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Multimodal decoding of human liver regeneration uncovers novel ANXA2$^+$ migratory hepatocytes for wound healing

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Acute liver failure (ALF) is the onset of severe liver injury without pre-existing chronic liver diseases.[1 2] The most common type of drug-induced ALF is caused by acetaminophen (APAP) overdose, which accounts for 45.7% of ALF cases in the US.[2] In severe cases of ALF, liver transplantation remains the only curative option.[1 2] Nonetheless, there is currently a shortage of liver donor organs available to meet the demands for liver transplantation needed to save the lives of patients with ALF. Due to the increasing prevalence of metabolic dysfunction-associated steatotic liver disease and metabolic and alcohol-associated liver disease,[3 4] many steatotic livers are discarded, further exacerbating the burden of liver shortages. Therefore, it is crucial to find therapeutic approaches that can potentially alleviate APAP-induced ALF (APAP-ALF). APAP-ALF occurs in three phases: metabolism, injury and recovery/regeneration. Before liver regeneration, emerging evidence shows that necrotic areas must be restricted/resolved, involving immune cells such as monocyte-derived macrophages (MoMFs) and migratory hepatocytes (figure 1A). Most patients with APAP-ALF in clinical settings have already progressed beyond the metabolic and injury phases, so enhancing the resolution of necrotic lesions as well as liver recovery and regeneration would be the most effective strategy to save their lives.

Hepatocytes, a liver cell type that accounts for approximately 70–80% of the liver mass, are the major hepatic parenchymal cells. They perform various metabolic and synthetic functions fundamental to proper liver functions.[5 6] Hepatocyte replenishment, the cells' ability to regenerate and compensate for the cell loss due to damages, has been demonstrated as the major contribution to hepatic wound closure after necroinflammatory liver injury.[7 8] There are several mechanisms to achieve hepatocyte replenishment on severe liver injury. It has been suggested that hepatocytes could be replenished by the transdifferentiation from cholangiocytes.[9] Moreover, hepatocyte replenishment can also be derived from the proliferation of pre-existing hepatocytes during liver homeostasis.[5 10 11] Current evidence indicates that hepatocyte proliferation plays a more significant role in liver regeneration and repair than cholangiocyte transdifferentiation during ALF. Despite the extensive proliferation of pre-existing hepatocytes, the wound area in human APAP-ALF remains significant in the explanted liver at the point of liver transplantation, which possibly reflects defective or insufficient repair as a cause of organ failure. Hence, identifying novel mechanisms to effectively promote hepatic wound closure and restore the liver's normal architecture following APAP-induced liver injury will have a significant impact on improving the survival of APAP-ALF. Although the mechanisms of how APAP induces hepatotoxicity are well established, the processes that limit necrotic areas to promote healing and liver regeneration remain largely unknown.

In a recent study published in *Nature*,[12] Matchett *et al* used a cross-species, integrative multimodal approach including single-nucleus RNA sequencing (snRNA-seq), spatial transcriptomics and multiplex single-molecule fluorescence in situ hybridisation (MsmFISH) to investigate the cellular and molecular mechanisms regulating liver regeneration using liver tissue samples from ALF human patients and experimental ALF mouse models. They first constructed the normal liver zonation gene modules using these multimodal approaches.

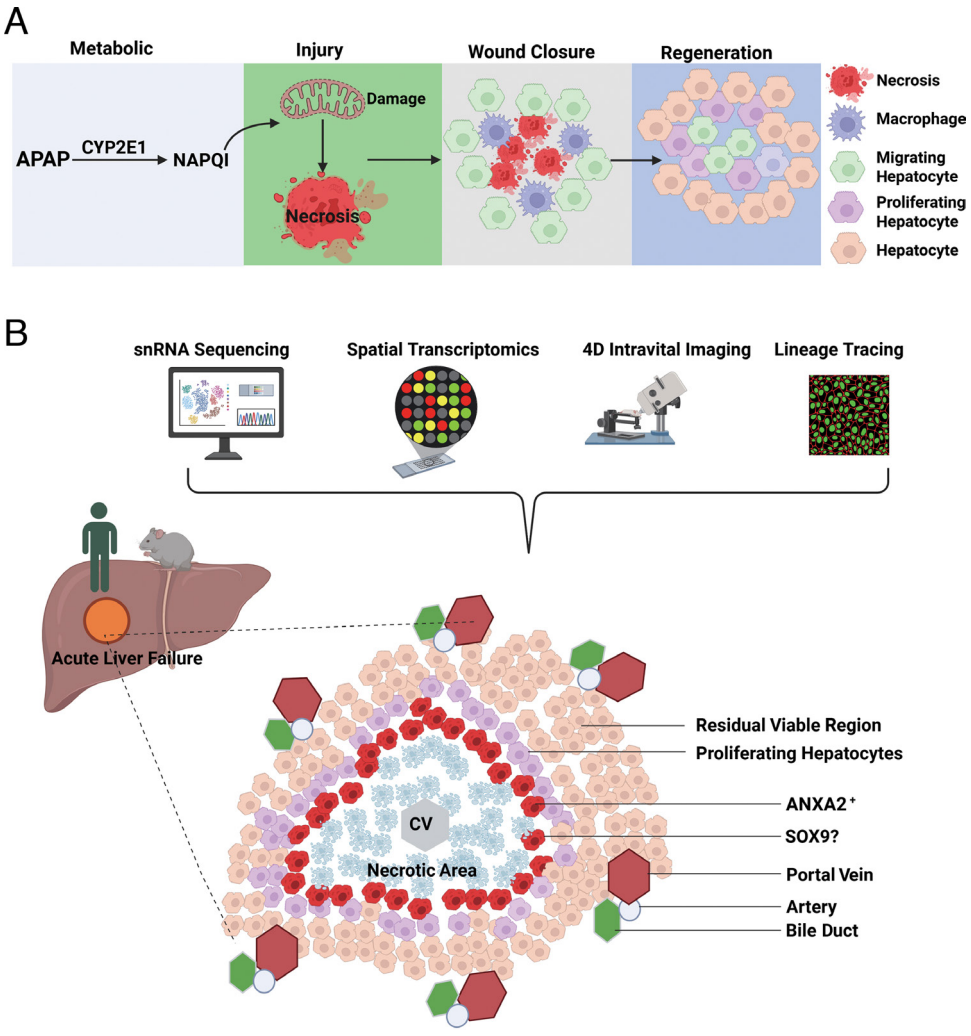

Figure 1 The scheme of using integrative multimodal approaches to dissect the cellular and molecular mechanisms regulating wound healing and liver regeneration in ALF. (A) Distinct phases of APAP-ALF. (B) Integrative multimodal approaches, including single-nucleus RNA sequencing spatial transcriptomics, four-dimensional intravital imaging and lineage tracing in both human and experimental mouse models of ALF, have led to the identification of a unique subpopulation of hepatocytes that express ANXA2. Although it is currently unclear whether ANXA2-positive cells also express SOX9, this subpopulation of hepatocytes may play a critical role in limiting and closing necrotic areas, potentially aiding in liver repair and regeneration to improve outcomes in ALF. ALF, acute liver failue; APAP, acetaminophen; CV, central vein; CYP2E1, Cytochrome P450 family 2 subfamily E member 1; NAPQI, N-acetyl-p-benzoquinone imine; SOX9, SRY-box transcription factor 9.

In comparison with healthy human liver zonation genes, Matchett *et al* showed that APAP-ALF human liver exhibited disrupted liver zonation with a loss of hepatocyte portal-central polarity, along with the appearance of hepatocytes exhibiting characteristics of both portal and central lobular zones. The residual viable region (RVR) displayed mixed portal and central Gene Ontology patterns that were distinct from those present in the perinecrotic (PNR) or necrotic regions, suggesting that RVR hepatocytes possessed functional plasticity and likely gained compensatory proliferation capacities for the cell loss following APAP-ALF. Notably, a hepatocyte subpopulation that forms a boundary surrounding the necrosis areas was abundantly expressing ANXA2 ($ANXA2^{+}$) with migratory cell phenotypes. Immunofluorescence (IF) and MsmFISH further confirmed that hepatocytes adjacent to the necrotic area in APAP-ALF had high migratory features, which were absent in healthy human liver tissue. $ANXA2^{+}$ hepatocytes exhibited migratory cell morphology, including ruffled membranes and extending lamellipodia, perhaps functioning to enclose and restrict the necrosis regions in coordination with other cells such as hepatic stellate cells (HSCs) and immune cells. Interestingly, a similar approach in APAP-induced mouse liver injury revealed a corresponding $ANXA2^{+}$ migratory hepatocyte subpopulation in mice, which displayed gene signatures and cellular morphology similar to those observed in human APAP-ALF.

To better understand the temporal dynamics of wound closure and hepatocyte proliferation, the authors investigated the necrotic wound closure and hepatocyte proliferation across different time points following APAP-induced mouse liver injury. They showed that hepatocyte necrosis peaked at 30 hours post-APAP-induced liver injury and

was subdued afterwards, whereas hepatocyte proliferation peaked at 72 hours. Using an elegant four-dimensional intravital microscopy approach with the lineage tracing fluorescent reporter mouse that can monitor hepatocyte migration in live mouse livers, they showed that the emergence of hepatocyte migration occurred between 36 hours and 42 hours *in vivo*, following APAP-induced mouse liver injury. Hepatocytes that showed high expression of ANXA2 exhibited a motile morphology, characterised by membrane ruffling and lamellipodia formation. These cells were located immediately adjacent to the wound area. Further analysis of PNR and RVR hepatocytes revealed that PNR hepatocytes displayed greater mobility, deviation, ellipticity and sphericity than RVR hepatocytes. Overall, the data suggests that hepatocyte migration was the primary mechanism driving wound closure following APAP-induced liver injury in mice.

Glutamine synthetase (GS) is exclusively expressed in hepatocytes located near the central vein. After 14 days following APAP-induced liver injury, 75.6% of GS-positive hepatocytes were found to be 5′-bromo-2′-deoxyuridine (BrdU)-negative. This suggests that the hepatocytes in the necrotic area directly adjacent to the central vein did not originate from hepatocyte proliferation but from hepatocyte migration. To further investigate the sources of these newly migratory hepatocytes, the authors performed an *in silico* analysis of a human snRNA-seq data set. They found that cholangiocytes are not a significant source of hepatocytes in human APAP-ALF. Additionally, using lineage tracing in hepatocytes with R26RLSLtdTomato reporter mice, the authors demonstrated that 100% of ANXA2-positive hepatocytes were derived from tdTomato-positive hepatocytes at all time points studied. To further validate the role of ANXA2 hepatocytes in regulating wound closure in APAP-induced liver injury, the authors investigated cell migration and liver injury by knocking down ANXA2 expression in both *in vitro* and *in vivo* studies. Using a cell scratch wound assay, the authors showed that ANXA2 knockdown led to decreased wound closure in the Huh7 cell line and hepatocyte growth factor-stimulated primary mouse hepatocytes, independent of hepatocyte proliferation. Furthermore, hepatocyte-specific ANXA2 knockdown using AAV8-shRNA-ANXA2 in mice showed a decrease in wound closure progression compared with controls following APAP-induced liver injury, independent of hepatocyte proliferation.

In summary, this study has successfully created a single-cell, pan-lineage atlas of human liver regeneration using a cutting-edge cross-species, multimodal approach (figure 1B). It has also advanced the liver regeneration field by identifying ANXA2$^+$ migratory hepatocytes that promote wound healing and limit necrotic areas in the liver. However, many questions remain unanswered. First, although the deletion of ANXA2 negatively affects the reduction of necrotic regions, the impact is only moderate. Notably, necrotic areas were still resolved in ANXA2 knockdown mouse livers after exposure to APAP for up to 72 hours. This suggests that other undiscovered molecules in these migratory hepatocytes may be more crucial for promoting hepatocyte migration and closing necrotic areas. Analysing the gene expression profiles of ANXA2-positive hepatocytes compared with ANXA2-negative ones could help identify potential targets that regulate the closure of the necrotic regions and facilitate injury resolution and hepatocyte proliferation. While knocking down ANXA2 had only moderate effects on the closure of necrotic regions, overexpressing ANXA2 could have more significant effects in promoting closure and hepatocyte proliferation. This hypothesis should be tested in future studies.

Second, it is still unclear whether the closure of the necrotic areas is directly mediated by the ANXA2 protein itself or by the population of ANXA2$^+$ hepatocytes. Future studies using a cell ablation approach to eliminate ANXA2$^+$ hepatocytes may provide more definitive evidence regarding their role in facilitating the closure of necrotic areas following an APAP overdose. Furthermore, it is well known that immune cells, specifically infiltrating MoMFs, are recruited to necrotic areas to clear dead hepatocytes and support liver regeneration.[13 14] In an immune-mediated ALF model induced by concanavalin A (ConA), it was demonstrated that MoMFs were rapidly recruited to and encapsulated necrotic regions. These MoMFs exhibited increased activation of Jagged1/Notch homolog protein 2 (JAG1/NOTCH2), which led to the formation of SRY-box transcription factor 9$^+$ (SOX9$^+$) hepatocytes. These SOX9$^+$ hepatocytes are derived from mature hepatocytes located near the necrotic lesions and act as a protective barrier against further injury. Additionally, MoMFs activated HSCs and increased HSC contraction, which helped to compress and ultimately eliminate the necrotic lesions.[13] It would be particularly interesting to investigate whether these SOX9$^+$ hepatocytes are the same population as ANXA2$^+$ hepatocytes in ALF. Exploring the relationship between ANXA2$^+$ hepatocytes and the infiltrating immune cells, as well as HSCs, could provide valuable insights into their collaborative role in closing necrotic areas and promoting hepatocyte proliferation.

Finally, the study discussed here primarily focuses on the resolution of liver necrotic lesions and liver regeneration, which are most commonly observed in cases of acute liver injury. The mechanisms discussed appear to be specific to areas of liver damage characterised by necrotic lesions rather than random single-cell death, such as apoptosis induced by TNFα. In chronic liver diseases, such as hepatitis B virus and hepatitis C virus infections, persistent hepatocyte injury often coexists with compensatory regeneration, which can lead to liver tumourigenesis. It would be

interesting to investigate whether ANXA2^{+} hepatocytes are involved in chronic liver diseases and if they contribute to liver tumourigenesis. Overall, these new studies have opened an exciting avenue for future therapeutic interventions aimed at improving ALF by targeting the closure of necrotic regions.

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