Immunomagneto-activated hepatocyte-specific tissue vesicle profiling reveals message diversification from regenerating liver

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Abstract

Background & Aims: The liver possesses a remarkable regenerative capacity which involves intricate intercellular communication. However, the mechanisms underlying the complex intercellular interactions remain incompletely understood. Extracellular vesicles (EVs) are emerging as important messengers in diverse pathophysiological conditions. Nevertheless, tissue-derived, cell-specific functional EV populations in regeneration have not been robustly analyzed.

Methods: Single-cell RNA sequencing (scRNA-seq) analysis of the regenerating liver after partial hepatectomy (PHx), ultrastructural examinations of *in situ* liver tissue extracellular vesicles (LT-EVs), and nanoscale and proteomic profiling of hepatocyte-specific EVs were integrated. Especially, a feasible approach for investigating antigen-specific endogenous EVs was developed after hepatic enzymatic digestion followed by differential centrifugation and immunomagnetic sorting of LT-EVs.

Results: scRNA-seq analysis revealed hepatocytes as the critical source of intercellular communication in the regenerating liver. In particular, hepatocytes are programmed to be enriched for biogenesis and release of EVs in liver regeneration. By analyzing *in situ* EVs using transmission electron microscope (TEM), LT-EVs were discovered to be emergingly present in the extracellular interstitial space adjacent to hepatocytes in the regenerating liver. Moreover, asialoglycoprotein receptor (ASGPR)-marked, magnetic microbead-sorted LT-EVs (named ASGPR^{+}mLT-EVs) were collected. These hepatocyte-specific LT-EVs were further demonstrated with strengthened release and distinct protein signatures during liver regeneration. Importantly, the EVs demonstrated parental hepatocyte-derived information

diversification, which not only inherited regulated proliferative and metabolic signals at the cellular level but also showed vesicular enrichment of organelle components.

Conclusions: These findings provide the first knowledge that hepatocyte-specific tissue vesicles are indeed phenotypically and functionally involved in liver regeneration. Our study paves an avenue for in-depth biological and mechanistic research of regenerative LT-EVs and sheds light on extensive dissection of antigen-marked, physiological, and endogenous tissue EV populations.

Key words

Liver regeneration; hepatocytes; extracellular vesicles; single-cell RNA sequencing; immunomagnetic sorting; proteomic analysis

1. Introduction

The liver is the largest solid organ in the body which critically maintains the metabolism and serves as the central hub for detoxification. In order to cope with daily challenges, the liver has developed a potent, robust, and fine-tuned regenerative capability, which can fully regenerate up-to-2/3 loss of the total parenchyma [1, 2]. The process of liver regeneration involves a complex cellular interplay primarily centered on hepatocytes but also including biliary cells and various non-parenchymal cells (NPCs) [3]. Particularly, in the healthy liver, hepatocytes are mitotically quiescent; whereas following toxic damage or surgical resection, hepatocytes can rapidly enter the cell cycle to proliferate or transdifferentiate from biliary epithelial cells (BECs) with orchestrating immune, endothelial, and stromal components to synergistically restore liver mass and function [4-8]. This hepatocyte expansion-correlated cellular coordination is acknowledged to be mediated by diverse paracrine cytokines, which safeguard efficient liver regeneration as a prerequisite for proper body functioning [4, 5, 9]. However, the liver regeneration capacity can be overwhelmed or suppressed under large injury or progressive damages, resulting in hepatic diseases remaining as a global medical challenge [10, 11]. Deciphering in-depth the mechanisms underlying liver regeneration will help to devise feasible strategies for hepatic disease therapy.

Extracellular vesicles (EVs) represent an emerging and alternative form of intercellular communication regulating a variety of biological processes, which are functionalized with surface molecules and encapsulated cargoes, such as proteins, nucleic acids, and lipids [12, 13]. Particularly, solid tissues are increasingly recognized as natural reservoirs of endogenous EVs, and tissue-derived EVs (Ti-EVs) play a putative role in reflecting and

regulating the tissue health and disease statuses [14]. A recent study has isolated Ti-EVs from the kidney and the skin, which have been used to promote target tissue repair in a donor tissue-specific manner after allogeneic transplantation [15]. It has also been reported that liver tissue EVs (denoted LT-EVs) isolated from both normal and carbon tetrachloride (CCl_4)-induced damaged liver accelerate the recovery of liver tissues from CCl_4-induced hepatic necrosis after administration [16]. However, owing to the heterogeneous nature of tissue resident cells and the imprecision property of the bulk isolation method of Ti-EVs, it remains an unmet need to clarify and characterize the detailed endogenous EV populations that contribute to tissue physiology and pathology [17-19]. With regard to liver regeneration, small EVs derived from the cultured hepatocyte supernatant have been shown to promote liver regeneration after infusion [20]. Nevertheless, whether LT-EVs from hepatocytes or any other liver cell types specifically participate in the liver regeneration process has not been evaluated.

In this study, we aimed to investigate *in situ* tissue-derived, cell-specific functional EV population(s) in liver regeneration. By performing an unbiased examination of the hepatic cell landscape *via* single-cell RNA sequencing (scRNA-seq), we revealed that hepatocytes serve as the key source of intercellular communication in the regenerating liver after partial hepatectomy (PHx), which are programmed to be enriched for biogenesis and release of EVs. Furthermore, we first conducted the ultrastructural observation of LT-EVs *in situ* and established an immunomagnetic sorting protocol analyzing the hepatocyte-specific LT-EVs marked by the featured asialoglycoprotein receptor (ASGPR). These $ASGPR^+$ magnetic microbead-sorted LT-EVs (hereby denoted mLT-EVs) were thoroughly characterized for the

physicochemical properties of morphology, size, particle content, and protein composition at the nanoscale or high throughput level, and the transcriptome of hepatocytes and the proteome of ASGPR^{+}mLT-EVs were integrated to understand their contributions to liver regeneration. We discovered that hepatocytes release a specific LT-EV population which transmits diversified messages and hallmarks liver regeneration. Our study thus paves an avenue for in-depth biological and mechanistic research of regenerative LT-EVs and sheds light on extensive investigation of antigen-marked, physiological, and endogenous tissue EV populations.

2. Results

2.1 scRNA-seq analysis reveals hepatocytes as the key source of intercellular communication in the regenerating liver

To begin, we select the PHx model which enables the study of liver regeneration in a setting without enormous necrosis and associated inflammation [2]. A 2/3 PHx surgery was performed in mice with laparotomy only as the Sham control [21]. Liver regeneration with hepatocyte proliferation was confirmed at 72 h after PHx by histological examination and immunofluorescence (IF) staining of phospho-histone H3 (PHH3), a mitosis marker [23] (Figure S1A and B). We then collected and reanalyzed two scRNA-seq data of the mouse liver respectively from Sham and PHx conditions (GSM4572241; GSM4572244). Cell clustering using principal component analysis and t-distributed stochastic neighbor embedding (tSNE) plot visualization identified 8 distinct liver cell types, in which the proportion of hepatocytes decreased after PHx (Figure 1A and B). Intriguingly, despite

decreased proportion, enrichment of intercellular communication originated from hepatocytes was revealed prominently after PHx by the Cellchat analysis (Figure 1C). Moreover, the number of differentially expressed genes (DEGs) in hepatocytes over other liver cell types was high (Figure 1D). These DEGs were further demonstrated by the Gene Ontology (GO) analysis to be associated with multiple terms, notably the “cellular anatomical entity” in the “Cellular Component” domain and the “cellular process” and “metabolic process” in the “Biological Process” domain (Figure 1E). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis also exhibited that hepatocyte DEGs were particularly associated with “Transport and catabolism” and “cell growth and death” in the “Cellular Processes” domain and were linked with the “Metabolism” domain (Figure 1F). These data were in accordance with hepatocyte turnover behaviors and metabolic function in liver homeostasis and regeneration, which additionally indicated the cellular transport-based information transmission as a characteristic of hepatocytes. Taken together, these results suggest that hepatocytes serve as the key source of intercellular communication in the regenerating liver.

Figure 1

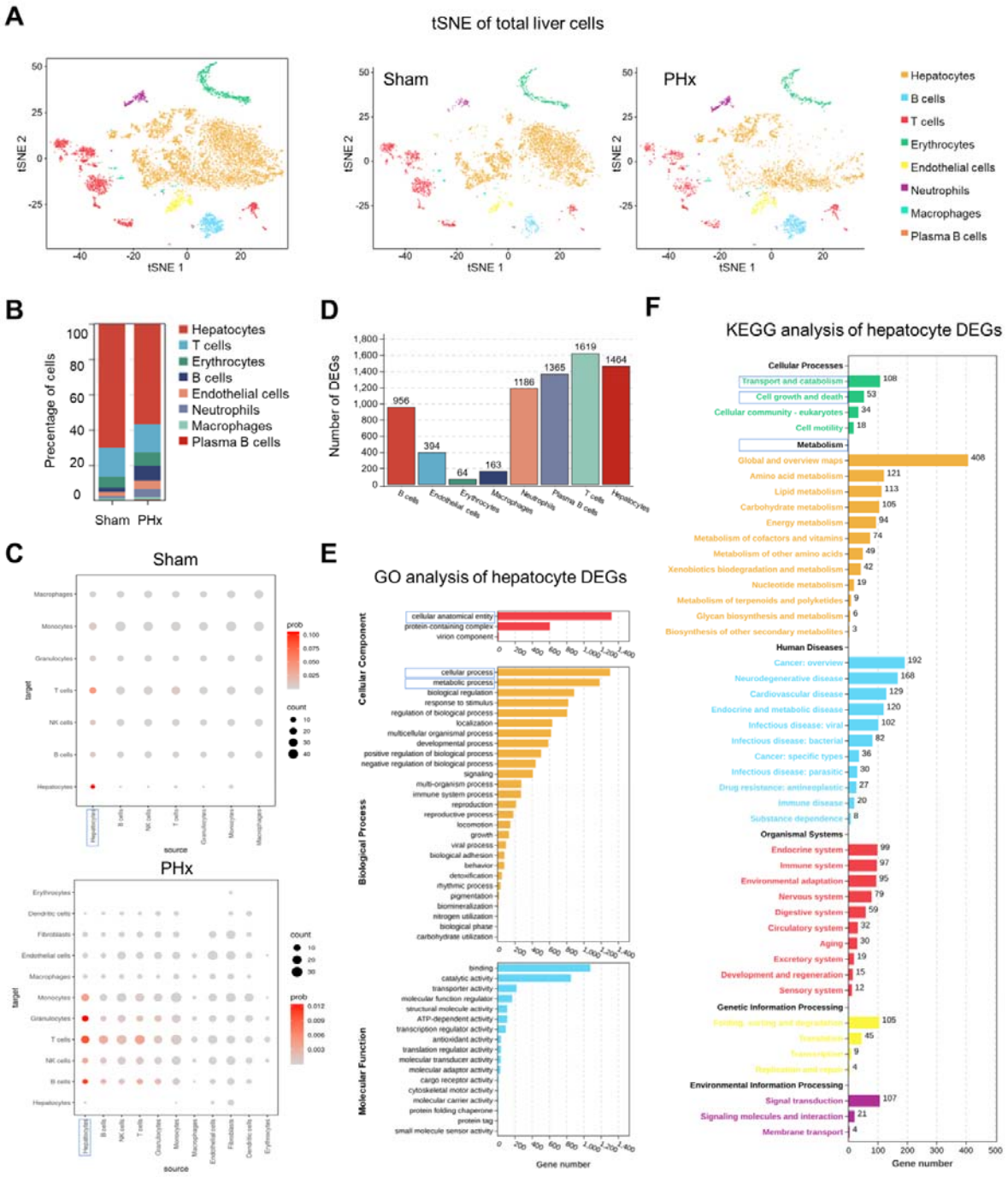

Figure 1. scRNA-seq analysis reveals hepatocytes as the key source of intercellular communication in the regenerating liver. **(A)** tSNE plots displaying the distribution of different cell types in Sham and PHx livers. **(B)** Cell type proportion in Sham and PHx livers. **(C)** Bubble maps showing intercellular interactions among Sham and PHx liver cells. **(D)**

The number of upregulated DEGs in respective liver cell clusters. **(E)** GO analysis of upregulated DEGs in hepatocytes. **(F)** KEGG analysis of upregulated DEGs in hepatocytes. Source data were derived from the GSM4572241 and GSM4572243 series in the GEO database.

2.2 Hepatocytes are programmed to be enriched for biogenesis and release of EVs in liver regeneration

Next, we investigated the detailed gene expression profiles of hepatocytes in Sham and PHx livers using the scRNA-seq data. In the pseudotime analysis, hepatocytes were ordered into seven stages (Figure 2A). Particularly, hepatocytes from Sham and PHx livers fell into distinct stage branches with a few transitioning cells, indicating a cellular state shift (Figure 2B). Accordingly, 3524 DEGs between Sham and PHx hepatocytes were identified, with respectively 1232 upregulated DEGs and 2292 downregulated DEGs in PHx hepatocytes compared to their Sham counterparts (Figure 2C). These DEGs, based on the functional annotations of the GO database, were associated with “cellular anatomical entity” in the “Cellular Component” domain and the “cellular process” and “metabolic process” in the “Biological Process” domain, which were consistent with the overall analysis of hepatocyte DEGs compared to other liver cells (Figure 2D). Notably, the PHx over Sham hepatocyte DEGs were associated with the “biological regulation”, “regulation of biological process” and “response to stimulus” terms in the “Biological Process” domain, indicating functional relationship with liver regeneration after injury (Figure 2D). Also, these DEGs corresponded with the overall analysis of hepatocyte DEGs in the KEGG analysis, which

were associated with the “Transport and catabolism” and “cell growth and death” in the “Cellular Processes” domain and were linked with the “Metabolism” domain (Figure 2E). Rapid entry of hepatocytes into the cell cycle with promotion of mitosis was further confirmed by the Cellcycle analysis (Figure S1C). To examine in-depth functional implications of the DEGs, enrichment analyses were performed based on GO and KEGG information, which showed the organelle-related terms, such as “mitochondrion” and “ribosome”, and detailed metabolic terms, were among the top enriched terms in PHx hepatocytes (Figure 2F and G). Particularly, considering above findings on the hepatocyte role of intercellular communication in liver regeneration, we focused on EV-related terms in the PHx over Sham hepatocyte DEGs, and the results demonstrated remarkable enrichment of potential EV function in PHx hepatocytes, including EV assembly and secretion (Figure 2H). Taken together, these results suggest that hepatocytes are programmed to be enriched for biogenesis and release of EVs in liver regeneration.

Figure 2

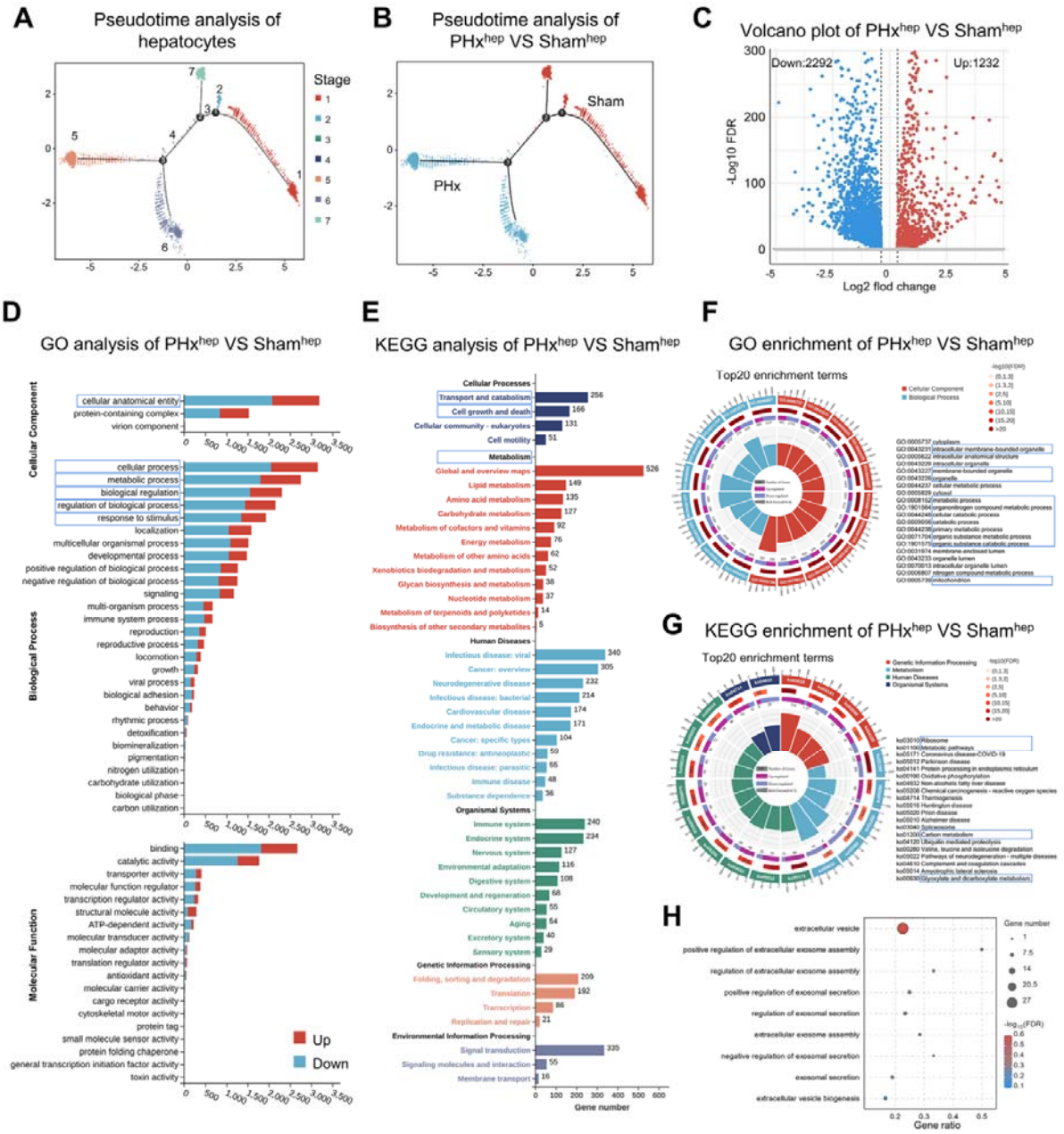

Figure 2. Hepatocytes are programmed to be enriched for biogenesis and release of EVs in liver regeneration. (A and B) Pseudotime analysis plots indicating cellular trajectories of hepatocytes in PHx and Sham livers. **(C)** Volcano plot showing DEGs between PHx and Sham hepatocytes. **(D)** GO analysis of DEGs between PHx and Sham hepatocytes. **(E)** KEGG analysis of DEGs between PHx and Sham hepatocytes. **(F)** GO enrichment analysis of DEGs between PHx and Sham hepatocytes shown by the circle

diagram. **(G)** KEGG enrichment analysis of DEGs between PHx and Sham hepatocytes shown by the circle diagram. **(H)** EV-related terms are enriched in PHx hepatocytes over Sham hepatocytes. Source data were derived from the GSM4572241 and GSM4572243 series in the GEO database.

2.3 LT-EVs are emergingly present in the extracellular interstitial space adjacent to hepatocytes in the regenerating liver

Next, we intended to investigate that whether EVs are indeed involved in the liver regeneration process. Ti-EVs putatively exist in the tissue extracellular space and reflect pathophysiological features of the tissue [14, 15]. However, current studies examine Ti-EVs mainly *via* enzymatic digestion and isolation, resulting in loss of spatiotemporal information and potential compromise of EV amount and integrity [17-19]. Existing literatures are also limited on the evaluation of *in situ* EVs in tissue regeneration. Therefore, to detect the *bona fide* EVs in the liver, we conducted transmission electron microscopy (TEM) analysis (Figure 3). Notably, in tissue sections of PHx, compared to those of Sham, hepatocytes were enlarged and the interstitial space was narrowed, in accordance with the hypertrophic phenotype in regeneration (Figure 3) [24]. Particularly, in the extracellular space adjacent to hepatocytes, vesicle-like nanosized particles was detected, which were presence in the Sham liver and demonstrated a dramatic increase in abundance in the PHx liver (Figure 3). These findings first indicate the active involvement of *in situ* EVs in liver regeneration.

Figure 3

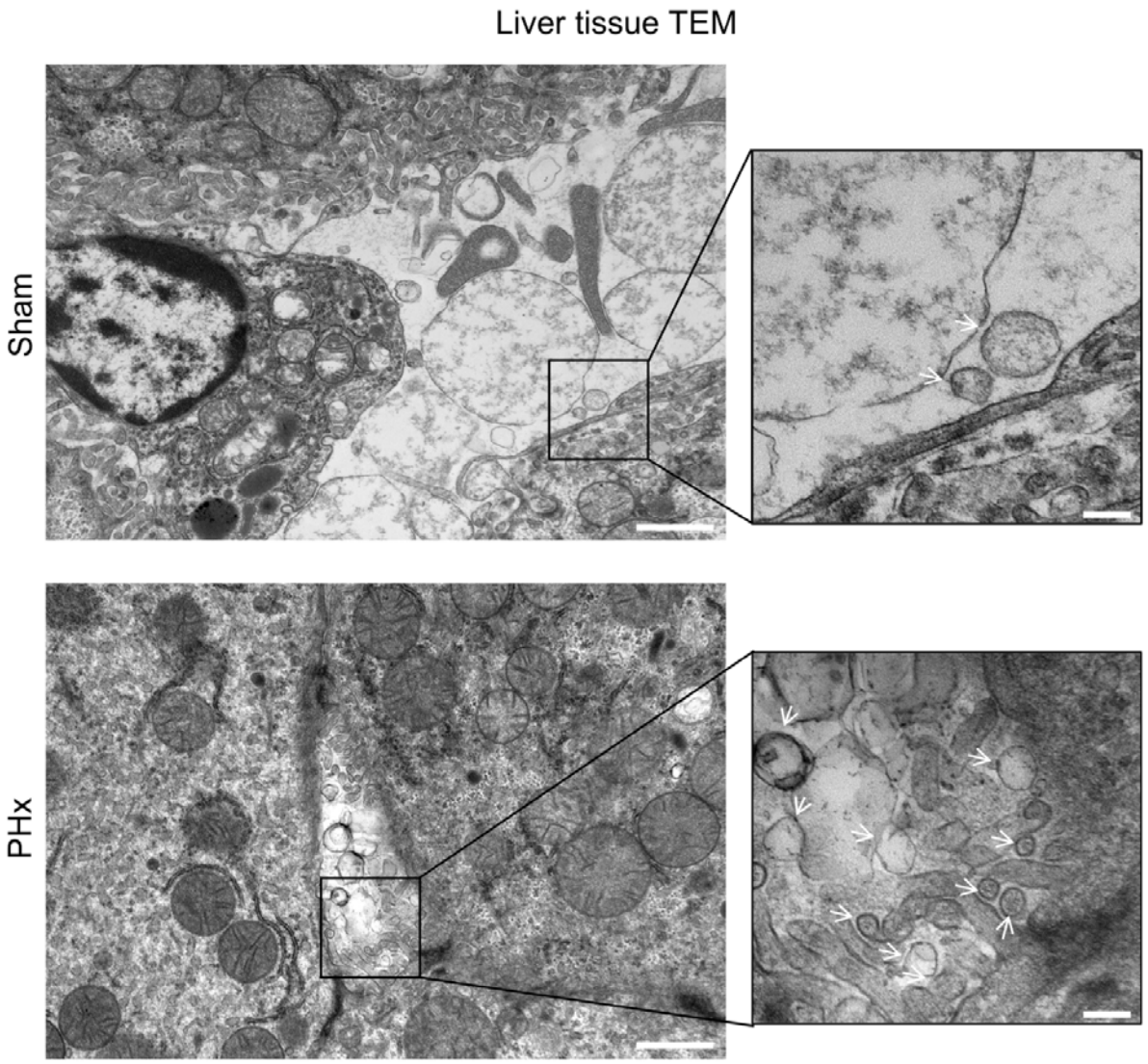

Figure 3. LT-EVs are emergingly present in the extracellular interstitial space adjacent to hepatocytes in the regenerating liver. Sham and PHx liver tissues were prepared and analyzed by TEM with showing representative images. Hepatocytes were identified with featured morphology. White arrows indicating EVs in the extracellular interstitial space. Bars: 1 µm (left) and 200 nm (right).

2.4 Protocol establishment for collecting and characterizing hepatocyte-specific LT-EVs

The above results inspired us to specifically investigate hepatocyte-derived EVs in liver regeneration. To fulfill this aim, we established a protocol for collecting and characterizing antigen-specific Ti-EVs by integrating our previous methods of immunomagneto-activated sorting of the marked EV population from cultured cells and isolation of traceable EVs from the tissue (Figure 4) [22, 25]. Particularly, ASGPR is primarily expressed on the surface of hepatocytes [26], which was utilized for sorting and collecting hepatocyte-specific LT-EVs (Figure 4). Briefly, liver tissues were disassociated, digested with Liberase, and subjected to differential centrifugation to pellet LT-EVs (Figure 4, left). LT-EVs were then incubated for the PE-conjugated ASGPR antibody followed by magnetic microbead incubation, which were subsequently sorted *via* the LD columns and collected by centrifugation (Figure 4, right). The hepatocyte-specific LT-EVs were accordingly denoted $ASGPR^+$ magnetic microbead-sorted LT-EVs (mLT-EVs), which can be characterized by TEM for the morphology, nanoparticle tracking analysis (NTA) for the size distribution and number, flow cytometric and Western blot assays for the surface and cargo profiles, and the mass spectrometry for multi-omic analyses (Figure 4, bottom). As expected, flow cytometric experiment data confirmed that the $ASGPR^+$ percentage of LT-EVs was 33.79% before magnetic sorting, while the percentage decreased to 0.10% in $ASGPR^-$LT-EVs and increased to 98.34% in $ASGPR^+$mLT-EVs, indicating the successful application of this approach (Figure 5A). The negative controls and gating strategy were additionally shown (Figure S2A and B). Together, we suggest that this approach will help assess the properties of hepatocyte-specific tissue EVs to enhance our understanding of liver regeneration.

Figure 4

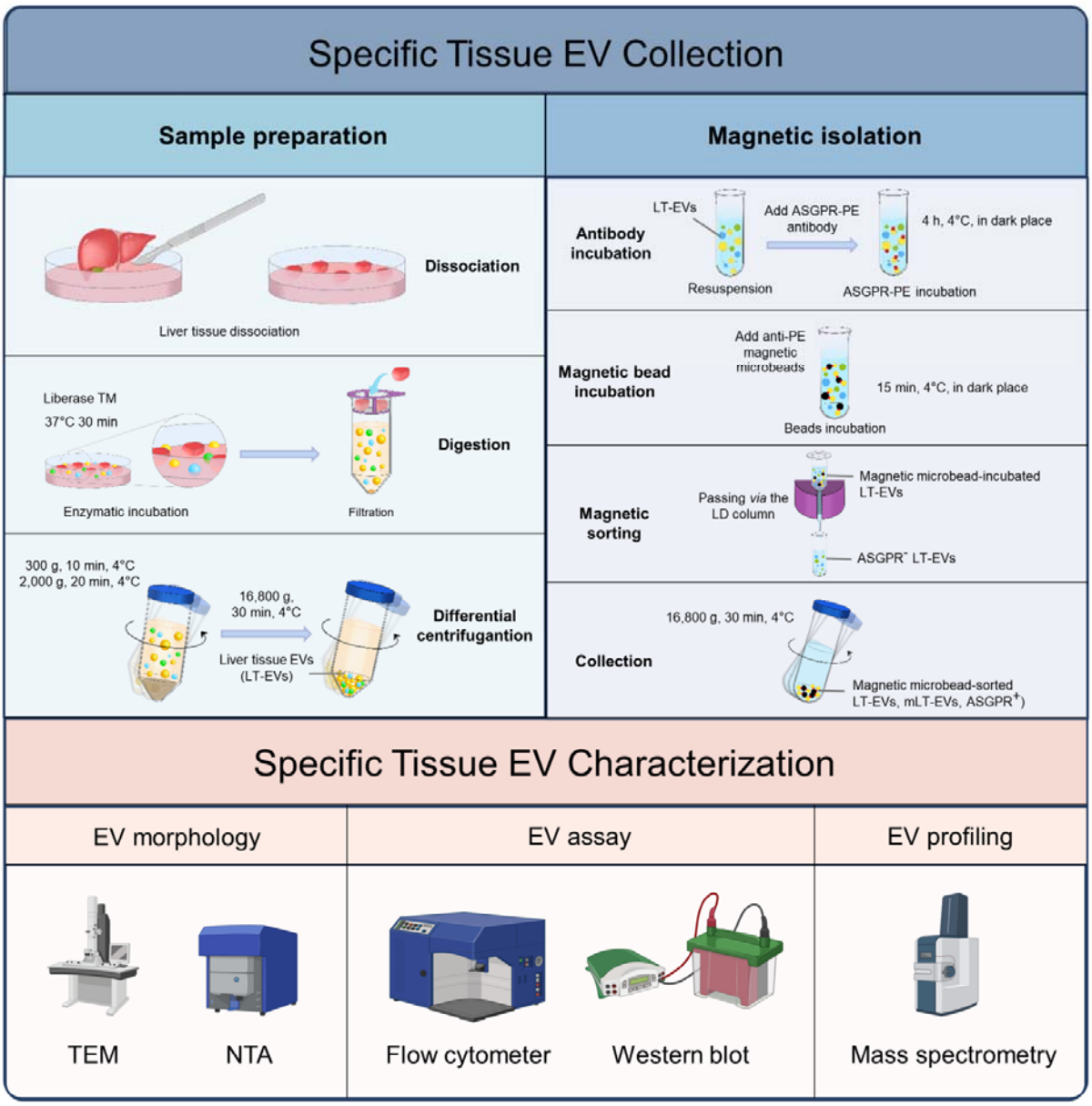

Figure 4. Protocol establishment for collecting and characterizing hepatocyte-specific LT-EVs. Schematic overview of the protocol employing enzymatic digestion, gradient centrifugation, immunomagnetic separation, and multiscale analysis for the isolation and characterization of hepatocyte-specific EVs (hereby denoted ASGPR^{+}mLT-EVs) from liver tissues.

2.5 Hepatocytes strengthen EV release in liver regeneration

After validation of the efficacy of immunomagnetic isolation, we adopted this approach to investigate LT-EVs and mLT-EVs derived from Sham and PHx mice, with the quality of isolated EVs evaluated according to the Minimal information for studies of extracellular vesicles 2018 (MISEV2018) [27]. TEM observation showed that LT-EVs exhibited typical cup-shape morphology with membranous structure in Sham and PHx (Figure 5B). Moreover, both LT-EVs and mLT-EVs from Sham and PHx mice expressed representative EV markers, such as the tetraspanin surface molecule CD63, the endosomal sorting complex required for transport (ESCRT)-associated protein tumor suppressor gene 101 (TSG101), and the lipid raft protein Flotillin-1 (Figure 5C). Meanwhile, the Golgi component protein Golgin84, a negative EV marker, was not detected in either LT-EVs or mLT-EVs (Figure 5C). Further measurement by NTA demonstrated that the diameters of LT-EVs from both Sham and PHx mice ranged from 50 to 500 nm and peaked at 150-200 nm (Figure 5D). Notably, compared to Sham mice, PHx mice produced significantly more amount of LT-EVs, as quantified by calculating the particle number by NTA (Fig. 5E) and the protein content by BCA assay (Figure 5F). After immunomagnetic sorting, ASGPR^{+}mLT-EVs from both Sham and PHx mice still showed characteristic particle distribution in NTA (Figure 5G), and PHx ASGPR^{+}mLT-EVs demonstrated higher yield than Sham ASGPR^{+}mLT-EVs (Figure 5H and I). Together, our results suggest that hepatocytes strengthen EV release in liver regeneration.

Figure 5

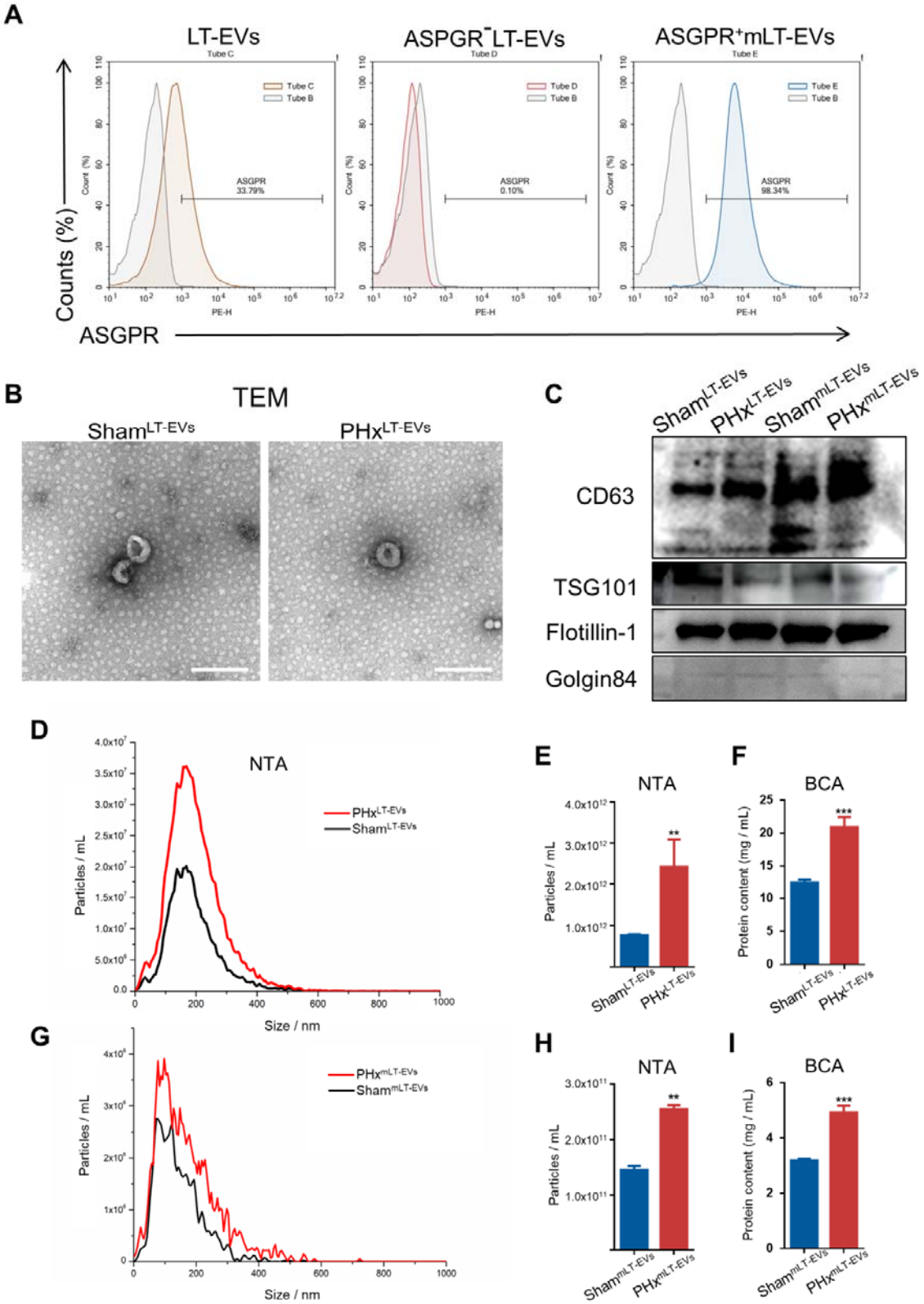

Figure 5. Hepatocytes strengthen EV release in liver regeneration. (A) Flow cytometric

analysis showing ASGPR surface expression on LT-EVs before and after immunomagnetic sorting. **(B)** Representative TEM image of collected LT-EVs from Sham and PHx livers. Bars, 250 nm. **(C)** Western blot analysis of protein marker expression of collected LT-EVs and ASGPR$^+$mLT-EVs from Sham and PHx livers. **(D and E)** Size distribution and particle number quantification of LT-EVs determined by NTA. **(F)** Quantification of protein amount of LT-EVs. **(G and H)** Size distribution and particle number quantification of ASGPR$^+$mLT-EVs determined by NTA. **(I)** Quantification of protein amount of ASGPR$^+$mLT-EVs. n = 3 per group. **, $p < 0.01$; ***, $p < 0.001$; two-tailed Student's unpaired *t* tests.

2.6 Proteomic analysis identifies liver regeneration-associated cargo profiles of hepatocyte-specific LT-EVs

Next, we investigated the protein cargo profiles of hepatocyte-specific LT-EVs from Sham and PHx by performing the liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis (Table S1). A total of over 3000 proteins were identified (Table S2). The Venn diagram illustrated the presence of 3357 shared proteins between Sham and PHx mLT-EVs, with respective 56 and 129 distinct proteins in each group (Figure 6A). Among the shared proteins, 387 proteins were identified as the differentially expressed proteins (DEPs; fold change > 1.5 and p value < 0.05), and 193 DEPs were upregulated in PHx ASGPR$^+$mLT-EVs with 184 DEPs downregulated (Figure 6B). Heatmap also showed clear separation and distinct protein signatures between Sham and PHx ASGPR$^+$mLT-EVs (Figure 6C). The top 30 DEPs of PHx over Sham ASGPR$^+$mLT-EVs was exhibited in the radar diagram (Figure 6D). With respect to their subcellular localization, most of the DEPs

existed in the cytoplasm region (32.1%) or located on the plasma membrane (20.42%), indicating transferred cargoes or potential surface markers (Figure 6E). GO analysis of DEPs further demonstrated that annotation related to metabolic and biosynthetic processes, as well as binding and transporter activities, were particular regulated terms in hepatocyte EVs during liver regeneration (Figure 6F). With regard to the functionality of DEPs, based on the KEGG database categories, DEPs were notably associated with “Transport and catabolism” and “Cell growth and death” in the “Cellular Processes” domain, as well as the “Environmental Information Processing” and the “Metabolism” domains (Figure 6G). Together, these findings identify liver regeneration-associated cargo profiles of hepatocyte-specific LT-EVs.

Figure 6

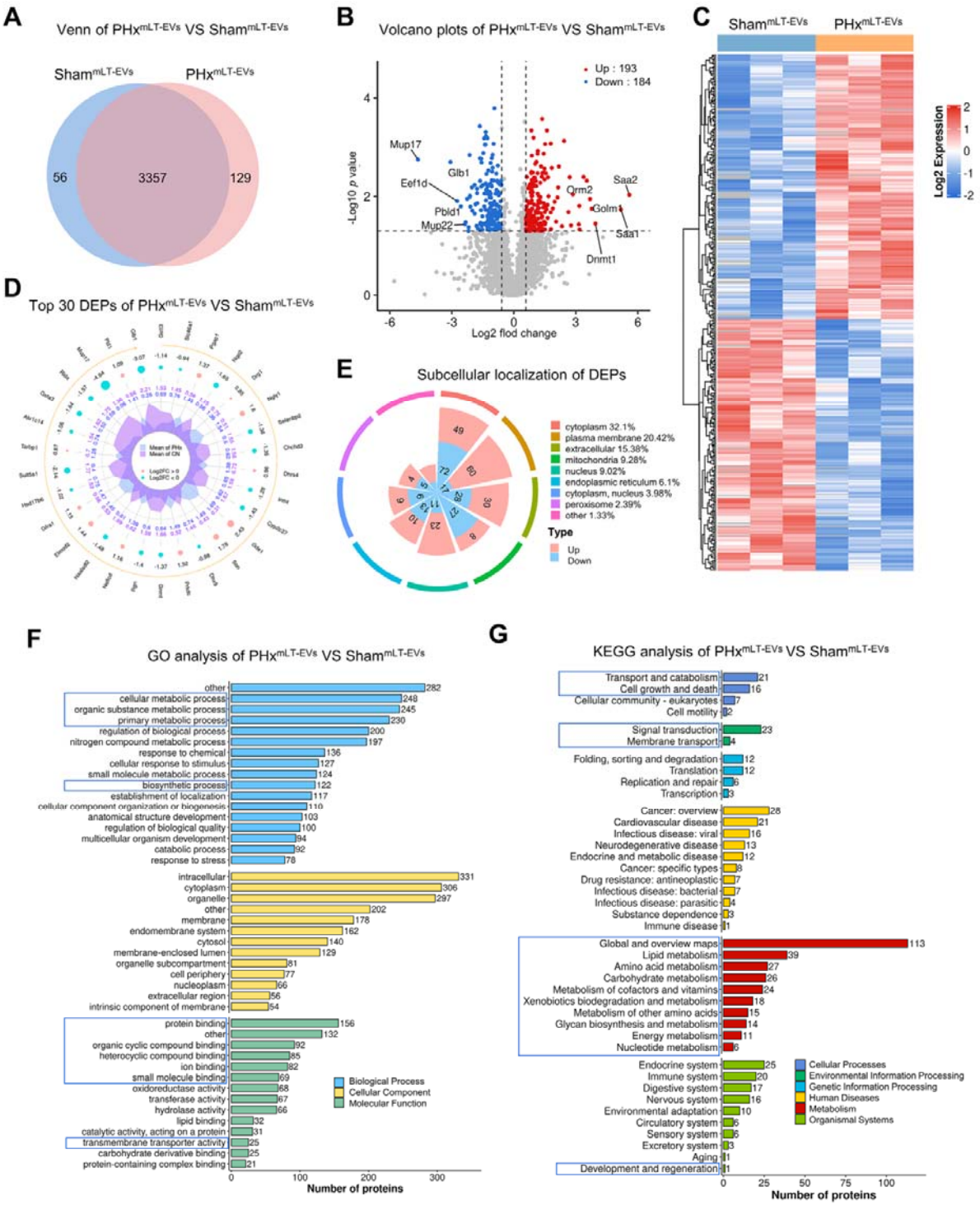

Figure 6. Proteomic analysis identifies liver regeneration-associated cargo profiles of hepatocyte-specific LT-EVs. (A) Venn analysis of mLT-EVs from PHx and Sham livers. **(B)** Volcano plot of DEPs between PHx and Sham mLT-EVs. **(C)** Hierarchical clustering of

DEPs between PHx and Sham mLT-EVs. **(D)** Radar diagram of DEPs between PHx and Sham mLT-EVs. **(E)** Distribution of DEPs in subcellular location categories retrieved from UniProt database according to GO annotation. **(F)** GO analysis of DEPs between PHx and Sham mLT-EVs. **(G)** KEGG analysis of DEPs between PHx and Sham mLT-EVs. n = 3 per group.

2.7 EVs diversify hepatocyte-derived information in liver regeneration

Next, we kept focusing on these DEPs and performed detailed enrichment analysis. GO enrichment analysis revealed that DEPs of PHx over Sham ASGPR^{+}mLT-EVs were particularly related to metabolic processes, which, however, were largely downregulated (Figure 7A). To further elucidate the functional disparities of DEPs, we classified the DEPs into four segments: Q1 (upregulated more than two folds), Q2 (upregulated less than two folds), Q3 (downregulated less than two folds), and Q4 (downregulated more than two folds). Each segment was then subjected to GO enrichment analysis, followed by functional cluster analysis. Notably, the “Biological Process” enrichment-based clustering analysis revealed that PHx mLT-EVs exhibited remarkable upregulated DEPs in the Q1 segment related to hepatocyte proliferation, including “telomere maintenance via recombination” and “mitotic recombination” (Figure 7B). On the contrary, downregulated DEPs in the Q4 segment were related to the metabolic processes, such as “gluconeogenesis” (Figure 7B). The “Cellular Component” enrichment-based clustering analysis also showed proliferation-related DEP upregulation in the Q1 segment, such as “chromosomes”, with organelle-related terms, including “endoplasmic reticulum” (Figure 7C). The

enrichment-based clustering analysis in “Molecular Function” subsequently identified specific upregulated DEPs in the Q1 segment linked to “epidermal growth factor receptor binding”, while demonstrating a significant DEP downregulation in “antioxidant activity” (Figure 7D). KEGG enrichment analysis of DEPs with functional clustering confirmed the trend of metabolic repression in PHx ASGPR^{+}mLT-EVs (Figure 7E and F). In the Q1 segment of KEGG enrichment-based DEP clustering analysis, regenerative proliferation-related terms were further notable, such as “DNA replication” and “Cell cycle” (Figure 7F). The correlations among the DEPs were discovered to be substantial (Figure S3A).

We intended to take a final step to dissect the potential relationship between proteomic alterations of hepatocyte-specific LT-EVs with transcriptomic changes of their parental cells. By integrating the scRNA-seq data and the proteomic data, we performed a nine-quadrant analysis to distinguish cellular and vesicular expression patterns under liver regeneration. The graph depicted the same expression patterns in particular interested quadrants of P3 (upregulation in both cases) and P7 (downregulation in both cases), meanwhile displaying different expression patterns in other quadrants, such as P6 (upregulation only in proteomic analysis) and P9 (upregulation in proteins despite downregulation in mRNAs) (Figure 7G). For P3, GO enrichment analysis revealed association of terms with cellular activity, indicating that EVs inherited active regulation from parental hepatocytes in liver regeneration (Figure 7H). It is especially notable that the term “epidermal growth factor receptor binding” term was enriched in P3, suggesting proliferation-related functionality (Figure 7H). Intriguingly, for P6 plus P9, where the mLT-EV proteins were uniquely

upregulated irrespective of the cellular transcriptome, plenty of organelle-related terms was enriched (Figure 7I). Expectedly, metabolism-related terms were enriched in P7, suggesting consistent downregulation in hepatocytes and their EVs during liver regeneration (Figure S3B). To verify the bioinformatic results, we additionally performed Western blot analysis on several organelle and cell cycle markers in mLT-EVs. Accumulation of Calnexin, an endoplasmic reticulum (ER) protein, and the presence of Cyclin-dependent kinase 1 (Cdk1), a cell cycle regulator, was detected in ASGPR^{+}mLT-EVs from the PHx mice (Figure S3C). Taken together, these results suggest that EVs diversify hepatocyte-derived information in liver regeneration.

Figure 7

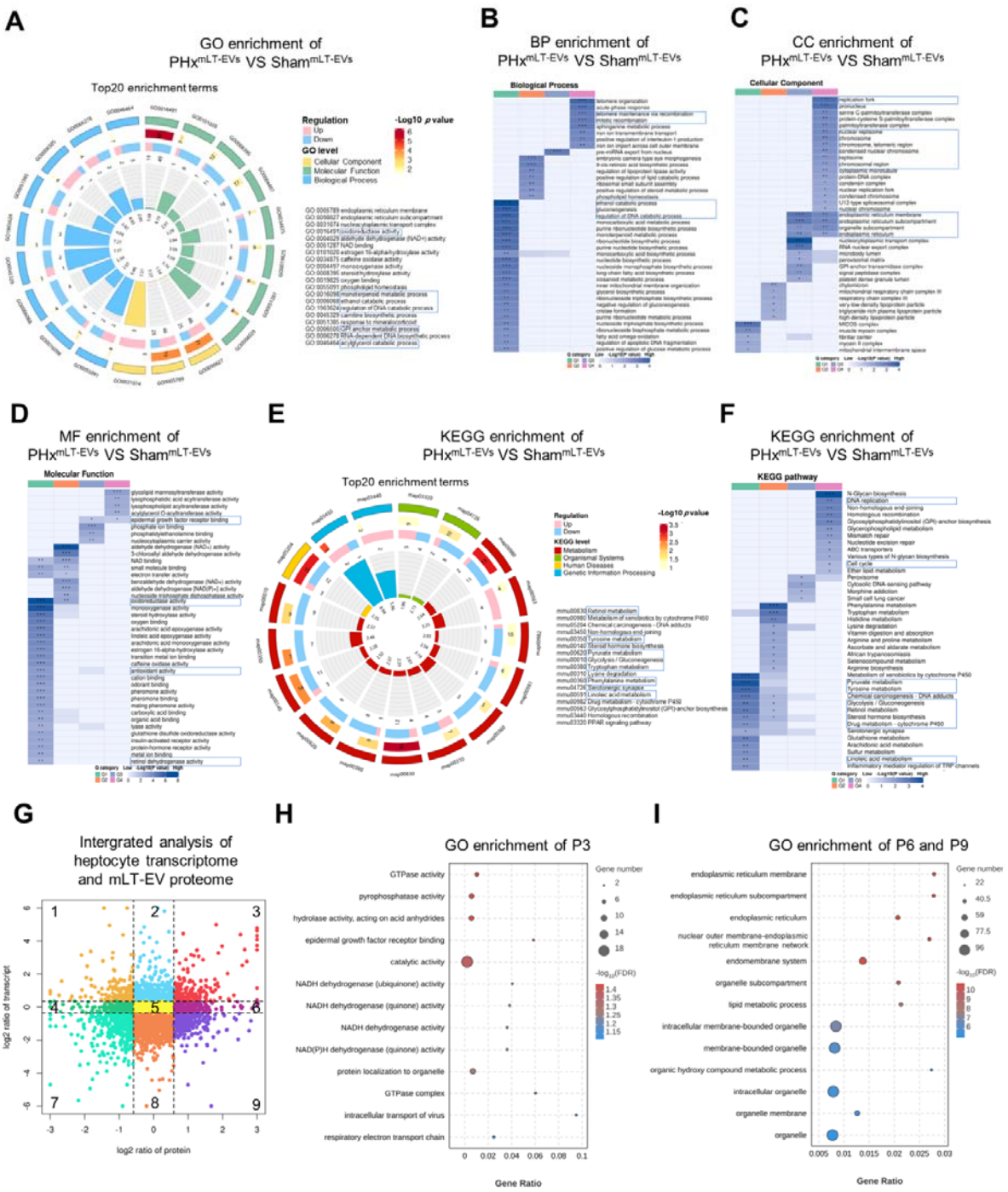

Figure 7. EVs diversify hepatocyte-derived information in liver regeneration. (A) GO enrichment analysis of DEPs between PHx and Sham mLT-EVs shown by the circle diagram. **(B-D)** GO enrichment analysis of DEPs between PHx and Sham mLT-EVs shown by functional cluster analysis. Q categories: Q1, upregulated more than two folds; Q2.

upregulated less than two folds; Q3, downregulated less than two folds; Q4, downregulated more than two folds. **(E)** KEGG enrichment analysis of DEPs between PHx and Sham mLT-EVs shown by the circle diagram. **(F)** KEGG enrichment analysis of DEPs between PHx and Sham mLT-EVs shown by functional cluster analysis. Q categories: Q1, upregulated more than two folds; Q2. upregulated less than two folds; Q3, downregulated less than two folds; Q4, downregulated more than two folds. **(G)** Nine-quadrant diagram showing integrated analysis of hepatocyte transcriptome from scRNA-seq and mLT-EV proteome. **(H)** GO enrichment analysis of the P3 quadrant showing consistent upregulation between the hepatocyte genes and the mLT-EV proteins. **(I)** GO enrichment analysis of the P6 plus P9 quadrants showing upregulation of the mLT-EV proteins despite unchange or downregulation of hepatocyte genes.

3. Discussion

The liver is responsible for maintaining metabolism and detoxification of the body and is highly regenerative [1, 2]. The process of liver regeneration involves complex intercellular communication between parenchymal cells and NPCs [3]. However, the mechanism of this elaborate functional interaction has not been thoroughly understood. EVs are biologically active paracrine messengers which act dynamically in various pathophysiological states [12, 13]. In the present study, we mapped the transcriptomic landscape of liver regeneration at the single cell level, which highlighted the crucial role of hepatocyte-centered intercellular communication based on EVs. Our investigation further uncovered that liver regeneration promoted the release of LT-EVs, particularly hepatocyte-derived $ASGPR^{+}$mLT-EVs, which

can be specifically collected from the liver tissue following a multi-step protocol involving enzymatic treatment, immunomagnetic separation, and differential centrifugation. Notably, ASGPR^{+}mLT-EVs exhibited diversified proteomic cargoes with their parental hepatocytes, which not only inherited regulated regenerative and metabolic signals at the cellular level but also showed vesicular enrichment of organelle components. For the first time, this study provides an integrated characterization of the hepatocyte transcriptome in related to the EV proteome in liver regeneration, offering insights into the biological and mechanistic aspects of the regenerating liver.

The liver possesses the remarkable ability to fully regenerate after undergoing partial resection [2]. Studies have documented that liver regeneration involves a combination of subsequent hepatocyte division, hypertrophy, and proliferation [8, 24]. In our scRNA-seq, data further suggested significant influences of liver regeneration on hepatocyte metabolic processes, which largely suppressed in the regenerating liver. Indeed, hepatic metabolic reprogramming after PHx represent an important event, and the transcriptome sequencing combined with metabolomic analysis has indicated detailed metabolic changes during liver regeneration [28, 29]. Our findings, together with others' findings and despite preliminary, suggest that hepatocytes might sacrifice metabolic activity for safeguarding regenerative expansion, and that hepatocytes are equipped with flexible metabolic machinery able to adapt dynamically to tissue regeneration [29]. It is also noteworthy that scRNA-seq analysis revealed a substantial involvement of the immune system in liver regeneration, which is in accordance with the current understanding [3, 10]. Our data further demonstrated that the proportions of certain immune cell types, such as B cells and neutrophils, were increased

after PHx, and that the communication between hepatocytes and various immune cells potentially contributed to liver regeneration. Future research is needed to examine in-depth the reciprocal regulation mechanisms between hepatocytes and detailed immune cells that safeguard liver regeneration.

Researchers worldwide have recently dedicated significant efforts to investigating EVs. These membranous nanoparticles are discovered to escape from the autophagy-lysosomal pathway and be release into the circulation and the extracellular space of tissues [30, 31]. Particularly, studies have shown the potential of Ti-EVs in promoting tissue regeneration, and LT-EVs have been employed to expedite the hepatic disease recovery process [15, 16]. However, the specific cellular origin of Ti-EVs responsible for the action remains unknown. In this study, we established an immunomagnetic microbead assay to specifically isolate LT-EVs derived from hepatocytes in the regenerating liver. As assessed by TEM and NTA, the isolation procedure did not exert significant impact on the LT-EV morphology and size distribution, suggesting that the properties of these Ti-EVs were largely maintained. Indeed, with advantages of potent specificity and efficiency, the immunomagnetic separation assay based on immunomagnetic particles has been acknowledged as a powerful system for the enrichment of target marked cells [32, 33]. Utilization of this approach in the EV research will benefit the in-depth biological and mechanistic investigations of endogenous Ti-EVs, as well as t diagnostic and therapeutic applications. In addition, this approach can be applied to gain profound insights into crucial subpopulations of EVs not only derived from specific cell sources but also possessing featured membrane proteins. Future examinations should elucidate the distinct functionalities of LT-EVs from diverse cellular sources in liver health

and disease. Moreover, the communications between hepatocytes and other liver cell types potentially mediated by ASGPR$^+$ LT-EVs and functionally regulating liver regeneration will require experimental dissection.

The pivotal role EVs play in regulating liver health and disease has been suggested, but the function of endogenous LT-EVs remains not fully understood [34]. It is worth noting that our integrated analysis of hepatocyte transcriptome and ASGPR$^+$mLT-EV proteome indicated two aspects of findings: on one hand, ASGPR$^+$mLT-EVs inherited proliferative messages and reduced metabolic cues from donor hepatocytes in liver regeneration; on the other hand, ASGPR$^+$mLT-EVs were particularly enriched for organelle components with no significant changes of hepatocyte transcription. For the former aspect, epidermal growth factor receptor (EGFR) is well known as a crucial regulator safeguarding liver regeneration, and multiple biological enzymes in the mitochondrial respiratory electron transport chain contribute to the cell cycle and proliferative regulation of hepatocytes [35, 36]. For the latter aspect, interestingly, ER represents the major site of protein synthesis in the cell and form the endosomal secretory pathway with Golgi apparatus [37]. Moreover, emerging studies have documented that the EV biogenesis and secretion can be dependent on ER, and that ER stress can regulate the release of EVs [38-40]. Additionally, ER proteostasis is essential to adapt to cell proliferation and regeneration in development [41]. It has recently been reported that the EV cargoes are not randomly but specifically selected [42]. Therefore, future research is needed to further investigate the function of ER in regulating hepatic regenerative EVs and orchestrating liver regeneration through EV-mediated intercellular communication.

In summary, our findings provide the first knowledge that hepatocyte-specific tissue vesicles are indeed phenotypically and functionally involved in liver regeneration. Our study paves an avenue for in-depth biological and mechanistic research of regenerative LT-EVs and sheds light on extensive dissection of antigen-marked, physiological, and endogenous tissue EV populations.

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Author contributions

S.Q.Y., Y.C., L.L., and Z.K.Z. contributed equally to the design and conduction of the study and drafted the manuscript. X.Y.L. analyzed and interpreted data. C.H.L. performed partial hepatectomy. S.Y.C. contributed to the scRNA-seq experiment. X.L. contributed to the proteome experiment. S.J.X. contributed to H&E and IF experiments. K.S. contributed to TEM and flow cytometry analysis. J.Y.Q. and Y.Y.L. contributed to NTA and WB experiments. C.X.Z. and F.J. contributed to the discussion and interpretation of data. Y.J. and B.D.S. conceived the project, designed and supervised the experiments. All authors revised the manuscript and approved the final version.

Competing interests

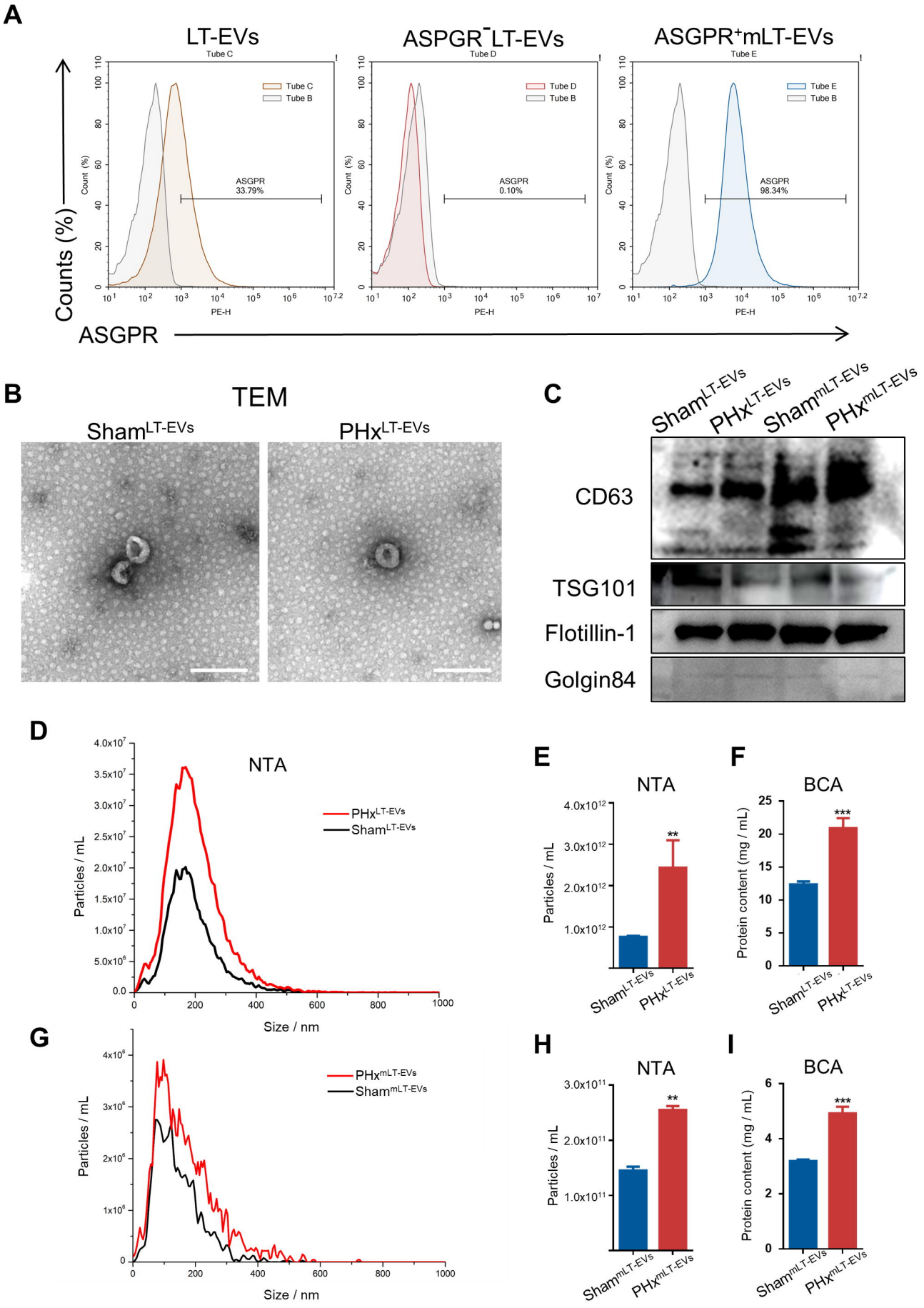
Authors declare that they have no competing interests.

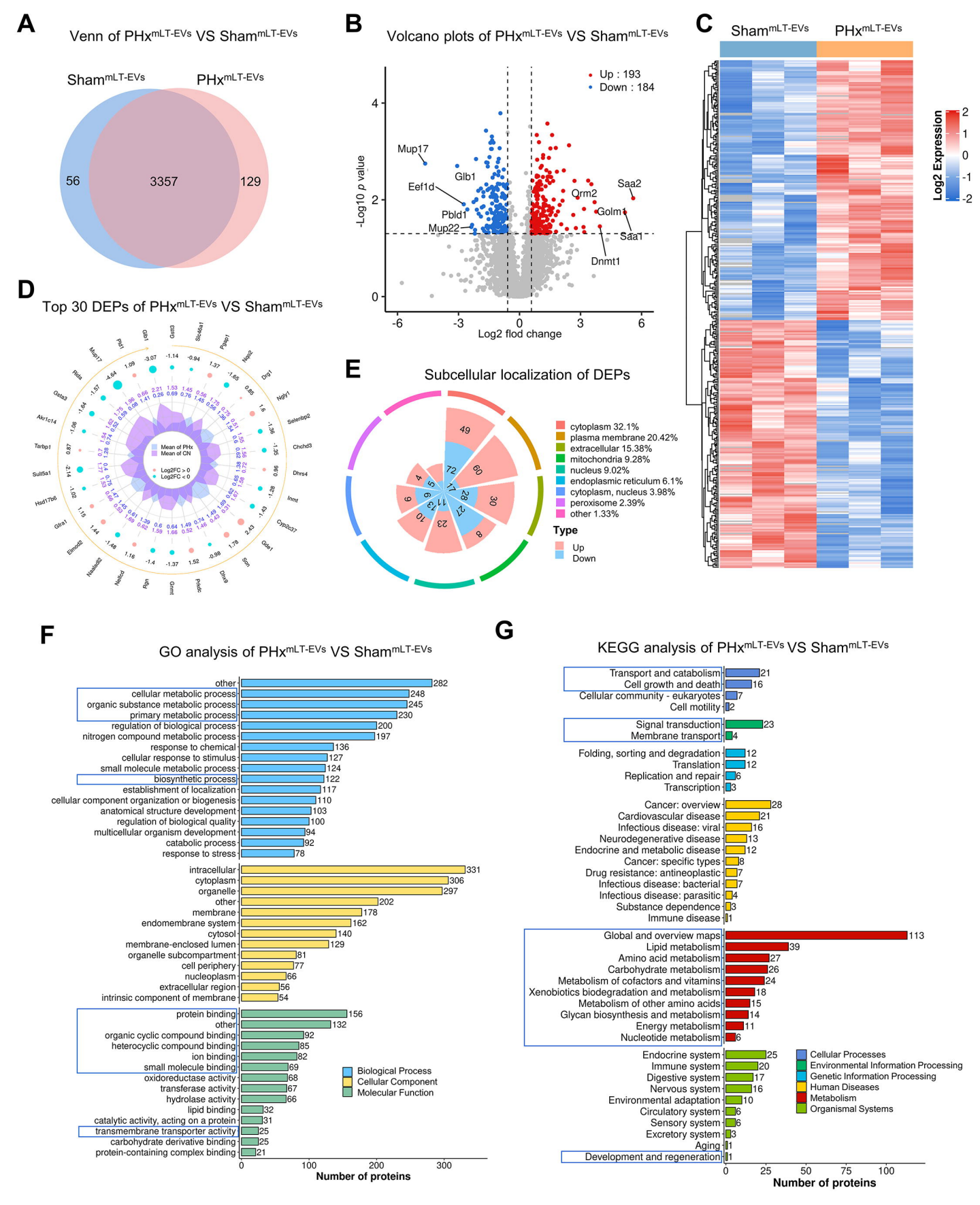
Data and materials availability

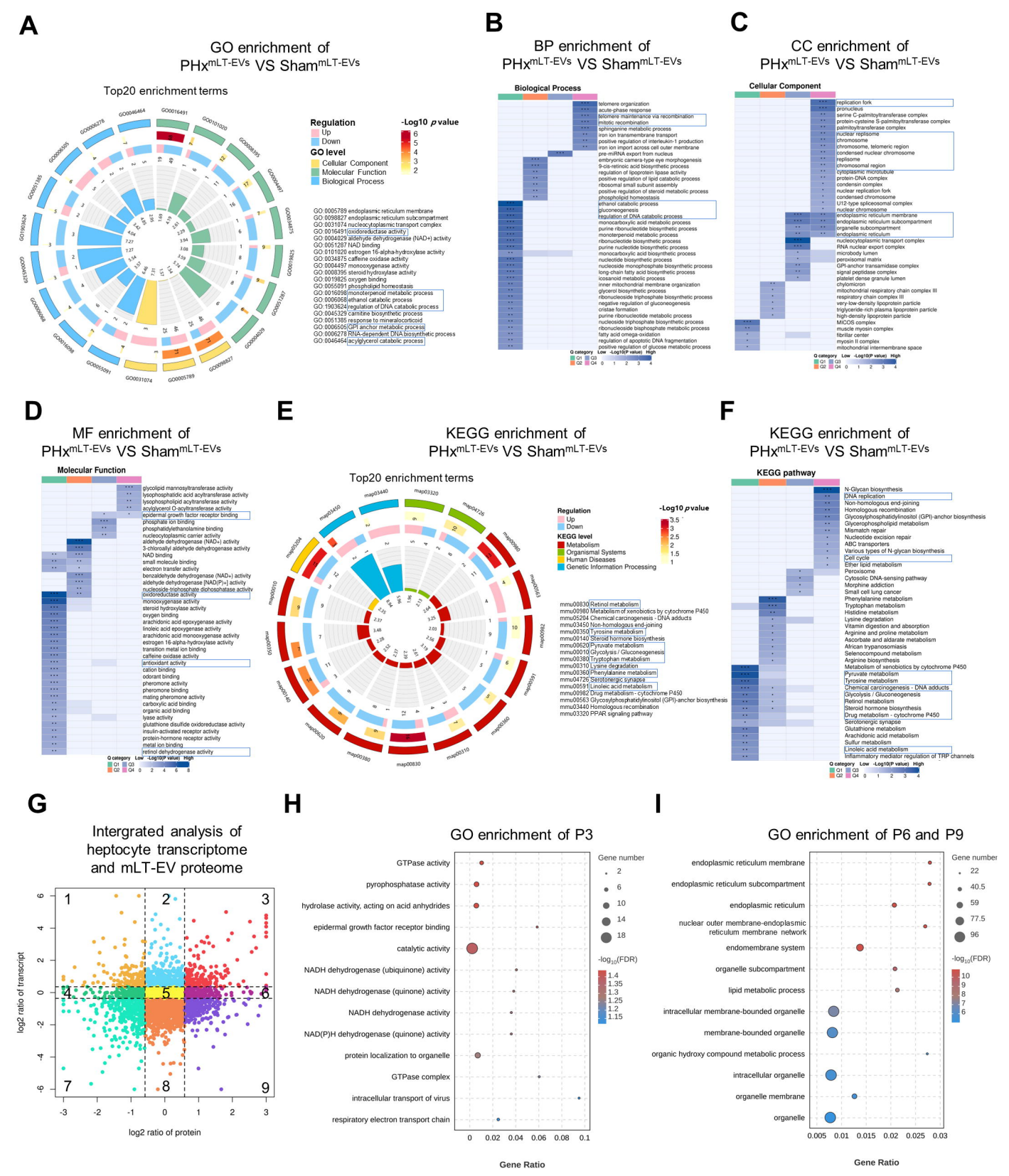
All data included in this study are available upon reasonable request of corresponding authors.

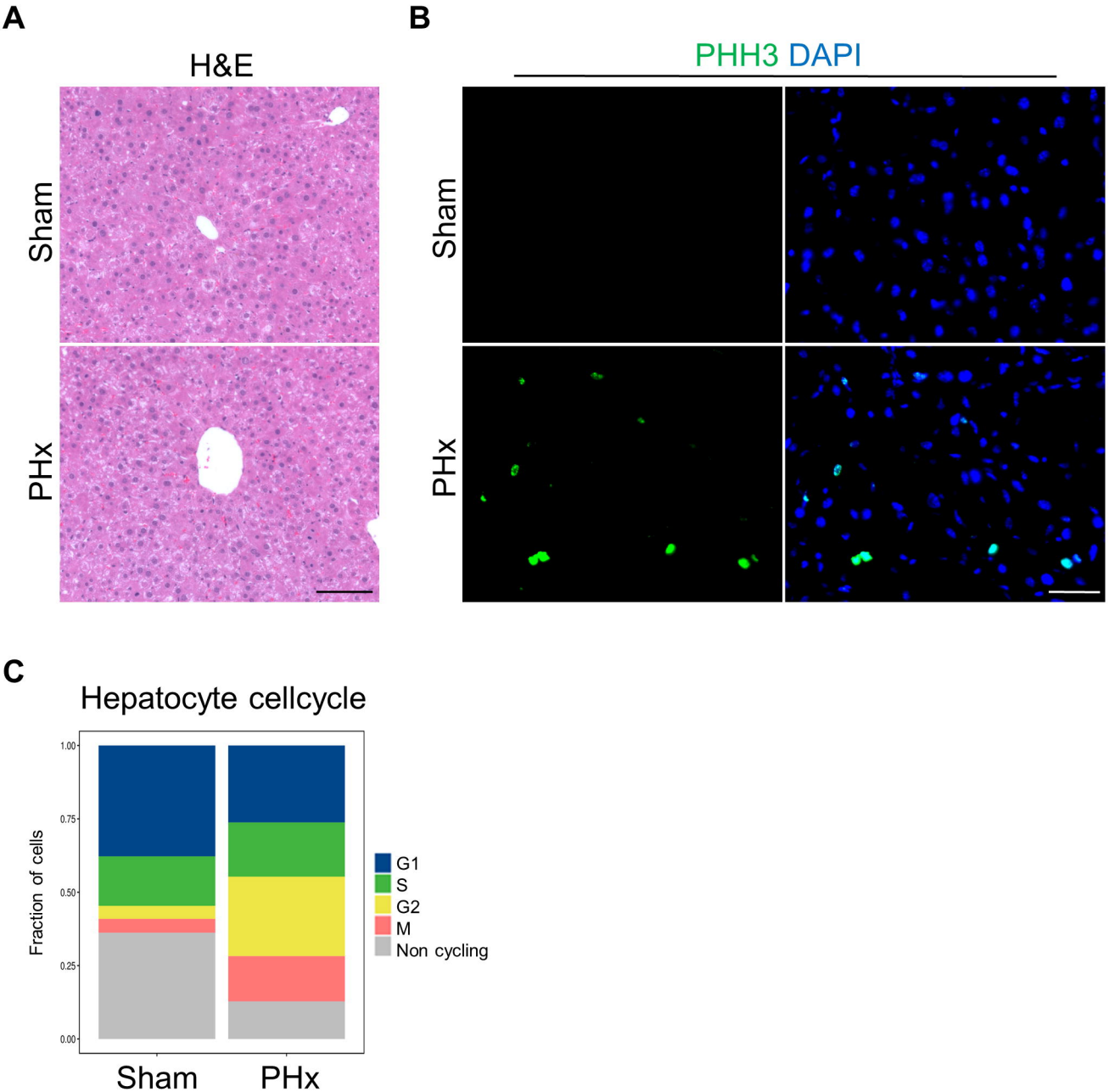
Abbreviations

ASGPR, asialoglycoprotein receptor; BECs, biliary epithelial cells; BSA, bovine serum albumin; CCl_4, carbon tetrachloride; Cdk1, cyclin-dependent kinase 1; DEGs, differentially expressed genes; DEPs, differentially expressed proteins; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; EVs, extracellular vesicles; GEO, Gene Expression Omnibus; GO, Gene Ontology; HRP, horseradish peroxidase; IF, immunofluorescence; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, Liquid chromatography with tandem mass spectrometry; LT-EVs, liver tissue extracellular vesicles; mLT-EVs, magnetic microbead-sorted liver tissue extracellular vesicles; NPCs, non-parenchymal cells; NTA, nanoparticle tracking analysis; PFA, paraformaldehyde; PHx, partial hepatectomy; PVDF, polyvinylidene difluoride; SD, standard deviation; TEM, transmission electron microscopy; Ti-EVs, tissue-derived EVs; TSG101, tumor suppressor gene 101; tSNE, t-distributed stochastic neighbor embedding.


A
LT-EVs
ASPGR⁻LT-EVs
ASGPR⁺mLT-EVs
Tube C
Tube B
Tube D
Tube E
ASGPR 33.79%
ASGPR 0.10%
ASGPR 98.34%
Count (%)
PE-H
Counts (%)
ASGPR
B
TEM
ShamLT-EVs
PHxLT-EVs
C
ShamLT-EVs
PHxLT-EVs
ShammLT-EVs
PHxmLT-EVs
CD63
TSG101
Flotillin-1
Golgin84
D
NTA
PHxLT-EVs
ShamLT-EVs
Particles / mL
Size / nm
E
NTA
Particles / mL
F
BCA
Protein content (mg / mL)
G
PHxmLT-EVs
ShammLT-EVs
H
NTA
I
BCA

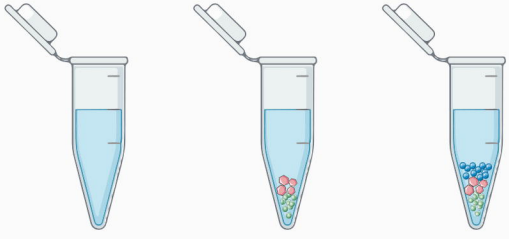

A
Venn of PHx^mLT-EVs VS Sham^mLT-EVs
Sham^mLT-EVs
PHx^mLT-EVs
56
3357
129
B
Volcano plots of PHx^mLT-EVs VS Sham^mLT-EVs
Up : 193
Down : 184
-Log10 p value
Log2 flod change
Mup17
Glb1
Eef1d
Pbld1
Mup22
Qrm2
Saa2
Golm1
Saa1
Dnmt1
C
Sham^mLT-EVs
PHx^mLT-EVs
Log2 Expression
D
Top 30 DEPs of PHx^mLT-EVs VS Sham^mLT-EVs
Mean of PHx
Mean of CN
Log2FC > 0
Log2FC < 0
E
Subcellular localization of DEPs
cytoplasm 32.1%
plasma membrane 20.42%
extracellular 15.38%
mitochondria 9.28%
nucleus 9.02%
endoplasmic reticulum 6.1%
cytoplasm, nucleus 3.98%
peroxisome 2.39%
other 1.33%
Type
Up
Down
F
GO analysis of PHx^mLT-EVs VS Sham^mLT-EVs
other 282
cellular metabolic process 248
organic substance metabolic process 245
primary metabolic process 230
regulation of biological process 200
nitrogen compound metabolic process 197
response to chemical 136
cellular response to stimulus 127
small molecule metabolic process 124
biosynthetic process 122
establishment of localization 117
cellular component organization or biogenesis 110
anatomical structure development 103
regulation of biological quality 100
multicellular organism development 94
catabolic process 92
response to stress 78
intracellular 331
cytoplasm 306
organelle 297
other 202
membrane 178
endomembrane system 162
cytosol 140
membrane-enclosed lumen 129
organelle subcompartment 81
cell periphery 77
nucleoplasm 66
extracellular region 56
intrinsic component of membrane 54
protein binding 156
other 132
organic cyclic compound binding 92
heterocyclic compound binding 85
ion binding 82
small molecule binding 69
oxidoreductase activity 68
transferase activity 67
hydrolase activity 66
lipid binding 32
catalytic activity, acting on a protein 31
transmembrane transporter activity 25
carbohydrate derivative binding 25
protein-containing complex binding 21
Biological Process
Cellular Component
Molecular Function
Number of proteins
G
KEGG analysis of PHx^mLT-EVs VS Sham^mLT-EVs
Transport and catabolism 21
Cell growth and death 16
Cellular community - eukaryotes 7
Cell motility 2
Signal transduction 23
Membrane transport 4
Folding, sorting and degradation 12
Translation 12
Replication and repair 6
Transcription 3
Cancer: overview 28
Cardiovascular disease 21
Infectious disease: viral 16
Neurodegenerative disease 13
Endocrine and metabolic disease 12
Cancer: specific types 8
Drug resistance: antineoplastic 7
Infectious disease: bacterial 7
Infectious disease: parasitic 4
Substance dependence 3
Immune disease 1
Global and overview maps 113
Lipid metabolism 39
Amino acid metabolism 27
Carbohydrate metabolism 26
Metabolism of cofactors and vitamins 24
Xenobiotics biodegradation and metabolism 18
Metabolism of other amino acids 15
Glycan biosynthesis and metabolism 14
Energy metabolism 11
Nucleotide metabolism 6
Endocrine system 25
Immune system 20
Digestive system 17
Nervous system 16
Environmental adaptation 10
Circulatory system 6
Sensory system 6
Excretory system 3
Aging 1
Development and regeneration 1
Cellular Processes
Environmental Information Processing
Genetic Information Processing
Human Diseases
Metabolism
Organismal Systems
Number of proteins


A
GO enrichment of
PHx^mLT-EVs VS Sham^mLT-EVs
Top20 enrichment terms
Regulation
Up
Down
GO level
Cellular Component
Molecular Function
Biological Process
-Log10 p value
GO:0005789 endoplasmic reticulum membrane
GO:0098827 endoplasmic reticulum subcompartment
GO:0031074 nucleocytoplasmic transport complex
GO:0016491 oxidoreductase activity
GO:0004029 aldehyde dehydrogenase (NAD+) activity
GO:0051287 NAD binding
GO:0101020 estrogen 16-alpha-hydroxylase activity
GO:0034875 caffeine oxidase activity
GO:0004497 monooxygenase activity
GO:0008395 steroid hydroxylase activity
GO:0019825 oxygen binding
GO:0055091 phospholipid homeostasis
GO:0016098 monoterpenoid metabolic process
GO:0006068 ethanol catabolic process
GO:1903624 regulation of DNA catabolic process
GO:0045329 carnitine biosynthetic process
GO:0051385 response to mineralocorticoid
GO:0006505 GPI anchor metabolic process
GO:0006278 RNA-dependent DNA biosynthetic process
GO:0046464 acylglycerol catabolic process
B
BP enrichment of
PHx^mLT-EVs VS Sham^mLT-EVs
Biological Process
telomere organization
acute-phase response
telomere maintenance via recombination
mitotic recombination
sphingamine metabolic process
iron ion transmembrane transport
positive regulation of interleukin-1 production
iron ion import across cell outer membrane
pre-miRNA export from nucleus
embryonic camera-type eye morphogenesis
9-cis-retinoic acid biosynthetic process
regulation of lipoprotein lipase activity
positive regulation of lipid catabolic process
ribosomal small subunit assembly
positive regulation of steroid metabolic process
phospholipid homeostasis
ethanol catabolic process
gluconeogenesis
regulation of DNA catabolic process
monocarboxylic acid metabolic process
purine ribonucleotide biosynthetic process
monoterpenoid metabolic process
ribonucleotide biosynthetic process
purine nucleoside biosynthetic process
monocarboxylic acid biosynthetic process
nucleotide biosynthetic process
nucleoside monophosphate biosynthetic process
long-chain fatty acid biosynthetic process
icosanoid metabolic process
inner mitochondrial membrane organization
glycerol biosynthetic process
ribonucleoside triphosphate biosynthetic process
negative regulation of gluconeogenesis
cristae formation
purine ribonucleotide metabolic process
nucleoside triphosphate biosynthetic process
ribonucleoside bisphosphate metabolic process
fatty acid omega-oxidation
regulation of apoptotic DNA fragmentation
positive regulation of glucose metabolic process
Q category Low -Log10(P value) High
Q1 Q3
Q2 Q4 0 1 2 3 4
C
CC enrichment of
PHx^mLT-EVs VS Sham^mLT-EVs
Cellular Component
replication fork
pronucleus
serine C-palmitoyltransferase complex
protein-cysteine S-palmitoyltransferase complex
palmitoyltransferase complex
nuclear replisome
chromosome
chromosome, telomeric region
condensed nuclear chromosome
replisome
chromosomal region
cytoplasmic microtubule
protein-DNA complex
condensin complex
nuclear replication fork
condensed chromosome
U12-type spliceosomal complex
nuclear chromosome
endoplasmic reticulum membrane
endoplasmic reticulum subcompartment
organelle subcompartment
endoplasmic reticulum
nucleocytoplasmic transport complex
RNA nuclear export complex
microbody lumen
peroxisomal matrix
GPI-anchor transamidase complex
signal peptidase complex
platelet dense granule lumen
chylomicron
mitochondrial respiratory chain complex III
respiratory chain complex III
very-low-density lipoprotein particle
triglyceride-rich plasma lipoprotein particle
high-density lipoprotein particle
MICOS complex
muscle myosin complex
fibrillar center
myosin II complex
mitochondrial intermembrane space
Q category Low -Log10(P value) High
Q1 Q3
Q2 Q4 0 1 2 3 4
D
MF enrichment of
PHx^mLT-EVs VS Sham^mLT-EVs
Molecular Function
glycolipid mannosyltransferase activity
lysophosphatidic acid acyltransferase activity
lysophospholipid acyltransferase activity
acylglycerol O-acyltransferase activity
epidermal growth factor receptor binding
phosphate ion binding
phosphatidylethanolamine binding
nucleocytoplasmic carrier activity
aldehyde dehydrogenase (NAD+) activity
3-chloroallyl aldehyde dehydrogenase activity
NAD binding
small molecule binding
electron transfer activity
benzaldehyde dehydrogenase (NAD+) activity
aldehyde dehydrogenase [NAD(P)+] activity
nucleoside-triphosphate diphosphatase activity
oxidoreductase activity
monooxygenase activity
steroid hydroxylase activity
oxygen binding
arachidonic acid epoxygenase activity
linoleic acid epoxygenase activity
arachidonic acid monooxygenase activity
estrogen 16-alpha-hydroxylase activity
transition metal ion binding
caffeine oxidase activity
antioxidant activity
cation binding
odorant binding
pheromone activity
pheromone binding
mating pheromone activity
carboxylic acid binding
organic acid binding
lyase activity
glutathione disulfide oxidoreductase activity
insulin-activated receptor activity
protein-hormone receptor activity
metal ion binding
retinol dehydrogenase activity
Q category Low -Log10(P value) High
Q1 Q3
Q2 Q4 0 2 4 6 8
E
KEGG enrichment of
PHx^mLT-EVs VS Sham^mLT-EVs
Top20 enrichment terms
Regulation
Up
Down
KEGG level
Metabolism
Organismal Systems
Human Diseases
Genetic Information Processing
-Log10 p value
mmu00830 Retinol metabolism
mmu00980 Metabolism of xenobiotics by cytochrome P450
mmu05204 Chemical carcinogenesis - DNA adducts
mmu03450 Non-homologous end-joining
mmu00350 Tyrosine metabolism
mmu00140 Steroid hormone biosynthesis
mmu00620 Pyruvate metabolism
mmu00010 Glycolysis / Gluconeogenesis
mmu00380 Tryptophan metabolism
mmu00310 Lysine degradation
mmu00360 Phenylalanine metabolism
mmu04726 Serotonergic synapse
mmu00591 Linoleic acid metabolism
mmu00982 Drug metabolism - cytochrome P450
mmu00563 Glycosylphosphatidylinositol (GPI)-anchor biosynthesis
mmu03440 Homologous recombination
mmu03320 PPAR signaling pathway
F
KEGG enrichment of
PHx^mLT-EVs VS Sham^mLT-EVs
KEGG pathway
N-Glycan biosynthesis
DNA replication
Non-homologous end-joining
Homologous recombination
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis
Glycerophospholipid metabolism
Mismatch repair
Nucleotide excision repair
ABC transporters
Various types of N-glycan biosynthesis
Cell cycle
Ether lipid metabolism
Peroxisome
Cytosolic DNA-sensing pathway
Morphine addiction
Small cell lung cancer
Phenylalanine metabolism
Tryptophan metabolism
Histidine metabolism
Lysine degradation
Vitamin digestion and absorption
Arginine and proline metabolism
Ascorbate and aldarate metabolism
African trypanosomiasis
Selenocompound metabolism
Arginine biosynthesis
Metabolism of xenobiotics by cytochrome P450
Pyruvate metabolism
Tyrosine metabolism
Chemical carcinogenesis - DNA adducts
Glycolysis / Gluconeogenesis
Retinol metabolism
Steroid hormone biosynthesis
Drug metabolism - cytochrome P450
Serotonergic synapse
Glutathione metabolism
Arachidonic acid metabolism
Sulfur metabolism
Linoleic acid metabolism
Inflammatory mediator regulation of TRP channels
Q category Low -Log10(P value) High
Q1 Q3
Q2 Q4 0 1 2 3 4
G
Intergrated analysis of
heptocyte transcriptome
and mLT-EV proteome
log2 ratio of transcript
log2 ratio of protein
H
GO enrichment of P3
GTPase activity
pyrophosphatase activity
hydrolase activity, acting on acid anhydrides
epidermal growth factor receptor binding
catalytic activity
NADH dehydrogenase (ubiquinone) activity
NADH dehydrogenase (quinone) activity
NADH dehydrogenase activity
NAD(P)H dehydrogenase (quinone) activity
protein localization to organelle
GTPase complex
intracellular transport of virus
respiratory electron transport chain
Gene number
-log10(FDR)
Gene Ratio
I
GO enrichment of P6 and P9
endoplasmic reticulum membrane
endoplasmic reticulum subcompartment
endoplasmic reticulum
nuclear outer membrane-endoplasmic reticulum membrane network
endomembrane system
organelle subcompartment
lipid metabolic process
intracellular membrane-bounded organelle
membrane-bounded organelle
organic hydroxy compound metabolic process
intracellular organelle
organelle membrane
organelle
Gene number
-log10(FDR)
Gene Ratio

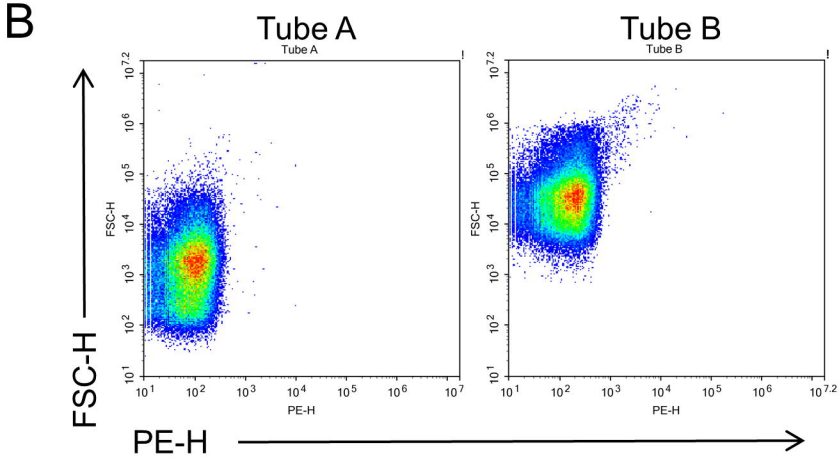

A
H&E
Sham
PHx
B
PHH3 DAPI
Sham
PHx
C
Hepatocyte cellcycle
Fraction of cells
1.00
0.75
0.50
0.25
0.00
Sham
PHx
G1
S
G2
M
Non cycling

A

	Tube A	Tube B	Tube C	Tube D	Tube E
ASGPR-PE antibody			+	+	+
PE isotype antibody		+	+	+	+
Anti-PE microbeads		+	+	+	+
PBS	+	+	+	+	+

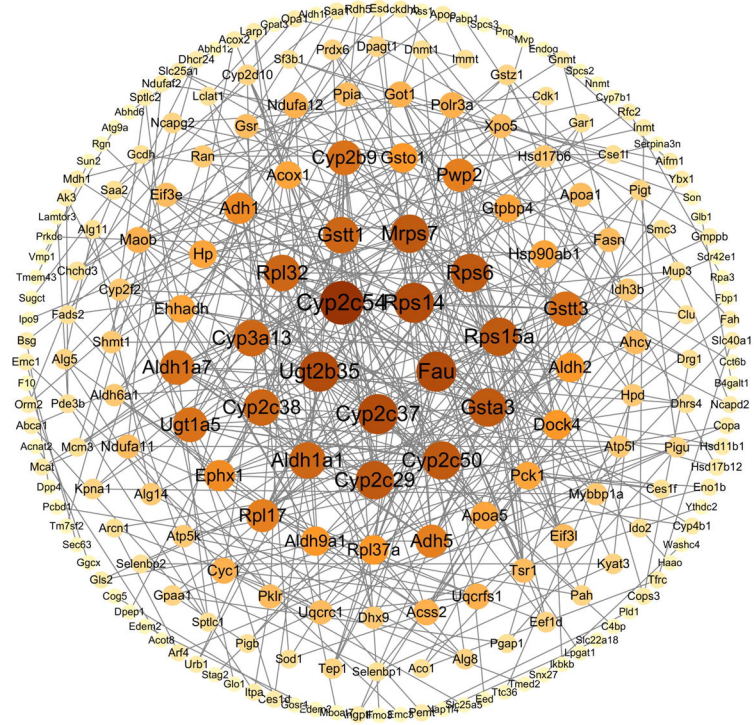


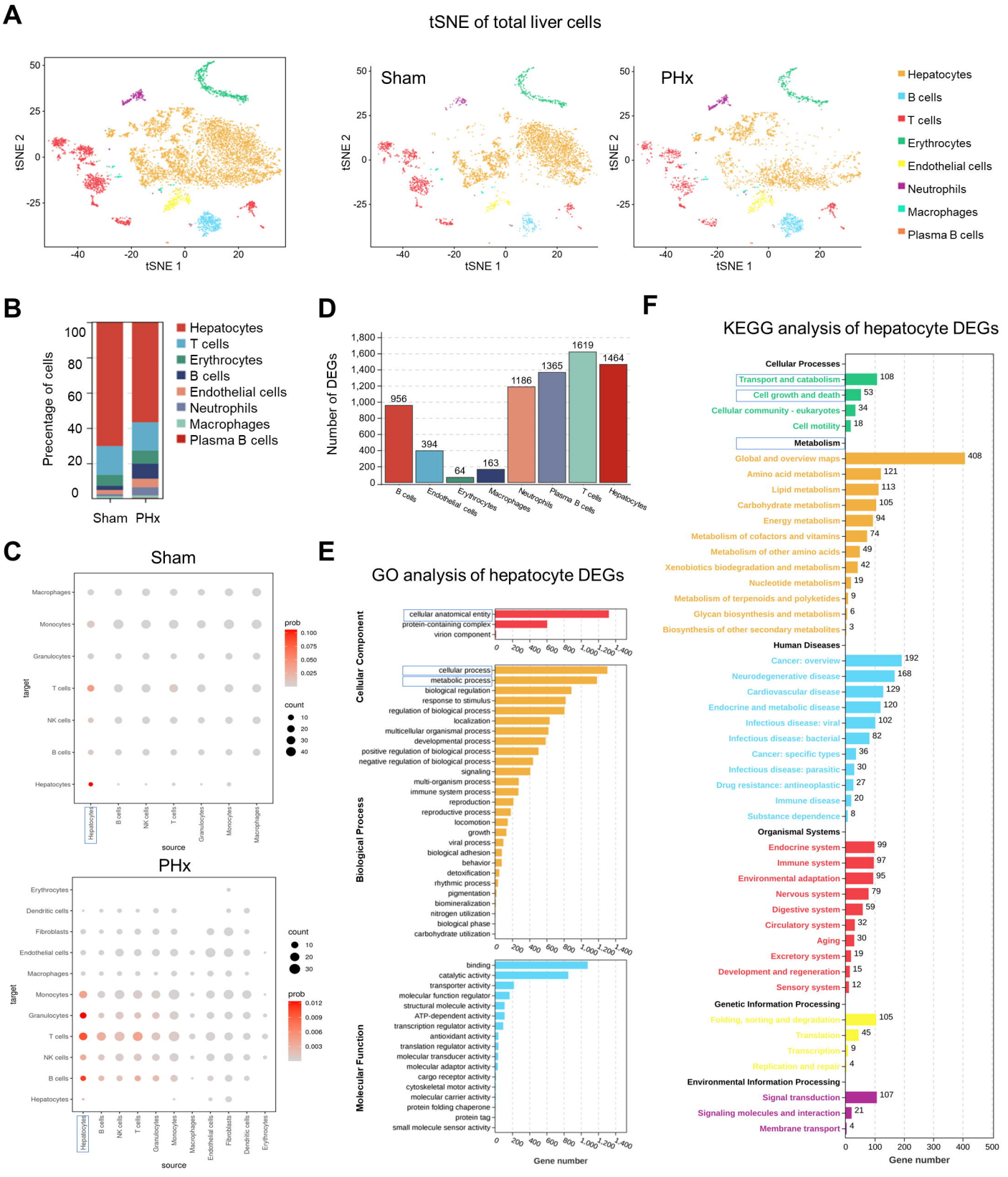
B



A

PPI network analysis of DEPs


Cyp2c54
Rps14
Cyp2b9
Gsto1
Mrps7
Gstt1
Rpl32
Rps6
Cyp3a13
Rps15a
Aldh1a7
Ugt2b35
Fau
Cyp2c38
Cyp2c37
Gsta3
Ugt1a5
Aldh1a1
Cyp2c50
Cyp2c29
Rpl17
Aldh9a1
Rpl37a
Adh5
Acox1
Pwp2
Adh1
Gstt3
Hp
Ehhadh
Ndufa12
Ppia
Got1
Polr3a
Xpo5
Gsr
Ran
Gtpbp4
Hsp90ab1
Idh3b
Ahcy
Aldh2
Dock4
Ephx1
Pck1
Apoa5
Tsr1
Pklr
Uqcrc1
Dhx9
Acss2
Uqcrfs1
Cyc1
Eif3l
Apoa1
Fasn
Maob
Alg5
Shmt1
Alg11
Eif3e
Cyp2f2
Fads2
Chchd3
Alg14
Kpna1
Atp5k
Selenbp2
Gpaa1
Sptlc1
Pigb
Sod1
Tep1
Selenbp1
Aco1
Alg8
Pgap1
Eef1d
Pld1
Cops3
Pah
Kyat3
Haao
Washc4
Cyp4b1
Ido2
Ythdc2
Eno1b
Ces1f
Mybbp1a
Hsd17b12
Hsd11b1
Pigu
Atp5l
Copa
Ncapd2
Dhrs4
Hpd
Drg1
Cct6b
B4galt1
Slc40a1
Clu
Fah
Fbp1
Rpa3
Mup3
Sdr42e1
Gmppb
Gib1
Son
Ybx1
Aifm1
Serpina3n
Inmt
Rfc2
Cyp7b1
Nnmt
Spcs2
Gnmt
Endog
Mvp
Pnp
Spcs3
Abpd1
Apoe
Hsd17b6
Cse1l
Pigt
Smc3
Gar1
Cdk1
Immt
Gstz1
Dnmt1
Dpagt1
Prdx6
Sf3b1
Dhcr24
Abhd14
Acox2
Larp1
Opa1
Aldh1a1
Saa1
Rdh5
Esdckdh
Hss1
Slc25a1
Ndufaf2
Sptlc2
Lclat1
Abhd6
Ncapg2
Atg9a
Rgn
Gcdh
Sun2
Mdh1
Ak3
Saa2
Lamtor3
Prkdc
Vmp1
Tmem43
Sugct
Ipo9
Bsg
Emc1
F10
Orm2
Pde3b
Abca1
Acnat2
Mcm3
Ndufa11
Mcat
Dpp4
Pcbd1
Tm7sf2
Arcn1
Sec63
Ggcx
Gls2
Cog5
Dpep1
Edem2
Acot8
Arf4
Urb1
Stag2
Glo1
Itpa
Ces1d
Gbsc1
Edem3
Mboat7
Tmo3
Emc8
Pemt
Hap1
Slc25a5
Eed
Ttc36
Tmed2
Snx27
Ikbkb
Lpgat1
Slc22a18
C4bp
Pld1
Tfrc
Cct6b
Slc40a1
Ncapd2
Copa
Hsd11b1
Hsd17b12
Eno1b
Ythdc2
Washc4
Haao


A
tSNE of total liver cells
Sham
PHx
tSNE 1
tSNE 2
Hepatocytes
B cells
T cells
Erythrocytes
Endothelial cells
Neutrophils
Macrophages
Plasma B cells
B
Precentage of cells
Sham
PHx
D
Number of DEGs
956
394
64
163
1186
1365
1619
1464
B cells
Endothelial cells
Erythrocytes
Macrophages
Neutrophils
Plasma B cells
T cells
Hepatocytes
C
Sham
PHx
target
source
prob
count
E
GO analysis of hepatocyte DEGs
Cellular Component
Biological Process
Molecular Function
Gene number
F
KEGG analysis of hepatocyte DEGs
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Nucleotide metabolism
Metabolism of terpenoids and polyketides
Glycan biosynthesis and metabolism
Biosynthesis of other secondary metabolites
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Infectious disease: bacterial
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Immune disease
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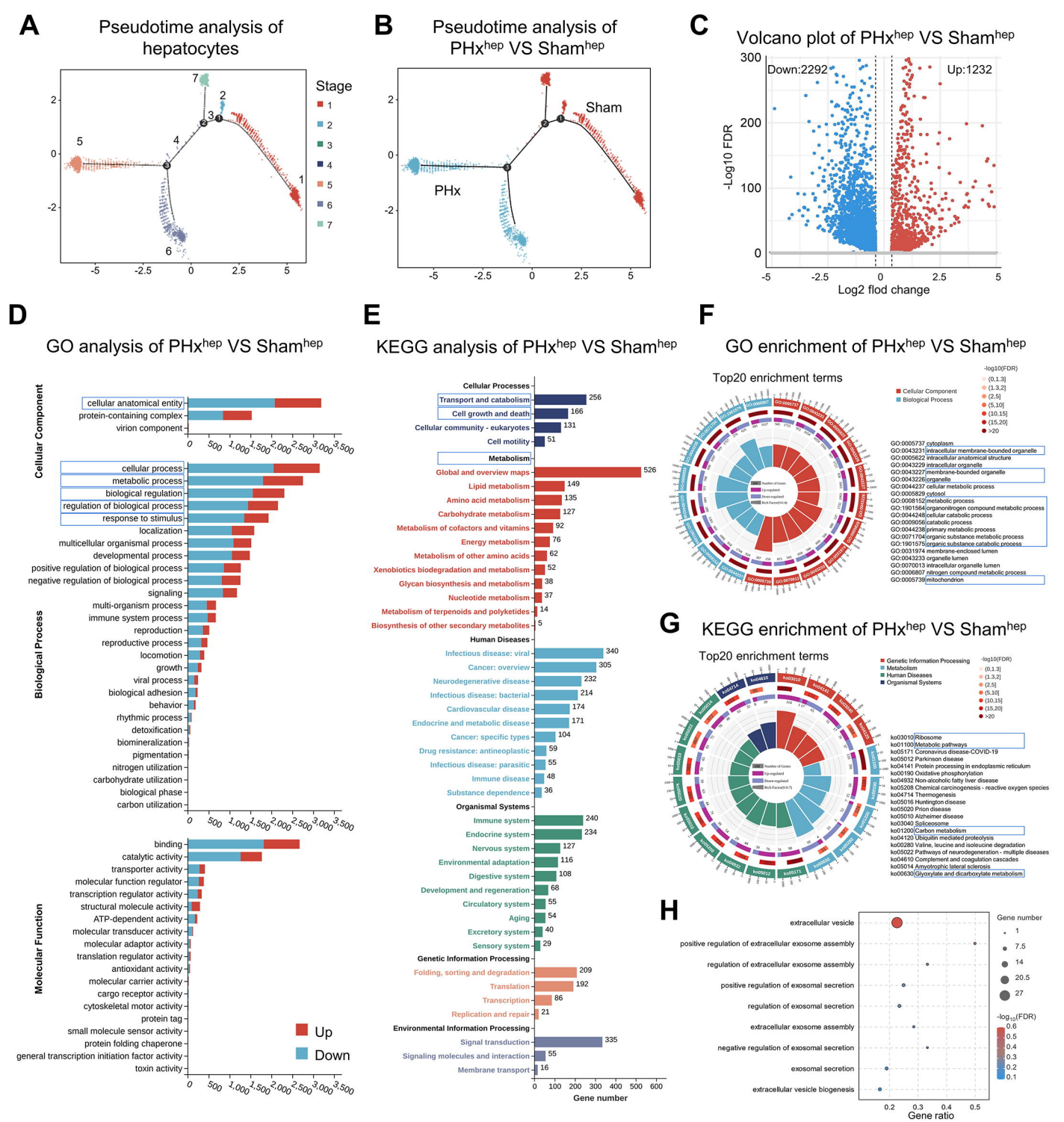
Immune system
Environmental adaptation
Nervous system
Digestive system
Circulatory system
Aging
Excretory system
Development and regeneration
Sensory system
Genetic Information Processing
Folding, sorting and degradation
Translation
Transcription
Replication and repair
Environmental Information Processing
Signal transduction
Signaling molecules and interaction
Membrane transport
Gene number


A
Pseudotime analysis of hepatocytes
Stage
1
2
3
4
5
6
7
B
Pseudotime analysis of PHxhep VS Shamhep
Sham
PHx
C
Volcano plot of PHxhep VS Shamhep
Down:2292
Up:1232
-Log10 FDR
Log2 flod change
D
GO analysis of PHxhep VS Shamhep
Cellular Component
cellular anatomical entity
protein-containing complex
virion component
Biological Process
cellular process
metabolic process
biological regulation
regulation of biological process
response to stimulus
localization
multicellular organismal process
developmental process
positive regulation of biological process
negative regulation of biological process
signaling
multi-organism process
immune system process
reproduction
reproductive process
locomotion
growth
viral process
biological adhesion
behavior
rhythmic process
detoxification
biomineralization
pigmentation
nitrogen utilization
carbohydrate utilization
biological phase
carbon utilization
Molecular Function
binding
catalytic activity
transporter activity
molecular function regulator
transcription regulator activity
structural molecule activity
ATP-dependent activity
molecular transducer activity
molecular adaptor activity
translation regulator activity
antioxidant activity
molecular carrier activity
cargo receptor activity
cytoskeletal motor activity
protein tag
small molecule sensor activity
protein folding chaperone
general transcription initiation factor activity
toxin activity
Up
Down
E
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Nucleotide metabolism 37
Metabolism of terpenoids and polyketides 14
Biosynthesis of other secondary metabolites 5
Human Diseases
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Cancer: overview 305
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Immune disease 48
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Immune system 240
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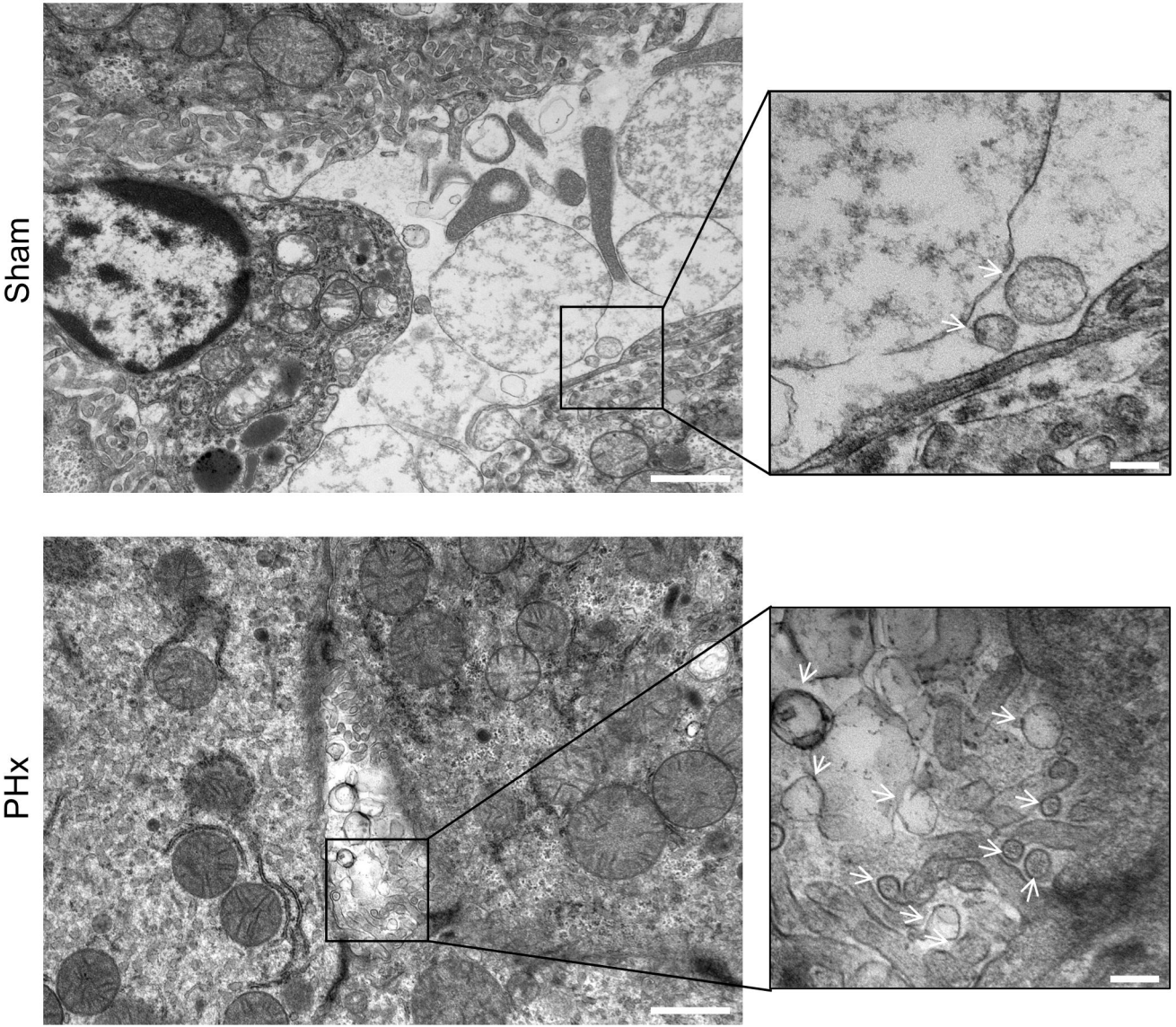
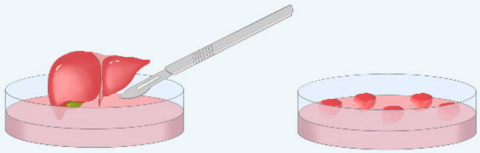
Aging 54
Excretory system 40
Sensory system 29
Genetic Information Processing
Folding, sorting and degradation 209
Translation 192
Transcription 86
Replication and repair 21
Environmental Information Processing
Signal transduction 335
Signaling molecules and interaction 55
Membrane transport 16
Gene number
F
GO enrichment of PHxhep VS Shamhep
Top20 enrichment terms
Cellular Component
Biological Process
-log10(FDR)
GO:0005737 cytoplasm
GO:0043231 intracellular membrane-bounded organelle
GO:0005622 intracellular anatomical structure
GO:0043229 intracellular organelle
GO:0043227 membrane-bounded organelle
GO:0043226 organelle
GO:0044237 cellular metabolic process
GO:0005829 cytosol
GO:0008152 metabolic process
GO:1901564 organonitrogen compound metabolic process
GO:0044248 cellular catabolic process
GO:0009056 catabolic process
GO:0044238 primary metabolic process
GO:0071704 organic substance metabolic process
GO:1901575 organic substance catabolic process
GO:0031974 membrane-enclosed lumen
GO:0043233 organelle lumen
GO:0070013 intracellular organelle lumen
GO:0006807 nitrogen compound metabolic process
GO:0005739 mitochondrion
G
KEGG enrichment of PHxhep VS Shamhep
Top20 enrichment terms
Genetic Information Processing
Metabolism
Human Diseases
Organismal Systems
-log10(FDR)
ko03010 Ribosome
ko01100 Metabolic pathways
ko05171 Coronavirus disease-COVID-19
ko05012 Parkinson disease
ko04141 Protein processing in endoplasmic reticulum
ko00190 Oxidative phosphorylation
ko04932 Non-alcoholic fatty liver disease
ko05208 Chemical carcinogenesis - reactive oxygen species
ko04714 Thermogenesis
ko05016 Huntington disease
ko05020 Prion disease
ko05010 Alzheimer disease
ko03040 Spliceosome
ko01200 Carbon metabolism
ko04120 Ubiquitin mediated proteolysis
ko00280 Valine, leucine and isoleucine degradation
ko05022 Pathways of neurodegeneration - multiple diseases
ko04610 Complement and coagulation cascades
ko05014 Amyotrophic lateral sclerosis
ko00630 Glyoxylate and dicarboxylate metabolism
H
extracellular vesicle
positive regulation of extracellular exosome assembly
regulation of extracellular exosome assembly
positive regulation of exosomal secretion
regulation of exosomal secretion
extracellular exosome assembly
negative regulation of exosomal secretion
exosomal secretion
extracellular vesicle biogenesis
Gene number
-log10(FDR)
Gene ratio

Liver tissue TEM



Specific Tissue EV Collection

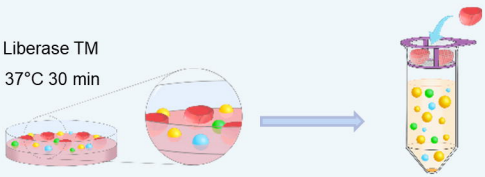
Sample preparation



Liver tissue dissociation

Dissociation

Liberase TM
37°C 30 min

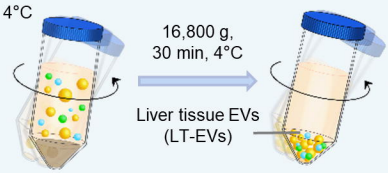


Enzymatic incubation

Filtration

Digestion

300 g, 10 min, 4°C
2,000 g, 20 min, 4°C



16,800 g,
30 min, 4°C

Liver tissue EVs
(LT-EVs)

Differential centrifugantion

Magnetic isolation

Antibody incubation

LT-EVs

Resuspension

Add ASGPR-PE antibody

ASGPR-PE incubation

4 h, 4°C, in dark place

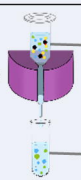
Magnetic bead incubation

Add anti-PE magnetic microbeads

Beads incubation

15 min, 4°C, in dark place

Magnetic sorting



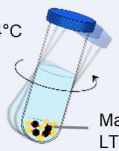
Passing *via* the LD column

Magnetic microbead-incubated LT-EVs

ASGPR^{-} LT-EVs

Collection

16,800 g, 30 min, 4°C

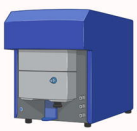


Magnetic microbead-sorted LT-EVs, mLT-EVs, ASGPR^{+})

Specific Tissue EV Characterization

EV morphology

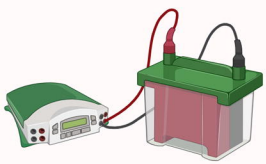
TEM



NTA

EV assay

Flow cytometer



Western blot

EV profiling

Mass spectrometry