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Engraftment and Repopulation Potential of Late Gestation Fetal Rat Hepatocytes

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Abstract

Background—The limited availability of donor organs has led to a search for alternatives to liver transplantation to restore liver function and bridge patients to transplantation. We have shown that the proliferation of late gestation (embryonic day 19; ED19) fetal rat hepatocytes is mitogen-independent, and that mechanisms regulating of mRNA translation, cell cycle progression and gene expression differ from those of adult rat hepatocytes. In the present study, we investigated whether E19 fetal hepatocytes can engraft and repopulate an injured adult liver.

Methods—Fetal hepatocytes were isolated using a monoclonal antibody against a hepatic surface protein, leucine amino peptidase (LAP). LAP+ and LAP– fractions were analyzed by immunofluorescence and microarray. Immunopurified E19 liver cells from DPPIV+ rats were transplanted via splenic injection into partial hepatectomized DPPIV– rats that had been pretreated with mitomycin C.

Results—More than a third of LAP+ fetal hepatocytes expressed ductal markers. Transcriptomic analysis revealed that these dual expressing cells represent a population of less well differentiated hepatocytes. Upon transplantation, LAP+ late gestation fetal hepatocytes formed hepatic, endothelial and ductal colonies within 1 month. By 10 months, colonies derived from LAP+ cells increased so that up to 35% of the liver was repopulated by donor-derived cells.

Conclusions—Late gestation fetal hepatocytes, despite being far along in the differentiation process, possess the capacity for extensive liver repopulation. This is likely related to the unexpected presence of a significant proportion of hepatocyte marker-positive cells maintaining a less well differentiated phenotype.

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AUTHOR CONTRIBUTIONS

JAS conceived the study. HFV and JAS performed the animal studies. HFV performed the staining. JMB performed the gene expression analysis of microarray data. JAS, JMB and PAG interpreted the data. JAS drafted the manuscript. All authors contributed to revisions of the manuscript and have read and approved the final version.

DISCLOSURES

The authors declare no conflicts of interest.

INTRODUCTION

Chronic liver disease is currently the 12th leading cause of death in the United States.¹ Liver transplantation is the only effective therapeutic option for patients with end-stage liver disease. The severe shortage of donor organs results in high morbidity and mortality with several thousand waitlisted patient deaths per year.² This has led to the search for novel therapies to restore liver function and bridge patients until a donor liver becomes available. Hepatic cell transplantation is an alternative strategy to generate new liver parenchyma.^{3,4} However, the successful, widespread application of this therapy will require a better understanding of the characteristics that make a particular cell type optimal for this use, and development of strategies to promote engraftment, expansion and long-term survival of the transplanted cells.

Rodent transplantation models have been established as 1 of the most definitive methods to assess the ability of cells to restore injured parenchyma.^{5,6} The majority of studies have focused on fetal liver stem/progenitor cells isolated from developing rats on embryonic day (ED) 12–14, a time in liver development prior to commitment to the hepatic or ductal lineage.^{7,8} These cells have been shown to repopulate adult liver through the process of cell competition.⁹ Previous studies in our laboratory have shown that late gestation fetal hepatocytes isolated on ED19 (term, ED21) are highly proliferative and relatively well-differentiated along a hepatic lineage, expressing albumin, glycolytic enzymes and other indicators of mature hepatocyte function.^{10–12} We have also shown that growth factor signaling pathways involving ERK1/2 and PI3K/Akt are uncoupled in late gestation fetal liver.^{13,14} In addition, fetal hepatocyte proliferation is resistant to rapamycin, the cognate inhibitor of the mTOR pathway.^{15,16} This signaling phenotype is in sharp contrast to that of adult hepatocytes and is a result of differences in gene expression, cell cycle control and regulation of protein translation.^{17–19} We hypothesized that the mitogen-independence ED19 fetal hepatocytes would provide them with a selective growth advantage and the ability to engraft and repopulate an injured adult liver.

In the present study, we have tested this hypothesis by isolating ED19 fetal hepatocytes using a monoclonal antibody against a hepatic surface protein, leucine amino peptidase (LAP), and assessing their repopulating ability using the dipeptidyl peptidase IV (DPPIV)/mitomycin C model.^{20,21} Phenotypic characterization of the LAP+ fetal hepatocytes revealed that more than a third expressed ductal markers. Our studies demonstrated the ability of these fetal cells to engraft, proliferate and repopulate injured adult liver suggesting that the fetal hepatocyte signaling phenotype is maintained following transplantation and that these cells may serve as a good model for understanding the factors necessary for optimal cell transplantation.

MATERIALS AND METHODS

Animals

Timed-pregnant and 5–6 week old male American DPPIV+ F344 rats were obtained from Charles River Laboratories (Wilmington, MA). Host German F334 rats (5–6 weeks of age) that express an inactive form of DPPIV (DPPIV-) were obtained from our breeding colony

maintained at Rhode Island Hospital. All animals were housed under standard conditions with access to food and water ad libitum. Adult rodents were euthanized by exsanguination under isoflurane anesthesia. All animal studies were performed in accordance within the guidelines of the National Institutes of Health and the Rhode Island Hospital Institutional Animal Care and Use Committee.

Cell Isolation

Adult hepatocytes were isolated from DPPIV+ rats by 2-step collagenase digestion as previously described.²² E19 fetal DPPIV+ liver cells were isolated by collagenase digestion.²³ The first 6 pups from each litter were utilized to avoid the effects of stress. E19 fetal hepatocytes were isolated as previously described²⁴ using a monoclonal antibody against LAP, a hepatic cell surface marker, in combination with micromagnetic beads. Labeled cells were sorted using an autoMACS separator with the autoMacS double positive selection in sensitive mode program. In a second selection strategy, antibodies against the ductal cell surface marker OC.2 and the hepatic marker LAP were utilized to isolate subpopulations of fetal hepatocytes. Briefly, E19 fetal liver cells were incubated with anti-OC.2 followed by incubation in antimouse IgM microbeads. The resulting autoMacS sorted OC.2- and OC.2+ fractions were incubated with anti-LAP followed by incubation in antimouse IgG microbeads. This selection resulted in the LAP+/OC.2-, LAP+/OC.2+ and LAP-/OC.2- fractions used for subsequent analysis. Viability of the cell isolates was determined by Trypan Blue dye exclusion. Purity of the cell fractions was verified by immunofluorescent staining of acetone-fixed cytopins.

Immunofluorescent Staining

Indirect immunofluorescence (IIF) was performed for each antigen on multiple acetone fixed cryosections (5µm) or fixed cells from a minimum of 3 biological replicates. Primary antibodies included hepatocyte markers LAP,²⁵ H4,²⁷ Cyp2E1 (Abcam, Cambridge MA) and albumin (Thermo Fisher Scientific, Waltham MA); cholangiocyte markers BD.2,²⁷ OC.2²⁶ and CK-19; and endothelial markers RECA1 (Abcam) and SE1 (Novus Biologics, Littleton CO). A mouse monoclonal against active DPPIV²¹ was used to identify transplanted fetal cells. Dual IIF was performed by sequential incubation in the first primary antibody and an Alexafluor-conjugated Ig secondary antibody (Thermo Fisher Scientific) followed by the second primary antibody and incubation in the appropriate Alexafluor-conjugated secondary antibody. Omission of the primary antibody was used as a negative control. As an additional control condition, the order of the primary antibodies was reversed to account for interference between the 2 antibodies. LAP+ cells were quantified from three 20× fields per slide.

Hepatocyte Transplantation and Liver Repopulation

Host DPPIV- rats were administered 2mg/kg mitomycin C by splenic injection as previously described.²⁰ One week after mitomycin C injection, DPPIV- hosts were subjected to 2/3 partial hepatectomy (PH). Immediately following PH, 5×10⁶ DPPIV+ mock-purified total, LAP+ or LAP- fetal liver isolates were transplanted via splenic injection.²⁴ In order to acquire an adequate number of fetal cells for transplantation, liver cells harvested from male and female fetuses derived from 5–8 dams were pooled prior to immunopurification. Host

animals were euthanized 1 or 10 months following transplantation. For 10 month time point, 2 independent transplantation experiments were performed. Liver repopulation was determined by enzyme histochemistry for DPPIV on acetone-fixed cryosections as previously described.²⁸ Slides were scanned using the Aperio ScanScope system (Aperio Technologies, Inc., Vista, CA). NIH Image J software was used to calculate total section area and the area occupied by DPPIV+ colonies. Image analysis was performed as described in the Supplemental Materials and Methods. All sections were analyzed in a blinded fashion.

Gene Expression Analysis

Total RNA was isolated from frozen cell pellets using the Mirvana Kit (Thermo Fisher Scientific) per the manufacturer's instructions and analyzed using Affymetrix Rat Gene ST 1.0 arrays (Affymetrix, Santa Clara, CA). Microarray data are accessible through GEO Series accession number GSE97897. Principal component analysis (PCA) was performed in R as described.²⁹ Identification of differentially expressed genes using a false discovery rate (FDR) of <0.05 ³⁰ was performed with Partek Genomic Suite version 6.6 (Partek, St. Louis, MO). Volcano plots used the adjusted p value from pair-wise analysis of variance (ANOVA) results and fold-change in gene expression. Ingenuity Pathway Analysis (IPA; Qiagen, www.qiagen.com/ingenuity) was performed as previously described.¹⁹ Input for IPA was significant genes (FDR <0.05) with a fold-change greater or equal to ± 3 . IPA categories were considered significant when the p value was below the p values obtained from 5 similarly sized sets of randomly selected genes with a fold-change in expression > 1.2 .

Statistics

Statistical analyses were performed in GraphPad Prism (San Diego, CA). For comparison of the percentage of LAP+ cells, results were analyzed by 1-way ANOVA with a posthoc Tukey test. A Student's t test was employed to compare the repopulating ability of LAP+ cells versus total liver isolate.

RESULTS

Isolation and Phenotypic Characterization of Late Gestation Fetal Hepatocytes

Hepatocytes from DPPIV+ ED19 fetal liver cell isolates were immunopurified using a monoclonal antibody for the hepatic marker LAP in combination with micromagnetic bead sorting. When we analyzed triplicate isolates enriched for LAP by immunofluorescence (Figure 1A), $94\% \pm 2.5\%$ of the cells showed reactivity with the LAP antibody used for selection. On average, 8% of the cells in the unbound LAP- fraction were positive. The purity of the LAP isolation is highly reproducible with similar results obtained in more than 25 experiments performed between 2004 and 2007. To further investigate the phenotype of the LAP+ and LAP- fractions, we carried out dual immunofluorescence for additional hepatic and ductal markers. The clear majority of cells in the LAP+ fraction was positive for the hepatocyte markers albumin and H4 (Figure 1B). On average about 50% of the LAP+ fetal hepatocytes also expressed 2 ductal markers, OC.2 and BD.2 (Figure 1B). There was variability between cell preparations from different litters where the percent of dual positive cells ranged from 27% to 67%. In contrast, very few cells ($<2\%$) in the LAP- fraction were

positive for albumin (Figure 1C). However, a subset of these cells expressed the ductal marker OC.2 (Figure 1C).

Isolation of Subpopulations of Late Gestation Fetal Hepatocytes

To further characterize the 2 subpopulations of LAP+ fetal hepatocytes, we immunopurified ED19 liver cells using a monoclonal antibody against OC.2 followed by selection with anti-LAP. Immunofluorescent analysis for LAP and OC.2 revealed the LAP+/OC.2+ fraction to be highly enriched in cells expressing both LAP and OC.2, while OC.2+ cells were only rarely seen in the LAP+/OC.2- fraction (Figure 2A). The LAP-/OC.2- fraction was depleted of cells positive for either marker. We hypothesized that the LAP+ cells coexpressing ductal markers would represent a less mature population of fetal liver cells. To test this hypothesis, we performed immunofluorescence for albumin as an indicator of differentiation status in the 3 subpopulations of fetal liver cells (Figure 2B). A few LAP+/OC.2+ cells expressed albumin while the majority of LAP+/OC.2- cells were intensely stained. As expected, the LAP-/OC.2- fraction was negative for albumin.

Gene Expression Profiling of Subpopulations of Late Gestation Fetal Hepatocytes compared to Adult Hepatocytes

To further characterize the differences in the LAP+/OC.2- and LAP+/OC.2+ cells, we profiled global gene expression using Affymetrix microarrays. RNA was extracted from the 2 populations of freshly isolated fetal cells and from adult hepatocytes. Principal component analysis (PCA) was performed to assess the degree of similarity between the 3 samples (Figure 3A). This analysis revealed differences between all 3 groups. The LAP+/OC.2+ and LAP+/OC.2- fetal cells were segregated from each other to a degree similar to their separation from adult hepatocytes.

We constructed volcano plots using ANOVA to identify genes that were significantly different in pair-wise comparisons. These volcano plots (Figure 3B) showed that the LAP+/OC.2- fetal cells differed from adult hepatocytes to a lesser degree than LAP+/OC.2+ fetal cells. This difference was accounted for by both fewer significant genes and a lesser fold-difference of both upregulated and downregulated genes. A comparison of the LAP+/OC.2- and LAP+/OC.2+ fetal cells showed far fewer significant genes and smaller fold-differences than the comparisons with adult hepatocytes. These results led us to hypothesize that the LAP+/OC.2- fetal cells exhibited a greater degree of functional hepatic differentiation than the LAP+/OC.2+ cells. We further hypothesized that the LAP+/OC.2+ cells may represent a residual bipotential cell population that had yet to commit to a hepatic or ductal lineage.

To test these hypotheses, we performed pathway analysis using IPA. The input for this analysis consisted of significant genes (FDR = 0.05) with a fold-difference between paired groups of ≥ 3 (Supplemental Table 1). A comparison of the 2 fetal groups with adult hepatocytes (Table 1, Supplemental Table 2) revealed upregulation of IPA canonical pathways associated with lipid metabolism, xenobiotic metabolism, plasma protein secretion and bile secretion in adult hepatocytes. The genes accounting for the identification of these pathways were mainly aldehyde dehydrogenases, fatty acid binding proteins, cytochrome

p450 enzymes, transporters and complement factors. There were no additional pathways identified in the adult versus LAP+/OC.2- comparison. However, additional IPA results related to amino acid metabolism and other functions of differentiated hepatocytes were upregulated in the adult compared to LAP+/OC.2+ cells.

Most canonical pathways identified as upregulated in the adult versus fetal cells were also upregulated in the LAP+/OC.2- compared to the LAP+/OC.2+ fetal hepatocytes (Table 1). The genes accounting for the pathways were also similar to those identified in the adult-fetal comparison with the exception of lipid metabolism. In the fetal comparison, differences in the expression of apolipoproteins and the enzymes and regulators of lipid metabolism accounted for identification of the pathway.

Whereas upregulated pathways were identified in comparisons of adult hepatocytes to either fetal cell fraction, pathways downregulated in the adult cells were only identified in the comparison with LAP+/OC.2+ fetal cells. These downregulated pathways were, in general, related to cell proliferation and were accounted for by cell cycle proteins and synthetic enzymes. Heme synthesis was also identified as being downregulated in the LAP+/OC.2- compared to the LAP+/OC.2+ fetal cells. We interpreted the IPA pathway results as consistent with the conclusion that the LAP+/OC.2- fetal cell population bears greater similarity to adult hepatocytes than the LAP+/OC.2+ population, consistent with the latter representing a less well differentiated, bipotential cell population.

The IPA results for upstream regulators mirrored the results for canonical pathways. Transcriptional regulators (Figure 4, Supplemental Table 2) with well-known roles in the expression of liver-specific genes (HNF1A, HNF4a), phase I and II enzymes (NR1I3, NR1I2, RORA, RXR) and lipid metabolism (PPARA, PPARG and PPARGC1A)^{31,32} were identified in the analysis of genes that were upregulated in the adult hepatocytes versus LAP+/OC.2- (Figure 4A) and LAP+/OC.2+ (Figure 4B) comparisons. The transcriptional regulators identified for genes downregulated in adult hepatocytes versus either fetal hepatocyte population included those involved in cell proliferation and growth (E2Fs, Myc, Jun). Comparison of the 2 fetal hepatocyte populations identified many of the same transcriptional regulators controlling hepatic differentiation and function for the genes upregulated in the LAP+/OC.2- compared to the LAP+/OC.2+ fraction (Figure 4C). GATA1 and 2, CEBPE, MLK1 and other transcriptional regulators associated with cellular proliferation and hematopoiesis were identified as upstream regulators for the genes upregulated in LAP+/OC.2+ versus LAP+/OC.2- cells.

We used gene set enrichment analysis (GSEA) as a complementary approach to characterizing the transcriptome in the 3 cell populations. Through examination of relative enrichment for sets of genes using all genes detected on the microarray, GSEA is well suited to detect numerous small differences in gene expression.³³ Results (Figure 5, Supplemental Table 3) reinforced the findings obtained using IPA. Using a stringent cut-off for significance (FDR = 0.001), there were far fewer KEGG gene sets enriched in adult hepatocytes compared to LAP+/OC.2- relative to the adult hepatocyte versus LAP+/OC.2+ fetal hepatocyte comparison (Figures 5A and B). The genes sets, enriched in adult cells compared to either fetal sample, largely reflected differences in cytochrome p450 enzymes,

fatty acid metabolism, steroid biosynthesis and bile acid biosynthesis. Additional genes sets, enriched in the adult versus LAP+/OC.2+, related to amino acid synthesis and intermediary metabolism. Gene sets reflecting cellular growth and proliferation were enriched in the 2 fetal cell fractions compared to adult hepatocytes. Results from the comparison of the 2 fetal hepatocyte samples showed enrichment of the sets related to functional differentiation and metabolism in the LAP+/OC.2- cells while sets related to growth and proliferation were enriched in the LAP+/OC.2+ cells.

Analysis for transcription factor targets (Supplemental Table 3) revealed enrichment of E2F in the LAP+/OC.2+ compared to LAP+/OC.2- cells and to adult hepatocytes. Members of the HNF family were enriched in the adult and LAP+/OC.2- hepatocytes relative to LAP+/OC.2+ cells. There were no significantly enriched transcription factor target gene sets in the adult versus LAP+/OC.2- hepatocytes. E2F family members were enriched in the LAP+/OC.2- relative to adult hepatocytes.

Engraftment and Liver Repopulation by LAP+ Fetal Hepatocytes

The proliferative and differentiation potential of late gestation LAP+ fetal hepatocytes was further investigated using the mitomycin C DPPIV transplant model.²⁰ DPPIV- adult rats were administered mitomycin C in order to inhibit adult hepatocyte proliferation. Hosts underwent 2/3 partial hepatectomy immediately prior to transplanting DPPIV+ total fetal liver isolates and immunopurified LAP+ and LAP- cell isolates via splenic injection. Liver repopulation was assessed by DPPIV enzyme histochemistry (Figure 6). At 1 month following transplantation, the LAP+ cells had formed numerous, small DPPIV+ colonies throughout the liver parenchyma (Figure 6A). Colony morphology indicated that the LAP+ fetal hepatocytes formed both hepatic and endothelial colonies. Rare, small DPPIV+ ductal colonies were also observed (Figure 6C, upper panel). In contrast, transplantation of equal numbers of unfractionated fetal liver cells or LAP- cells did not result in repopulation of the liver parenchyma. The rare colonies formed by these 2 cell populations displayed endothelial morphology.

To assess the long-term repopulating potential of LAP+ fetal hepatocytes, we maintained adult hosts for 10 months after transplantation (Figure 6B). Repopulation accounted for up to 35% of the liver surface area, with LAP+ fetal hepatocytes forming large hepatic and endothelial colonies that were indistinguishable from the surrounding host parenchyma. Mixed colonies consisting of donor-derived hepatocytes and endothelial cells were frequently observed. The number of well-organized DPPIV+ ductal structures increased substantially at 10 months compared to 1 month posttransplant (Figure 6C, bottom panel).

Immunofluorescent analysis was performed to determine the degree of functional differentiation of the cells within the colonies derived from LAP+ cells (Figure 7). The hepatic colonies showed canalicular localization of LAP and staining for H4 and CYP2E1 similar to that of the surrounding host hepatocytes. The DPPIV+ hepatocytes did not display positive staining for the ductal marker OC.2. Double IIF labeling showed that the DPPIV+ endothelial colonies were strongly positive for the pan-endothelial marker RECA-1 and the sinusoidal endothelial marker SE-1. Depending on the area examined, the donor-derived cells completely or partially lined the sinusoids of the host liver. The DPPIV+ ductal

structures displayed strong reactivity with CK-19 and OC.2 and were negative for hepatic markers. These structures were found both adjacent to donor-derived hepatic colonies and within the host parenchyma.

DISCUSSION

Cellular transplantation as an alternative to whole liver transplantation has great potential as a clinical therapy. However, the lack of understanding of the mechanisms involved in cell expansion, maintenance of differentiated function and survival have hindered the clinical application of this therapeutic strategy. Our studies on the mechanisms involved in fetal liver development indicate that the signaling pathways involved in the regulation of cell proliferation, growth and function differ from those active in adult hepatocytes.¹¹ More specifically, late gestation fetal hepatocyte proliferation is independent of key mitogenic signaling pathways, including mTOR and Erk.^{15,34} This unique signaling phenotype led us to hypothesize that late gestation fetal hepatocytes would have a selective growth advantage upon transplantation to the adult liver.

For the present study, we isolated ED19 fetal hepatocytes using a monoclonal antibody against the hepatic cell surface marker LAP. Phenotyping revealed that a third of these cells express both ductal and hepatic markers. Bipotential hepatoblasts that form the liver bud express both hepatic and cholangiocyte markers.⁷ The proportion of bipotential cells is thought to dramatically decrease at E16 as the cells become unipotent and commit to either a hepatic or cholangiocyte lineage.^{7,8,35} The continued expression of hepatic and ductal markers by a significant proportion of E19 hepatocytes suggest that these cells may retain bipotentiality. To determine whether the dual expressing cells represented a population with a distinct cellular phenotype, we profiled the transcriptome.

PCA showed that the 2 fetal cell fractions clustered separately from each other indicating that the LAP+/OC.2- and LAP+/OC.2+ fractions represent 2 distinct cell populations. IPA showed upregulation of pathways and transcription factors related to functional maturation in adult hepatocytes compared to the 2 fetal cell populations.³⁶ This was accounted for by differences in the expression of cytochrome p450 enzymes and other phase I/II enzymes that are induced during liver development.^{37,38} Further analysis of the 2 fetal cell populations compared to adult hepatocytes revealed many fewer changes in the LAP+/OC.2- cells. LAP+/OC.2+ cells were less well differentiated in terms of the expression of genes involved in amino acid metabolism, fatty acid oxidation and synthesis of plasma proteins. Direct comparison of the 2 fetal populations showed increased expression of genes related to functional differentiation in the LAP+/OC.2- cells, while genes involved in cellular proliferation were increased in the dual positive cells. In contrast to prior studies, our data suggest that not all hepatoblasts undergo a definitive switch in gene expression at E16. Instead a third of the cells appear to be relatively less mature while maintaining expression of both hepatic and ductal cells markers. This heterogeneity in cell types has not been addressed in past studies profiling the transcriptional changes that occur during liver development as whole liver was used for analysis.^{8,38}

Transplantation of immunopurified LAP+ ED19 hepatocytes resulted in repopulation of up to 35% of mitomycin C-treated host liver by 10 months posttransplant. Transplanted fetal cells formed hepatic, ductal and endothelial colonies. The presence of dual staining bipotential liver cells in the LAP+ population may be responsible for the ability of the fetal cells to form both ductal and hepatic colonies. This conclusion is consistent with the findings of Hixson et al.,³⁹ who reported that immunopurified late gestation ED18/19 cholangiocytes expressing OC.2 were capable of liver repopulation forming both hepatic and ductal colonies. However, fully mature adult hepatocytes have been shown to dedifferentiate into cholangiocyte-like cells and express ductal markers.^{40,41} Thus we cannot exclude the possibility that the LAP+/OC.2- cells are also capable of differentiating along a ductal lineage. One limitation of our current study is that we were not able to directly determine whether the different LAP+ subpopulations vary in their ability to repopulate. The dual antibody immunopurification process we utilized to isolate the LAP+/OC.2- and LAP+/OC.2+ cell populations resulted in a significant decrease in cell survival following transplantation, precluding interpretation of transplantation results. We were also unable to directly test the bipotentiality of the LAP+/OC.2+ fetal cells as the fetal hepatocyte signaling phenotype is not maintained when fetal liver cells are cultured under our standard conditions.^{15,42}

Most previous liver cell transplantation studies have utilized ED12-ED14 fetal hepatic stem/progenitor cells. These cells have the ability to extensively repopulate injured and normal adult liver.^{9,43,44} The present observation that late gestation fetal hepatocytes, despite their being far along in the differentiation process, possess a similar capacity for repopulation was unexpected. This is likely related to the presence of a significant proportion of hepatocyte marker-positive cells maintaining a less well differentiated phenotype. We hypothesize that late gestation liver contains a bipotential cell population with an epigenotype that allows the cells to sustain their proliferative phenotype over many months. Characterization of the epigenome of these cells may yield important information regarding the cellular mechanisms that can support engraftment and sustained cellular expansion. It is our hope that this information, in combination with studies on the response of these cells to the adult liver microenvironment, will assist in the identification of an ideal cell population for use in clinical transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ANOVA	analysis of variance
DPPIV	dipeptidyl peptidase IV
ED	embryonic day
GSEA	gene set enrichment analysis
IIF	indirect immunofluorescence
IPA	Ingenuity Pathway Analysis
LAP	leucine amino peptidase
PCA	principal component analysis

References

1. Kochanek KD, Murphy SL, Xu J, Tejada-Vera B. Deaths: final data for 2014. *Natl Vital Stat Rep*. 2016; 65(4):1–122.
2. Kim WR, Lake JR, Smith JM, et al. OPTN/SRTR 2015 Annual Data Report: Liver. *Am J Transplant*. 2017; 17(Suppl 1):174–251. [PubMed: 28052604]
3. Pietrosi G, Vizzini G, Gerlach J, et al. Phases I–II matched case-control study of human fetal liver cell transplantation for treatment of chronic liver disease. *Cell Transplant*. 2015; 24(8):1627–1638. [PubMed: 24971879]
4. Hansel MC, Gramignoli R, Skvorak KJ, et al. The history and use of human hepatocytes for the treatment of liver diseases: the first 100 patients. *Curr Protoc Toxicol*. 2014; 62(14):12.1–23. [PubMed: 25378242]
5. Forbes SJ, Gupta S, Dhawan A. Cell therapy for liver disease: from liver transplantation to cell factory. *J Hepatol*. 2015; 62(1 Suppl):S157–169. [PubMed: 25920085]
6. Rezvani M, Grimm AA, Willenbring H. Assessing the therapeutic potential of lab-made hepatocytes. *Hepatology*. 2016; 64(1):287–294. [PubMed: 27014802]
7. Shiojiri N, Lemire JM, Fausto N. Cell lineages and oval cell progenitors in rat liver development. *Cancer Res*. 1991; 51:2611–2620. [PubMed: 1708696]
8. Petkov PM, Zavadil J, Goetz D, et al. Gene expression pattern in hepatic stem/progenitor cells during rat fetal development using complementary DNA microarrays. *Hepatology*. 2004; 39(3):617–627. [PubMed: 14999680]
9. Menthena A, Koehler CI, Sandhu JS, et al. Activin A, p15INK4b signaling, and cell competition promote stem/progenitor cell repopulation of livers in aging rats. *Gastroenterology*. 2011; 140(3):1009–1020. [PubMed: 21147108]
10. Gruppuso PA, Brautigan DL. Induction of hepatic glycogenesis in the fetal rat. *Am J Physiol Endocrinol Metab*. 1989; 256(1 Pt 1):E49–E54.
11. Gruppuso PA, Sanders JA. Regulation of liver development: implications for liver biology across the lifespan. *J Mol Endocrinol*. 2016; 56(3):R115–125. [PubMed: 26887388]
12. Gruppuso PA, Bienieki TC, Faris RA. The relationship between differentiation and proliferation in late gestation fetal rat hepatocytes. *Pediatr Res*. 1999; 46(1):14–19. [PubMed: 10400128]
13. Boylan JM, Gruppuso PA. In vitro and in vivo regulation of hepatic mitogen-activated protein kinases in fetal rats. *Am J Physiol Gastrointest Liver Physiol*. 1994; 267(6):G1078–G1086.
14. Anand P, Boylan JM, Ou Y, Gruppuso PA. Insulin signaling during perinatal liver development in the rat. *Am J Physiol Endocrinol Metab*. 2002; 283(4):E844–852. [PubMed: 12217903]

15. Boylan JM, Anand P, Gruppuso PA. Ribosomal protein S6 phosphorylation and function during late gestation liver development in the rat. *J Biol Chem.* 2001; 276(48):44457–44463. [PubMed: 11574531]
16. Sanders JA, Lakhani A, Phornphutkul C, Wu KY, Gruppuso PA. The effect of rapamycin on DNA synthesis in multiple tissues from late gestation fetal and postnatal rats. *Am J Physiol Cell Physiol.* 2008; 295(2):C406–C413. [PubMed: 18550700]
17. Gruppuso PA, Tsai SW, Boylan JM, Sanders JA. Hepatic translation control in the late-gestation fetal rat. *Am J Physiol Regul Integr Comp Physiol.* 2008; 295(2):R558–R567. [PubMed: 18565838]
18. Boylan JM, Gruppuso PA. D-type cyclins and G1 progression during liver development in the rat. *Bio Biochem Biophys Res Comm.* 2005; 330(3):722–730.
19. Boylan JM, Sanders JA, Neretti N, Gruppuso PA. Profiling of the fetal and adult rat liver transcriptome and translome reveals discordant regulation by the mechanistic target of rapamycin (mTOR). *Am J Physiol Regul Integr Comp Physiol.* 2015; 309(1):R22–R35. [PubMed: 25924882]
20. Brilliant KE, Mills DR, Callanan HM, Hixson DC. Engraftment of syngeneic and allogeneic endothelial cells, hepatocytes and cholangiocytes into partially hepatectomized rats previously treated with mitomycin C. *Transplantation.* 2009; 88(4):486–495. [PubMed: 19696631]
21. Thompson NL, Hixson DC, Callanan H, et al. A Fischer rat substrain deficient in dipeptidyl peptidase IV activity makes normal steady-state RNA levels and an altered protein. Use as a liver-cell transplantation model. *Biochem J.* 1991; 273(Pt 3):497–502. [PubMed: 1705112]
22. Seglen PO. Preparation of rat liver cells. *Exp Cell Res.* 1972; 74:450–454. [PubMed: 4343020]
23. Curran J, Thomas R, Bahner J, Roderick I, Oh W, Gruppuso PA. Mitogen-independent DNA synthesis by fetal rat hepatocytes in primary culture. *Exp Cell Res.* 1993; 209(1):53–57. [PubMed: 8224006]
24. Sanders JA. Late gestation fetal hepatocytes for liver repopulation in the rat. *Methods Mol Biol.* 2017; 1506:45–60. [PubMed: 27830544]
25. Mowery J, Hixson DC. Detection of cell-CAM 105 in the pericanalicular domain of the rat hepatocyte plasma membrane. *Hepatology.* 1991; 13(1):47–56. [PubMed: 1988344]
26. Hixson DC, Allison JP. Monoclonal antibodies recognizing oval cells induced in the liver of rats by N-2-fluorenylacetamide or ethionine in a choline-deficient diet. *Cancer Res.* 1985; 45:3750–3760. [PubMed: 2410100]
27. Faris RA, McBride A, Yang L, Affigne S, Walker C, Cha C-J. Isolation, propagation, and characterization of rat liver serosal mesothelial cells. *Am J Pathol.* 1994; 145(6):1432–1443. [PubMed: 7992846]
28. Piazza GA, Callanan HM, Mowery J, Hixson DC. Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix. *Biochem J.* 1989; 262(1):327–334. [PubMed: 2573346]
29. Adebayo Michael AO, Ahsan N, Zabala V, et al. Proteomic analysis of laser capture microdissected focal lesions in a rat model of progenitor marker-positive hepatocellular carcinoma. *Oncotarget.* 2017; 8(16):26041–26056. [PubMed: 28199961]
30. Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A.* 2003; 100(16):9440–9445. [PubMed: 12883005]
31. Costa RH, Kalinichenko VV, Holterman AX, Wang X. Transcription factors in liver development, differentiation, and regeneration. *Hepatology.* 2003; 38(6):1331–1347. [PubMed: 14647040]
32. Vacca M, Degirolamo C, Massafra V, et al. Nuclear receptors in regenerating liver and hepatocellular carcinoma. *Mol Cell Endocrinol.* 2013; 368(1–2):108–119. [PubMed: 22789748]
33. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A.* 2005; 102(43):15545–15550. [PubMed: 16199517]
34. Boylan JM, Gruppuso PA. A comparative study of the hepatic mitogen-activated protein kinase and Jun-NH2-terminal kinase pathways in the late-gestation fetal rat. *Cell Growth Differ.* 1996; 7:1261–1269. [PubMed: 8877106]

35. Zorn, AM. StemBook. , editor. Liver development. The Stem Cell Research Community, StemBook. Oct 31. 2008 doi/10.3824/stembook.1.25.1, <http://www.stembook.org>
36. Kymrzi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. *Genes Dev.* 2006; 20(16):2293–2305. [PubMed: 16912278]
37. Moscovitz JE, Aleksunes LM. Establishment of metabolism and transport pathways in the rodent and human fetal liver. *Int J Mol Sci.* 2013; 14(12):23801–23827. [PubMed: 24322441]
38. Lee JS, Ward WO, Knapp G, et al. Transcriptional ontogeny of the developing liver. *BMC Genomics.* 2012; 13:33. [PubMed: 22260730]
39. Simper-Ronan R, Brilliant K, Flanagan D, et al. Cholangiocyte marker-positive and -negative fetal liver cells differ significantly in their ability to regenerate the livers of adult rats exposed to retrorsine. *Development.* 2006; 133(21):4269–4279. [PubMed: 17021035]
40. Michalopoulos GK, Bowen WC, Mule K, Lopez-Talavera JC, Mars W. Hepatocytes undergo phenotypic transformation to biliary epithelium in organoid cultures. *Hepatology.* 2002; 36(2): 278–283. [PubMed: 12143035]
41. O'Neill KE, Thowfeequ S, Li WC, et al. Hepatocyte-ductal transdifferentiation is mediated by reciprocal repression of SOX9 and C/EBPalpha. *Cell Reprogram.* 2014; 16(5):314–323. [PubMed: 25153359]
42. Boylan JM, Gruppuso PA. Uncoupling of hepatic, epidermal growth factor-mediated mitogen-activated protein kinase activation in the fetal rat. *J Biol Chem.* 1998; 273:3784–3790. [PubMed: 9452512]
43. Oertel M, Rosencrantz R, Chen YQ, et al. Repopulation of rat liver by fetal hepatoblasts and adult hepatocytes transduced ex vivo with lentiviral vectors. *Hepatology.* 2003; 37(5):994–1005. [PubMed: 12717380]
44. Oertel M, Menthe A, Dabeva MD, Shafritz DA. Cell competition leads to a high level of normal liver reconstitution by transplanted fetal liver stem/progenitor cells. *Gastroenterology.* 2006; 130(2):507–520. [PubMed: 16472603]

Figure 1.

Enrichment of LAP⁺ cells. Late gestation E19 fetal hepatocytes were enriched by immunopurification using an antibody against the hepatic marker LAP in combination with micromagnetic beads. **A.** Acetone fixed cytopins of total liver isolates (Unfrac), and the LAP⁺ and LAP⁻ fractions derived from 3 litters were stained for LAP (green) and counterstained with DAPI (blue). Representative 20 $\times$ images are shown. Scale bar = 50 μ m. The graph depicts the percent of LAP⁺ cells in each fraction. Data are expressed as the mean + 1SD. a, Unfrac vs. LAP⁺, $p = 0.001$; b, LAP⁺ vs. LAP⁻, $p = 0.0001$; c, Unfrac vs. LAP⁻,

p 0.01. Acetone-fixed cytopins of immunopurified LAP+ (**B**) and LAP– (**C**) fractions were dual labeled with the indicated antibodies. Cytopins were counterstained with DAPI. Representative 20× confocal images are shown. Similar results were obtained in 3 independent experiments. Scale bar = 50µm.

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Figure 2.

Enrichment of E19 fetal cells expressing hepatic and ductal markers. E19 fetal liver cells were isolated using antibodies against the hepatic marker LAP and the ductal marker OC.2 in combination with micromagnetic beads. **A.** Dual immunostaining was performed with antibodies against LAP and OC.2. **B.** Cells were immunostained with an antibody against albumin. Representative 20× images are shown. Scale bar = 50μm. Similar results were obtained in a second independent experiment.

Figure 3. Microarray analysis of triplicate E19 fetal LAP+/OC.2-, LAP+/OC.2+ cells and adult hepatocytes. **A.** Principal component analysis (PCA) showing the first, second, and third components (PC1, PC2, PC3) representing 42.7%, 16.6%, and 12% of the variance in the data, respectively. Adult hepatocytes, black circles; LAP+/OC.2- fetal hepatocytes, red circles; LAP+/OC.2+ fetal hepatocytes, green circles. **B.** Volcano plots for the 3 pair-wise comparisons showing the negative log₁₀ of the p value versus the log₂ fold-change in gene expression. The red line represents the cut-off for statistical significance, a FDR p value of 0.05. Red dots represent significant genes with a fold difference ≥ 3 .

Figure 4. IPA results for transcriptional regulators of upregulated and downregulated genes comparing E19 LAP+/OC.2- cells, E19 LAP+/OC.2+ cells and adult hepatocytes. Significant genes (FDR p value<0.05) with a fold-difference greater than or equal to ± 3 comprised the input for IPA. Data are shown as the negative log₁₀ of unadjusted p values. Filled bars, regulators for upregulated genes; open bars, regulators for downregulated genes. Only categories for which p was less than 10^{-5} are shown. This threshold for significance was based on analysis

of control data sets. **A.** Adult vs. LAP+/OC.2-. **B.** Adult vs. LAP+/OC.2-. **C.** LAP+/OC.2- vs. LAP+/OC.2+.

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Figure 5. Gene Set Enrichment Analysis (GSEA) of the transcriptome from E19 LAP+/OC.2- cells, E19 LAP+/OC.2+ cells and adult hepatocytes. The graphs depict the positive and negative enrichment score for KEGG pathways with a FDR q-value 0.001 for each comparison. The x-axis represents the GSEA enrichment score. **A.** Adult vs. LAP+/OC.2-. **B.** Adult vs. LAP+/OC.2+. **C.** LAP+/OC.2- vs. LAP+/OC.2+.

Figure 6.

Liver repopulation by DPPIV+ E19 fetal LAP+ cells at 1 and 10 months after transplantation into DPPIV- adult hosts. DPPIV+ cells derived from male and female fetuses pooled from 5–8 litters were incubated with LAP or mock purified and sorted on an AutoMacs cell separator. Mock purified total cell isolates (unfractionated), LAP+ and LAP- fractions were transplanted via splenic injection into partial hepatectomized DPPIV- adult hosts that were pretreated with mitomycin C. Histochemistry for DPPIV enzyme activity was performed on acetone fixed cryosections prepared from liver 1 month (**A**) or 10 months (**B**) following transplantation. Images were acquired at 4× (top panels, Scale bar = 1mm.) and 20 × (bottom panels, Scale bar = 100 μm.). Repopulation was determined using a minimum of 5 sections from 5 different areas/lobes of each rat liver. DPPIV- hosts from 2 independent

experiments were analyzed at 10 months posttransplant. Data are graphed as individual animals with the mean for each cell transplantation group shown as a horizontal line. *, $p < 0.05$, LAP+ vs. Unfrac.

Figure 7.

Phenotypic analysis of LAP⁺ donor colonies present at 10 months following transplantation into DPPIV⁻ adult hosts. Acetone fixed cryosections from triplicate DPPIV⁻ adult hosts were double labeled with an antibody specific for enzymatically active DPPIV (red) and the following markers. **A.** H4: DPPIV⁺/H4⁻ donor ductal colonies (arrows) are located adjacent to a cluster of DPPIV⁻/H4⁺ host hepatocytes. DPPIV⁺/H4⁺ donor hepatocytes are also shown (arrow heads). **B.** H4, DPPIV⁺/H4⁺ donor hepatic colony with a small DPPIV⁺/H4⁻ endothelial colony (arrow). **C.** LAP: DPPIV⁺ colony displaying strong canalicular LAP reactivity **D.** Cyp2E1. **E.** RECA-1 **(F)** SE-1 **(F)**. **G.** OC.2 **(G)** and CK-19 **(H)** show donor-derived DPPIV⁺ ductal structures. Scale bars = 100 μ m.

Table 1

Up and downregulated functional processes based on IPA Canonical Pathways comparing adult hepatocytes (AdH), LAP+/OC.2- and LAP+/OC.2+ fetal cells.

Comparison	Functional Process	IPA Pathways	Representative Genes
Upregulated in AdH reative to LAP+/OC.2- and LAP+/OC.2+	Lipid Metabolism (p value 10^{-39} to 10^{-9})	LPS/IL-1 Mediated Inhibition of RXR Function, FXR/RXR Activation, LXR/RXR Activation	Acetyl coA-oxidases, aldehyde dehydrogenases, fatty acid binding proteins
	Xenobiotic Metabolism (p value 10^{-15} to 10^{-6})	Nicotine Degradation III, Melatonin Degradation I, Superpathway of Melatonin Degradation, Xenobiotic Metabolism Signaling, Bupropion Degradation, Acetone Degradation I (to Methylglyoxal), Estrogen Biosynthesis, Serotonin Degradation, Superpathway of Citrulline Metabolism, Superpathway of Melatonin Degradation, PXR/RXR Activation	Phase I/II ezymes: transporters, transferases, dehydrogenases, Cyps
	Plasma Protein Secretion (p value 10^{-14} to 10^{-6})	Complement System, Acute Phase Response Signaling	Complement factors & components, serpin peptidase inhibitors
	Bile Secretion (p value 10^{-39} to 10^{-16})	Bile Acid Biosynthesis, Neutral Pathway	Cyps, alpha-keto reductases, solute carriers
Upregulated in AdH compared to LAP +/OC.2-	N/A	No significant pathways	N/A
Upregulated in AdH compared to LAP +/OC.2+	Xenobiotic Metabolism (p value 10^{-15} to 10^{-6})	Ethanol Degradation II, Aryl hydrocarbon receptor signaling, dopamine degradation, Glutathione-mediated Detoxification, Noradrenaline & Adrenaline Degradation	Dehydrogenases, monoamine oxidases, transferases,
	Amino Acid Metabolism (p value 10^{-9} to 10^{-6})	Arginine Biosynthesis IV, Tryptophan Degradation III (eukaryotic), Valine Degradation, Superpathway of Methionine Degradation, Glycine Betaine Degradation	Hydratases, methyltransferases, aminotransferases
	Plasma Protein Secretion (p value 10^{-11} to 10^{-8})	Coagulation System, Complement System, Extrinsic Prothrombin Activation Pathway, Intrinsic Prothrombin Activation Pathway	Complement factors & components, coagulation factors, serpin family members
	Lipid Metabolism (p value 10^{-9})	Fatty acid β oxidation	Acetyl coA-acyltransferase, dehydrogenases, solute carriers
	Other (p value 10^{-6})	NRF2-mediated oxidative stress response, TR/RXR Activation	Aldo-keto reductase, glutathione transferases, metabolic enzymes
Upregulated in LAP +/OC.2- compared to LAP+/OC.2+	Lipid Metabolism (p value 10^{-34} to 10^{-6})	LPS/IL-1 Mediated Inhibition of RXR Function, LXR/RXR Activation, FXR/RXR Activation, Atherosclerosis Signaling, Superpathway of Cholesterol Biosynthesis, Cholesterol Biosynthesis I, II & III	Apolipoproteins, lipoprotein lipases, acetyl CoA-synthetase, solute carriers

Comparison	Functional Process	IPA Pathways	Representative Genes
	Plasma Protein Secretion (p value 10^{-22} to 10^{-9})	Coagulation System, Complement System, Acute Phase Response Signaling, Extrinsic Prothrombin Activation Pathway, Intrinsic Prothrombin Activation Pathway	Complement factors & components, coagulation factors, serpin family members
	Xenobiotic Metabolism (p value 10^{-7})	Ethanol Degradation II, Aryl hydrocarbon receptor signaling	Phase I/II enzymes: transferases, aldehyde dehydrogenases
Downregulated in AdH relative to LAP +/-OC.2- and LAP +/-OC.2+	N/A	No significant pathways	N/A
Downregulated in AdH compared to LAP+/OC.2-	N/A	No significant pathways	N/A
Downregulated in AdH compared to LAP+/OC.2+	Proliferation (p value 10^{-9} to 10^{-7})	Cell Cycle Control of Chromosomal Replication, Mitotic Roles of Polo-Like Kinase, Cyclins and Cell Cycle Regulation	Cyclins, CDKs, MCMs
Downregulated in LAP+/OC.2- compared to LAP +/-OC.2+	Heme Synthesis (p value 10^{-6})	Heme Biosynthesis II	Synthetic enzymes