NOVEL COMPOSITIONS FOR TREATING DISEASE

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0001] The official copy of the Sequence Listing is submitted concurrently with the specification as an xml file, made with WIPO Sequence Version 2.1.0, via EFS-Web, with a file name of "HPX004.xml", a creation date of February 11, 2025, and a size of 7 kilobytes. The Sequence Listing filed via EFS-Web is part of the specification and is incorporated in its entirety by reference herein.

BACKGROUND

[0002] Morbidity and mortality from liver disease are very high. Mortality from liver disease ranks 12th in the United States. Nearly 4% of hospitalizations in the US are for chronic liver disease at a cost of nearly $20 billion. Alcohol-related hepatitis (AH) and metabolic associated steatohepatitis (MASH), previously referred to as non-alcoholic steatohepatitis (NASH), contribute more than 50% to the burden of chronic liver disease in the United States. Orthotopic liver transplantation is an effective treatment but is severely limited in availability.

[0003] It is estimated that fully one-third of the population of the United States of America has some type of liver disease. Alcohol-related hepatitis, metabolic dysfunction associated steatohepatitis, viral hepatitis, and autoimmune hepatitis all contribute to the burden of chronic liver disease.

[0004] Hepatocyte loss, inflammation, and fibrosis are characteristic of the pathology of chronic liver disease regardless of the etiology. It is an object to infuse suspensions of mesenchymal stromal cells (MSCs) to address multiple aspects of this pathology. Our data shows that this type of cell therapy can 1) reduce inflammatory macrophage activity, 2) promote reparative macrophage activity, 3) suppress CD4+ and CD8+ T cell activation, and 4) stimulate Treg differentiation. It is a further object to use MSC therapy to promote the resolution of liver fibrosis in both animals and humans by influencing hepatic stellate cell activity, increased vascularization, and extracellular matrix remodeling. It is further object to make hepatocyte-like cells (HLCs) prior to treatment so the above properties are amplified by increasing secretion of hepatotropic factors and promoting homing to the liver.

SUMMARY

[0005] Mesenchymal stem/stromal cells (MSCs) and cells derived from MSCs have emerged as promising therapeutic modalities in multiple chronic diseases, including liver disease. However, despite numerous clinical evaluations, only mixed success has been reported when cell therapies with these cell compositions have been used in several types of clinical applications. There seem to be two main sources of this variability. The first is donor to donor variability in MSC

function (intrinsic variability). The second is a lack of clear understanding of process variables for MSCs production that affect potency (i.e. critical quality attributes (CQAs)) and consequently variable potency of the clinical product (extrinsic variability).

[0006] Cell compositions disclosed herein include MSC derived cells such as, for example, Hx-001 cells which are allogeneic adipose stem cell derived hepatocyte-like cells. Hx-001 cells have a phenotype intermediate between MSCs from which they are derived and hepatocytes. This intermediate phenotype for Hx-001 cells is demonstrated, in part, by low to no expression of the hepatocyte markers Albumin (ALB) and KRT18. HX-001 cells also express the markers PROS1, SOD2 and GLUL which are not expressed in ASCs (Adipose-derived MSC) showing that Hx-001 is not an MSC (e.g., ASC). Hx-001 also does not express (or has low expression) of EPCAM and FOXA2.

[0007] MSC and cell compositions derived from MSCs offer the opportunity to treat liver disease and other diseases. Ample evidence from published studies demonstrate the potential for cell therapy to have a positive clinical benefit in NASH and AH. The cell compositions disclosed herein (e.g., Hx-001) can be used to treat NASH, AH, severe metabolic dysfunction-related or alcohol-related liver disease. This approach offers several advantages compared to liver transplant, including greater availability, lower morbidity, and dramatically lower cost.

[0008] The disclosure herein provides manufacturing methods which reproducibility produce cell compositions of desired potency for treatment of liver disease. These methods can reduce both extrinsic (production-related) variability and intrinsic (donor-to-donor) variability in the final product. These methods can also improve potency and reduce variability between batches and between donors.

BRIEF DESCRIPTION OF THE FIGURES

[0009] FIG. 1 shows a bar graph for expression of certain cytokines from macrophages grown with and without Hx-001.

[0010] FIG. 2A, FIG. 2B and FIG. 2C show results from a mouse model for liver injury. FIG. 2A shows ALT levels in the blood of mice treated with vehicle or Hx-001. FIG. 2B shows glutathione levels in liver tissue of mice treated with vehicle or Hx-001. FIG. 2C shows hematoxylin and eosin stain of fixed liver tissue from mice treated with vehicle or Hx-001.

[0011] FIG. 3 shows the release of oxygen from islets in a cryogel-CPO bioscaffold.

[0012] FIG. 4A and FIG. 4B shows results from a co-culture of macrophages with Hx-001. FIG. 4A shows the relative expression of TNFα (macrophages) and PROS1 (Hx-001). FIG. 4B shows the relative expression of TNFα (macrophages) and IDO1 (Hx-001).

[0013] FIG. 5A, FIG. 5B, FIG. 5C, FIG. 5D, FIG. 5E and FIG. 5F show results from a mouse model for liver injury from alcohol. FIG. 5A shows ALT levels in the blood for mice treated with vehicle or Hx-001 6 hours after treatment. FIG. 5B shows cytokine levels in the liver tissue of mice treated with vehicle or Hx-001. . FIG. 5C shows glutathione content of liver tissue after treatment with vehicle or Hx-001. FIG. 5D shows ALT levels in the blood for mice treated with vehicle or Hx-001 48 hours after treatment. FIG. 5E shows the expression of smooth muscle actin and collage in liver tissue of mice treated with vehicle or Hx-001. FIG. 5F shows the proliferation of hepatocytes in liver tissue after treatment with vehicle or Hx-001.

DETAILED DESCRIPTION

[0014] Before the various embodiments are described, it is to be understood that the teachings of this disclosure are not limited to the particular embodiments described, and as such can, 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 teachings will be limited only by the appended claims.

[0015] 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 disclosure belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present teachings, some exemplary methods and materials are now described.

[0016] 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 can 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 teachings. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

[0017] As used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to “a polypeptide” includes more than one polypeptide.

[0018] The section headings used herein are for organizational purposes only and not to be construed as limiting the subject matter described.

Definitions

[0019] As used herein, the terms “protein”, “polypeptide,” and “peptide” are used interchangeably and are defined to mean a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (*e.g.*, glycosylation,

phosphorylation, lipidation, myristilation, ubiquitination, etc.). Included within this definition are D- and L-amino acids, and mixtures of D- and L-amino acids. In some embodiments of the descriptions of polypeptides, the standard single or three letter abbreviations are used for the genetically encoded amino acids (see, *e.g.*, IUPAC-IUB Joint Commission on Biochemical Nomenclature, "Nomenclature and Symbolism for Amino Acids and Peptides," *Eur. J. Biochem.* 138:9-37, 1984).

[0020] As used herein, the terms "polynucleotide" or "nucleic acid' are used interchangeably and are defined to mean two or more nucleosides that are covalently linked together. The polynucleotide may be wholly comprised ribonucleosides (*i.e.*, an RNA), wholly comprised of 2' deoxyribonucleotides (*i.e.*, a DNA) or mixtures of ribo- and 2' deoxyribonucleosides. While the nucleosides will typically be linked together via standard phosphodiester linkages, the polynucleotides may include one or more non-standard linkages. The polynucleotide may be single-stranded or double-stranded, or may include both single-stranded regions and double-stranded regions. Moreover, while a polynucleotide will typically be composed of the naturally occurring encoding nucleobases (*i.e.*, adenine, guanine, uracil, thymine and cytosine), it may include one or more modified and/or synthetic nucleobases, such as, for example, inosine, xanthine, hypoxanthine, *etc*. Preferably, such modified or synthetic nucleobases will be encoding nucleobases.

[0021] As used herein, the term "coding sequence" is defined to mean a portion of a nucleic acid (*e.g.*, a gene) that encodes an amino acid sequence of a protein.

[0022] As used herein, the terms "wild-type" is defined to mean the form found predominantly in nature. For example, a wild-type polypeptide or polynucleotide sequence is a sequence predominantly present in an organism that can be isolated from a source in nature and which has not been intentionally modified by human manipulation.

[0023] As used herein, the terms "recombinant" or "engineered" or "non-naturally occurring" are used interchangeably and are defined to mean modified polypeptides or nucleic acids which polypeptides or nucleic acids are modified in a manner that would not otherwise exist in nature, or is produced or derived from synthetic materials and/or by manipulation using recombinant techniques. Non-limiting examples include, among others, recombinant cells expressing genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise expressed at a different level.

[0024] As used herein, the terms "percentage of sequence identity" and "percentage homology" are used interchangeably and are defined to mean comparisons among polynucleotides or polypeptides, and are determined by comparing two optimally aligned sequences over a

comparison window, where the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence for optimal alignment of the two sequences. The percentage may be calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Alternatively, the percentage may be calculated by determining the number of positions at which either the identical nucleic acid base or amino acid residue occurs in both sequences or a nucleic acid base or amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Those of skill in the art appreciate that there are many established algorithms available to align two sequences. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, *Adv Appl Math.* 2:482, 1981; by the homology alignment algorithm of Needleman and Wunsch, *J Mol Biol.* 48:443, 1970; by the search for similarity method of Pearson and Lipman, *Proc Natl Acad Sci. USA* 85:2444, 1988; by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package), or by visual inspection (see generally, Current Protocols in Molecular Biology, F. M. Ausubel et al., eds., Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement). Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., *J. Mol. Biol.* 215:403-410, 1990; and Altschul et al., *Nucleic Acids Res.* 25(17):3389-3402, 1977; respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. BLAST for nucleotide sequences can use the BLASTN program with default parameters, *e.g.*, a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. BLAST for amino acid sequences can use the BLASTP program with default parameters, *e.g.*, a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, *Proc Natl Acad Sci. USA* 89:10915, 1989). Exemplary determination of sequence alignment and % sequence identity can also employ the BESTFIT or GAP programs in the GCG Wisconsin Software package (Accelrys, Madison WI), using default parameters provided.

[0025] As used herein, the term "reference sequence" is defined to mean a defined sequence used as a basis for a sequence comparison. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence is at least 20 nucleotide or amino acid residues in length, at least 25 residues in length, at least 50 residues in length, or the full length of the nucleic acid or polypeptide. Since two polynucleotides or polypeptides may each (1) comprise a sequence (*i.e.*, a portion of the complete sequence) that is similar between the two sequences, and (2) may further comprise a sequence that is divergent between the two sequences, sequence comparisons between two (or more) polynucleotides or polypeptide are typically performed by comparing sequences of the two polynucleotides or polypeptides over a "comparison window" to identify and compare local regions of sequence similarity. In some embodiments, a "reference sequence" can be based on a primary amino acid sequence, where the reference sequence is a sequence that can have one or more changes to the primary sequence.

[0026] As used herein, the term "substantial identity" refers to a polynucleotide or polypeptide sequence that has at least 80 percent sequence identity, at least 85 percent identity and 89 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 residue positions, frequently over a window of at least 30-50 residues, wherein the percentage of sequence identity is calculated by comparing the reference sequence to a sequence that includes deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. In specific embodiments applied to polypeptides, the term "substantial identity" means that two polypeptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using standard parameters, *i.e.*, default parameters, share at least 80 percent sequence identity, preferably at least 89 percent sequence identity, at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

[0027] As used herein, the terms "corresponding to", "reference to" or "relative to" are used interchangeably when used in the context of the numbering of a given amino acid or polynucleotide sequence and are defined in this context to mean the numbering of the residues of a specified reference sequence when the given amino acid or polynucleotide sequence is compared to the reference sequence. In other words, the residue number or residue position of a given polymer is designated with respect to the reference sequence rather than by the actual numerical position of the residue within the given amino acid or polynucleotide sequence. For example, a given amino acid sequence, can be aligned to a reference sequence by introducing

gaps to optimize residue matches between the two sequences. In these cases, although the gaps are present, the numbering of the residue in the given amino acid or polynucleotide sequence is made with respect to the reference sequence to which it has been aligned. As such, the term "corresponding to", "reference to" or "relative to" also refers to a residue that is analogous, homologous, or equivalent to an enumerated residue in a reference polypeptide. In addition, in some embodiments, crystal structure coordinates of a reference sequence may be used as an aid in determining a homologous polypeptide residue's three dimensional structure and location of equivalent residues.

[0028] As used herein, the terms "consensus sequence" and "canonical sequence" are defined to mean an archetypical amino acid sequence against which all variants of a particular protein or sequence of interest are compared. The terms also refer to a sequence that sets forth the nucleotides that are most often present in a DNA sequence of interest. For each position of a gene, the consensus sequence gives the amino acid that is most abundant in that position in a multiple sequence alignment (MSA).

[0029] As used herein, the terms "optimal alignment" or "optimally aligned" are defined to mean the alignment of two (or more) sequences giving the highest percent identity score. For example, optimal alignment of two polypeptide sequences can be achieved by aligning the sequences such that the maximum number of identical amino acid residues in each sequence are aligned together or by using software programs or procedures described herein or known in the art. Optimal alignment of two nucleic acid sequences can be achieved by aligning the sequences such that the maximum number of identical nucleotide residues in each sequence are aligned together. Two sequences (*e.g.*, polypeptide sequences) may be deemed "optimally aligned" when they are aligned using defined parameters, such as a defined amino acid substitution matrix, gap existence penalty (also termed gap open penalty), and gap extension penalty, so as to achieve the highest similarity score possible for that pair of sequences. Optimal alignment can be done manually or by using software programs or procedures described herein or known in the art. *e.g.*, the BLASTP program for amino acid sequences and the BLASTN program for nucleic acid sequences.

[0030] [0023] As used herein, the terms "amino acid substitution" or "amino acid difference" are defined to mean a change in the amino acid residue at a position of a polypeptide sequence relative to the amino acid residue at a corresponding position in a reference sequence. The positions of amino acid differences generally are referred to herein as "Xn," where n refers to the corresponding position in the reference sequence upon which the residue difference is based.

[0031] As used herein, the terms "conservative amino acid substitution" or "conservative amino acid difference" are defined to mean a change in the amino acid at a residue position to a different residue having a similar side chain, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar defined class of amino acids. By way of example and not limitation, an amino acid with an aliphatic side chain may be substituted with another aliphatic amino acid, *e.g.*, alanine, valine, leucine, and isoleucine; an amino acid with hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain, *e.g.*, serine and threonine; an amino acid having aromatic side chains is substituted with another amino acid having an aromatic side chain, *e.g.*, phenylalanine, tyrosine, tryptophan, and histidine; an amino acid with a basic side chain is substituted with another amino acid with a basic side chain, *e.g.*, lysine and arginine; an amino acid with an acidic side chain is substituted with another amino acid with an acidic side chain, *e.g.*, aspartic acid or glutamic acid; and a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively. Exemplary conservative substitutions are provided in **Table 1** below.

Table 1

Residue	**Possible Conservative Substitutions**
A, L, V, I	Other aliphatic (A, L, V, I) Other non-polar (A, L, V, I, G, M)
G, M	Other non-polar (A, L, V, I, G, M)
D, E	Other acidic (D, E)
K, R	Other basic (K, R)
N, Q, S, T	Other polar
H, Y, W, F	Other aromatic (H, Y, W, F)
C, P	None

[0032] As used herein, the terms "non-conservative substitution" or "non-conservative amino acid difference" are defined to mean a change in the amino acid at a residue position to a different residue with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and affects (a) the structure of the peptide backbone in the area of the substitution (*e.g.*, proline for glycine), (b) the charge or hydrophobicity, or (c) the bulk of the side chain. By way of example and not limitation, an exemplary non-conservative substitution can be an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

[0033] As used herein, the term "deletion" is defined to mean a modification of a polypeptide by removal of one or more amino acids from the reference polypeptide or modification of a nucleic acid by removal of one or more nucleotides from the reference nucleic acid.

[0034] As used herein, the term "insertion" is defined to mean a modification to a polypeptide by addition of one or more amino acids from the reference polypeptide, or modification of a nucleic acid by addition of one or more nucleic acids.

[0035] As used herein, the term "gene" is defined to mean a polynucleotide (*e.g.*, a DNA segment) that encodes a polypeptide. The term includes regions preceding and following the coding regions as well as any intervening sequences when present (*e.g.*, introns) between individual coding segments (exons).

[0036] As used herein, the term "homologous genes" is defined to mean a pair of genes which correspond to each other and which are identical or similar to each other. The term encompasses genes that are separated by speciation (*i.e.*, the development of new species) (*e.g.*, orthologous genes), as well as genes that have been separated by genetic duplication (*e.g.*, paralogous genes).

[0037] As used herein, the terms "ortholog" and "orthologous genes" are defined to mean genes in different species that have evolved from a common ancestral gene (*i.e.*, a homologous gene) by speciation. Typically, orthologs retain the same function during the course of evolution. Identification of orthologs finds use in the reliable prediction of gene function in newly sequenced genomes.

[0038] As used herein, the terms "paralog" and "paralogous genes" are defined to mean genes that are related by duplication within a genome. Generally, paralogs tend to evolve into new functions, even though some functions are often related to the original one.

[0039] As used herein, the term "chromosomal integration" is defined to mean the process whereby an incoming sequence is introduced into the chromosome of a host cell. The homologous regions of the transforming DNA align with homologous regions of the chromosome. Subsequently, the sequence between the homology boxes is replaced by the incoming sequence in a double crossover (*i.e.*, homologous recombination). In some embodiments, homologous sections of an inactivating chromosomal segment of a DNA construct align with the flanking homologous regions of the indigenous chromosomal region of a host cell chromosome. Subsequently, the indigenous chromosomal region is deleted by the DNA construct in a double crossover (*i.e.*, homologous recombination).

[0040] As used herein, the term "homologous recombination" is defined to mean the exchange of DNA fragments between two DNA molecules or paired chromosomes at the site of identical

or nearly identical nucleotide sequences. In some embodiments, chromosomal integration is homologous recombination.

[0041] As used herein, the term "stringent hybridization conditions" is defined to mean hybridizing in 50% formamide at 5XSSC at a temperature of 42 °C and washing the filters in 0.2XSSC at 60 °C. (1XSSC is 0.15M NaCl, 0.015M sodium citrate.) Stringent hybridization conditions also encompasses low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50 °C; hybridization with a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42 °C; or 50% formamide, 5XSSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42 °C, with washes at 42 °C in 0.2XSSC (sodium chloride/sodium citrate) and 50% formamide at 55 °C, followed by a high-stringency wash consisting of 0.1XSSC containing EDTA at 55 °C.

[0042] As defined herein, the term "heterologous" polynucleotide or polypeptide is defined to mean any polynucleotide or polypeptide that is not naturally found in a host cell. As such, the term includes polynucleotides that are removed from a host cell, subjected to laboratory manipulation, and then reintroduced into a host cell. In some embodiments, the introduced polynucleotide expresses the heterologous polypeptide.

[0043] As used herein, the term "codon optimized" is defined to mean changes in the codons of the polynucleotide encoding a protein to those preferentially used in a particular organism such that the encoded protein is efficiently expressed in the organism of interest. Although the genetic code is degenerate in that most amino acids are represented by several codons, called "synonyms" or "synonymous" codons, it is well known that codon usage by particular organisms is nonrandom and biased towards particular codon triplets. This codon usage bias may be higher in reference to a given gene, genes of common function or ancestral origin, highly expressed proteins versus low copy number proteins, and the aggregate protein coding regions of an organism's genome.

[0044] As used herein, the term "control sequence" is defined to include all components, which are necessary or advantageous for the expression of a polynucleotide and/or polypeptide of the present disclosure. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and

transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and where appropriate, translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

[0045] As used herein, the term "operably linked" is defined to mean a configuration in which a control sequence is appropriately placed (*i.e.*, in a functional relationship) at a position relative to a polynucleotide of interest such that the control sequence directs or regulates the expression of the polynucleotide and/or polypeptide of interest.

[0046] As used herein, the term "promoter sequence" is defined to mean a nucleic acid sequence that is recognized by a host cell for expression of a polynucleotide of interest, such as a coding sequence or gene. The promoter sequence contains transcriptional control sequences, which mediate the expression of a polynucleotide of interest. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Compositions with Mesenchymal Stem Cells (MSC), MSC Derived Cells, and/or Bioscaffolds

[0047] Mesenchymal stem cells are stem cells that have self-renewal, immunomodulatory, anti-inflammatory, signaling, and differentiation properties. Mesenchymal stem cells (MSCs) self-renewal capacity is characterized by their ability to divide and develop into multiple specialized cell types present in a specific tissue or organ. Mesenchymal stem cells (MSCs) can be sourced from a variety of tissue including adipose tissue (fat), bone marrow, umbilical cord tissue, blood, liver, dental pulp, and skin.

[0048] Compositions with mesenchymal stem/stromal cells (MSCs) or cells derived from MSCs have emerged as promising therapeutic modalities in multiple chronic diseases, including liver disease. MSCs and/or cells derived from MSCs disclosed herein are multipotent cells that can be capable of transdifferentiating into the hepatocyte lineage when exposed to WNT pathway agonists and/or compounds including dimethyl sulfoxide and dexamethasone and cytokines like hepatocyte growth factor. MSCs can be bone-marrow derived MSCs (BM-MSC), adipose derived MSC (ASC), umbilical cord MSCs (USC), placenta MSCs (PSC), cord blood MSC (CBSC), induced pluripotent derived MSC (iPSC), dental pulp MSC (DPSC), or liver MSC (LSC).

[0049] BM-MSC are found in bone marrow and can be collected through a bone marrow aspiration procedure. ASC are obtained from fat tissue and can be acquired in large numbers through liposuction. ASCs from younger donors can have a higher proliferation rate and can be more viable than those from older donors. USC, PSC and CBSC are derived from tissues associated with neonatal birth.

[0050] MSC derived cells used herein can secrete factors that (1) reduce inflammatory damage (reduce activity of inflammatory macrophages, suppress activation of CD4+ and CD8+ T-cells, and stimulate Treg differentiation), and/or (2) promote reparative activity by macrophages. One such MSC-derived cell is Hx-001. Hx-001 cells can be derived from any of the types of MSCs by treating the MSCs to cause them to differentiate towards hepatocyte cells.

[0051] Hx-001 cells have a phenotype intermediate between adipose tissue stem/stromal cells from which they are derived and hepatocytes. This intermediate phenotype for Hx-001 cells is demonstrated, in part, by low to no expression of the hepatocyte markers Albumin (ALB) and KRT18. Hx-001 cells also have higher expression of the markers PROS1, SOD2 and GLUL which have lower expression in ASCs (Adipose-derived MSC) showing that Hx-001 are not ASCs. Hx-001 also does not express (or has low expression) EPCAM and FOXA2 (which are expressed in Pelz cells).

[0052] Hx-001 cells are different from ASCs or cells made by Peltz (see Table 2). The phenotype of Hx-001 cells differ from Peltz or ASCs in at least the following markers:

Table 2. Phenotype Differences

Gene expression differences	Peltz cells	Hx-001	ASCs
EPCAM	+	-	-
FOXA2	+	-	-
ALB	+	-	-
TDO2	+	+	-
SERPINE1	+	+	+
KRT18	++	+/-	+
ASGR1	+	+	-
SOX17	+	-	-
PROS1	UNK	+	-
SOD2	UNK	++	-
GLUL	UNK	++	-

Functional differences			
Urea synthesis	++	+	-
Glycogen synthesis	++	++	+/-
LDL uptake	+	+	-

* Less endodermal phenotype in Hx-001 results in a mixed phenotype that is unique and retains higher immunomodulatory capacity.

The Peltz Method is reported in Xu et al, Enabling Autologous Human Liver Regeneration With Differentiated Adipocyte Stem Cells, 2014, Cell Transplant 23:1573-84, which is incorporated by reference in its entirety for all purposes.

The Banas Method is reported in Banas et al, Rapid hepatic fate specification of adipose-derived stem cells and their therapeutic potential for liver failure, 2009, J. Gastroenterol. Hepatol. 24:70-77, which is incorporated by reference in its entirety for all purposes.

[0053] EPCAM (Epithelial Cell Adhesion Molecule) acts as a differentiation marker, particularly in epithelial tissues, as its expression is often high in progenitor cells and decreases as cells differentiate, indicating a role in regulating the transition from a proliferative state to a differentiated one; essentially, high EpCAM expression is associated with less differentiated cells, while lower expression signifies more differentiated cells.

[0054] FOXA2 (Forkhead box protein A2) also known as hepatocyte nuclear factor 3-beta (HNF-3B), is a transcription factor that inhibits adipocyte differentiation, and plays an important role during differentiation of cells to hepatocytes. FOXA2 can be a transcriptional activator for liver-specific genes such as albumin and transthyretin, and can also interact with chromatin.

[0055] ALB (Albumin) can be a marker specifically for the differentiation of cells into mature hepatocytes (liver cells), meaning that when a cell starts expressing high levels of albumin, it is a strong indication that it has successfully differentiated into a functional liver cell.

[0056] KRT18 (Keratin-18) can be a differentiation marker for epithelial cells, meaning its expression is often used to identify and distinguish cells of epithelial origin. Its presence indicates a differentiated epithelial cell state.

[0057] ASGR1 (Asialoglycoprotein Receptor 1) can be a hepatic surface marker. ASGR1 may promote liver injury by modulating monocyte to macrophage differentiation during sepsis. This receptor is a transmembrane protein that plays a critical role in serum glycoprotein homeostasis by mediating the endocytosis and lysosomal degradation of glycoproteins with exposed terminal galactose or N-acetylgalactosamine residues.

[0058] SOD2 (Superoxide dismutase 2) can be a negatively correlated marker for ASCs. As ASCs differentiate SOD2 expression can be increased. SOD2 promotes the immunosuppressive function of mesenchymal stem cells at the expense of adipocyte differentiation. SOD2 is a member of the iron/manganese superoxide dismutase family. It encodes a mitochondrial protein that forms a homotetramer and binds one manganese ion per subunit. This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen. Blocking of adipogenic differentiation by mitochondrial antioxidant may represent a novel strategy to enhance the immunosuppressive activity of MSCs in the inflammatory microenvironment.

[0059] GLUL (glutamine synthetase) is considered a differentiation marker for hepatocytes because its expression is highly specific to mature hepatocytes, particularly those located around the central vein of the liver lobule, indicating a marker for a differentiated state within the hepatic lineage. Expression of GLUL is also negatively correlated with differentiation away from an ASC.

[0060] Other MSC cell markers that are downregulated in Hx-001 include, for example, CD73, CD90, and CD105. CD73 also known as ecto-5'-nucleotidase, is a glycosyl-phosphatidylinositol (GPI)-linked 70-kDa cell surface enzyme that can play a role in immune regulation and cancer progression. CD90 also known as Thy-1, is a glycosylphosphatidylinositol-anchored glycoprotein on the cell surface that is used as a marker for stem cells and neurons. It can also be a marker for some types of cancer. CD105 also known as endoglin is an accessory receptor for transforming growth factor beta (TGF-β) and its expression can be up-regulated in actively proliferating endothelial cells.

[0061] PROS1 (Protein S) encodes a vitamin K-dependent plasma protein that functions as a cofactor for the anticoagulant protease, activated protein C (APC) to inhibit blood coagulation. Secreted PROS1 effectively dampen macrophage M1-associated gene expression through Mer and Tyro3. PROS1 expression by Hx001 correlates with reduced expression of proinflammatory cytokines by macrophages. The nucleic acid sequence for PROS1 (Accession No. NM_001314077.2 at ncbi.nlm.nih.gov) is as follows:

GTTCCGCCGAGGCTCGCTGGGTCGCTGGCGCCGCCGCGCAGCACGGCTCAGACCGA
GGCGCACAGGCTCGCAGCTCCGCGGCGCCTAGCGCTCCGGTCCCCGCCGCGACGCG
CCACCGTCCCTGCCGGCGCCTCCGCGCGCTTCGAAATGAGGGTCCTGGGTGGGCGC
TGCGGGGCGCTGCTGGCGTGTCTCCTCCTAGTGCTTCCCGTCTCAGAGGCAAACTTT
TGTTTATATTTTAGAAATGATTTTATATACAACCGTGCATGCATTTCTGTATTGGTCG
GCTTATCTGGATGCAATTTTTTCTATTCTATATGCTTTTTGTCAAAGCAACAGGCTTC

ACAAGTCCTGGTTAGGAAGCGTCGTGCAAATTCTTTACTTGAAGAAACCAAACAGG
GTAATCTTGAAAGAGAATGCATCGAAGAACTGTGCAATAAAGAAGAAGCCAGGGA
GGTCTTTGAAAATGACCCGGAAACGGATTATTTTTATCCAAAATACTTAGTTTGTCT
TCGCTCTTTTCAAACTGGGTTATTCACTGCTGCACGTCAGTCAACTAATGCTTATCCT
GACCTAAGAAGCTGTGTCAATGCCATTCCAGACCAGTGTAGTCCTCTGCCATGCAAT
GAAGATGGATATATGAGCTGCAAAGATGGAAAAGCTTCTTTTACTTGCACTTGTAA
ACCAGGTTGGCAAGGAGAAAAGTGTGAATTTGACATAAATGAATGCAAAGATCCCT
CAAATATAAATGGAGGTTGCAGTCAAATTTGTGATAATACACCTGGAAGTTACCAC
TGTTCCTGTAAAAATGGTTTTGTTATGCTTTCAAATAAGAAAGATTGTAAAGATGTG
GATGAATGCTCTTTGAAGCCAAGCATTTGTGGCACAGCTGTGTGCAAGAACATCCC
AGGAGATTTTGAATGTGAATGCCCCGAAGGCTACAGATATAATCTCAAATCAAAGT
CTTGTGAAGATATAGATGAATGCTCTGAGAACATGTGTGCTCAGCTTTGTGTCAATT
ACCCTGGAGGTTACACTTGCTATTGTGATGGGAAGAAAGGATTCAAACTTGCCCAA
GATCAGAAGAGTTGTGAGGTTGTTTCAGTGTGCCTTCCCTTGAACCTTGACACAAAG
TATGAATTACTTTACTTGGCGGAGCAGTTTGCAGGGGTTGTTTTATATTTAAAATTTC
GTTTGCCAGAAATCAGCAGATTTTCAGCAGAATTTGATTTCCGGACATATGATTCAG
AAGGCGTGATACTGTACGCAGAATCTATCGATCACTCAGCGTGGCTCCTGATTGCAC
TTCGTGGTGGAAAGATTGAAGTTCAGCTTAAGAATGAACATACATCCAAAATCACA
ACTGGAGGTGATGTTATTAATAATGGTCTATGGAATATGGTGTCTGTGGAAGAATTA
GAACATAGTATTAGCATTAAAATAGCTAAAGAAGCTGTGATGGATATAAATAAACC
TGGACCCCTTTTTAAGCCGGAAAATGGATTGCTGGAAACCAAAGTATACTTTGCAG
GATTCCCTCGGAAAGTGGAAAGTGAACTCATTAAACCGATTAACCCTCGTCTAGAT
GGATGTATACGAAGCTGGAATTTGATGAAGCAAGGAGCTTCTGGAATAAAGGAAAT
TATTCAAGAAAAACAAAATAAGCATTGCCTGGTTACTGTGGAGAAGGGCTCCTACT
ATCCTGGTTCTGGAATTGCTCAATTTCACATAGATTATAATAATGTATCCAGTGCTG
AGGGTTGGCATGTAAATGTGACCTTGAATATTCGTCCATCCACGGGCACTGGTGTTA
TGCTTGCCTTGGTTTCTGGTAACAACACAGTGCCCTTTGCTGTGTCCTTGGTGGACTC
CACCTCTGAAAAATCACAGGATATTCTGTTATCTGTTGAAAATACTGTAATATATCG
GATACAGGCCCTAAGTCTATGTTCCGATCAACAATCTCATCTGGAATTTAGAGTCAA
CAGAAACAATCTGGAGTTGTCGACACCACTTAAAATAGAAACCATCTCCCATGAAG
ACCTTCAAAGACAACTTGCCGTCTTGGACAAAGCAATGAAAGCAAAAGTGGCCACA
TACCTGGGTGGCCTTCCAGATGTTCCATTCAGTGCCACACCAGTGAATGCCTTTTAT
AATGGCTGCATGGAAGTGAATATTAATGGTGTACAGTTGGATCTGGATGAAGCCAT
TTCTAAACATAATGATATTAGAGCTCACTCATGTCCATCAGTTTGGAAAAAGACAA

AGAATTCTTAAGGCATCTTTTCTCTGCTTATAATACCTTTTCCTTGTGTGTAATTATA
CTTATGTTTCAATAACAGCTGAAGGGTTTTATTTACAATGTGCAGTCTTTGATTATTT
TGTGGTCCTTTCCTGGGATTTTTAAAAGGTCCTTTGTCAAGGAAAAAAATTCTGTTG
TGATATAAATCACAGTAAAGAAATTCTTACTTCTCTTGCTATCTAAGAATAGTGAAA
AATAACAATTTTAAATTTGAATTTTTTTCCTACAAATGACAGTTTCAATTTTTGTTTG
TAAAACTAAATTTTAATTTTATCATCATGAACTAGTGTCTAAATACCTATGTTTTTTT
CAGAAAGCAAGGAAGTAAACTCAAACAAAAGTGCGTGTAATTAAATACTATTAATC
ATAGGCAGATACTATTTTGTTTATGTTTTTGTTTTTTTCCTGATGAAGGCAGAAGAG
ATGGTGGTCTATTAAATATGAATTGAATGGAGGGTCCTAATGCCTTATTTCAAAACA
ATTCCTCAGGGGGAACAGCTTTGGCTTCATCTTTCTCTTGTGTGGCTTCACATTTAAA
CCAGTATCTTTATTGAATTAGAAAACAAGTGGGACATATTTTCCTGAGAGCAGCAC
AGGAATCTTCTTCTTGGCAGCTGCAGTCTGTCAGGATGAGATATCAGATTAGGTTGG
ATAGGTGGGGAAATCTGAAGTGGGTACATTTTTTAAATTTTGCTGTGTGGGTCACAC
AAGGTCTACATTACAAAAGACAGAATTCAGGGATGGAAAGGAGAATGAACAAATG
TGGGAGTTCATAGTTTTCCTTGAATCCAACTTTTAATTACCAGAGTAAGTTGCCAAA
ATGTGATTGTTGAAGTACAAAAGGAACTATGAAAACCAGAACAAATTTTAACAAAA
GGACAACCACAGAGGGATATAGTGAATATCGTATCATTGTAATCAAAGAAGTAAGG
AGGTAAGATTGCCACGTGCCTGCTGGTACTGTGATGCATTTCAAGTGGCAGTTTTAT
CACGTTTGAATCTACCATTCATAGCCAGATGTGTATCAGATGTTTCACTGACAGTTT
TTAACAATAAATTCTTTTCACTGTATTTTATATCACTTATAATAAATCGGTGTATAAT
TTTAAAATGCATGTGAATATCTTTATTATATCAACTGTTTGAATAAAACAAAATTAC
ATAATAGA (SEQ ID NO: 1)

The amino acid sequence for PROS1 (Accession No. XP_054203239.1 at ncbi.nlm.nih.gov) is as follows:

MRVLGGRCGALLACLLLVLPVSEANFCLYFRNDFIYNRACISVLVGLSGCNFFYSICFLS
KQQASQVLVRKRRANSLLEETKQGNLERECIEELCNKEEAREVFENDPETDYFYPKYLV
CLRSFQTGLFTAARQSTNAYPDLRSCVNAIPDQCSPLPCNEDGYMSCKDGKASFTCTCK
PGWQGEKCEFDINECKDPSNINGGCSQICDNTPGSYHCSCKNGFVMLSNKKDCKDVDE
CSLKPSICGTAVCKNIPGDFECECPEGYRYNLKSKSCEDIDECSENMCAQLCVNYPGGY
TCYCDGKKGFKLAQDQKSCEVVSVCLPLNLDTKYELLYLAEQFAGVVLYLKFRLPEISR
FSAEFDFRTYDSEGVILYAESIDHSAWLLIALRGGKIEVQLKNEHTSKITTGGDVINNGL
WNMVSVEELEHSISIKIAKEAVMDINKPGPLFKPENGLLETKVYFAGFPRKVESELIKPIN
PRLDGCIRSWNLMKQGASGIKEIIQEKQNKHCLVTVEKGSYYPGSGIAQFHIDYNNVSS
AEGWHVNVTLNIRPSTGTGVMLALVSGNNTVPFAVSLVDSTSEKSQDILLSVENTVIYRI

QALSLCSDQQSHLEFRVNRNNLELSTPLKIETISHEDLQRQLAVLDKAMKAKVATYLGG
LPDVPFSATPVNAFYNGCMEVNINGVQLDLDEAISKHNDIRAHSCPSVWKKTKNS
(SEQ ID NO: 2)

[0062] PROS1 nucleic acids include those that hybridize under stringent hybridization conditions with SEQ ID NO: 1. PROS1 nucleic acids include nucleic acids that are 70%, 80%, 90%, 95%, and/or 99% identical to SEQ ID NO: 1. PROS1 nucleic acids include nucleic acids that are 70% identical to SEQ ID NO: 1. PROS1 nucleic acids include nucleic acids that are 80% identical to SEQ ID NO: 1. PROS1 nucleic acids include nucleic acids that are 90% identical to SEQ ID NO: 1. PROS1 nucleic acids include nucleic acids that are 95% identical to SEQ ID NO: 1. PROS1 nucleic acids include nucleic acids that are 99% identical to SEQ ID NO: 1. The PROS1 nucleic acid sequence can encode a protein having one or more amino acid substitutions, deletions, or insertions compared to SEQ ID NO: 2. The PROS1 nucleic acid sequence can encode a protein having the amino acid sequence of SEQ ID NO: 2.

[0063] PROS1 polypeptides include polypeptides that are 70%, 80%, 90%, 95%, and/or 99% identical to SEQ ID NO: 2. PROS1 polypeptides include polypeptides that are 70% identical to SEQ ID NO: 2. PROS1 polypeptides include polypeptides that are 80% identical to SEQ ID NO: 2. PROS1 polypeptides include polypeptides that are 90% identical to SEQ ID NO: 2. PROS1 polypeptides include polypeptides that are 95% identical to SEQ ID NO: 2. PROS1 polypeptides include polypeptides that are 99% identical to SEQ ID NO: 2. The PROS1 polypeptide can have one or more amino acid substitutions, deletions, or insertions compared to SEQ ID NO: 2. The PROS1 polypeptide can have the amino acid sequence of SEQ ID NO: 2.

[0064] IDO1 (Indoleamine 2,3-dioxygenase 1) expression by Hx001 also correlates with decreased expression of inflammatory cytokines by macrophages. The IDO pathway is also immunomodulatory, with IDO1 well-characterized as a mediator of tumor immune evasion. IDO1 is necessary for the differentiation of regulatory T cells. IDO1 has been shown to inhibit T cell activation and induce T regulatory cell development in vitro. In vivo, IDO1 is best known for its immunoregulatory role in mediating tumor immune evasion. Elevated IDO1 expression has been described in several human tumors and mouse tumor models and IDO1 deficient mice are resistant to tumor formation in preclinical models. This inhibitory function of IDO1 is thought to primarily be through the induction of T regulatory cells, although recent studies have described a novel function for IDO1 in inflammatory neovascularization, that could be just as, if not more, important in some tumor settings. In contrast to its link to regulating immune responses in cancer, the effect of IDO1 on autoimmune responses has been less clear. Some studies describe a regulatory function, while others suggest a pro-inflammatory role. Human

embryonic stem cells (hESCs) depend on glycolysis for energy and pluripotency. Here, we demonstrate that indoleamine 2,3-dioxygenase 1 (IDO1) is expressed in primed hESCs and its expression rapidly downregulated upon hESC differentiation. IDO1 is required to maintain pluripotency by suppressing mitochondria activity and promoting glycolysis through the increase of NAD+/NADH ratio. The upregulation of IDO1 during hESC differentiation suppresses the differentiation of hESCs into certain lineages of cells such as cardiomyocytes, which depend on oxidative phosphorylation to satisfy their high energy demand. Therefore, IDO1 plays important roles in maintaining the pluripotency of hESCs.

[0065] Other genes expressed by Hx001 that positively correlate with decreased expression of inflammatory cytokines by macrophages are AGT, HGF, and VEGFA. Genes that negatively correlate with decreased expression of inflammatory cytokines by macrophages are LIF and KRT18.

[0066] LIF (Leukemia inhibitory factor) is a key factor in maintaining the undifferentiated state of stem cells, including mouse embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). LIF promotes self-renewal by recruiting signal transducer and activator of transcription 3 (Stat3). LIF can be both pro-and anti-inflammatory depending on the context.

[0067] VEGFA (vascular endothelial growth factor A) is a growth factor that induces proliferation and migration of vascular endothelial cells, and is essential for both physiological and pathological angiogenesis. Lamin A and VEGFA control osteoblast and adipocyte differentiation by regulating the levels of the osteoblast and adipocyte transcription factors RUNX2 and PPARγ, respectively.

[0068] HGF (hepatocyte growth factor) is a potent factor for hepatocytes in primary culture and can trigger liver regeneration. It's released in the liver after injury, such as hepatitis, ischemia, or physical damage. HGF binds to the c-Met receptor on cells, which triggers a cascade of events that affects cell growth, proliferation, and survival.

[0069] ALB, KRT18, FOXA2 and ASGR1 are all markers associated with hepatocyte differentiation expressed in the cells made by Peltz. The reduced expression of these markers in Hx-001 cells compared to the cells made by Peltz show that Hx-001 is a more immature cell than the Peltz cells.

[0070] PROS1, SOD2 and GLUL are markers for hepatocytes and upregulation of these markers correlates with differentiation into hepatocytes. The increased expression of these markers in Hx-001 compared to ASCs shows that Hx-001 is not an ASC.

[0071] Hx-001 cells have a phenotype intermediate between adipose tissue stem/stromal cells from which they are derived and hepatocytes. Expression of pluripotency genes usually

expressed in pluripotent cells is reduced, e.g., GDF6, SOX9, NOTCH1, NES, CASC3. Expression of genes characteristic of MSCs is reduced, e.g., ENG, THY1, NT5E (aka CD73, CD90 and CD105). Expression of some genes characteristic of hepatocyte phenotype are increased. Examples include genes for lipid metabolism (LDLR), urea metabolism (ASS1, NAGS), glycogen synthesis (GYS1), coagulation (PROS1, PLAU, A2M), and drug transporters (SLC2A3, TAP1, SLC19A2, SLC19A3, SLC22A7, SLC2A1).

[0072] Functional characteristics of Hx-001 include upregulation of biochemical functions typical of hepatocytes, including upregulation of glycogen synthesis, increased uptake of LDL, urea synthesis, and uptake of indocyanine green. Other upregulated functions detected in Hx-001 include secretion of certain molecules, including IDO1, VEGFA, and CCL2. Compared to starting ASCs, Hx-001 cells exhibit increased activity in suppressing the inflammatory activity of activated macrophages in vivo as measured by IL1 beta, IL6, and TNF alpha synthesis in the macrophages.

[0073] Release criteria for Hx-001 can include: growth rate (doubling time), increases in expression of PROS1 and/or IDO1, suppression of TNFa and IL-1b expression from macrophages stimulated with LPS and IFNg when co-cultured with Hx-001, and/or absence of undifferentiated ASC.

[0074] MSC therapies delivered as cell suspensions are cleared within several days after administration. This may limit the potency of the therapy and necessitate multiple infusions. Lack of trophic factors that promote cell survival, lack of access to appropriate cellular niches, and acute hypoxia may contribute to cell loss. Strategies to overcome cell loss that have been tried including the formation of cellular aggregates (spheroids) and the combination of cells or spheroids with biocompatible materials (e.g., scaffolds) to improve survival in vivo. Both of these strategies seek to provide trophic factors for survival and/or shielding from immune clearance.

[0075] Hx-001 (or other MSCs or cells derived from MSCs) can be combined with bioscaffolds that provide an environment conducive to cell survival, engraftment, and extended function. Hx-001 cells have repeatedly demonstrated promise in treating multiple types of liver disease. These cells represent a novel treatment approach to liver disease that acts by multiple mechanisms, including secreting anti-inflammatory and pro-regenerative factors. We have shown that administration of a suspension of Hx-001 cells has a positive impact on clinically relevant parameters in multiple liver disease models in mice, including carbon tetrachloride treatment, alcohol feeding, and acetaminophen overdose. Hx-001 is a cell suspension that is scalable and cost-effective to produce.

[0076] A suspension of Hx-001 cells in buffer can be almost entirely cleared in mice within 48 hours. Similarly, in humans, cell suspensions given IV can be cleared within 1-2 days. By extending the viability of cell therapies in vivo one can increase their potency. Given our goal of extended viability in vivo, collagen, which has been shown to facilitate the survival of MSCs and works well with Hx-001, is an excellent choice that has been shown to facilitate in vivo survival. Hepatocyte viability in vitro for up to 7 days has been demonstrated with fluorocarbon-based oxygen carrier in gelled collagen. Collagen-based bioscaffolds can improve long-term survival of cells in vivo for up to 30 days and lead to an improved therapeutic effect. These bioscaffolds can address the need for structural and trophic support for Hx-001 compositions by providing an attachment matrix and providing long-term trophic support through improved oxygen generation during vascularization.

[0102] Combining trophic and structural support to Hx-001 can improve the survival of the Hx-001 compositions in vivo, allowing them to secrete therapeutic factors over a longer time, and thereby improve their therapeutic effect. Combination with a bioscaffold can also enable localized treatment, further increasing therapeutic effect.

[0103] Biomaterials for improving cell therapies can be used with cell therapies, including pancreatic islet transplantation to treat diabetes, hepatocytes to treat acute liver failure, and MSCs to treat inflammatory diseases. For example, bioscaffolds can provide physical support, protection and, in some cases, trophic molecules to enhance the effectiveness/potency of transplanted MSCs or cells derived from MSCs. A variety of materials can be used including collagen, and poly(dimethylsiloxane) (PDMS). Biomaterials can also be used to deliver mature hepatocytes as a treatment for liver disease to increase half-life of MSCs following intravenous injection, and improve the survival and function of MSC cell compositions in vivo.

[0104] A collagen-based bioscaffold can enhance MSC and/or cells derived from MSCs (e.g., Hx-001) survival in vivo. What makes this approach unique is that the technology enables titration of calcium peroxide (CPO) into the matrix during the cryogelation step ensuring that all cells seeded into the cryogel-CPO bioscaffold will have, for the first time, a uniform supply of oxygen. This has advantages over other technologies which (i) cannot ensure an even and uniform delivery of oxygen (i.e. the PDMS-CaO2 (Oxysite), (ii) use platforms that induce inflammatory reactions (i.e. perfluorodecalin (PFD)–plasma constructs), (iii) involve the addition of microorganisms (i.e. Chlorella sorokiniana) or need oxygen reservoirs to be continually replenished.

[0105] Bioscaffolds can be synthesized with 0 wt%, 0.25 wt%, 0.5 wt%, and 1.0 wt% CPO, the range of CPO concentrations used in previous studies. Aggregations averaging approximately

2000 Hx-001 cells formed in suspension can be used. Cell loading will be performed in microwell plates using cell compositions with Hx-001. Cell compositions that have been incubated in the absence of bioscaffolds can be used as a control for viability.

[0106] Bioscaffold synthesis: bioscaffolds can be synthesized from bovine Achilles tendon collagen (Sigma-Aldrich) as described. Briefly, collagen is dispersed in 5mM hydrochloric acid (HCl) overnight at 4°C, then mixed with CPO (Sigma Aldrich) to achieve the desired wt%, placed in molds, and crosslinking initiated by addition of N-hydroxysuccinimide and 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide hydrochloride. The mixtures in the molds are placed at -20°C until solid. Subsequently, the collagen gel-CPO mixtures are thawed and washed with distilled H_2O and cut to size. These bioscaffolds with 300 um pores will be used for the studies described. Bioscaffold porosity, density, structure, and mechanical properties can be analyzed by using the Equation S1.

$$\text{Porosity (\%)} = \frac{\frac{W_s - W_d}{\rho_w}}{\frac{W_s - W_d}{\rho_w} + \frac{W_d}{\rho_s}}$$

Equation S1

[0107] W_s is the weight of the saturated bioscaffold, W_d is the weight of the dried bioscaffold, ρ_w is the density of water and ρ_s is the density of the bioscaffold. The volume of each bioscaffold was calculated using the height (thickness) and diameter of sectioned samples. The weight to volume ratio was then used to obtain each bioscaffold density (g.cm-3) using the Equation S2.

$$\rho = \frac{W}{\pi \times \frac{D^2}{4} \times H}$$

Equation S2

[0108] ρ is the density, W is the dry weight in grams, D is the diameter in cm, and H is the thickness of a bioscaffold in cm.

[0109] Oxygen release from bioscaffolds (discs measuring 0.5mm thick x 1mm diameter) can be measured using a Dissolved Oxygen (DO) Meter (YSI™ Pro2030, USA; DO range of 0-1.5mM) connected to a YSI™ 2003 Pro Series Polarographic DO Sensor. Bioscaffolds are immersed in a sealed vial with PBS (10mL) and incubated in a sealed and controlled atmosphere glove box (Labconco, USA). Reactive oxygen species can be measured using a fluorometric assay using 2′,7′-Dichlorofluorescin diacetate (DCFH-DA; Sigma–Aldrich). DCFH-DA is a cell-permeable non-fluorescent probe which is hydrolyzed by intracellular esterases, thereby

trapping it within the cell. This non-fluorescent molecule can then be oxidized by ROS, which then turns it into fluorescent dichlorofluorescin (DCF).

Methods for Making Novel MSC Compositions

[0110] Methods disclosed herein accelerate production, produce a more consistent phenotype, and improve scalability. For example Hx-001 can be made by differentiating MSCs (e.g., ASC) into HLCs (hepatocyte like cell). Hx-001 cells have a phenotype intermediate between transitional hepatocytes and MSCs, retaining desirable properties of both.

[0111] Hepatocyte like cells can be made by in vitro differentiating MSCs (e.g., ASC) using certain culture methods. ASCs can be isolated from human lipoaspirate, then cultured in MesenPRO RS™ Medium (Gibco, Gaithersburg, MD, USA; Cat: 12746-012), passaged at 90% confluence at a 1:4 ratio, and the medium was changed every other day. For preparation of Chi-Heps, a modification of the two-stage protocol of Ochiya and colleagues (Banas et al., Rapid hepatic fate specification of adipose-derived stem cells and their therapeutic potential for liver failure, 2009, J. Gastroenterol. Hepatol. 24(1):70–77, which is incorporated by reference in its entirety for all purposes) was used to induce ASC differentiation into iHeps. After two to five passages, the cells were plated on Matrigel (BD Biosciences, San Jose, CA, USA; Cat: 354277)-coated dishes. After the cells reached 50% confluence, endodermal transdifferentiation was induced over 3 days of culture in the stage 1 medium: Roswell Park Memorial Institute medium (RPMI-1640; Gibco; Cat: 12633-012) supplemented with 100 ng/ml activin A (R&D Systems, Minneapolis, MN, USA; Cat: 338-AC-010), 50 ng/ml wingless-type mouse mammary tumor virus (MMTV) integration site family, member 3 a (Wnt3a; StemRD, Burlingame, CA, USA; Cat: W3A-H-100), 20 ng/ml fibroblast growth factor 4 (FGF4; PeproTech, Rocky Hill, NJ, USA; Cat: 100-31), and 20% B27 (Gibco; Cat: 17504-044). Hepatic differentiation was then induced over a 13- to 15-day period by culture in the stage 2 medium: hepatocyte culture medium (HCM; Lonza, Walkersville, MD, USA; Cat: cc-3198) supplemented with 150 ng/ml hepatocyte growth factor (HGF; PeproTech; Cat: 100-39), 25 ng/ml FGF4, 30 ng/ml oncostatin M (OSM; PeproTech; Cat: 300-10), $2 \times 10{-}5$ M dexamethasone (Dex; Sigma, St. Louis, MO, USA; Cat: D4902), and 0.1% dimethyl sulfoxide (DMSO; Sigma; Cat: C6164).

[0112] Alternatively, ASCs can be isolated from human lipoaspirate, then placed in suspension culture in Dulbecco's modified Eagle medium + 10% fetal bovine serum (FBS) and stirred at 40 rpm. Following overnight culture, sphere formation can be confirmed and the spheres transferred to stage 1 medium for differentiation into definitive endoderm. After two days of culture, the spheres are transferred to stage 2 medium (William's E with hepatocyte growth factor (HGF), fibroblast growth factor 4 (FGF4), epidermal growth factor (EGF),

dexamethasone, and dimethyl sulfoxide) and cultured for an additional 6 days. Hepatic differentiation can be confirmed by quantitative PCR for selected hepatocyte specific genes and function can be evaluated by ability to suppress inflammatory cytokine expression in co-cultures with human activated macrophages as described. Hx-001 cells can be produced that meet certain criteria: expression of benchmark genes (AAT, AGT, KRT18, PROS1, SOD2, HGF, GLUL) within 2-fold of average from our database of >30 differentiations and >50% reduction in expression of IL-1 beta and TNF alpha in macrophage co-cultures. Further methods for preparing HLCs (e.g., Hx-001) are described below.

[0113] HLCs (e.g., Hx-001) have been prepared from the lipoaspirates of more than 70 donors. ASCs can be expanded by 4-6 doublings to generate a seed and/or a working cell bank (WCB). Immunophenotype data for 10 preparations demonstrate the appropriate immunophenotype and differentiation capability of the banked ASCs. The cells prepared using the methods herein can undergo an average of 25 population doublings prior to senescence. ASCs can be grown for 16 population doublings prior to differentiation. Using an estimate of 7.5×10^6 ASCs obtained per L of lipoaspirate, this will yield approximately 4.9×10^{11} cells.

[0114] Table 3 below shows some of the differences between the methods herein and those of Peltz and Banas, as well as the impact of those differences on the HLCs made:

Table 3. Process Differences

	Peltz Method	Banas Method	Hx-001	Impact:
Starting material	Stromal vascular fraction, not placed in culture	adipose tissue MSCs expanded in culture then selected for CD105 expression using MACS system.	Adipose tissue MSCs, expanded in culture	Peltz method uses a highly variable cell population. The Banas method may be selecting for an uncharacterized cell population based on the antibody they used.
Day 1 culture	Suspension	adherent	adherent	Affects endodermal differentiation*

Stage 1 medium for endoderm commitment	RPMI 1640, ITS, activin A (high conc.), FGF4, CHIR99021	DMEM, Activin A (low conc.), FGF4	RPMI 1640, ITS, activin A (high conc.), FGF4, CHIR99021 (CHIR only on day 1)	Lack of WNT agonist in Banas may inhibit differentiation. Use of WNT agonist for two days in Peltz may encourage cholangiocyte differentiation.
Use of Wnt agonist	Continuous for days 2-4	No WNT agonist used	Only during the first 24 hours	Affects endodermal differentiation*
Use of Matrigel	Used to coat surfaces for endodermal differentiation	No use of Matrigel	No use of Matrigel	Affects endodermal differentiation*
Stage 2 medium for hepatocyte differentiation	Williams E, HGF, Oncostatin M, FGF4, EGF, Dexamethasone (Dex), Insulin-Transferrin-Selenium (ITS), dimethylsulfoxide	Hepatocyte culture medium (similar to Williams E), HGF, FGF1, FGF4, dex, ITS, oncostatin M, dimethylsulfoxide	Williams E, HGF, Oncostatin M, FGF4, EGF, Dexamethasone (Dex), Insulin-Transferrin-Selenium (ITS), dimethylsulfoxide	Banas uses FGF1, which may slow differentiation.
Stage 2 differentiation timeframe	6 days or less	10+ days	6 days or less	Hepatocyte phenotype develops faster with the Peltz/Hx-001 method

* Less endodermal phenotype in Hx-001 results in a mixed phenotype that is unique and retains higher immunomodulatory capacity.

The Peltz Method is reported in Xu et al, Enabling Autologous Human Liver Regeneration With Differentiated Adipocyte Stem Cells, 2014, Cell Transplant 23:1573-84, which is incorporated by reference in its entirety for all purposes.

The Banas Method is reported in Banas et al, Rapid hepatic fate specification of adipose-derived stem cells and their therapeutic potential for liver failure, 2009, J. Gastroenterol. Hepatol. 24:70-77, which is incorporated by reference in its entirety for all purposes.

[0115] HLCs (e.g., Hx-001) can be seeded into sterilized bioscaffolds, achieving a desired density in complete medium per bioscaffold; these can be placed in each well of a 96-well plate. Cell compositions seeded into bioscaffolds can be cultured in a humidified incubator with 20% O2 and 5% CO_2 at 37°C for 7 days prior to analysis.

[0116] The phenotype of the Hx-001 cells in the bioscaffolds can be compared to those without bioscaffolds by RNA sequencing followed by principle component (PC) analysis using JMP 14.0. Averages of the PC1 and PC2 dimensions can be compared across samples using Student's T test with $p<0.05$ considered statistically significant. The viability and function of Hx-001 in the scaffolds can be determined using 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as directed by the manufacturer (Abcam). Unloaded bioscaffolds can be used as negative controls and equal numbers of Hx-001 cell compositions cultured in the absence of bioscaffolds can be used as positive controls. Live/dead imaging can be performed by incubating bioscaffold-cell composition combination with fluorescein diacetate and propidium iodide (Thermo Fisher Live-Dead stain) for 20 minutes followed by confocal imaging. Triplicate samples of cell-loaded bioscaffolds can be tested and the experiment can be run twice to confirm results.

[0117] Using known methods for the cryogel bioscaffold formation should produce similar characteristics, physical properties, and oxygen/oxygen radical generation for bioscaffolds. The gene expression of PC1 and PC2 for Hx-001 in bioscaffolds should be within one standard deviation of cells without bioscaffolds. Cell composition formation should be predictable and yield a uniform composition of Hx-001 cells. Hx-001 cell compositions should readily attach to bioscaffolds since the cell compositions readily attach to collagen coated surfaces and that viability can be comparable to cell compositions in the absence of cryogels. The CPO wt%-cell number combination that maximizes number of viable cells as measured by MTT assay can be used. If similar maximum viable cell number is seen with multiple combinations, the combination with the lowest CPO wt% can be used. The target viability can be 90% of controls. Means for MTT assay can be compared by Student's T test with $p=0.05$ used as the level of

significance. Hx-001 cells can also be used with graphene scaffolds that have been shown to accommodate MSCs effectively.

[0118] The functional properties of Hx-001 cells in the loaded bioscaffolds can be assessed. Hx-001 cells manufactured from ASCs can be loaded into CPO bioscaffolds using optimal conditions, and then tested for cytokine secretion and immunomodulatory properties in macrophage co-cultures.

[0119] Hx-001 loaded bioscaffolds can be incubated in complete Hx-001 stage 2 medium without hepatocyte growth factor in microwell plates for 48 hours. Medium can then be collected, centrifuged to remove debris, and snap frozen in liquid nitrogen and stored at -80°C. Aliquots will be submitted for cytokine measurement. Cytokine concentrations can be measured using Luminex technology using the Luminex Human 80-plex assay (custom from EMD-Millipore). Equivalent numbers of Hx-001 cells without bioscaffolds can be incubated in parallel as controls in triplicate.

[0120] Hx-001-loaded or empty bioscaffolds, as well as Hx-001 cells without bioscaffolds can be co-cultured with U937 monocyte cultures to evaluate the effects of the scaffolds on synthesis of cytokines by macrophages. U937 cells can be differentiated to M0 macrophages using 4α-phorbol 12-myristate 13-acetate (PMA) overnight in Iscove's Modified Dulbecco Medium plus 10% fetal bovine serum (complete macrophage medium) at 37°C. To induce the inflammatory (M1) macrophage phenotype, the M0 macrophage cultures can be incubated with complete macrophage medium containing 100 ng/mL lipopolysaccharide and 20 ng/mL interferon gamma. After a 3 hour incubation the Hx-001 loaded bioscaffolds can be added to the macrophage cultures using Transwells. Incubation will continue for an additional 21 hours. Co-cultures can be terminated by lysis of the macrophages in RNA lysis buffer. RNA purified from the co-cultures will be analyzed for human cytokine expression by qPCR.

[0121] Loading of Hx-001 cell compositions onto scaffolds containing CPO should not adversely affect the functional properties of the cells as measured by cytokine secretion profile and co-cultures with macrophages. Secretion of HGF, VEGFA, and CCL2 can be at similar levels as Hx-001 cells free of bioscaffold material. Expression can be within 1 standard deviation of scaffold-free cell compositions. In macrophage co-cultures, reductions in IL-1 beta can be 76%, and TNF alpha by 82% after 21 hours of co-culture, similar results can be obtained with Hx-001 cells loaded on bioscaffolds. A reduction of 50% can be obtained in both IL-1 beta and TNF alpha from the M1 macrophages using cell compositions with and without bioscaffolds. Variations include, for example, differentiation of the Hx-001 cells under

conditions with 5-aza cytidine known to enhance differentiation, derivatizing the scaffold material with trophic factors, or use of graphene bioscaffolds.

[0122] Hx-001 cells embedded in bioscaffolds can secrete therapeutic molecules and impact pathophysiological processes in liver disease. Bioscaffolds with or without Hx-001 cells can be implanted in the inguinal fat pad of NSG mice chronically treated with carbon tetrachloride. At 48 hours and 7 days post-implantation blood samples will be analyzed for human cytokines and explanted bioscaffolds can be analyzed for functional Hx-001 cells and host response.

Combination Therapy

[0123] Disclosed herein are compositions and methods for providing HLCs (e.g., Hx-001) in combination with another therapy. The HLC therapy (e.g., Hx-001) and the other therapy can be provided to a subject at the same time, or one can be provided to the subject before the other, or the HLC therapy and the other therapy can be provided in alternating cycles, or the HLC therapy together with the other therapy can be provided in cycles, or other combinations of administration of the two can be used.

[0124] The other therapy can include, for example, anti-inflammatory medications, antiviral medications, corticosteroids, immunosuppressants, surgery, antioxidant, anti-HSCs (anti-hepatic stellate cells) therapy, anti-fibrotic medications, autophagy targeted medicines, gene therapy, cell therapy, gut microbiota, and nanoparticles. Anti-inflammatory medications can, for example, reduce liver inflammation. Antiviral medications can treat viral hepatitis. Corticosteroids and immunosuppressants can, for example, treat autoimmune liver diseases.

[0125] Anti-inflammatories can include, for example, prednisolone, gabapentin, pregabalin, certain COX-2 inhibitors (e.g., celecoxib), oxicams, ibuprofen, and certain other NSAIDS. Antivirals can include, for example, interferons (e.g., Intron A), lamivudine, 3TC, adefovir, entecavir, telbivudine, tenofovir, and emtricitabine. Corticosteroids can include, for example, prednisolone, methylprednisolone, hydrocortisone, and dexamethasone. Antioxidants can include, for example, silymarin, selenium, Vitamin E, N-acetylcysteine, and/or MitoQ. Anti-HSC therapy can include, for example, imatinib, sorafenib, paclitaxel, ferulic acid, methyl ferulic acid, and/or curcumin. Anti-fibrotic medications can include, for example, Pirfenidone, statins, and/or anti-NADPH oxidases. Gene therapy can include, for example, HGF, matrix metalloproteinase-1, siRNA (targeting CTGF, TGF-β, NF-κB target gene A, galectin-3, and αvβ3 integrin). Cell therapies can include, for example, MSCs, BMSCs, Matrix metalloproteinase 2, tissue inhibitor of metalloproteinase 1, and growth arrest-specific 6. Gut microbiota treatments can include, for example, Baicalin, and probiotics. Nanoparticles can include, for example, gold, Phosphatidylserine-decorated nanoparticles, and liposome

nanoparticles (e.g., deliver payload via integrins of activated hepatic stellate cells, facilitating gene therapy using siRNAs and mRNAs to modulate gene expression of hepatocytes). Autophagy inhibitors can include, for example, Becn 1 knockdown, carvedilol, doxazosin, and resolvin D1.

[0126] FGF19 and/or FGF21 can be used to reduce inflammation and fibrosis in MASH. Cells can be engineered to secrete these proteins. The cells become a slow-release system/time release system for these therapeutics. The cells can be used to alter the PK of the therapeutics. Cells can be engineered to control secretion of these therapeutics by controlling transcription or other parts of the synthesis and secretion process. Options for controlling transcription are many and may include: ultrasound controlled promoter, heat shock controlled promoter, optogenetic control of a promoter, ligand induced promoter (e.g., Tet promoter). Optionally, the engineered cells can be combined with a material to facilitate survival in vivo.

Uses of Novel Cell Compositions

[0127] The novel cell compositions disclosed herein can be used to treat liver disease including, for example, Alcohol-related hepatitis, metabolic dysfunction associated steatohepatitis, viral hepatitis, and autoimmune hepatitis. Alcohol-related hepatitis (AH) and non-alcoholic steatohepatitis (NASH), contribute more than 50% to the burden of chronic liver disease in the United States.

[0128] Damage to Hepatocytes can be caused by multiple agents, including alcohol, fats, and viruses. Dead Hepatocytes become damage associated molecular patterns (DAMPs). DAMPs activate an inflammatory response in macrophages (Kupffer cells in the liver). The macrophage inflammatory response stimulates secretion of cytokines, including tumor necrosis alpha (TNFa), IL-1 beta, and others. The cytokines recruit additional inflammatory cells. The resulting inflammation results in additional tissue damage and release of additional DAMPs, forming a positive feedback loop that results in additional liver damage. To resolve the inflammation macrophages have a time-resolved negative feedback loop that shifts the macrophage phenotype from inflammatory to resolving. PROS1 is a key part of the negative/resolving feedback loop and can reduce the inflammatory phenotype of macrophages.

[0129] The novel cell compositions disclosed herein can have multiple mechanisms of action including, for example, replacing dead or damaged cells in the liver, immunomodulation, trophic effects on damaged tissues, and cytoprotective effects. The novel cell compositions can reduce inflammatory cytokine expression by macrophages and reduce proliferation of reactive T cells.

[0130] Novel cell compositions such as Hx-001 can reduce inflammatory cytokines from macrophages. This reduction in cytokine production from macrophages can be a dose dependent

reduction. The expression of PROS1 and IDO1 in Hx-001 was highly correlated with reduction in inflammatory cytokines (e.g., TNFa and IL1b) from macrophages. PROS1 (SEQ ID NO: 1 and 2) and related polypeptides (e.g., sharing sequence identity with SEQ ID NO: 1 or 2 of 70%, 80%, 90%, 95%, and/or 99%) can be used to immunomodulate macrophages reducing the production of inflammatory cytokines (e.g., in liver disease). This PROS1 can be recombinantly produced, and such recombinant PROS1 can be derivatized in manners known in the art. E.g., moieties such as PEG or other water-soluble polymers can be derivatized to the PROS1 so that the serum half-life of PROS1 is increased. PROS-1 can also be derivatized to and/or fused with a carrier polypeptide such as, for example, albumin, an Fc polypeptide, etc.

[0131] Furthermore, the cytoprotective effects of the novel cell compositions (e.g., Hx-001 cells) was evaluated by measuring glutathione (GSH) content in hepatoma cells following oxidative injury. Co-culture with Hx-001 cells was shown to preserve cellular GSH, indicating a cytoprotective effect.

[0132] The novel cell compositions (e.g., Hx-001) can be allogeneic to a subject. The novel cell composition can be, for example, a GMP-compatible Hx-001 production process that could be used to establish dose, safety and efficacy information for treatment of patients.

[0133] HGF, VEGFA, and CCL2 can be detected in serum from subjects treated with Hx-001. The expression of these cytokines can be compared using Student's T test with $p=0.05$ used as a significance level. Immunostain sections of the Hx-001 for the cytokines above can determine if the cytokines are being synthesized but are not entering the bloodstream. Liver sections can be scored for inflammatory infiltrate and fibrosis. Pearson's Chi^2 test can be used to evaluate between group differences in liver histology with $p=0.05$ as the significance level.

[0134] Various features and embodiments of the disclosure are illustrated in the following representative examples, which are intended to be illustrative, and not limiting. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the inventions as described more fully in the claims which follow thereafter. Unless otherwise indicated, the disclosure is not limited to specific procedures, materials, or the like, as such may 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.

EXAMPLES

Example 1. Hx-001 Cell Compositions

[0135] Lipoaspirates were obtained from human donors. ASCs were prepared from these samples.

[0136] The ASCs were cultured in MesenPRO RS™ Medium (Gibco, Gaithersburg, MD, USA; Cat: 12746-012). After two to five passages, the cells were plated on collagen coated dishes. Endodermal transdifferentiation was induced in the Stage 1 medium: Roswell Park Memorial Institute medium (RPMI-1640; Gibco; Cat: 12633-012) supplemented with 100 ng/ml activin A (R&D Systems, Minneapolis, MN, USA; Cat: 338-AC-010), 3 μM CHIR99021 (Biogems #2520691), 20 ng/ml fibroblast growth factor 4 (FGF4; PeproTech, Rocky Hill, NJ, USA; Cat: 100-31), and 2% B27 minus insulin (Gibco; Cat: Gibco A1895601) for 24 hours. The medium is removed and replaced with the same medium without CHIR99021 for an additional 24 hours.

[0137] Hepatic differentiation was then induced by culture in the Stage 2 medium: Williams E medium (Gibco 12551032) supplemented with 150 ng/ml hepatocyte growth factor (HGF; PeproTech; Cat: 100-39), 25 ng/ml FGF4, 30 ng/ml oncostatin M (OSM; PeproTech; Cat: 300-10), 2×10^{-5} M dexamethasone (Dex; Sigma, St. Louis, MO, USA; Cat: D4902), 1X insulin-transferrin-selenium solution (ITS) (Corning 25-800-CR) and 0.1% dimethyl sulfoxide (DMSO; Sigma; Cat: C6164). The cells were then cultured in Stage 2 medium.

[0138] Initial exposure to an endoderm medium containing activin A and a WNT agonist was followed by induction of hepatic differentiation by exposure to HGF-containing medium. Importantly, initial formation of ASC spheres has been shown to both accelerate differentiation, as well as yield a more mature phenotype than HLCs derived from induced pluripotent cells. Single cell RNA sequencing of HLCs revealed that the resulting cells resemble transitional hepatoblasts or early hepatocytes rather than mature hepatocytes. Expression of genes characteristic of hepatocyte lineage cells, including LDL receptor, GLUL and other genes involved in glycogen synthesis, and certain drug transporters are upregulated, while genes characteristic of the ASC phenotype, including ENG and NT5E are downregulated during differentiation (not shown). Mature hepatocyte genes, such as ALB or ASGR1, are not expressed at this stage. Extensive characterization of these cells in vitro revealed that the cells retain some phenotypic characteristics of the parent ASCs, including the expression of immunomodulatory and trophic genes such as HGF, VEGFA, and indoleamine 2,3-dioxygenase.

Example 2: Making Hx-001 from a WCB

[0139] A working cell bank of ASCs is expanded typically to 12 PD before differentiation to Hx-001. Conventional planar surface culture and microcarriers are used to expand ASCs for differentiation. Differentiation is performed by seeding the cells on planar surfaces and exposing them to media as described above.

[0140] The expression of both PROS1 and IDO1 in Hx-001 are highly significantly correlated with expression of TFNα and IL-1β in macrophages co-cultured with Hx-001.

Immunomodulation of macrophages is an important mechanism of action, and so, PROS1 and IDO1 gene expression can be assessed during Hx-001 production. Doubling time is an additional putative parameter for characterization of Hx-001 as it influences the rate of growth and yield of Hx-001. Macrophage co-culture can also be used as a potency assay.

[0141] Table 1 below shows the use of these markers to assess exemplary batches of Hx-001. Each batch was made using the methods described above and yielded in excess of 2 x 10^8 cells.

Table 1. Hx-001 Preps

Exp ID	Doubling time	PROS1 (dCT)	IDO1 (dCT)
YB89	1.16	5.41	12.14
RA70	1.52	5.81	13.01
RA78	1.09	5.46	15.65
ES191 (RA79)	1.54	6.01	12.33
Average	1.33	5.673	13.283
Std Dev	0.236	0.285	1.619

[0142] Gene expression of PROS1 and IDO1 are consistent from batch to batch.

Example 3: ASC Growth

[0143] Aspirate medium from ASCs, rinse with PBS, and aspirate PBS. Add TrypLE (2.5 mL for T-75, 5 mL for T-175/T-182, 7 mL for 15 cm dish), swirl flask/dish to make sure TrypLE covers entire growth surface, and incubate for 5 minutes. Check that ASCs have detached - gentle tapping or pipetting may be required to dislodge cells. Quench TrypLE with equal volume ASC medium (plain DMEM is okay for this step) and transfer to a conical tube. Count ASCs. Seed ASCs at a density of 8,045 cells/cm^2 in multilayer flasks in 150 mL ASC medium a. Separate desired number of ASCs (7M per multilayer flask), centrifuge at 300G for 5 minutes, and resuspend in ASC medium. Transfer a volume of cell suspension with 7 million ASCs to a 200-250mL bottle/centrifuge tube and add ASC medium to a final volume of 150 mL ASC medium, pipette up and down or invert/swirl to create an even cell suspension, and transfer to flask. Place in a 37°C 5% CO_2 incubator. Allow cells to grow for 72 hours, with a media change to fresh ASC medium after 24 hours.

Example 4: Co-culture with Hx-001 reduces inflammatory cytokine expression from macrophages

[0144] U937 cells were differentiated to macrophages, then treated with interferon gamma and lipopolysaccharide to induce an inflammatory phenotype. Hx-001 or ASCs were added to the cultures using Transwells. After 21 hours of co-culture macrophage RNA was analyzed by qPCR. Expression normalized to M1 inflammatory macrophages is shown in FIG. 1.

[0145] When co-cultured with activated macrophages, Hx-001 cells can significantly reduce expression of key inflammatory cytokines, including IL-1 alpha, IL-1 beta, IL-6, and TNF alpha (FIG. 1). Differentiation significantly increases this immunomodulatory activity (FIG. 1). Similarly, in co-cultures with HepG2 hepatoma cells that have experienced oxidative damage, Hx-001 cells show the ability to mitigate loss of glutathione and thus oxidative damage.

Example 5: Hx-001 cells reduce liver damage, inflammation, and fibrosis and increase hepatocyte proliferation in a murine model of liver injury

[0146] C57bl/6 mice were received 1 x 10^6 Hx-001 cells IV, then 0.5 hours later received an injection of acetaminophen (400 mg/kg) intraperitoneally (IP). Two hours after the acetaminophen injection the mice received a second injection of 4 x 10^6 Hx-001 IP or vehicle IP. The mice were sacrificed at 6 hours and blood and liver tissue were harvested for analysis. FIG. 2A) ALT and AST measured in serum; FIG. 2B) glutathione content in liver tissue; C) hematoxylin and eosin stain of fixed liver tissue.

Example 6: Biomaterials to improve cell therapy

[0147] Collagen based cryogel bioscaffolds have the following attributes: (i) macropores that can accommodate cellular aggregates; (ii) co-existing micropores that can facilitate vascular ingrowth; (iii) high surface area-to-volume ratio so a large number of cellular aggregates can be accommodated in a small "footprint"; (iv) 3D matrix that enables an even distribution of cells, thereby preventing clumping and clustering which increase the risk of cell necrosis; and (v) modular rigidity to provide mechanical support for cell, while ensuring sufficient deformability to facilitate implantation at extrahepatic sites; (vi) highly biocompatible with minimal foreign body reaction. The Thakor Group has been able to incorporate additional features into the matrix of the collagen bioscaffold, including CPO. CPO is an oxygen generator that produces oxygen as it gets hydrolyzed. When tested with islets seeded into the cryogel-CPO bioscaffolds, oxygen was shown to be released in a controlled and sustained manner over several days as shown in FIG. 3.

[0148] Hx-001 can be combined with CPO cryogel bioscaffolds that support and extend the therapeutic function of the cells. Iterative cycles of bioscaffold loading with Hx-001 cells and testing. The scaffold:Hx-001 can be 1) evaluated for optimal oxygen generation and loading of bioscaffolds with HLCs, 2) evaluated for cell function in combination with bioscaffolds in vitro, and 3) evaluated for the activity of the combination in a mouse model of liver disease.

Example 7: Hx-001 and Macrophage Co-culture

[0149] ASCs or Hx-001 were co-cultured with activated macrophages derived from U937 monocytes. U937 cells were differentiated to quiescent M0 macrophages using phorbol 12-

myristate 13-acetate (PMA, 20 ng/mL) overnight at 37°C. The M0 macrophages were activated to M1 using lipopolysaccharide (100 ng/mL) plus interferon γ (20ng/mL) for three hours prior to cell addition. Total RNA is harvested from macrophages and analyzed for IL1β and TNFα. Total RNA was harvested from ASCs or Hx-001 and analyzed for expression of a number of genes.

[0150] Expression by Hx-001 of PROS1 and/or IDO1 correlated with reduced expression of IL1β and TNFα by macrophages (FIG. 4A and FIG. 4B). Seven ASC lines (blue) and Hx-001 (orange) produced from the same ASC lines were compared for PROS1 (A) and IDO1 (B) expression and their effect on expression of TNFα in M1 macrophages.

[0151] Other genes expressed in Hx-001 that correlated with reduced expression of IL1β and TNFα by macrophages included, for example, AGT (angiotensinogen), ALB (albumin), KRT18 (keratin 18), HGF (hepatocyte growth factor), VEGFA (vascular endothelial growth factor A), and/or LIF (leukemia inhibitory factor).

Example 8: Treatment of Liver Injury with Hx-001

[0152] Mice were fed ethanol (EtOH) for 10 days, with a binge on day 11. On days 9-11 the mice also received galactosamine to potentiate inflammation. The Early Group received Hx-001 or vehicle 6 hours after binge and were sacrificed at 24 hours; the Late Group received Hx-001 at 6 hours and 24 hours after the binge and were sacrificed at 48 hours.

[0153] Lower level of alanine aminotransferase (ALT) was measured in the peripheral blood of the cell-treated mice at 6 hours (Figure 5A) and at 48 hours (Figure 5D), indicating a reduction in liver damage compared to vehicle treated mice. Expression of inflammatory genes is reduced in Hx-001 treated mice compared to controls at 6 hours (Figure 5B). Cryoprotection is also evidenced by increased glutathione content of the liver tissue at 6 hours versus vehicle treated mice (Figure 5C). Markers of fibrosis were reduced in Hx-001 treated mice at 48 hours (Figure 5E) and increased proliferation of hepatocytes was observed at 48 hours (Figure 5F). Similar patterns of reduction in liver damage, increased GSH, and reduced inflammation have been observed in an acetaminophen overdose model and acute carbon tetrachloride toxicity model in mice.

[0154] Hx-001 treatment of mice in this alcohol induced liver injury model showed reduced liver damage, reduced expression of inflammatory genes, cryoprotection, reduced fibrosis, and growth of hepatocytes.

Example 9: Dose Optimization in the Treatment of Alcohol-Related Hepatitis

[0155] Using the hybrid intragastric (IG) and ad lib HFD feeding model (IG-HFD) model, mice are dosed via a surgically implanted intragastric catheter, achieving blood alcohol levels approximately double that obtained using ad libitum feeding of EtOH, resulting in elevated ALT

levels (250 U/L). Incorporating a high fat (Western) diet (HFD) and a weekly binge of EtOH adds key MetALD histologic features including neutrophilic inflammation, cellular ballooning degeneration, apoptosis, and ductular reaction.

[0156] Hx-001 cells in HBSS can be injected IP 24 hours after the ethanol binge on week 8 (Study 1), or week 6; week 6 and 7; or week 6, 7, and 8 (Study 2). At week 10 the mice can be sacrificed. Blood can be obtained by terminal bleed and livers will be excised. Body weight, spleen weight, and liver weight can be recorded. Samples of liver tissue can be fixed in 4% PFA for immunohistochemistry and histology and additional samples can be snap frozen for biochemical analysis.

[0157] The primary analysis variables can include mortality, serum ALT, liver TNFα, and PCNA immunohistochemistry. Secondary endpoints can include liver panel blood chemistry (AST, alkaline phosphatase, and total bile acids), human cytokines CCL2 and HGF (ELISA), Sirius red staining for fibrosis, myeloperoxidase and CD68 staining for neutrophil and macrophage infiltration in liver tissue. Other endpoints: qPCR for IL-1 beta, IL-6 bacterial 16S/host 18S rRNA in liver tissue.

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[0158] All publications, patents, patent applications and other documents cited in this application are hereby incorporated by reference in their entireties for all purposes to the same extent as if each individual publication, patent, patent application or other document were individually indicated to be incorporated by reference for all purposes.

[0159] While various specific embodiments have been illustrated and described, it will be appreciated that various changes can be made without departing from the scope of the invention(s) of the disclosure.

CLAIMS

What is claimed is:

1. A method for treating a liver disease, comprising: administering a cell composition to a patient with the liver disease, wherein the cell composition is derived from a mesenchymal stem cell.

2. The method of claim 1, wherein the cell composition derived from the mesenchymal stem cell is a hepatocyte-like cell.

3. The method of claim 1, further comprising the step of differentiating a mesenchymal stem cell to a hepatocyte-like cell.

4. The method of claims 2 or 3, wherein the hepatocyte-like cell compared to the mesenchymal stem cell from which the hepatocyte-like cell is derived has increased expression of TDO2, PROS1, SOD2, and GLUL.

5. The method of any one of claims 2-4, wherein the hepatocyte-like cell compared to a Peltz cell has decreased expression of EPCAM, FOXA2, ALB, and KRT18.

6. The method of any one of claims 2-5, wherein the hepatocyte-like cell compared to the mesenchymal stem cell from which the hepatocyte-like cell is derived has increased urea synthesis, glycogen synthesis, and LDL uptake.

7. The method of any one of claims 1-6, wherein the mesenchymal stem cell is a bone marrow derived mesenchymal stem cell, an adipose derived mesenchymal stem cell, an umbilical cord mesenchymal stem cell, a placenta mesenchymal stem cell, a cord blood mesenchymal stem cell, a dental pulp mesenchymal stem cell, a liver mesenchymal stem cell, or an induced pluripotent stem cell derived mesenchymal stem cell.

8. The method of claim 7, wherein the mesenchymal stem cell is an adipose mesenchymal stem cell.

9. The method of any of claims 1-8, wherein the liver disease is an alcohol-related hepatitis, a metabolic dysfunction associated steatohepatitis, a viral hepatitis, or an autoimmune hepatitis.

10. The method of claim 16, wherein the liver disease is an alcohol-related hepatitis.

11. The method of claim 16, wherein the liver disease is a metabolic dysfunction associated steatohepatitis.

12. The method of claim 16, wherein the liver disease is a viral hepatitis.

13. The method of any of claims 1-12, further comprising the step of reducing the expression of inflammatory cytokines by a macrophage.

14. The method of claim 13, wherein the inflammatory cytokine is an IL-1α, an IL-1β, an IL-6, or a TNFα.

15. The method of any one of claims 2-14, further comprising the step of replacing a dead cell or a damaged cell in a liver with a cell derived from the hepatocyte-like cell.

16. The method of any one of claims 1-15, further comprising the step of increasing a cytoprotection of cells in a liver.

17. The method of any one of claims 2-16, wherein the hepatocyte-like cell is allogeneic to the patient.

18. An isolated cell composition comprising a hepatocyte-like cell derived from a mesenchymal stem cell.

19. The isolated cell composition of claim 18, wherein the mesenchymal stem cell is a bone marrow derived mesenchymal stem cell, an adipose derived mesenchymal stem cell, an umbilical cord mesenchymal stem cell, a placenta mesenchymal stem cell, a cord blood mesenchymal stem cell, a dental pulp mesenchymal stem cell, a liver mesenchymal stem cell, or an induced pluripotent stem cell derived mesenchymal stem cell.

20. The isolated cell composition of any one of claims 18-19, wherein the mesenchymal stem cell is an adipose derived mesenchymal stem cell.

21. The isolated cell composition of any one of claims 18-20, wherein the hepatocyte-like cell compared to the mesenchymal stem cell from which the hepatocyte-like cell is derived has increased expression of TDO2, PROS1, SOD2, and GLUL.

22. The isolated cell composition of any one of claims 18-21, wherein the hepatocyte-like cell compared to a Peltz cell has decreased expression of EPCAM, FOXA2, ALB, and KRT18.

23. The isolated cell composition of any one of claims 18-22, wherein the hepatocyte-like cell compared to the mesenchymal stem cell from which the hepatocyte-like cell is derived has increased urea synthesis, glycogen synthesis, and LDL uptake.

24. The isolated cell composition of any one of claims 18-23, wherein the cell composition can reduce the expression of inflammatory cytokines by a macrophage.

25. The isolated cell composition of claim 24, wherein the inflammatory cytokine is an IL-1α, an IL-1β, an IL-6, or a TNFα.