Online Supplement

Additional Information on the Methods

Table 1. Sourcing of Reagents

Factor or Reagent		*Company*	*Catalog Number*
Fetal bovine serum (FBS)		Gibco, Carlsbad, CA	26140-079
Kubota's medium	RPMI 1640	Gibco, Carlsbad, CA	11875-093
	Insulin		I5500
	Transferrin	Sigma, St. Louis, MO	T1283
	Free fatty acids		
	High-density lipoprotein	Sigma, St. Louis, MO	L8039
	Beta-mercaptoethanol	Sigma, St. Louis, MO	M6250
	Fatty acid–free albumin	Sigma, St. Louis, MO	A8806-5G
	Selenium	Sigma-Aldrich, St. Louis, MO	211176
	Zinc	Specpure-Alfa AESAR, Ward Hill, MA	JMC156
Collagens	Type I	Collagen, Palo Alto, CA	Vitrogen 100
	Type III	Sigma, St. Louis, MO	C3511
	Type III	Becton Dickinson, Franklin, NJ	354244
	Type IV	Becton Dickinson, Franklin, NJ	354245
Laminin		Sigma, St. Louis, MO	L2020
Fibronectin		Sigma, St. Louis, MO	F0895
Transwell inserts		Becton Dickinson, Franklin, NJ	3095

Nomenclature

Self-replication, also called self-renewal, is cell division in which daughter cells are identical phenotypically to the parent cell. It is associated with ease of subculturing, ability to expand cells clonogenically, and ability to maintain cells with a stable phenotype. All diploid cells are able to form colonies when seeded at low seeding densities. Those diploid cells that are not stem cells will go through transient expansion and then become more differentiated; they are unable to self-replicate indefinitely.

Human Liver Sourcing

Liver tissue was provided by an accredited agency (Advanced Biological Resources, San Francisco, CA) from fetuses between 16 and 20 weeks' gestational age obtained by elective pregnancy terminations. The research protocol was reviewed and approved by the Institutional Review Board for Human Research Studies at the University of North Carolina at Chapel Hill.

Fetal Liver Processing

Fetal liver processing was done as reported previously.[1,2] Estimated cell viability by trypan blue exclusion was routinely higher than 95%.

Matrix Components

Collagens

Sigma's type III collagen (Sigma; cat #C3511) is a mixture of type III and some type I collagen and prepared by the method of Bornstein and Traub.[33] Becton Dickinson's collagens were highly purified type III (BD #354244) or type IV collagen (BD #354245). Analyses of three collagen preparations from the commercial sources by a collagen chemist, Dr. Mitsuo Yamauchi (School of Dentistry, UNC-Chapel Hill), and his student, Marnisa Sricholpech, indicated that the Becton Dickinson preparations are reasonably pure, whereas that from Sigma varied in its chemistry from prep to prep but was predominantly type III collagen with variable amounts of type I (Yamauchi, personal communication). We refer to the collagens as Sigma's type III collagen or BD type III or IV. The collagen coating process incorporated a 10-h incubation and 2-h UV sterilization followed by three 1× phosphate-buffered saline (PBS) washes for ph 7.4 neutralization. Vitrogen's type I collagen gels (Collagen) were also used according to the same protocol.

Seeding Densities

The cells seeded are the parenchymal cells in the original cell suspension. The majority of these are hepatoblasts, and

only 0.5–1.5% of the parenchyma are human hepatic stem cells (hHpSCs). The ratios were defined in our prior studies utilizing flow cytometric analyses.[1] Cells were seeded either at low (3820 cells/cm^2) or high (83,100 cells/cm^2) density onto tissue culture plastic (TCP) dishes with or without extracellular matrix coatings or onto Transwell inserts, porous (0.4 μm) inserts (Becton Dickinson; cat #3095). The inserts were placed into six-well Petri dishes (Fisher; cat #08-771-24).

Immunohistochemistry

Cells were fixed with acetone/methanol (1:1) for 2 min at ambient temperature, rinsed with 1× PBS, incubated with 10% goat serum for 45 min, and rinsed again. Fixed cells were incubated with a primary human antibody conjugated with a fluorescent probe for 1–8 h at room temperature. Stained cells were preserved with 2% formaldehyde in Hank's Buffered Saline Solution (HBSS). As controls, some cells were labeled with antibodies that are isotype controls to the antibody used (see Table 2).

Albumin Expression

Media supernatants were analyzed at 24-h intervals for albumin secretion and the levels normalized per cell. Culture media were used to assay for human serum albumin content by enzyme-linked immunosorbent assays.

In Vivo Engraftment of hHpSCs in Mouse Liver

To assess the ability of hHpSCs to engraft *in vivo*, cells were introduced into the livers of immune-deficient SCID/nod mice via the splenic circulation. Freshly isolated human EpCAM+ cells or cells from hHpSC colonies were suspended in KM and transplanted into the livers of 5-week-old mice. Half of the mice were treated with carbon tetrachloride (0.6 μL/g body weight), a treatment that results in loss of pericentral cells, zone 3 in the liver acinus, and creates a cellular vacuum permissive for expansion and engraftment of transplanted cells. One week, 2 weeks, and 40 days after transplantation, the mice were sacrificed, and the livers fixed, embedded, and sectioned. The sections were stained for expression of human-specific proteins using one of the antibodies noted in Table 2.

Quantification

Cell numbers and colony area sizes

To quantify total areas and proliferation dynamics of hHpSCs, micrographs were analyzed daily using Metamorph software as highlighted below:

- Method-1 incorporates "thresholding" to isolate entwined cell borders with integrated computational meshes. These meshes were automatically interpreted as cell objects.
- Method-2 incorporates a semiautomated approach. Operators physically select hHpSC colonies while information is networked into spreadsheet formats.

Estimates of hHpSC Growth Within Colonies

To determine proliferation states of hHpSC colonies, Metamorph "sketching tools" were used to monitor hHpSCs in culture. Subsequently, total area dimensions of the hHpSC colonies were calculated and used to define the doubling time, saturation density kinetics, and stability at confluence.

Cell doubling times and stability of cells at confluence were defined by assuming hHpSCs have stable cell diameters, an assumption supported by our findings that cell morphologies remained uniform throughout culture periods. Image analysis using Metamorph Tracking Software enabled us to generate data on hHpSC proliferation characteristics.

1. Cultures were analyzed daily using an IX70 microscope to determine the initiation of hHpSC colony growth and then follow the growth of each hHpSC colony throughout the study.

Table 2. Antibodies Utilized

Antibody	*Titer*	*Company (catalog #)*
α-Fetoprotein (AFP)	1:500	Sigma (#8452), St. Louis, MO
Albumin	1:800	Sigma (#A6684), St. Louis, MO
Cytokeratin (CK) 19	1:200	NovaCastra, Newcastle upon Tyne, United Kingdom (#NCL-CK19)
EpCAM (CD 326). Also known as tumor-associated calcium signal transducer, GA733-2, KSA, ESA, or 17-1A antigen	1:800	Neomarkers, Fremont, CA (#MS-155-P1ABX)
ICAM-1 (CD54)	1:200	Pharmingen, San Diego, CA (#664970), or Bender MedSystems, Burlingame, CA (#BMS108)
NCAM (CD56)	1:250	Becton Dickinson (#340363) or NovoCastra (#CD56-1B6)
Goat anti-mouse IgG2a (Alex Fluor 568)	1:200	Molecular Probes (#A21124), Eugene, OR
Goat anti-mouse IgG1 (Cy5)	1:200	Southern Biotec, Birmingham, AL (#1070-15)
Isotype controls		Becton Dickinson, Franklin, NJ (#349041 and #349043)
Mouse IgG-FITC and IgG-PE		

2. All colonies were counted and the findings presented graphically in Figures 3–5.
3. Daily, and from cultures under each condition, 10 random 10× images were obtained and used for analyses that yielded the data in Figures 3–5.
4. At the end of the culture studies, Metamorph Tracking Software was used to define exterior borders of the colonies to allow precise measurements of the dimensions of the colonies. This was facilitated by incorporating hemocytometer grid images that were used on 10 random images of hHpSC colonies. Automated colony outlining was accomplished by image thresholding. An average area of a colony was calculated as:

$$\text{Average colony area} = \frac{\text{Individual colony area}}{10}$$

The average area was extrapolated onto the total culture surface area represented by a 35 mm Petri dish ($r = 1.75\,\text{cm}$ $A = 9.625\,\text{cm}^2$). Thus, a direct "area" relationship was achieved.

$$\frac{\text{Micrograph total area}}{\text{Average colony area}} = \frac{\text{Total culture surface area}}{X} = \frac{9.625\,\text{cm}^2}{X}$$

5. Cell count determination: Metamorph software was used to interpret the dimensions of a single cell. Ten random cell images were quantified, on a daily basis, by outlining cell borders and doing Metamorph calibrations.
   - From all images, individual radii (μm) and area (μm^2) measurements were used to establish "average" cell geometries. The cell radius of an hHpSC was found to be $8 \pm 1\,\mu m$ with an area of $2.0114E\text{-}6\,\text{cm}^2$.
   - Cell areas were used to determine total cell numbers within a colony.

$$\text{Cell Numbers} = \frac{\text{Average colony area}}{\text{Average cell area}}$$

Data Analysis

Each data point was derived from the average of a minimum of three tissue isolations and with triplicate measurements per experiment. Functional data values were analyzed with Microsoft Excel (Microsoft, Seattle, WA). Proliferation measurements were determined using Metamorph software (Universal Imaging, Downingtown, PA). All data sets were plotted using Sigma Plot (SPSS Science, Chicago, IL) where error bars indicate the standard deviation.

Telomerase

Indirect measurements of telomerase activities were accomplished using a modified telomeric repeat amplification protocol (TRAP).[13–16] The TRAP assays for telomerase activities were measured ($n = 4$) for hHpSC colonies and compared against control cells, HeLa, used routinely as a standard in these assays. Two-step PCR was performed with 10-min denaturation at 95°C and 35 cycles at 95°C, 20 s and 60°C, 90 s. Lineage standard curves were created using extracts from 1, 10, 100, 1000, and 10,000 HeLa cells.