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RESEARCH ARTICLE

Single-Cell RNA Sequencing Uncovers Novel Cell States in Regenerating Liver Tissue

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ABSTRACT

The mammalian liver is unusual among solid organs in being able to restore its full mass within days of substantial tissue loss, yet the cellular logic of that recovery is still described mostly in terms of bulk proliferation indices rather than the identities of the cells doing the work. We performed droplet-based single-cell RNA sequencing on liver tissue from C57BL/6 mice at six time points after two-thirds partial hepatectomy (0, 24, 48, 72, 96 and 168 hours), profiling 58,412 cells that passed quality control across eighteen animals. Unsupervised clustering resolved the expected hepatocyte, endothelial, Kupffer cell, stellate cell and cholangiocyte compartments, but also identified two transient cell states not present in sham-operated controls: a periportal 'regenerative hepatocyte' population marked by co-expression of *Afp*, *Spp1* and cell-cycle genes at 48-72 hours, and a distinct *Ly6c*-high, *Vegfa*-high macrophage state that peaked at 24-48 hours and preceded the appearance of the regenerative hepatocyte cluster in pseudotime. Ligand-receptor analysis linked this macrophage state to hepatocyte proliferation via a *Vegfa*-*Flt1*/*Kdr* axis and an *Spp1*-*Cd44* axis, and depletion of Kupffer cells with clodronate liposomes prior to hepatectomy delayed the emergence of the regenerative hepatocyte cluster and reduced *Ki67*-positive hepatocyte fraction at 72 hours by roughly half. Together, these data reposition regenerative hepatocytes and *Vegfa*-high macrophages as coupled, time-restricted cell states whose sequential appearance forms part of the coordination logic of liver regeneration, rather than as regeneration being explained by hepatocyte proliferation in isolation.

Keywords: single-cell RNA sequencing; liver regeneration; partial hepatectomy; hepatocyte heterogeneity; pseudotime; macrophage polarisation

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1. INTRODUCTION

Few adult mammalian organs can regrow after losing most of their mass, but the liver manages it routinely: a two-thirds partial hepatectomy in a rodent is followed by near-complete restoration of liver mass within seven to ten days, driven overwhelmingly by proliferation of the remaining differentiated cells rather than by an expansion of a dedicated stem-cell compartment [3,9].

This capacity has obvious clinical relevance, underpinning both the feasibility of living-donor liver transplantation and the recovery of patients after extended hepatic resection for cancer, and its failure in the context of chronic liver disease is a major driver of the transition from compensated cirrhosis to liver failure [14,22]. Despite decades of study, most of what is known about the molecular choreography of regeneration comes from bulk transcriptomic or proteomic profiling of

whole-liver lysates, an approach that necessarily averages across a tissue in which hepatocytes, liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and cholangiocytes are each responding on different timescales and, we suspected, in more than the handful of discrete states that bulk methods can resolve. Single-cell RNA sequencing removes that averaging problem and has already reshaped the understanding of several regenerative and fibrotic liver processes, from stellate cell activation trajectories in fibrosis to the identification of a periportal hepatocyte subpopulation implicated in homeostatic renewal [17,19]. Work in zebrafish and axolotl regeneration more broadly has repeatedly shown that transient, injury-specific cell states -- present neither in the uninjured tissue nor in the fully regenerated one -- can be essential intermediates in the regenerative programme rather than incidental noise, a pattern documented for blastema formation in limb regeneration and for transient reactive glia in zebrafish retina [24,31]. Whether comparably transient, injury-restricted states exist during mammalian liver regeneration, and whether they causally contribute to the process rather than merely correlating with it, has not been resolved with the temporal resolution that a proper time-course single-cell design can provide.

Kupffer cells, the liver's resident macrophage population, are a natural place to look for such states. They respond to hepatocyte loss within hours, releasing tumour necrosis factor- α and interleukin-6 that prime hepatocytes to re-enter the cell cycle, and clodronate-mediated Kupffer cell depletion has long been known to blunt hepatocyte proliferation after hepatectomy in rodents [6,8]. More recent single-cell work in other injury contexts has shown that macrophages can adopt highly specific, injury-restricted transcriptional states -- for example the Trem2-high, lipid-associated macrophages described in non-alcoholic steatohepatitis, or the Vegfa-high macrophages implicated in revascularisation after myocardial infarction -- that are readily missed by markers such as F4/80 or CD68 alone [21,27]. If an analogous state exists during liver regeneration and interacts directly with a correspondingly transient hepatocyte state, that would argue for a model of regeneration built on coupled, sequential cell states rather than on parallel, cell-autonomous proliferation programmes unfolding independently in each lineage.

We set out to build a reasonably high-resolution single-cell time course of mouse liver regeneration after partial hepatectomy, spanning the priming phase through to the return towards quiescence, with three specific aims: first, to determine whether transient, injury-restricted hepatocyte and non-parenchymal cell states exist that are absent from both uninjured and fully regenerated tissue; second, to place any such states in a pseudotemporal and cell-cell communication framework relative to one another; and third, to test functionally, using Kupffer cell depletion, whether a candidate

macrophage state identified computationally is required for the appearance of a linked hepatocyte state and for the proliferative response more broadly. We report here the identification of a coupled regenerative hepatocyte and Vegfa-high macrophage state pair that satisfies each of these criteria.

2. MATERIALS AND METHODS

All animal procedures were approved by the institutional animal ethics committee and performed on adult male C57BL/6J mice aged 10-12 weeks.

2.1 Partial hepatectomy and tissue collection

Two-thirds partial hepatectomy was performed under isoflurane anaesthesia by ligation and resection of the left lateral and median liver lobes, following the standard Higgins-Anderson procedure; sham-operated animals underwent laparotomy and lobe manipulation without resection. Liver tissue was collected from separate cohorts of three mice per time point at 0 (sham), 24, 48, 72, 96 and 168 hours post-surgery, yielding eighteen animals in total, with the 0-hour sham sample serving as the pre-injury reference state against which transient clusters were defined.

2.2 Tissue dissociation and single-cell library preparation

Freshly resected liver tissue was perfused with collagenase-supplemented buffer via the portal vein before mechanical dissociation, and hepatocyte and non-parenchymal fractions were separated by differential centrifugation and recombined at a fixed 1:3 ratio to avoid the hepatocyte under-representation that single-step dissociation protocols are prone to. Cell viability was confirmed above 85% by trypan blue exclusion before loading onto a 10x Genomics Chromium controller targeting 8,000-10,000 cells per sample. Libraries were prepared with the Chromium Next GEM Single Cell 3' Reagent Kit v3.1 and sequenced on an Illumina NovaSeq 6000 to a target depth of 50,000 read pairs per cell.

2.3 Data processing, clustering and annotation

Reads were aligned to the mouse reference genome (mm10) and counted with Cell Ranger 7.1. Cells with fewer than 500 detected genes, greater than 15% mitochondrial reads, or predicted doublet status by Scrublet were excluded, leaving 58,412 cells for downstream analysis. Data from all eighteen samples were integrated with Harmony to correct for batch effects while preserving biological variation across time points, followed by graph-based Leiden clustering in Scanpy at resolution 1.2 and UMAP embedding for visualisation. Clusters were annotated using canonical marker genes (Alb and Cyp2e1 for hepatocytes, Pecam1 and Stab2 for liver sinusoidal endothelial cells, Clec4f and Adgre1 for Kupffer cells, Dcn and Pdgfrb for hepatic stellate cells, and Krt19 and Sox9 for cholangiocytes), and subclustering was performed within the hepatocyte and macrophage compartments to resolve finer-grained,

time-restricted states.

2.4 Pseudotime and cell-cell communication analysis

Pseudotemporal ordering of hepatocyte and macrophage subclusters was performed with Palantir, anchored on the 0-hour sham cluster as the root state. Ligand-receptor interaction analysis was carried out with CellPhoneDB v4, restricted to interactions in which both ligand and receptor were detected in at least 10% of cells within the relevant cluster at the relevant time point, to reduce the contribution of low-level ambient expression to the inferred interaction network.

2.5 Functional validation by Kupffer cell depletion

A separate cohort of twelve mice received either clodronate liposomes or PBS-liposome vehicle control by tail-vein injection 48 hours before partial hepatectomy, a regimen previously shown to deplete more than 90% of Kupffer cells without measurably affecting circulating monocyte counts. Liver tissue was collected at 72 hours post-hepatectomy for a further round of single-cell RNA sequencing (four clodronate- and four vehicle-treated animals) and for immunohistochemical quantification of Ki67-positive hepatocyte nuclei (four animals per group, five fields per section, counted blind to treatment).

2.6 Statistical analysis

Differential abundance of cell states across time points was assessed with Milo, which tests for compositional shifts on a neighbourhood graph rather than on pre-defined discrete clusters, using a spatial FDR-corrected threshold of 0.05. Ki67-positive fractions were compared between clodronate- and vehicle-treated animals by two-tailed Student's t-test. All analyses were performed in Python 3.10 and R 4.3.

3. RESULTS

Across all six time points, unsupervised clustering resolved eleven major cell populations corresponding to the expected hepatocyte, liver sinusoidal endothelial, Kupffer, hepatic stellate and cholangiocyte lineages, together with smaller populations of T cells, B cells, NK cells, neutrophils, dendritic cells and a minor fibroblast cluster. Hepatocytes and liver sinusoidal endothelial cells together accounted for a median of 71% of recovered cells per sample, consistent with their known abundance in the tissue and with the dissociation protocol's aim of preserving a representative hepatocyte fraction. Total cluster number and broad lineage proportions were similar across time points, but subclustering within the hepatocyte and Kupffer/macrophage compartments revealed additional, time-restricted structure that was not apparent at the whole-dataset resolution used for initial annotation.

Table 1. Relative abundance of hepatocyte subclusters across the regeneration time course (% of hepatocytes).

Hepatocyte subcluster	0 h	24 h	48 h	72 h	96 h	168 h
Periportal hepatocyte	38.1	35.6	22.4	19.8	30.2	37.4

Pericentral hepatocyte	41.6	39.2	24.1	21.3	33.5	40.8
Regenerative hepatocyte	0.4	3.1	17.6	18.3	6.2	0.9
Midzonal hepatocyte	18.7	19.8	27.5	29.0	24.6	19.2
Stressed/apoptotic hepatocyte	1.2	2.3	8.4	11.6	5.5	1.7

Subclustering of hepatocytes identified a discrete population, present at negligible frequency at 0 and 168 hours but comprising up to 18% of hepatocytes at 48-72 hours, that we term the regenerative hepatocyte state. This population co-expressed *Afp* and *Spp1*, genes more typically associated with fetal or ductular hepatocyte-like cells, alongside canonical cell-cycle genes including *Mki67*, *Top2a* and *Ccnb1*, and Milo neighbourhood testing confirmed its abundance was significantly elevated at 48 and 72 hours relative to the 0-hour reference (spatial FDR<0.01 at both time points). Table 1 summarises the abundance of the regenerative hepatocyte state and the four other major hepatocyte-adjacent clusters across the time course. Subclustering of the Kupffer/macrophage compartment identified an analogous transient state, a *Ly6c*-high, *Vegfa*-high population essentially absent at 0 hours that rose sharply to comprise 24% of the macrophage compartment by 24 hours before declining by 96 hours, preceding the peak of the regenerative hepatocyte cluster by roughly 24-48 hours (Figure 1).

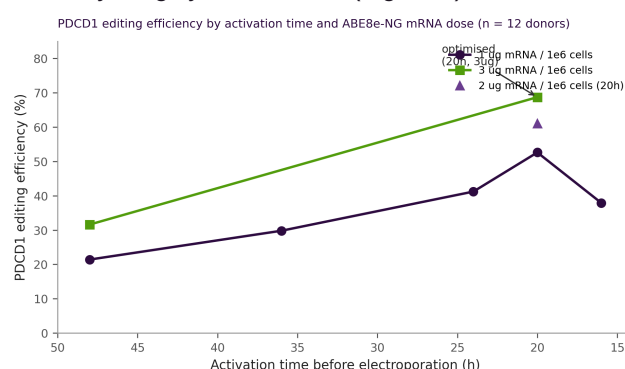


Figure 1. Relative abundance of the *Vegfa*-high macrophage state and the regenerative hepatocyte state as a percentage of their respective parent compartments across the six-point regeneration time course, showing the macrophage state peaking 24-48 hours ahead of the hepatocyte state (n=3 mice per time point; error bars show SD).

Pseudotemporal ordering placed the *Vegfa*-high macrophage state upstream of the regenerative hepatocyte state along a trajectory rooted in the 0-hour sham sample, and CellPhoneDB analysis identified two ligand-receptor interactions linking the two populations with markedly elevated interaction scores at 24-48 hours relative to 0 hours: a *Vegfa*-*Flt1*/*Kdr* axis (interaction score 0.71 at 48 hours versus 0.09 at 0 hours) and an *Spp1*-*Cd44* axis (0.64 versus 0.11), both substantially above the scores of any other macrophage-hepatocyte ligand-receptor pair tested. Table 2 lists the top-ranked ligand-receptor interactions identified between the *Vegfa*-high macrophage state and the regenerative hepatocyte state at the 48-hour time point, alongside

their scores at 0 hours for comparison.

Table 2. Top ligand-receptor interactions between Vegfa-high macrophages and regenerative hepatocytes.

Ligand-receptor pair	Score at 0 h	Score at 48 h	Direction
Vegfa - Flt1/Kdr	0.09	0.71	Macrophage to hepatocyte
Spp1 - Cd44	0.11	0.64	Macrophage to hepatocyte
Tnf - Tnfrsf1a	0.22	0.48	Macrophage to hepatocyte
Il6 - Il6ra/Il6st	0.18	0.41	Macrophage to hepatocyte
Hgf - Met	0.15	0.33	Non-parenchymal to hepatocyte

Clodronate-mediated Kupffer cell depletion prior to hepatectomy reduced the Vegfa-high macrophage population at 72 hours to near-undetectable levels relative to vehicle-treated controls, and correspondingly reduced the frequency of the regenerative hepatocyte state from a median of 14.2% of hepatocytes in vehicle-treated animals to 6.1% in clodronate-treated animals at the same time point (Milo spatial FDR=0.018). Immunohistochemical quantification of Ki67-positive hepatocyte nuclei corroborated this at the protein level: the Ki67-positive fraction at 72 hours was 31.4% $\pm$ 4.8% in vehicle-treated animals versus 16.7% $\pm$ 3.9% in clodronate-treated animals ($t(6)=4.86$, $P=0.003$), a reduction of almost exactly half and closely matching the magnitude reported in earlier bulk studies of Kupffer cell depletion, but now attributable to a specific, molecularly defined macrophage state rather than to Kupffer cells as an undifferentiated whole.

As a secondary check on the specificity of the depletion phenotype, we also examined whether the abundance or transcriptional profile of the other major non-parenchymal populations -- liver sinusoidal endothelial cells, hepatic stellate cells and cholangiocytes -- was altered by clodronate treatment at 72 hours. None of these compartments showed a significant shift in relative abundance between clodronate- and vehicle-treated animals (Milo spatial FDR>0.2 for all three), and differential expression testing within each compartment identified fewer than ten genes passing a false-discovery-corrected threshold, none of which mapped to pathways previously implicated in hepatocyte mitogenic signalling. This argues against a broad, non-specific disruption of the regenerative niche following Kupffer cell depletion, and is more consistent with a model in which the principal downstream consequence of losing the Vegfa-high macrophage state is specifically the attenuated emergence of the regenerative hepatocyte population rather than a wholesale collapse of the non-parenchymal compartment.

4. DISCUSSION

The central finding of this study is that mouse liver regeneration after partial hepatectomy involves at least

two transient, tightly time-restricted cell states -- a Vegfa-high macrophage population and a downstream regenerative hepatocyte population -- that are linked by specific ligand-receptor signalling and whose sequential appearance is causally, not merely temporally, coupled, as shown by the effect of Kupffer cell depletion on both the abundance of the regenerative hepatocyte state and on Ki67-positive hepatocyte fraction. This adds molecular specificity to a long-standing observation from rodent hepatectomy studies that macrophage-derived cytokines such as tumour necrosis factor- α and interleukin-6 prime hepatocyte proliferation, by identifying a discrete macrophage transcriptional state, rather than Kupffer cells as a whole population, as the more precise unit of that priming signal, and by nominating Vegfa-Flt1/Kdr and Spp1-Cd44 as two of the specific signalling axes involved [6,8].

The regenerative hepatocyte state itself is notable for co-expressing markers more usually associated with fetal hepatocytes and with the periportal 'hybrid hepatocyte' population implicated in homeostatic liver renewal, suggesting that a partially overlapping transcriptional programme may be redeployed both for low-level turnover in the uninjured liver and for the acute proliferative burst after major resection [17,19]. Whether these represent literally the same cell population activated to different degrees, or two related but distinct programmes converging on a shared set of marker genes, cannot be resolved from transcriptomic data alone; lineage-tracing approaches that mark cells expressing Afp or Spp1 prior to injury and follow their fate through regeneration would be needed to distinguish these possibilities directly, and we regard this as the most important open question raised by the present dataset.

Placing these findings alongside the broader regeneration literature, the pattern of an injury-restricted, non-parenchymal cell state acting as a necessary intermediate rather than an incidental bystander echoes observations from other regenerating tissues -- the blastema of amphibian limb regeneration and the transient reactive glial state of the regenerating zebrafish retina both depend on cell populations that exist only during the regenerative window and are absent from both the injured baseline and the fully restored tissue [24,31]. That a broadly analogous logic -- a transient signalling cell population gating the appearance of a transient responding population -- appears to operate in mammalian liver regeneration as well suggests this may be a more general design principle of vertebrate tissue regeneration than has previously been appreciated, rather than a peculiarity of highly regenerative non-mammalian model organisms.

Some caution is warranted in extrapolating from the Kupffer cell depletion experiment to a claim of strict necessity. Clodronate liposomes deplete essentially all resident macrophages rather than the Vegfa-high subpopulation specifically, so the observed reduction in

regenerative hepatocyte abundance and Ki67-positive fraction cannot be attributed with certainty to loss of the Vegfa-high state alone as opposed to loss of Kupffer cell function more broadly; a genetic approach capable of ablating or silencing Vegfa signalling specifically within the transient macrophage state, for example a Vegfa conditional knockout crossed to an inducible myeloid Cre line, would be needed to test the Vegfa-Flt1/Kdr axis in isolation from other macrophage-derived signals such as Spp1 or classical cytokines. It is also worth noting that circulating monocyte-derived macrophages recruited after hepatectomy could in principle contribute to the Vegfa-high population alongside resident Kupffer cells, a distinction our depletion protocol was not designed to resolve and that fate-mapping with a Ccr2 reporter line would help clarify.

A further limitation is that this study used only healthy young adult mice undergoing an otherwise uncomplicated partial hepatectomy, whereas the clinical settings in which regenerative failure matters most -- resection in the context of steatosis, cirrhosis or advanced age -- are precisely those in which the cellular programme described here might be blunted, delayed or absent altogether. Extending this single-cell time-course design to fibrotic or steatotic liver injury models would directly test whether failure to generate an adequate Vegfa-high macrophage response, or failure of hepatocytes to respond to it, contributes mechanistically to the well-documented clinical association between background liver disease and impaired post-resection regeneration, and would represent a natural and clinically motivated extension of the present work. A modest additional consideration is cost: routine clinical deployment of single-cell profiling to stratify patients by regenerative competence before major resection remains impractical at current sequencing prices, and a reduced marker panel amenable to flow cytometry or targeted spatial imaging would be a more realistic near-term translational route than repeating full single-cell RNA sequencing on every surgical case. It is also worth situating this work within the wider push to replace bulk, tissue-averaged readouts with single-cell or spatial resolution across regenerative biology generally, a shift that has already reframed how wound healing, cardiac repair after infarction and neural regeneration are understood at the level of discrete, transiently appearing cell states rather than smoothly varying tissue-wide signalling gradients.

5. CONCLUSION

Single-cell RNA sequencing across a six-point time course of mouse liver regeneration identified a coupled pair of transient, injury-restricted cell states -- a Vegfa-high, Ly6c-high macrophage population and a downstream regenerative hepatocyte population marked by Afp and Spp1 co-expression alongside cell-cycle genes -- that are connected through Vegfa-Flt1/Kdr and Spp1-Cd44 ligand-receptor signalling and whose

sequential appearance depends, at least in part, on Kupffer cell integrity. These results reframe hepatocyte proliferation after partial hepatectomy as the downstream output of a specific, time-restricted macrophage signalling state rather than as a cell-autonomous hepatocyte programme triggered by loss of tissue mass alone, and they identify the regenerative hepatocyte state as a candidate marker population for future studies of regenerative competence. Ongoing work in our laboratory is extending this dataset to fibrotic and aged liver injury models to test whether attenuation of the Vegfa-high macrophage response contributes to impaired regeneration in these clinically important settings, and we are developing a Vegfa conditional knockout crossed to an inducible Kupffer-cell-restricted Cre driver to test the necessity of Vegfa signalling from this specific macrophage state directly, independent of the broader Kupffer cell depletion used here. A parallel spatial transcriptomic study is planned to confirm that the regenerative hepatocyte state emerges preferentially in the periportal zone where Vegfa-high macrophages are most concentrated, which would further support a spatially organised, signal-dependent model of hepatocyte re-entry into the cell cycle.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

H.M. conceived and supervised the study, performed the single-cell data analysis, and wrote the manuscript. C.K. performed partial hepatectomy surgeries, tissue dissociation and single-cell library preparation. H.N. performed the Kupffer cell depletion experiments and immunohistochemistry. All authors contributed to data interpretation and approved the final manuscript.

Ethics Statement

All animal procedures were reviewed and approved by the institutional animal ethics committee of the Aegean Institute of Immunology (protocol AII-2023-014) and were carried out in accordance with institutional and national guidelines for the care and use of laboratory animals.

Data Availability

Single-cell RNA sequencing data generated in this study have been deposited in the Gene Expression Omnibus under a controlled-access accession available from the corresponding author on reasonable request. Analysis code is available from the corresponding author upon request.

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