Fat-associated lymphoid clusters as expandable niches for ectopic liver development

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Abstract
Background and Aims: Hepatocyte transplantation holds great promise as an alternative approach to whole-organ transplantation. Intraportal and intrasplenic cell infusions are primary hepatocyte transplantation delivery routes for this procedure. However, patients with severe liver diseases often have disrupted liver and spleen architectures, which introduce risks in the engraftment process. We previously demonstrated i.p. injection of hepatocytes as an alternative route of delivery that could benefit this subpopulation of patients, particularly if less invasive and low-risk procedures are required; and we have established that lymph nodes may serve as extrahepatic sites for hepatocyte engraftment. However, whether other niches in the abdominal cavity support the survival and proliferation of the transplanted hepatocytes remains unclear.

Approach and Results: Here, we showed that hepatocytes transplanted by i.p. injection engraft and generate ectopic liver tissues in fat-associated lymphoid clusters (FALCs), which are adipose tissue–embedded, tertiary lymphoid structures localized throughout the peritoneal cavity. The FALC-engrafted hepatocytes formed functional ectopic livers that rescued tyrosinemic mice from liver failure. Consistently, analyses of ectopic and native liver transcriptomes revealed a selective ectopic compensatory gene expression of hepatic function–controlling genes in ectopic livers, implying a regulated functional integration between the two livers. The lack of FALCs in the abdominal cavity of immunodeficient tyrosinemic mice hindered ectopic liver development, whereas the restoration of FALC formation through bone marrow transplantation restored ectopic liver development in these mice. Accordingly,

Abbreviations: CD, cluster of differentiation; CK18, cytokeratin 18; DPPIV, dipeptidyl peptidase IV; FAH, fumarylacetoacetate hydrolase; FALC, fat-associated lymphoid cluster; FRGN, Fah−/−/Rag2−/−/Il2 receptor gamma−/− mice on the nonobese diabetic background; GFP, green fluorescent protein; GS, glutamine synthetase; HBSS, Hanks’ balanced salt solution; LN, lymph node; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; OCT, optimal cutting temperature compound; omFALC, omental FALC; PBS-T, PBS supplemented with 0.05% Tween 20; Rag2, recombination activating 2; RNA-seq, RNA sequencing; RT, room temperature.

Maria Giovanna Francipane and Amin Cheikhi contributed equally to this work.

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INTRODUCTION

Liver transplantation is currently the only effective treatment for patients with severe hepatic failure. Unfortunately, there is an exacerbating liver transplant shortage due to many factors ranging from the limited number of liver donors to the increasing number of patients waiting for transplantation.\(^1\) Therefore, the development of therapies is a critical and urgent need. Hepatocyte transplantation into the native liver has been proposed as an alternative to liver transplantation for treating patients with liver failure.\(^2–4\) However, most patients with severe liver disease have fibrotic and cirrhotic liver obstructing blood flow, which constitutes an adverse environment for liver regeneration. Our group has previously demonstrated that lymph nodes (LN) are excellent extrahepatic sites for ectopic liver development\(^5–8\) as well as for other tissues,\(^9–12\) and direct injection of hepatocytes into LNs resulted in the formation of a functional ectopic liver.\(^6\)

When transplanted into the peritoneal cavity, hepatocytes migrated to the abdominal LNs to generate ectopic livers.\(^6\) Surprisingly, many ectopic hepatic nodules formed throughout the peritoneal cavity. A closer examination revealed that most of these hepatic nodules are actually selectively associated with adipose tissues such as the omentum, the portion of the mesentery near the intestine, and the gonadal gland. These observations raised the question as to whether any other unidentified sites within the abdominal cavity could also facilitate transplanted hepatocytes to engraft and proliferate. Fat-associated lymphoid clusters (FALCs) are recently identified leukocyte aggregates,\(^13\) mainly B and T lymphocytes, as well as an abundant blood supply surrounded by stromal cells.\(^14\) In contrast to LNs, FALCs have no surrounding capsule or distinct compartmentalization zones for B and T cells and, as such, are highly vascularized, permissive microenvironments.\(^13\) Collectively, these features ensure prompt and sufficient immunologic response to environmental cues.\(^15,16\) The observation that the distribution of engrafted hepatocytes in the abdominal cavity mirrors that of abdominal FALCs is reminiscent of tumor cell migration to FALCs following i.p. injection.\(^17–19\) We, therefore, hypothesized that hepatocytes injected i.p. engraft in FALCs and upon growth stimulation, such as in tyrosinemic mice, proliferate to form functional ectopic livers.

MATERIALS AND METHODS

Animals

Fumarylacetoacetate hydrolase knockout (\(Fah^{-/-}\)) mice on the 129sv background (a kind gift from Dr. Markus Grompe, Oregon Health & Science University, Portland, OR), \(Fah^{-/-}\) mice backcrossed on the C57BL/6J background, and \(Fah^{-/-}/\text{recombination activating 2 (Rag2}\ ^{-/-}/\text{Il2 receptor gamma}\ ^{-/-}\) mice on the nonobese diabetic background (FRGN mice; generously provided by Dr. Andrew Duncan, University of Pittsburgh, Pittsburgh, PA) were used as recipients. The 129sv mouse, C57BL/6J mice (catalog no. 000664; The Jackson Laboratory, Bar Harbor, ME), and green fluorescent protein (GFP) C57BL/6J mice (catalog no. 004353; The Jackson Laboratory) were used as donors. Freshly isolated hepatocytes were obtained from mice 8–12 weeks old and transplanted into 8-week-old to 12-week-old \(Fah^{-/-}\) mice. The protocol followed the National Institutes of Health guidelines for animal care and was approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee.

Hepatocyte isolation, transplantation, and induction of hepatocyte proliferation

Hepatocytes were harvested using a two-step collagenase perfusion technique.\(^20\) The yield and viability of isolated hepatocytes were determined by trypan blue exclusion. Hepatocytes with a viability of \(\geq 80\%\) were used for transplantation. One million viable hepatocytes were suspended in 50 \(\mu\)l Hanks’ balanced salt solution (HBSS; catalog no. 14-175-095; Thermo Fisher Scientific, Pittsburgh, PA) and kept on ice until transplantation. Hepatocytes were then injected into the left lower quadrant of the peritoneal cavity with a 28-gauge needle. For splenic transplantation, mice were anesthetized, and a small surgical incision was made in the left flank. The spleen was exposed,
and hepatocytes were injected into the splenic inferior pole using a 28-gauge needle. The injection site was ligated after the transplantation to prevent cell leakage and bleeding. All \( \text{Fah}^{-/-} \) mice were fed with drinking water containing 8 mg/L 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) until transplantation. NTBC inhibits the upstream enzyme 4-hydroxyphenylpyruvate dioxygenase and prevents liver injury, allowing \( \text{Fah}^{+/+} \) mice to be maintained in a healthy state.\(^{[21]} \) NTBC in the drinking water was discontinued just after hepatocyte transplantation to induce hepatic failure and hepatocyte proliferation. The weight of experimental mice was monitored weekly. When the mice lost >25% of their initial body weight, generally 3–4 weeks off NTBC, NTBC was added back to the drinking water to restore liver function. This was considered one cycle of selection. NTBC was discontinued again to initiate another cycle of selection once the weight of the mice returned to normal.

**Bone marrow cell isolation and transplantation**

Bone marrow cells were isolated from mouse femur and tibia according to an established protocol.\(^{[22]} \) Twelve million cells were suspended in 100 \( \mu \)l HBSS and transplanted into FRGN mice both i.p. from the left lower quadrant of the peritoneal cavity and i.v. through the retro-orbital venous sinus. Bone marrow transplantation through the retro-orbital venous sinus was added because it is the method used routinely to transplant bone marrow cells in mice. Hepatocyte transplantation was performed 3 weeks after bone marrow cell transplantation.

**Induction of sterile abdominal inflammation by zymosan**

To induce abdominal inflammation, zymosan (catalog no. Z4250; Sigma-Aldrich, St. Louis, MO) was suspended in PBS (catalog no. 21040CV; Thermo Fisher Scientific) at a concentration of 5 mg/ml. Two hundred microliters of this solution (1 mg zymosan) were injected into the peritoneal cavity of each mouse. Hepatocyte transplantation was performed 1 week after zymosan injection.

**Tissue preparation and fluorescent immunohistochemistry staining of frozen tissue sections**

After collection, tissues were preserved in two ways: (1) tissues were embedded in OCT and directly frozen in liquid nitrogen. The OCT frozen tissue blocks were cut into 4-\( \mu \)m-thick to 7-\( \mu \)m-thick sections for further analysis. Frozen sections which were prepared with method 1 were equilibrated at room temperature (RT) for 15 min and then rehydrated in PBS for 10 min. After three washes in PBS supplemented with 0.05% Tween 20 (PBS-T), a solution of 2.5% bovine serum albumin (BSA; catalog no. 9048468; Thermo Fisher Scientific) in PBS-T was applied to the sections for 30 min to prevent nonspecific binding. Sections were then incubated with specific primary antibodies for at least 1 h at RT or overnight at 4°C. After three washes in PBS-T, sections were incubated with Alexa Fluor 488 or 594 secondary antibodies (Thermo Fisher Scientific) for 30 min. Sections were washed three times, and the nuclei were counterstained with 1 \( \mu \)g/mL Hoechst 33342 (catalog no. H3570; Life Technologies, Frederick, MD). A solution of 50% glycerol in HBSS was used as the mounting medium. Frozen sections prepared with method 2 were fixed with acetone at −20°C for 5 min and air-dried for at least 20 min. The staining procedure was the same as above except using PBS to replace PBS-T. Acetone causes GFP bleaching; thus, in acetone-fixed frozen sections, engrafted hepatocytes were highlighted through the use of hepatocyte-specific markers.

A list of antibodies used is provided in Table 1. Isotype-matched antibodies were used as negative controls. Images were obtained with the Olympus IX71 inverted fluorescence microscope (Olympus America, Center Valley, PA).

**Whole-mount immunofluorescent staining of omentum**

Whole-mount staining was performed in wells of a 24-well plate with constant slow agitation. After dissection, the whole omentum was fixed in 4% paraformaldehyde in PBS for 1 h at 4°C. The omentum was then blocked in 2.5% BSA in PBS for 1 h at RT. After blocking, the tissue was stained with Alexa Fluor 594 antimouse cluster of differentiation 45 (CD45R/B220) antibody (1:100; catalog no. 103254; BioLegend, San Diego, CA) for 2 h at RT. After washing the tissue, the nuclei were counterstained with 1 \( \mu \)g/mL Hoechst 33342 in PBS for 1 h at RT. A solution of 50% glycerol in HBSS was used as the mounting medium. Images were obtained with the Olympus IX71 inverted fluorescence microscope.

**RNA preparation and RNA sequencing**

Total RNA was isolated from tissues stored in RNAlater reagent (catalog no. 76104; Qiagen, Valencia, CA)
using the RNeasy Mini kit (catalog no. 74104; Qiagen), according to the manufacturer's instructions. Potentially contaminating genomic DNA was digested using deoxyribonuclease (catalog no. 79254; Qiagen) at 37°C for 30 min using 1500 Kunitz units in 550 μl of the ribonuclease-free water provided with the kit. The purity of isolated RNA was determined by measuring the ratio of the optical density of the samples at 260 and 280 nm using a NanoDrop 2000/c spectrophotometer (Thermo Fisher Scientific). The 260/280 ratio ranged from 2.07 to 2.13 for all samples. RNAs were therefore shipped on dry ice to MedGenome (Foster City, CA) for library preparation and sequencing. All samples successfully passed MedGenome's internal quality control. Clean reads were obtained from FASTQ files by removing low-quality reads by trimming. High-quality reads of guanines and cytosines were aligned to the mouse genome (downloaded from the National Center for Biotechnology Information database CLC Genomic). RNA-sequencing (RNA-seq) data were mapped with the default parameters for the maximum number of allowed mismatches, minimum length and similarity fraction, and minimum number of hits per read. The threshold p value was determined according to the false discovery rate (FDR). In this study, genes that were identified as being differentially regulated met the following criteria: FDR $p \leq 0.05$. Because the sequencing depth might differ between samples, a per-sample library size normalization was automatically performed before sample comparison using the trimmed mean of M values (TMM) normalization method, which adjusts library sizes based on the assumption that most genes are not differentially expressed $^{23}$ to calculate effective library sizes, which are then used as part of the per-sample normalization (EdgeR package, Bioconductor). For the expression visualization using a heat map and principal component analysis, log counts per million (CPM) values were calculated for each gene. The CPM calculation uses the effective library sizes as calculated by the TMM normalization. After this first normalization, a second one is performed across samples for each gene: The counts for each gene are mean-centered and scaled to unit variance. Genes or transcripts with zero expression across all samples or invalid values (i.e., ±infinity) are removed.

Statistical analysis

Statistical significance was calculated by Prism 8.0.a (GraphPad Inc., San Diego, CA) using an unpaired two-tailed Student t test. $p < 0.05$ was considered significant.

RESULTS

Hepatocytes engraft in abdominal FALCs after i.p. transplantation

We first sought to directly examine the distribution of FALCs within the abdominal cavity. More specifically, we confirmed their presence throughout five major areas of adipose tissue: the omentum, the perisplenic adipose tissue, the periportal adipose tissue, the mesentery, and the gonadal adipose tissue (Figure 1A). Peripheral omental FALCs (omFALCs, or “milky spots”), which represent >80% of the FALCs, $^{24}$ were located at the end of each blood vessel branch (Figure 1B, I and II) and exhibited B cell–rich clusters, as confirmed by whole-mount B220 staining (Figure 1B, III and IV) and highlighted by the CD45 pan-leukocyte marker (Figure 1C). Additionally, they showed abundant vasculature (CD31/CD105 stain) and dense stroma (ER-TR7 stain) (Figure 1C; Figure S9), which likely enable a rapid immunologic response to abdominal insults $^{24}$.

Next, 1 million GFP+ C57BL/6J hepatocytes were transplanted into the peritoneal cavity of wild-type C57BL/6J mice. After 1 week, recipients were sacrificed, and fluorescence microscopy revealed that

### Table 1: List of antibodies used in this study

| Antibody   | Catalog number | Supplier                            | Dilution |
|------------|----------------|-------------------------------------|----------|
| CK18       | 10830-1-AP     | Proteintech, Chicago, IL            | 1:100    |
| CD31       | ab28364        | Abcam, Cambridge, MA                | 1:100    |
| CD105      | AF1320         | R&D, Minneapolis, MN                | 1:100    |
| Desmin     | ab80503        | Abcam                               | 1:200    |
| F4/80      | 70076S         | Cell Signaling Technology, Boston, MA| 1:250    |
| DPPIV (CD26)| MCA2345A488   | Bio-Rad, Hercules, CA               | 1:50     |
| GS         | ab49873        | Abcam                               | 1:500    |
| ER-TR7     | ab51824        | Abcam                               | 1:500    |
| CD45       | ab10558        | Abcam                               | 1:100    |
| CD3        | ab16669        | Abcam                               | 1:100    |
| B220       | 103254         | BioLegend, San Diego, CA            | 1:100    |
transplanted GFP+ hepatocytes engrafted in all five major areas of abdominal adipose tissue (Figure 1D). Importantly, engrafted GFP+ hepatocytes were found to be predominantly located in FALCs (Figure 1D–F; Figure S1), and only a few cells were engrafted in gonadal adipose tissue, based on the reported number of FALCs in these tissues [24] and our own observation (Table S4). To assess the relative abundance of GFP+ hepatocytes in omFALCs, we quantified the whole-mount B220 fluorescence (Figure 1E). Over 89% of hepatocytes were partitioned into the omFALCs of wild-type C57BL/6J mice (Figure 1G). Further immunofluorescence characterization of omFALCs showed close contact of engrafted hepatocytes (defined as GFP+ or cytokeratin 18–positive [CK18+] cells) with stromal cells (ER-TR7 stain) and endothelial cells (CD31+ and CD105+ cells) (Figure 1F; Figure S9). Next, we asked whether hepatocytes survived long-term without growth stimuli in wild-type C57BL/6 mice. Hepatocytes survived several months, but their number declined, indicating a progressive loss of the transplanted cells (Table S1). Finally, a low number of hepatocytes were transplanted (100 and 1000 cells) in wild-type C57Bl/6 mice and observed after 2 weeks (Table S2), indicating a high efficiency for FALC engraftment sites. In conclusion, hepatocytes were engrafted in abdominal FALCs after i.p. transplantation in mice.

**Hepatocytes in abdominal FALCs form ectopic livers that rescued mice from liver failure**

Tyrosinemic Fah−/− mice are commonly used in liver regeneration studies [21] with their normal liver function maintained by NTBC in drinking water. Withdrawal of NTBC results in progressive liver injury and eventually liver failure, with most mice dying 4–8 weeks later. A near-complete repopulation of the native liver’s hepatocytes can be achieved in Fah−/− mice after intrasplenic hepatocyte transplantation followed the same day by NTBC withdrawal. To test whether FALC-engrafted hepatocytes could proliferate and generate functional ectopic hepatic tissues after NTBC withdrawal, 1 million
syngeneic wild-type hepatocytes were transplanted i.p. into Fah−/− mice. As a positive control experiment, an equal number of hepatocytes were transplanted into the spleen of Fah−/− mice to enable their migration and engraftment into the native liver parenchyma, as demonstrated previously. Two weeks after i.p. hepatocyte transplantation and NTBC withdrawal, multiple small GFP+ hepatocellular aggregates were found in the omentum (Figure 2A, left panels). These aggregates grew over time, further increased in size 6 weeks post-transplantation (Figure 2A, right panels), and contained abundant blood supply, as observed under a light microscope and confirmed by CD31 immunofluorescence stain (Figure 2A, bottom panels). At 10–12 weeks post-transplantation, all female Fah−/− mice in the splenic injection group achieved long-term survival (5/5) with just one cycle of selection (Figure 2B, black line). Although not as efficient as with splenic injection, long-term survival of female Fah−/− mice was also achieved in the i.p. injection groups, with 25% (2/8) under one cycle of selection (Figure 2B, red line) and 67% (4/6) under two cycles of selection (Figure 2B, blue line). Importantly, in splenic injection, hepatocytes replace damaged Fah−/− hepatocytes dying in the native liver. In i.p. injection, hepatocytes need to rebuild a whole liver in FALCs, with vascularization and recruitment of other cells. This takes time, and the strong selection imposed on Fah−/− mice decreases their survival rate.

While long-term survival of male Fah−/− mice was not achieved in the i.p. injection groups with one or two cycles of selection, 70% (7/10) were rescued with three cycles of selection (Figure S2A). Laparotomy was performed in mice with long-term survival. Multiple ectopic hepatic nodules, which we are now calling “ectopic livers,” were observed in all five major abdominal adipose areas that contained FALCs (Figure 2C). The size and number of ectopic livers varied among rescued Fah−/− mice (Figure S2B). Interestingly, when we compared the mass of ectopic liver derived from FALCs with ectopic liver derived from LNs, we found that FALC-derived ectopic livers are generally larger than LNs derived from a single ectopic liver per animal.

**Figure 2** Hepatocytes engrafted in abdominal FALCs eventually formed functional ectopic livers and rescued Fah−/− mice with liver failure. (A) Macroscopic appearance of omentum from Fah−/− mice 2 and 6 weeks after i.p. transplantation of GFP+ hepatocytes. (Upper panels) Under light microscope. (Middle panels) Macroscopic appearance under fluorescence microscopy with GFP+ hepatocytes (green). (Lower panels) CD31 immunofluorescence staining (red) of omenta frozen sections with engrafted GFP+ hepatocytes (green) and nuclei counterstained with Hoechst (blue). Higher magnification of the region outlined with a yellow square is shown in the right panels. (B) Kaplan-Meier survival curve up to 80 days of Fah−/− female mice after intrasplenic or i.p. transplantation of 1 million wild-type hepatocytes followed by one or two cycles of selection (NTBC withdrawal). See Figure S2 for Fah−/− male mice data. (C) Gross pictures showing the formation, in rescued Fah−/− mice, of multiple ectopic hepatic nodules in the five major abdominal adipose areas containing FALCs. Dissected areas are shown together with a ruler (centimeters). (D) Immunofluorescence staining of ectopic livers in rescued Fah−/− mice for CD105 (red)/CK18 (green), desmin (red)/CK18 (green), CD45 (red)/CK18 (green), ER-TR7 (red)/CK18 (green), F4/80 (red), CK18 (red)/DPPIV (green), or GS (red). Nuclei were counterstained with Hoechst (blue). Scale bars, 250 μm. Abbreviations: IP, intraperitoneal; 1M, 1 million; SP, intrasplenic; Tx, transplantation.
Ectopic liver does not form after i.p. transplantation of hepatocytes into mice lacking abdominal FALCs but formed after FALC restoration by bone marrow cell transplantation

No FALCs were reported in FALCs, but a further study of FALCs injected directly. In consequence, more hepatic tissues are generated into FALCs, but a further study of this unexpected result needs to be done in the future. Coimmunostaining of CK18+ hepatocytes with other cell lineage markers demonstrated the presence of endothelial cells (CD105 stain), stellate cells (desmin stain), hematopoietic cells (CD45 stain), and stromal cells (ER-TR7 stain) (Figure 2D, upper panels). While there were F4/80+ macrophages within the lymphoid clusters, in the ectopic livers no positive cells were seen in the liver parenchyma (Figure 2D, lower left panel). Biliary cells were not present in the ectopic livers (data not shown), but the expression of bile canalicular enzyme dipeptidyl peptidase IV (DPPIV) stain was observed between hepatocytes, indicating the presence of bile canaliculi (Figure 2D, lower middle panel). Finally, the presence of glutamine synthetase (GS) stain in hepatocytes around the blood vessels indicated zonation in the ectopic hepatic tissue (Figure 2D, lower right panel), and complementary staining showed expression of other protein markers such as cytochrome P450 2E1 and phosphoenolpyruvate carboxykinase in addition to albumin, cytochrome P450 3A1, and hepatocyte nuclear factor 4A (HNF4a; Figure S10). Finally, serum markers such as alanine aminotransferase or total and direct bilirubin improved in Fah−/− mice rescued by hepatocyte engraftment into FALCs over untreated Fah−/− mice (Table S5).

Ectopic livers restore hepatic functions in tyrosinemic mice

We then proceeded to determine how FALC-associated ectopic livers have restored hepatic functions. We performed RNA-seq on rescued Fah−/− mice, contrasting the ectopic livers to control tissues (i.e., healthy control [native] livers and control [mesenteric] LNs) as well as to the native (tyrosinemic) livers originating from the same rescued mice where the ectopic livers were collected. Real-time PCR was then used on four genes (albumin, FAH, multidrug resistance–associated protein 2, and HNF4a) to validate the sequencing data (Figure S8). Data from principal component analysis and a heat map of transcriptomic data clearly showed an overall congruent transcriptomic profile of ectopic and control native livers, which did not cluster with their normal lymphoid tissues and native tyrosinemic liver counterparts (Figure 3A,B). Furthermore, FALC-derived ectopic livers are very similar to LN-derived hepatocytes (Figure S7). Wishing to elucidate in more detail the molecular mechanism(s) underlying the hepatic identity of the ectopic livers, we compared the expression profiles of 72 genes that were previously established as liver-specific (Table S6). Indeed, the hierarchical clustering of this liver-specific gene panel, as visualized by the heat map (Figure 3C; Figure S11), further demonstrates the convergence of the ectopic livers and control native livers and their divergence from native tyrosinemic livers and normal control lymphoid tissues.

The next step was to contrast the expression profile of genes that govern essential hepatic functions among the four different conditions. First, we checked the expression levels of five genes coding for enzymes in the tyrosine catabolism pathway (Figure 4A). As expected, Fah gene expression was minimal to nonexistent in native tyrosinemic livers, while its expression levels in the ectopic livers and control native livers were similar. Significantly, the expression levels of the other four genes, notably tyrosine aminotransferase and 4-hydroxyphenylpyruvate dioxygenase, were dramatically repressed in native tyrosinemic livers compared to the ectopic livers and control native liver counterparts. The Child-Pugh score is a system for assessing the prognosis of patients with chronic liver disease, primarily cirrhosis, and provides a forecast of the increasing severity of liver disease and expected survival rate. Genes regulating the coagulation system, urea cycle, and albumin synthesis were also substantially downregulated in the native tyrosinemic livers, while their expression in both ectopic livers and control native livers was comparably maintained (Figure 4B). In contrast, there was no differential expression of genes responsible for bilirubin transport and conjugation (Figure 4B) or bile acid synthesis and transport (Figure 4C) in control native livers and ectopic livers. Similarly, there was no significant difference in gene expression levels of several key transcription factors that control bile acid metabolism of ectopic livers and native livers and were comparable to those of control native livers (Figure 4C). The expression of genes involved in other important liver functions is shown in Figure S3. Collectively, these data suggest that ectopic livers behaved like auxiliary livers, which adaptively and specifically compensate for native tyrosinemic liver deficiencies to rescue the tyrosinemic mice from liver failure.
of FRGN mice (Figure S4A). One million GFP+ hepatocytes from wild-type C57BL/6J were transplanted into FRGN mice by peritoneal injection. Subsequently, NTBC was withdrawn from the drinking water, and FRGN mice were weighed every 2–3 days. NTBC was administered again once the weights decreased by 25%. Of the 29 transplanted FRGN mice, some of which underwent up to six cycles of selection for as long as 156 days, none were rescued by the transplanted hepatocytes (Figure S4B). The omenta were
collected 2, 8, or 12 weeks after i.p. transplantation. While the transplanted hepatocytes were still able to engraft in the omentum early after transplantation, they barely grew over time and lacked the abundant blood supply necessary to form ectopic liver (Figure 5A). Even 23 weeks after transplantation and six cycles of selection, ectopic livers could not be observed macroscopically in the omentum or other abdominal adipose tissues (Figure 5B). GFP⁺ hepatocytes were only seen microscopically with very limited growth (Figure 5C).

Next, C57BL/6J GFP⁺ bone marrow cells were transplanted into FRGN mice to test whether abdominal FALCs could be reconstituted. Three weeks later, GFP⁺ hematopoietic cell clusters were seen in all five major adipose areas (Figure 6A). Under a light microscope, these GFP⁺ clusters manifested as round-shaped gray structures that colocalized with abdominal FALCs in wild-type mice (Figure S5). Immunofluorescence staining of the omentum with transplanted bone marrow cells showed multiple restored CD45⁺ cell clusters (Figure 6B). The majority of these were composed of mainly B220⁺ B lymphocytes, as well as some CD3⁺ T cells (Figure 6C), indicating that bone marrow cell transplantation successfully restored abdominal FALCs. After 12 weeks of NTBC selection, there was only limited growth of hepatocytes in omentum without abdominal FALCs, whereas hepatic nodules were formed in the omentum with restored abdominal FALCs (Figure 6D). At 23 weeks after transplantation of hepatocytes and six cycles of NTBC selection, ectopic livers associated with restored FALCs in the abdominal adipose tissue contained abundant vasculature (Figure 6E). CD105 staining highlighted abundant newly formed vasculature in ectopic livers in the omentum, perisplenic adipose tissue, and periportal adipose tissue (Figure 6F).

Collectively, these data indicate that abdominal FALCs present a unique and highly favorable microenvironment for the development of ectopic livers after i.p. injection of hepatocytes.

**Induction of abdominal inflammation increased FALC numbers and ectopic liver development after hepatocyte transplantation**

Induction of abdominal inflammation results in increased size and number of FALCs in the mesentery. For this reason, we sought to investigate whether more ectopic livers could form by inducing the development of more abdominal FALCs with inflammation.

To do so, we used zymosan, an insoluble yeast polysaccharide widely used as a self-resolving model of peritoneal inflammation in mice to induce abdominal inflammation through i.p. injection into wild-type C57BL/6 mice. One week after zymosan treatment, 1 million C57BL/6 GFP⁺ hepatocytes were transplanted i.p., and the five major areas of abdominal adipose tissue containing FALCs were collected after 1 additional week. The initial engraftment of hepatocytes increased significantly in all five areas (Figure 7A,B). Notably, while
engrafted hepatocytes are located mainly in the jejunal portion of the mesentery without inflammation, after the induction of inflammation, hepatocytes engrafted throughout the mesentery (Figure S6, upper panels). Moreover, while there were only a few hepatocytes engrafted in gonadal adipose tissue without inflammation, more hepatocytes were engrafted upon induction of an inflammation response (Figure 7A; Figure S6, lower panels). Induced abdominal FALCs were visibly engrafted with hepatocytes (Figure S6). Immunofluorescence staining confirmed that transplanted hepatocytes were located within abdominal FALCs (Figure 7C).

Next, we asked whether more ectopic livers would form in the abdominal cavity of Fah−/− mice upon induction of abdominal inflammation. Female Fah−/− mice were used in this experiment. One week after i.p. injection of zymosan to Fah−/− mice, 1 million syngeneic hepatocytes were transplanted i.p., and NTBC selection was started posttransplantation. As expected, between 10 and 12 weeks posttransplantation better long-term survival was observed in both the group with one cycle of selection (4/6) and the group with two cycles of selection (5/5) (Figure 8A, red line and blue line, respectively) compared to the groups without inflammation (Figure 2B). Not only did the survival rate increase upon induction of inflammation after two cycles of selection but the weights of the mice in the inflammation group did not drop as much during the second selection as opposed to the control group, indicating faster ectopic liver growth that allowed faster recovery from liver failure (Figure 8B). Laparotomy was performed in Fah−/− mice with long-term survival without NTBC. Multiple ectopic livers were generated in all five major areas of abdominal adipose tissue containing FALCs, and more nodules were seen in the mesentery and gonadal adipose tissue in mice with inflammation (Figure 8C). Because the ectopic livers were fused in the omentum, perisplenic adipose tissue, and periportal adipose tissue, which made it difficult to count the exact number of the ectopic hepatic nodules in these areas, we...
counted the number of ectopic hepatic nodules in the mesentery and gonadal adipose tissue. The number of ectopic livers in the mesentery and gonadal adipose tissue significantly increased after inducing inflammation (Figure 8D).

Taken together, these data show that induction of abdominal inflammation increased the abundance of FALC sites and the success rate of initial engraftment of i.p. transplanted hepatocytes, thus resulting in the formation of more ectopic livers, which in turn enabled a more effective rescue of mice from liver failure.

**DISCUSSION**

In this study, we demonstrated that hepatocytes engraft in abdominal FALCs early after i.p. transplantation. Engrafted hepatocytes proliferated over time and formed functional ectopic livers, rescuing tyrosinemic mice that would have otherwise died of liver failure.

Our previously reported study using hepatocyte injection into the peritoneal cavity identified peritoneal LNAs as a favorable site of cell engraftment. Only recently were FALCs identified as lymphoid tissues.
we have carefully analyzed the engraftment of hepatocytes into the major LNs of mice (mesenteric, renal, inguinal, lumbar, and caudal) after i.p. injection. We were not able to confirm the presence of hepatocytes in these LNs in wild-type mice or Fah−/− mice after selection. If we compare our current study with our previous study, the same strains of mice were used between these two studies. However, the mouse facilities used previously had chronic infection issues. We know that inflammation can dramatically affect lymphatic sites, such as increasing FALC numbers and size, as well as LN sizes, and stimulation of lymphatic vessels and vasculature. We hypothesize that the chronic inflammatory conditions induced by the environment in our old facility made it more possible for hepatocytes to engraft in LNs. Furthermore, we can't rule out the possibility that in our earlier report some LNs engrafted by hepatocytes after i.p. transplantation were actually FALCs that were not yet identified and characterized at that time in the literature. Interestingly when we compared the mass of ectopic liver derived from the LNs versus FALCs, we found that FALC-derived liver masses are generally larger than the LN-derived liver mass per animal (Table S3). We believe that the reason may be the number of ectopic liver sites that can be generated after i.p. transplantation into FALCs compared to a single LN injected directly. In consequence, more hepatic tissues are generated into FALCs, but further study of this unexpected result needs to be done in the future.

While conventional secondary lymphoid organs, such as LNs, are developed during embryogenesis under the control of the lymphotxin pathway, FALCs are not. They are developed postnatally, possibly induced by inflammatory stimuli related to the presence of commensal microbes. Upon acute or chronic inflammation, FALC numbers are increased dramatically.
presenting more opportunities for hepatocyte engraftments and the generation of ectopic livers. Our study links the presence and abundance of FALCs to the rescue of the tyrosinemic mice from liver disease after peritoneal transplantation of hepatocytes. The repurposed unique property of FALCs as inducible sites for hepatocyte engraftment presents a great opportunity for hepatocyte transplantation and the regeneration of hepatic function. It is important to note that the liver is believed to be an immunological organ that consists of both innate and adaptive immune cells. These immune cells play an important role in native liver regeneration after hepatic injury. Abdominal FALCs, as a tertiary lymphoid site, may provide similar survival signals to engrafted hepatocytes and further serve as a niche for liver regeneration when appropriate growth stimulus occurs. Also, we recently demonstrated that lymphotxin beta-receptor signaling is crucial for the angiogenesis of metanephros transplanted in both the omentum and LNs. It will be interesting to know if a similar signal in the rich vascularized network of FALCs is necessary for hepatocyte remodeling into ectopic liver tissue.

A major unanswered question is how the ectopic and native livers differ, considering that ectopic liver development is conditioned by the diseased native liver. Transcriptomic analysis uncovered the gene expression profile of ectopic livers as quite divergent from their diseased counterparts while significantly converging toward the healthy control livers. There is a down-regulation of genes in the tyrosine catabolism pathway within diseased native livers that may result in decreased accumulation of toxic metabolites within defective hepatocytes, which may be an adaptive response to the Fah deficiency and the underlying reason for the better survival of Fah−/− hepatocytes under these diseased conditions. Several critical hepatic elements, such as coagulation factors, urea cycle, and albumin, were functionally reduced in the native tyrosinemic liver but compensated for by the ectopic livers. The native tyrosinemic liver was still highly involved in bilirubin and bile acid metabolism and transport. Given that the ectopic livers did not have a biliary system connected to the intestine and no accumulation of bile, the evidence suggested that hepatocytes in the ectopic livers were actively transporting bile to the intestine, possibly through the bloodstream using the remnant native tyrosinemic liver. Our previous work in the mouse and our two recent reports in the pig on the ectopic liver concurred with the notion that bile acids and bilirubin are transported outside of the ectopic livers.

Accordingly, our data indicated that ectopic livers behave like an auxiliary liver, a well-known concept developed by others, and that the coordination between native tyrosinemic liver and ectopic auxiliary livers rescued the tyrosinemic mice. But further investigations are needed to elucidate how bilirubin and bile acids are metabolized and transported in the rescued Fah−/− mice with ectopic livers.

In our study, a slower rate of ectopic hepatic regeneration was observed in male mice than female mice (Figure 2; Figure S2). This is consistent with other studies showing the involvement of sex hormones (estrogen and androgen) in liver regeneration. It was recently reported that estrogen regulates liver growth during development, regeneration, and tumorigenesis through the activation of G protein–coupled estrogen receptor 1.

FALCs are present in the human greater omentum. Compared to the mouse omentum, which is a narrow strip of adipose tissue located in the upper left quadrant of the abdomen, the human greater omentum represents a much larger adipose tissue with an abundant blood supply that covers the whole intestine. Transplanting hepatocytes i.p. could potentially treat patients with liver disease, knowing that cells would migrate to abdominal FALCs and generate new hepatic tissues under regenerative stimuli. This approach would be minimally invasive. Repeated transplantation could be readily performed. This, alongside the large increase of initial engraftment of hepatocytes post–i.p. transplantation, as a result of inducing inflammation in abdominal FALCs, could also be translated into clinical application. In our mouse study, the initial engraftment of hepatocytes after i.p. transplantation following induction of abdominal inflammation was estimated to exceed 1% of the transplanted cells, which was 50 times more of the initial engraftment of hepatocytes without inflammation. However, patients with serious liver disease may already have abdominal inflammation. Therefore, a safe method to monitor and increase the number of abdominal FALCs, including but not limited to induction of abdominal inflammation, needs to be developed and optimized for successful hepatic engraftment. Current development of hepatocyte i.p. injections has included a few clinical trials using abdominal hepatocyte transplantation which have reported promising results. Unfortunately, the results of the transplanted hepatocytes in these patients were never further investigated.

In conclusion, we discovered that abdominal FALCs are optimal niches for hepatocytes. Abdominal FALCs have the potential to provide sufficient blood supply and regenerative capacity to support functional ectopic liver development. Future studies will focus on methods for safety and efficacy to induce more abdominal FALCs, as well as the cellular and molecular mechanisms supporting the engraftment and proliferation of transplanted hepatocytes in abdominal FALCs.

**CONFLICT OF INTEREST**
Dr. Lagasse consults for and owns stock in LyGenesis.
Bing Han provided the experimental approaches, performed experiments and analyses, and drafted the manuscript and figures. Maria Giovanna Francipane isolated the RNA and provided critical feedback as well as assistance with manuscript writing and figure preparation. Amin Cheikhi performed RNA-sequencing analyses, provided critical feedback, and assisted with the manuscript writing. Joycelyn Johnson performed reverse-transcription PCR analyses and paraffin section immunostaining. Fei Chen weighed transplanted mice and performed some immunofluorescent staining. Ruoyu Chen generated the heatmap for the liver-mice and performed some immunofluorescent staining. Eric Lagasse supervised the project, conceived of the study design, and assisted with manuscript writing.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher's website.

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