et al, J Gastroenterol Hepatol. 2009 Jan;24(1):70-7, which is hereby incorporated by reference in its entirety). The ASC were cultured in MESENPRO RS™ Medium (Gibco, 12746-012), passaged at 90% confluence at a 1:4 ratio, and the medium was changed every other day. For preparation of Chi-Heps, after 2-5 passages, the ASC cells were plated on Martrigel (BD Biosciences, Cat: 354277) coated dishes. After the cells reached 50% confluence, endodermal trans-differentiation was induced over 3 days of culture in the stage 1 medium: RPMI1640 (Gibco, Cat: 12633-012) supplemented with 100 ng/mL ActivinA (R&D, 338-AC-010), 50ng/mL Wnt3a (StemRD, Cat: W3A-H-100), 20 ng/mL FGF4 (PeproTech, Cat: 100-31) and 20% B27 (Gibco Cat: 17504-044). Hepatic differentiation was then induced over a 13-15 day period by culture in the stage 2 medium: hepatocyte culture medium (HCM) (Lonza, Cat: cc-3198) supplemented with 150 ng/mL hepatocyte growth factor (HGF) (PeproTech, Cat: 100-39), 25 ng/mL FGF4, 30 ng/mL oncostatinM (OSM, PeproTech, Cat: 300-10), $2X10^{-5}$ M dexamethasone (Dex, Sigma, Cat: D4902), and 0.1% DMSO (Sigma, Cat: C6164). The cells were then cultured in HCM alone for 2 days prior to transplantation into TK-NOG mice.

Preparation of SCi-Hep. Primary ASC were isolated from lipo-aspirates and suspended in MesenPRO RS™ Medium (Gibco, Cat: 12746-012) at $5x10^4$ cells/ml. Then, the cells were cultured by the hanging drop method, which resulted in the formation of

spherical cellular aggregates ('spheres') that resembled embryoid bodies. Briefly, ASC were plated as individual drops (each ~40 ul) in rows on 150 mm petri dishes (BD Biosciences, Cat 354551) using an 8-channel pipette. After two days, the dishes were rinsed with PBS, and the spheres were collected by centrifugation at 150 x g at 37°C, re-suspended at 30 spheres/ml in the stage 1 medium, and seeded on matrigel-coated dishes (BD Biosciences, Cat: 354262). The sphere derived cells were then induced to differentiate into hepatocytes by a 2-stage process. Endodermal transdifferentiation was induced over a 2-day period by culturing the spheres in the stage 1 medium. Hepatic differentiation was induced over the subsequent 2-9 day period by culture in the stage 2 medium. The cells were then cultured in HCM alone for 2 days prior to transplantation into TK-NOG mice.

Preparation of iPS-Heps. ASCs were transfected with Lentivirus encoding Oct4, Sox2, Klf4 and c-MYC to generate iPS cells. Four days after infection, the cells were re-plated at 5x104 cells per 100-mm matrigel (BD Biosciences, Cat: 354277) coated dish in mTeSR-1 medium (StemCell Technologies Inc, Cat: 05850), and the media was changed every other day. Fifteen days after infection, individual colonies were manually picked, and passaged onto new matrigelcoated dishes. After the cells reached 50% confluence, a three-stage hepatic differentiation protocol was then used. (i) iPS cells were cultured for 4 days in the stage 1 medium. (ii) Over the next 7 days, the cells were induced in hepatocyte progenitor (HP) medium containing RPMI1640 (Gibco, Cat: 12633-012) with 20% B27, 20 ng/ml BMP4 (Pepro Tech, Cat: 120- 05ET) and 20 ng/ml FGF4 (PeproTech, Cat: 100-31). (iii) The cells were then placed for 15 days in a hepatocyte maturation (HM) medium, which had hepatocyte growth medium (Lonza, Cat: cc-3198) with 10 ng/ml HGF (PeproTech, Cat: 100-39), 10 ng/ml Oncostatin M (OSM, PeproTech, Cat: 300-10) and 0.1uM dexamethasone (Dex, Sigma, Cat: D4902). The iPS cells used for differentiation were between passages 10 and 20.

Immunofluorescence staining. Cells were fixed in 4% paraformaldehyde for 10 min, followed by blocking with 10% chicken serum for 30 min. After washing 3 times in PBS with 0.05% Triton X- 100) (TPBS), the cells were incubated with anti-human albumin (Bethyl Laboratories, Cat: A80- 129A, 1:100) or anti-human CK8/18 (abcam, Cat: ab17139, 1:100) primary antibodies overnight at 4°C, and then washed three times with TPBS. Alexa Fluor 488 (Invitrogen, Cat: A21200, 1:1000) or Alexa Fluor 594 (Invitrogen, Cat: A21201, 1:1000)-conjugated secondary antibodies were then applied for one hour in the dark. Nuclear staining was assessed using 4,6-diamidino- 2-phenylindole (DAPI, Sigma, Cat: D9542). The images were acquired using a Nikon Eclipse Ni- E imaging system.

For assessment of ASGR1 expression *in vitro*, the cells were permeablized with 0.2% Triton X- 100 for 10 min followed by washing three times in PBS with 0.05% Triton X-

100 (TPBS). The cells were pre-incubated with 10% chicken serum for 30 min, followed by incubation with a 1:50 dilution of Anti-ASGR1 antibody (Sigma-Aldrich cat #: HPA012852) primary antibody overnight at 4°C, and then washed three times with TPBS. Then, a 1:1000 dilution of Alexa Fluor 488 (Invitrogen, Cat #: A21200,)-conjugated secondary antibody was applied for one hr in the dark. Nuclear staining was assessed using 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Cat#: D9542). The images were acquired using a Nikon Eclipse Ni-E imaging system.

Periodic Acid-Schiff (PAS) Staining. PAS staining was performed according to the manufacturer's (Sigma, 395B) instructions. Briefly, the cells were fixed with 4% paraformaldehyde for 1 min. After rinsing with distilled water, the cells were stained with PAS solution for 5 min, then washed with distilled water, and stained using Schiff's reagent for 15 min. The cells were then washed once with water and counter-stained in hematoxylin solution for 90 sec; followed by rinsing 3 times with distilled water prior to analysis.

LDL endocytosis. LDL uptake was assessed using the Dil-LDL assay (Biomedical Technologies Inc., BT-904) according to the manufacturer's instruction. The cells were pre-incubated in serum-free medium with 1% BSA for 24 hr. Then 10ug/mL Dil-LDL was added for at least five hours at 37°C. After washing 3 times with DPBS, the cells were imaged using were imaged using Nikon Eclipse Ni-E imaging system.

Flow cytometry. CK8/18 expression was analyzed by flow cytometry (FACS, Aida, Software Flowjo). The cells were first blocked with an Fc receptor-blocking reagent (Miltenyi Biotech, Germany) according to the manufacturer's instructions, and then stained with primary antibody anti-human CK8/18 (abcam, Cat: ab17139, 1:200) for 30 min at 4°C, which was followed by incubation with a secondary antibody Alexa Fluor 488 (Invitrogen, Cat: A21200, 1:1000). Appropriately diluted isotype-matched antibodies (Ebioscience) were used as controls. The data from 10,000 analyzed events were stored and analyzed.

Human albumin production. Human albumin production was evaluated using enzyme linked immunosorbent assay (ELISA, E80-129; Bethyl Laboratories, Montgomery, TX, USA), which uses a human-specific antibody that does not cross-react with mouse albumin. Briefly, cell culture supernatants were collected on day 0 and every three days afterwards. The supernatants were concentrated using Amicon® Ultra-4 centrifugal filter units (EMD Millipore, UFC810024). Blood samples were first centrifuged at 2000 g for 5 min to isolate sera, and then diluted at 1:10000 for assay. Each data point is the mean±SE of 4 biologically independent samples analyzed.

Urea production and CYP450 activity. Cellular urea production was detected in un-diluted cell culture supernatants using a urea assay kit (abcam, ab83362) according to the manufacturer's instructions. Cellular CYP450 activity was measured using the P450-GloTM

CYP3A4 assay (Promega, Cat: V9002) according to the manufacturer's instructions. In brief, the cells were cultured with medium containing a luminogenic CYP substrate at 37°C for 60 min. Then, 25 µl of supernatant was transferred to 96-well opaque white luminometer plate at room temperature, and 25 µl of luciferin detection reagent was added to initiate the luminescent reaction. After a 20 min incubation, luminescence was measured using an IVIS 100 Imaging System.

RT-PCR analysis. Total RNA was extracted using RNeasy Plus kit (Qiagen, Cat: 74134) and (0.5µg) was reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Cat # 11754-050) according to the manufacturer's guidelines. PCR analyses were performed using the following TaqMan gene expression assays: FOXA2 (Hs00232764_m1), AFP (Hs00173490_m1), ALB (Hs99999922_s1), AAT (Hs01097800_m1), A1AT (Hs00165475_m1), TDO2 (Hs00194611_m1), CD37 (Hs01099648_m1), and CD29 (Hs00559595_m1), and CD105 (Hs00923996_m1). For statistical analysis of the RT-PCR data, the measured expression levels for each of the analyzed genes on the indicated day were normalized relative to its level of expression in ASC. Then, a one-sample t-test was applied using the log-transformed expression level to assess the statistical significance of the expression changes.

Microarray analysis of gene expression. Total RNA was harvested and separately processed from 3 independently prepared cultures of each cell type according to the manufacturer's instructions. In brief, cells were detached from the dish using the StemPro Accutase® Cell Dissociation Reagent (Invitrogen, Cat: A11105-01). RNA was extracted using the RNeasy Mini Kit (QIAGEN, Cat: 74104), and 10 ug RNA was labeled, and hybridized to Affymetrix Human Genome U219 Arrays, and processed according to the manufacturer's instructions. The resulting image files were analyzed using Affymetrix Microarray Analysis Suite version 5.0 software. Three biological replicates were analyzed for ASC, Chi-Hep, SCi-Hep, iPS-Hep and hepatocyte cells. The probe intensity data generated from all arrays were read into the R software environment (“”"www." followed by “R-project.org”, version 2.15.1) directly from the .CEL files using the R/affy package (Gautier et al, Bioinformatics. 2004 Feb 12;20(3):307-15), which was also used to extract and manipulate probe level data to assess data quality and to create expression summary measures. Normalization was carried out using the robust multiarray average (RMA) method (Irizarry et al, Biostatistics. 2003 Apr;4(2):249-64) to generate one expression measure for each probe set on each array. Student's t-test was applied to identify the expression changes in iHep, iPS-Hep and hepatocyte cells as compared to the ASC cells. The empirical Bayes method (Smyth et al, Stat Appl Genet Mol Biol. 2004;3:Article3) was applied to adjust the standard deviation estimation for each probe, and a multipletesting

adjustment was applied to generate the final the p-values using the R/limma package. Gene expression changes meeting pre-determined criteria (fold-change > 2 and adjusted pvalue <0.01) were selected for further analysis.

For illustration of the spatial relationship of the gene expression profiles of these cells, we defined the distance between any pair of array data as the squared root of the sum of the squares of the difference in the level of expression for each gene on the microarray:

$$\mathrm{Dist}(\mathrm{Array1}, \mathrm{Array2}) = \sqrt{\sum_{i=1}^{N} (E_{1i} - E_{2i})^2}$$

where N represents the total number of probes on the array and E_{1i} and E_{2i} present the log2 of the normalized expression level for probe i on array 1 and 2, respectively. In other words, the distance for a pair of profiles is the Euclidean distance between the two profiles treated as a vector of size N. Since there are 3 replicated arrays for each cell type, we defined the distance between any two cell types as the average of the distances measured when each of the 3 arrays for one cell type was compared with each of the 3 arrays for the other cell type (which produces a total of 9 array pairs) The 2-dimensional illustration of the spatial relationship among the 4 cell types was created by putting two triangles, which share the common ASC-hepatocyte axis next to each other. The length of each side of a triangle is proportional to the distance between the cell types at the indicated vertices.

Principle component analysis (PCA) was also used to visualize the relatedness of the overall patterns of the gene expression in the different types of cells analyzed. In brief, the dimension that explained the largest amount of the variation in the profiles was identified, and this became the 1st principle component (PC) shown on the x-axis. Then the dimension that was orthogonal to PC 1 and explained the largest amount of the remaining variation was identified, which became the 2nd PC shown on the y-axis. As a result, the first two components captured the majority of the variation in the data. Therefore, visualization could be focused on these components to reduce dimension without losing a significant amount of information present in the original data.

Preparation of TK-NOG mice. All animal experiments were performed according to protocols approved by the Stanford Institutional Animal Care and Use Committee. To prepare TK-NOG mice for transplantation, 8-10 week old TK-NOG mice were treated (i.p.) with 25 mg/kg ganciclovir (GCV) on day -7 and -5 prior to transplantation. Then, a 20 µl blood sample was collected 6 days after the 1st GCV treatment, diluted 1:3 into water, and 10 µl of the diluted sample was used to measure the serum ALT level using a Fuji dry-chem 7000 instrument as described (Hasegawa et al, Biochem Biophys Res Commun. 2011 Feb 18;405(3):405-10, which is hereby incorporated by reference in its entirety). Only mice with

an ALT level >200 U/L were used for cellular transplantation on day 7. Mice with ALT levels <200 U/L were treated with an additional dose of 25mg/kg GCV on day 7, the ALT level was re-examined on day 13, only mice whose repeat ALT level was >200 U/L were used for cell transplantation on day 14.

Ultrasound-guided liver injection. Transplanted cells were directly placed into a liver lobe under ultrasound-guided injection using a small animal ultrasound system Vevo 2100 (Visualsonics Canada). Brightness mode (B-mode) was used to acquire two-dimensional images for an area of interest with MS550s transducer. The mice were placed under 1.5% isofluorane anesthesia during this procedure using a single animal vaporizer unit (EZ-Systems Corp, EZ-108SA). Then, $5x10^6$ cells were suspended in 200 µl of William's E medium (Invitrogen, A12176-01), which was injected into 10 distinct sites in the liver using a 30G needle.

Analysis of tumor formation. To assess tumor formation, $5x10^4$ Chi-Hep, SCi-Hep or iPS-Hep were harvested and mixed with 50 µl of matrigel (BD Biosciences, Cat: 354277). NOG mice were anesthetized using 1.5% isoflurane using individual vaporizer unit (EZ-Systems Corp, EZ- 108SA). An incision was made, and a slight pressure to both sides of the incision was applied to expose the kidney. The cells were then injected under the kidney capsule using a syringe with a 27-gauge needle. After slowly delivering the cells, a dry swab was placed over the injection site to prevent leakage. During the procedure, the kidney was kept moist by application of saline with a cotton-tipped swab. After three to 8 weeks, the mice were sacrificed and the injected kidney was harvested for histological analysis.

Results

Example 1 – *Production of hepatocyte-like cells from adipocyte-derived stem cells*

Provided is a method for differentiating adipocyte-derived stem cells (ASCs) into hepatocyte-like cells (iHeps) (**Figure 1A**). ASCs are first cultured using the 'hanging drop' method to produce spherical cellular aggregates ('spheres') (**Figure 1B**). As shown by analysis of SRY-related HMGbox transcription factor 17 (Sox17) expression, spherical culture doubles the number of ASCs in a lipo-aspirate that differentiate into endodermal precursor cells (**Figure 5**). Since the ability of mesenchymal-derived ASCs to differentiate into endoderm may be rate limiting for hepatocyte generation, *wnt3*a was added to the (stage 1) differentiation medium. Relative to a previous described method of generating iHeps (Banas et al., J Gastroenterol Hepatol. 2009 Jan;24(1):70-7), the subject method produces a 2.3-fold increase in the number of ASCs obtained from a lipo-aspirate and a 3-fold increase in the efficiency (% of hepatocyte-like cells obtained, as assayed using the

CK8/18 marker) after the 2-stage differentiation process is completed (**Figure 1C** and **Table 1**). This method increases the number of iHeps obtained from a liter of lipo-aspirate by 7-fold and reduces the period of in *vitro* culture required to obtain biochemically defined hepatocytes at >37% purity to 9 days or less. Like Chi-Heps, the cells produced by spheroid culture, which we refer to as **SCi-Heps**, developed a hepatocyte-like morphology (**Figure 1B**), and exhibited many properties of mature hepatocytes. They expressed proteins (CK8/18) that are found on hepatocytes (**Figure 1C**), and had multiple metabolic properties of hepatocytes, including: LDL endocytosis, glycogen synthesis (**Figure 1D**), albumin secretion, and urea production (**Figure 1E**). Moreover, SCi-Heps expressed multiple hepatocyte specific mRNAs, and had markedly reduced levels of expression of multiple adipocyte-specific mRNAs (**Figure 2A**), along with reduced expression of an ASC-specific cell surface protein (CD105) (**Figure 6**; **Figure 12**).

Table 1. ASCs prepared from 3 different donors were induced to differentiate into Chi-Heps using a previous method (Banas et al., J Gastroenterol Hepatol. 2009 Jan;24(1):70-7) or into SCi-Heps (Spherical Culture iHeps) using the subject method. The number of ASCs obtained after 3 days (± SEM), the percentage of cells (± SEM) expressing a hepatocyte marker (CK8/18+) after differentiation for 12 (Chi-Hep) or 9 (SCi-Hep) days, the number of days required to complete the hepatocyte differentiation process and the estimated number of iHeps obtained per liter of lipo-aspirate are shown.

Table 1

Method	Day 3 Cell #	% CK8/18$^+$	Days of Differentiation	Days Total	Estimated Cells/Liter
Chi-Hep	(2.7 ± 0.3) x 10^4	12.3 ± 2.8	12	18-30	3.3 x 10^8
SCi-Hep	(6.1 ± 0.2) x 10^4	37.7 ± 7.7	9	12	2.3 x 10^9

We also compared the properties of Chi- and SCi-Heps with iPS-Heps, which are ASCs (obtained from the same donor) that were re-programed into iPS cells after transfection of four genes *(Oct4, Sox2, Klf4 and c-Myc*), and then induced to differentiate into hepatocytes using the Ochiya protocol (Banas et al., J Gastroenterol Hepatol. 2009 Jan;24(1):70-7). The iPS-Heps expressed a protein (CK8/18) found on hepatocytes (**Figure 1C**); could endocytose LDL, synthesize glycogen (**Figure 7**) secrete albumin, produce urea, and had CYP450 activity (**Figure 8**). Of importance, SCi-Heps produced albumin and urea after only 3 days of in vitro differentiation, which was 3-6 days before Chi-Heps (**Figure 1E**)

and 12 days before iPS-Heps (**Figure 8**) produced these analytes. ASCs must first be reprogramed into and then exit from the pluripotent state before they can differentiate into iPSHeps. In contrast, SCi-Heps are produced by direct differentiation of ASCs into endoderm, which explains why they can more quickly produce these analytes. Consistent with the more rapid differentiation process, SCi-Heps expressed mRNAs for endodermal (epithelial cell adhesion molecule, Epcam) and hepatocyte-specific (Albumin, Foxa2) genes within 3 days after initiation of hepatic differentiation (**Figure 2B**). Also, SCi-Heps had the highest level of albumin production (on a per cell basis) among the 3 types of ASC-derived cells tested. Their increased level of albumin production is consistent with the FACS results indicating that 37% of SCi-Heps expressed a mature hepatocyte marker, while only ~12% and 20% of Chi-Heps and iPS-Heps, respectively, expressed this marker (**Figure 1C**). Thus, the SCi-Heps method produces an increased number of hepatocyte like cells from a lipoaspirate, and the cells are prepared within a timeframe that makes it possible that they could be used in an acute clinical situation, such as would occur after an overdose of acetaminophen.

To further characterize these cells, gene expression profiling was performed in ASCs, Chi-Heps, SCi-Heps, iPS-Heps and hepatocytes using microarrays, and the data analysis is described in the supplemental information. In brief, multiple comparisons indicated that in vitro differentiation significantly altered the gene expression pattern in ASCs, and that Chi- and SCi-Heps expressed a very large number of hepatocyte-specific genes (e.g,. as published in Table S2 in Xu et al., Cell Transplantation, 2013 Oct 21; DOI: 10.3727/096368913X673432; "Enabling Autologous Human Liver Regeneration With Differentiated Adipocyte Stem Cells"; the entirety of which, including figures, tables, supplementary figures, supplementary tables, etc., is hereby incorporated by reference). Moreover, two analyses (a space diagram of the gene expression differences (**Figure 2C**) and principal component analysis (**Figure 9**)) indicate that the Chi- and SCi-Heps had a gene expression profile that was closer to that of hepatocytes than the iPS-Hep profile. iPS-Heps expressed a larger number of genes that were not expressed in adipocytes or hepatocytes (**Figure 11**). In summary, SCi- Heps have a gene expression pattern that resembles, but it does not fully mirror, that of hepatocytes. Since only 37% of the Sci-Hep cells expressed a mature hepatocyte marker (**Figure 1C**), it is possible that the gene expression pattern in fully differentiated SCi-Heps could more closely resemble that of hepatocytes than is suggested by this analysis, since some of the gene expression changes could be masked (diluted) by the preponderance of less differentiated cells in the population. Although SCi-Heps were produced by a different method and were cultured for a much shorter time period than were Chi-Heps, their gene expression profiles were

extremely similar: 48165 of 49395 probes did not show a significant expression difference (adjusted p-value > 0.01 or fold-change <2) between these two types of iHeps. The level of expression of 306 genes (0.6%) had a >3-fold (adjusted p<0.01) and only 44 genes (0.08%) had a >10-fold (adjusted p<0.01) difference in expression in these two cell types (e.g,. as published in Table S4 in Xu et al., Cell Transplantation, 2013 Oct 21; DOI: 10.3727/096368913X673432; "Enabling Autologous Human Liver Regeneration With Differentiated Adipocyte Stem Cells"; the entirety of which, including figures, tables, supplementary figures, supplementary tables, etc., is hereby incorporated by reference). Although a few liver-specific genes (e.g. CYP3A4) were differentially expressed, the vast majority of genes had a similar level of expression in Chi-Heps and SCi-Heps, which indicates that both types of cells have a similar liver-specific gene expression profile.

Analysis of gene expression data. A micro-array-based analysis of global gene expression was performed on ASCs, Chi-Heps, SCi-Heps, iPS-Heps and hepatocytes. To eliminate the effect that inter-individual differences could have on the gene expression profile, ASCs, Chi-Heps, SCi- Heps and iPS-Heps were prepared from the same individuals. For our initial analysis, the hepatocyte and ASC gene expression profiles were compared to enable the selection of a set of genes that met pre-determined criteria (fold-change > 5 and adjusted p value <0.01) for differential expression. To ensure that the subsequent comparisons were evaluating robust expression differences between ASCs and hepatocytes, we selected genes whose expression differences were greater than 5-fold. Based upon these criteria, this analysis identified 1,129 and 1,437 genes whose expression was increased or decreased, respectively, in hepatocytes relative to ASCs (e.g,. as published in Table S2 in Xu et al., Cell Transplantation, 2013 Oct 21; DOI: 10.3727/096368913X673432; "Enabling Autologous Human Liver Regeneration With Differentiated Adipocyte Stem Cells"; the entirety of which, including figures, tables, supplementary figures, supplementary tables, etc., is hereby incorporated by reference). To quantitatively assess the similarity between the 3 different types of iHeps and hepatocytes, we investigated how many of these selected genes also had an altered expression pattern after ASCs underwent the 3 different differentiation processes. For example, we found that the expression of 494 (or 44%) and 341 (or 24%) of the selected up- or down-regulated genes, respectively, were similarly altered in Chi-Heps (fold change >2 and adjusted p value < 0.01) (**Figure 11**). However, this result indicates that the level of expression of 1731 (or 67%) of these selected 2566 genes was either not significantly changed in Chi-Heps (relative to ASCs) or changed in a different direction (compared to hepatocytes). Similarly, we found using the same criteria that the expression of 565 (or 50%) and 449 (or 31%) of

the selected up- or down-regulated genes, respectively, were similarly altered in SCi-Heps (**Figure 11**); which indicates that the level of expression of 1552 (or 60%) of the selected 2566 genes was either not significantly changed in SCi-Heps or changed in a different direction. These results indicate that Chi- and SCi-Heps have a similar gene expression profile, which resembles, but certainly does not fully mirror, that of hepatocytes. Similar results emerged when this type of comparison was made using the iPS-Hep gene expression profile (**Figure 11**). We also used a second approach to compare the global gene expression profiles measured in the 3 different types of iHeps. This time, we directly compared the gene expression profiles in Chi-Heps, SCi-Heps and iPS-Heps with that in ASCs, and examined the number of gene expression changes that were also consistently present in hepatocytes (relative to ASCs). This approach differs from that used above, since this comparison examines all genes that are differentially expressed in iHeps relative to ASCs. Out of 1487 genes that were differentially expressed (fold-change >2 and adjusted p value <0.01) in Chi-Heps relative to ASCs, 835 (56%) genes also showed consistent (and significant) expression changes in hepatocyte cells relative to ASCs (fold change >5 and p value <0.01). Similarly, out of 2372 genes that were differentially expressed in SCi-Heps, 1014 (43%) of these genes also showed consistent (and significant) expression changes in hepatocyte cells relative to ASCs. However, the same comparison performed using the iPS-Hep expression data revealed that only 31% (1372 out of 4456) of the differentially expressed genes in iPS-Heps showed consistent changes in hepatocytes. The differences in the iPS-Hep gene expression pattern are also exemplified by analysis of the genes whose expression pattern was not changed in hepatocytes relative to ASCs. Of the 16446 genes whose expression pattern was unchanged in hepatocytes relative to ASCs, the expression of 96% and 92% of these genes were also unchanged in Chi- and SCi-Heps, respectively (**Figure 11**). However, the level of expression of 18% (or 2969) of these 16446 genes was altered in iPS-Heps. In summary, these analyses indicate that although their expression pattern does not fully reflect that of hepatocytes, Chi- and SCi-Heps have a gene expression pattern that better resembles that of hepatocytes than iPS-Heps. Moreover, there are a significant number of gene expression changes in iPS-Heps that are not reflective of that associated with hepatocyte differentiation.

Example 2 – Human liver regeneration using SCi-Heps

To determine whether SCi-Heps could reconstitute human liver *in vivo*, 5x106 SCi-Heps were transplanted by ultrasound-guided injection directly into the liver of four gancyclovir-conditioned TK-NOG mice (**Figure 3A**). We previously demonstrated that the

amount of human albumin in the sera of chimeric mice is an indicator of the extent of liver humanization (Hasegawa et al, Biochem Biophys Res Commun. 2011 Feb 18;405(3):405-10), so the serum human albumin level was serially assessed over an 8-week period after SCi-Heps transplantation. All four of these mice produced substantial and increasing amounts of human serum albumin when monitored over an 8-week period after transplantation. They had an average human albumin concentration of 0.29 (± 0.09) mg/ml in their sera at 4 weeks, which increased to 0.82 (± 0.41) at 8 weeks after SCi-Heps transplantation (**Figure 3B**). In contrast, none of the 3 mice that were transplanted with the same number of undifferentiated ASCs produced detectable human serum albumin.

Liver histology revealed that the transplanted SCi-Heps integrated into the liver, produced human albumin, and expressed markers found on mature human hepatocytes (ASGR1, CK8/18) (**Figure 3C**). Since SCi-Heps did not express ASGR1 in vitro just prior to transplantation (**Figure 10**), ASGR1 expression indicated that the SCi-Heps continued to differentiate after liver engraftment. Since it was important to determine if the engrafted SCi-Heps would proliferate in the liver, we investigated whether the SCi-Heps expressed the Ki67 nuclear protein, which is a specific marker for cellular proliferation. A significant percentage of the engrafted SCi-Heps were Ki67 positive (**Figure 3D**), which indicates that they proliferate in situ in the liver. In addition, a significant percentage of the engrafted SCi-Heps cells also expressed tight junction protein ZO-1 (**Figure 3D**). This indicates that they have established tight junction interactions with one another, which is required for human bile duct formation. In contrast, livers obtained from mice that were transplanted with undifferentiated ASCs did not have any Ki67 or ZO-1 positive cells (**Figure 3D**).

SCi-Heps do not form tumors. Because malignant potential is a critical determinant of whether SCi-Heps could be used for liver regeneration in human subjects, we examined whether SCi- Heps would form tumors after implantation under the kidney capsule of immunocompromised NOG mice. No tumors were formed over a 2-month observation period after 5 x104 Chi- or SCi- Heps were implanted into each of 5 NOG mice. Moreover, analysis of tissue sections indicated that only normal tissue was present in the area of implantation. In contrast, implantation of the same number of iPS-Heps into each of 4 NOG mice resulted in the formation of multiple tumors within 3 weeks, which could be palpated through the body wall (**Figure 4A**). Analysis of the tumor tissue obtained two months after iPS-Heps transplantation revealed that the tumors contained tissue derived from all 3 germ cell layers from all 4 mice (**Figure 4B**).

Example 3 – ASC culture and iHep formation using stirred suspension culture

ASCs were placed in spinner flask culture (in the absence of microcarriers) using numerous different rates of rotation, ranging from 0 to 150 rotations per minute (rpm). Culture conditions were identified that allowed ASCs to form aggregates and to proliferate. After 24 hours in the spinner flask system, the number of ASCs increased from $5x10^5$ (starting cell number) to $7.7\pm 0.3 \times 10^6$ (n=4 independent replicates) which is approximately a 14-fold increase in the number of cells. In contrast, a 6-fold increase in the number of ASCs (from $1x10^4$ to $6.1 +0.2 \times 10^4$) was obtained when ASCs were cultured for a 48-hour period using the hanging drop method (i.e., hanging drop suspension culture). Moreover, the morphology of the ASC cellular aggregates in the spinner flasks resembled the 'spheres' formed using the hanging drop method (**Figure 13**).

Furthermore, the cellular aggregates were able to be differentiated to endoderm at similar efficiency as using the hanging drop method. These results indicate that high density culture (e.g., stirred suspension culture, e.g., spinner flask culture, bioreactor culture, etc.) will support the growth and proliferation of ASCs, that ASCs form cellular aggregates (e.g., spheres) even in the absence of microcarriers, and that ASCs can proliferate at a substantially greater rate in stirred suspension culture (e.g., spinner flasks). Thus, stirred suspension culture can provide a greater starting number of ASCs (e.g., prior to contact with a differentiation media), and a similar (or greater) differentiation efficiency as seen with hanging drop suspension culture (e.g., spinner flask culture, bioreactor culture, etc.), thereby producing a much larger total number of iHeps.

ASCs cultured by attachment, and cellular aggregates (spheres) produced using (i) spinner flask culture (one day culture, in the absence of microcarriers), or (ii) hanging drop suspension culture (two days culture), were either: (1) stained with the ASC surface marker CD34, then analyzed for CD34 positive cells using fluorescent activated cell sorting (FACS) (**Figure 14A**, **Table 4**); or (2) cultured in stage 1 medium for two days, them stained with endoderm marker Sox17 and analyzed for positive cells using FACS (**Figure 14B**, **Table 4**). **Figures 14 A-B** demonstrate that ASCs cultured by stirred suspension culture (in this case, spinner flask culture in the absence of microcarriers) form a greater percentage (A) of CD34+ cells (adipose stem cells) and a roughly equal percentage (B) of SOX17+ cells (endodermal precursor cells) compared to ASCs cultured by the hanging-drop method.

Table 4. This table demonstrates that ASCs cultured by stirred suspension culture (spinner flask culture in this case) form a greater percentage of CD34+ cells (adipose stem cells) and a roughly equal percentage of SOX17+ cells (endodermal precursor cells)

compared to ASCs cultured by the hanging-drop method. Percentage is the percent of cells positive for expression of the marker.

[Table 4]	Attached ASC	Hanging Drop	Spinner flask
CD34+ cells	52.4%	62.8%	87.5%
SOX17+ cells	2.4%	63.3%	61.2%

Figures 15 A-B demonstrate that iHeps produced using spinner flask culture (SS-Hep) had the specific cytochrome CYP enzymes CYP3A4 and CYP1A1, and the activity of CYP3A4 was strongly induced by dexamethasone (Dex) treatment. YJM is a human hepatocyte cell line. CYP activity was normalized to cell viability. Results are presented with (+) and without (-) Dex induction.

Figure 16 and **Table 5** demonstrate that iHeps produced using spinner flask culture (SS-Hep) secreted an increased level of human albumin (hAlb) compared to Chi-Heps and SCi-Heps (approximately 16-fold relative to SCi-Heps and approximately 96-fold relative to Chi-Heps).

Table 5. This table demonstrates that iHeps produced using spinner flask culture (SS-Hep) secreted an increased level of human albumin (hAlb) compared to Chi-Heps and SCi-Heps (hAlb is in units of ng/ml/10^4 cells).

[Table 5]	ASCs	Chi-Heps	SCi-Heps	SS-Heps
hAlb secretion	0	22	124	2019

The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the

exemplary embodiments shown and described herein. Rather, the scope and spirit of the present invention is embodied by the appended claims.

CLAIMS

That which is claimed is:

1. A method of producing a population of hepatocyte-like cells from a population of adipocyte-derived stem cells (ASCs), the method comprising:

(a) placing a population of ASCs into a three dimensional culture for a period of time sufficient to produce an ASC-derived cellular aggregate;

(b) contacting cells of the ASC-derived cellular aggregate with a first culture medium comprising Activin A and a fibroblast growth factor (FGF) to produce a precursor cell population; and

(c) contacting cells of the precursor cell population with a second culture medium comprising hepatocyte growth factor (HGF) to produce an induced cell population that comprises induced hepatocyte-like cells (iHeps),

wherein the total time elapsed from beginning step (a) to the production of an induced cell population in step (c) is less than 13 days.

2. The method of claim 1, wherein the three dimensional culture is a spinner flask culture.

3. The method of claim 1, wherein the three dimensional culture is a microcarrier culture.

4. The method of claim 1, wherein the three dimensional culture is a hanging drop suspension culture.

5. The method of claim 1, wherein the total time elapsed from beginning step (a) to the production of an induced cell population in step (c) is 10 days or less.

6. The method of claim 1, wherein the period of time sufficient to produce an ASC-derived cellular aggregate is 3 days or less.

7. The method of claim 1, wherein the cells of the ASC-derived cellular aggregate are contacted with the first culture medium for 7 days or less.

8. The method of claim 1, wherein the cells of the ASC-derived cellular aggregate are contacted with the first culture medium for 7 days or less.

9. The method of claim 1, wherein the FGF is FGF 4.

10. The method of claim 1, wherein the first culture medium further comprises a Wnt signaling agonist.

11. The method of claim 3, wherein the Wnt signaling agonist is Wnt3a.

12. The method of claim 1, wherein the second culture medium further comprises a compound selected from the group consisting of: oncostatinM (OSM), dexamethasone (Dex), Dimethyl sulfoxide (DMSO), and a combination thereof.

13. The method of claim 1, wherein the second culture medium further comprises oncostatinM (OSM), dexamethasone (Dex), and Dimethyl sulfoxide (DMSO).

14. The method of claim 1, further comprising determining the percentage of cells of the induced cell population that are iHeps.

15. The method of claim 14, wherein determining the percentage of cells comprises:

contacting cells of the induced cell population with a specific binding agent for a hepatocyte marker molecule; and

determining the percentage of cells positive for expression of the hepatocyte marker molecule,

wherein cells positive for expression of the hepatocyte marker molecule are iHeps.

16. The method of claim 14, wherein 11% or more of the cells of the induced cell population are iHeps.

17. The method of claim 16, wherein 25% or more of the cells of the induced cell population are iHeps.

18. The method of claim 1, further comprising enriching the induced cell population for iHeps.

19. The method of claim 18, wherein the enriching comprises fluorescent activated cell sorting (FACS).

20. A method of treating an individual with reduced liver function, the method comprising:

(a) producing a population of hepatocyte-like cells from a population of adipose-derived stem cells (ASCs) by a method comprising:

(i) placing a population of ASCs into a three dimensional culture for a period of time sufficient to produce an ASC-derived cellular aggregate,

(ii) contacting cells of the ASC-derived cellular aggregate with a first culture medium comprising Activin A and a fibroblast growth factor (FGF) to produce a precursor cell population, and

(iii) contacting cells of the precursor cell population with a second culture medium comprising hepatocyte growth factor (HGF) to produce an induced cell population that comprises induced hepatocyte-like cells (iHeps),

wherein the total time elapsed from beginning step (i) to the production of an induced cell population in step (iii) is less than 13 days; and

(b) administering an effective number of iHeps into the individual to improve liver function.

21. The method of claim 20, wherein the three dimensional culture is a spinner flask culture.

22. The method of claim 20, wherein the three dimensional culture is a microcarrier culture.

23. The method of claim 20, wherein the three dimensional culture is a hanging drop suspension culture.

24. The method of claim 20, wherein the total time elapsed from beginning step (a) to the production of an induced cell population in step (c) is 10 days or less.

25. The method of claim 20, wherein the period of time sufficient to produce an ASC-derived cellular aggregate is 3 days or less.

26. The method of claim 20, wherein the cells of the ASC-derived cellular aggregate are contacted with the first culture medium for 7 days or less.

27. The method of claim 20, wherein the cells of the ASC-derived cellular aggregate are contacted with the first culture medium for 7 days or less.

28. The method of claim 20, wherein the FGF is FGF 4.

29. The method of claim 20, wherein the first culture medium further comprises a Wnt signaling agonist.

30. The method of claim 29, wherein the Wnt signaling agonist is Wnt3a.

31. The method of claim 20, wherein the second culture medium further comprises a compound selected from the group consisting of: oncostatinM (OSM), dexamethasone (Dex), Dimethyl sulfoxide (DMSO), and a combination thereof.

32. The method of claim 20, wherein the total time elapsed from beginning step (i) to producing an induced cell population that comprises induced hepatocyte-like cells (iHeps) in step (iii) is less than 13 days.

33. The method of claim 20, further comprising determining the percentage of cells of the induced cell population that are iHeps.

34. The method of claim 33, wherein determining the percentage of cells comprises:

contacting cells of the induced cell population with a specific binding agent for a hepatocyte marker molecule; and

determining the percentage of cells positive for expression of the hepatocyte marker molecule,

wherein cells positive for expression of the hepatocyte marker molecule are iHeps.

35. The method of claim 20, wherein 15% or more of the cells of the induced cell population are iHeps.

36. The method of claim 35, wherein 25% or more of the cells of the induced cell population are iHeps.

37. The method of claim 20, further comprising, prior to step (b), enriching the induced cell population for iHeps.

38. The method of claim 37, wherein enriching comprises fluorescent activated cell sorting (FACS).

39. The method of claim 20, wherein at least $1x10^4$ iHeps are administered.

40. The method of claim 20, wherein the iHeps are transplanted into the liver.

41. The method of claim 40, wherein the iHeps are transplanted into the liver using ultrasound guided injection.

42. The method of claim 20, wherein the individual is a mammal.

43. The method of claim 42, wherein the mammal is a human.

44. The method of claim 20, wherein the ASCs are ASCs isolated from the individual.

45. The method of claim 44, further comprising, prior to step (a), isolating the ASCs from the individual.