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Characterization of the scavenger cell proteome in mouse and rat liver

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Abstract: The structural-functional organization of ammonia and glutamine metabolism in the liver acinus involves highly specialized hepatocyte subpopulations like glutamine synthetase (GS) expressing perivenous hepatocytes (scavenger cells). However, this cell population has not yet been characterized extensively regarding expression of other genes and potential subpopulations. This was investigated in the present study by proteome profiling of periportal GS-negative and perivenous GS-expressing hepatocytes from mouse and rat. Apart from established markers of GS⁺ hepatocytes such as glutamate/aspartate transporter II (GLT1) or ammonium transporter Rh type B (RhBG), we identified novel scavenger cell-specific proteins like basal transcription factor 3 (BTF3) and heat-shock protein 25 (HSP25). Interestingly, BTF3 and HSP25 were heterogeneously distributed among GS⁺ hepatocytes in mouse liver slices. Feeding experiments showed that RhBG expression was increased in livers from mice fed with high protein diet compared to standard chow. While spatial distributions of GS and carbamoylphosphate synthetase 1 (CPS1) were unaffected, periportal areas constituted by glutaminase 2 (GLS2)-positive hepatocytes were enlarged or reduced in response to high or low protein diet, respectively.

The data suggest that the population of perivenous GS⁺ scavenger cells is heterogeneous and not uniform as previously suggested which may reflect a functional heterogeneity, possibly relevant for liver regeneration.

Keywords: glutaminase; glutamine synthetase; liver zonation; proteomics; scavenger cells.

Introduction

There is a sophisticated structural-functional organization in the liver acinus with regard to ammonium and glutamine metabolism (Frieg et al. 2021; Gebhardt and Mecke 1983; Häussinger 1983, 1990). Periportal hepatocytes express enzymes required for urea synthesis such as the rate-controlling enzyme carbamoylphosphate synthetase 1 (CPS1) and liver-type glutaminase 2 (GLS2) (for review see Häussinger (1990)). GLS2 is activated by its product ammonium and therefore acts as a pH-regulated mitochondrial ammonium amplifier (Häussinger 1983; Häussinger and Sies 1979; Häussinger et al. 1984). This amplification is required for efficient ammonium elimination via urea synthesis in view of the low affinity of CPS1 for ammonia and the physiologically low ammonium ion concentrations in the portal blood (for review see Häussinger (1990)). Whereas periportal urea synthesis reflects a high capacity, but low affinity-system for ammonium disposal, ammonia escaping periportal urea synthesis reaches a small perivenous cell population at the acinar outflow, which removes ammonium ions with high affinity through glutamine synthesis. These GS⁺ hepatocytes were also called perivenous ‘scavenger cells’, because they remove not only ammonium ions, but also other compounds with high affinity, before the sinusoidal blood reaches the hepatic veins (Häussinger 1990; Häussinger and Stehle 1988). These scavenger cells exclusively express not only glutamine synthetase (GS) in the liver but also proteins supporting glutamine synthesis, such as the glutamate/aspartate transporter II (GLT1), ornithine aminotransferase (OAT1), the ammonium transporter Rh type B (RhBG) or uptake systems for dicarboxylates (Boon et al. 1999; Häussinger and Gerok 1983; Stoll and Häussinger 1991; Weiner et al. 2003). The important role of these perivenous GS⁺ (scavenger) hepatocytes for ammonium homeostasis is

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underlined by the finding that deletion of GS in mouse liver triggers systemic hyperammonemia (Qvartskhava et al. 2015). Hyperammonemia was also observed in taurine transporter knockout mice, which exhibit impaired perivenous glutamine synthesis due to an inactivating tyrosine nitration of GS and downregulation of RhBG (Qvartskhava et al. 2019).

Earlier studies suggest that metabolic liver zonation is not static, but rather dynamic and may change in response to nutrients, metabolites or hormones and under pathological conditions such as liver cirrhosis or hepatocellular carcinoma (Boon et al. 1999; Gebhardt and Matz-Soja 2014; Jungermann 1995).

Two recent studies reported spatial transcriptome profiles in mouse liver (Ben-Moshe et al. 2019; Halpern et al. 2017) and established a detailed gene landscape across the liver acinus in spatially defined areas by means of single-cell sequencing. Due to limited resolution, cellular heterogeneity within a defined area was not taken into account in these studies (Ben-Moshe et al. 2019; Halpern et al. 2017).

In the present study, we characterized the proteome of perivenous GS⁺ scavenger cells and compared it to the proteome of GS⁻ hepatocytes (which include periportal and midzonal hepatocytes) from both, mouse and rat liver. In a second approach, we compared the proteome of perivenous scavenger cells and periportal hepatocytes who

express the liver-type glutaminase (GLS2). For this, scavenger cells were labeled using antibodies directed against the glutamate transporter 1 (GLT1) (Cadoret et al. 2002), while periportal GLS2-expressing hepatocytes were labeled using antibodies directed against the periportal hepatocyte marker E-cadherin (E-Cad) (Hempel et al. 2015). Moreover, we investigated effects of dietary protein load on the levels of GS and GLS2 in the liver.

Our study identifies new proteins being enriched in perivenous scavenger cells and gives evidence for cell heterogeneities among GS⁺ scavenger cells.

Results

Distribution of ammonium metabolism-related proteins in rodent livers

The distribution of GS, GLS2, CPS1, GLT1 and RhBG protein in liver sections was investigated by immunofluorescence analyses (Figure 1). GLS2⁺ hepatocytes were confined to the periportal zone, GS⁺ scavenger cells surrounded the central vein and both subpopulations were clearly demarked by a mid-zone constituted by GLS2⁻/GS⁻ hepatocytes in rodent liver slices. CPS1 was found in GLS2⁺ and in hepatocytes of the transitional zone but not in GS⁺ scavenger cells. As shown in Figure 1, GS strongly colocalized with RhBG as

Figure 1: Metabolic zonation of the liver.

Immunofluorescence analyses of glutamine synthetase (GS), glutaminase 2 (GLS2), carbamoylphosphate synthetase 1 (CPS1), glutamate/aspartate transporter II (GLT1) and ammonium transporter Rh type B (RhBG) protein in (A) mouse and (B) rat liver sections. Cell nuclei were counterstained with Hoechst 34580.

well as with GLT1 in scavenger cells from both, rat and mice. These data suggest that the localization of the ammonium metabolism-related proteins GLS2, CPS1 and GS is similar in mouse and rat livers. Furthermore, the clear and specific labeling of perivenous scavenger cells by the antibodies directed against GLT1 and GS confirmed their suitability for isolating perivenous scavenger cells from the liver.

Characterization of the cellular proteome of scavenger cells from rat and mouse livers

For the characterization of the proteome of GS-expressing scavenger cells in rat and mouse livers, GS⁺ (perivenous) cells and GS⁻ (periportal and midzonal) hepatocytes were purified by means of FACS sorting (Supplementary Figure 1). Protein lysates of the separated cells were then subjected to mass spectrometry in order to identify individual protein profiles of GS⁺ scavenger hepatocytes (GS⁺ HCs) and GS⁻ hepatocytes (GS⁻ HCs). As illustrated in Figure 2, a total of 1717 and of 1263 proteins was quantified in isolated mouse or rat hepatocytes, respectively. Among the 1717 quantified proteins in mouse, 1503 (87.5%) proteins showed comparable abundances in both, GS⁺ and GS⁻ hepatocytes. In GS⁺ hepatocytes, 130 (7.6%) proteins showed a significantly higher abundance compared to GS⁻ hepatocytes (*q*-value < 0.05), whereas 84 (4.9%) proteins were higher abundant in GS⁻ HCs (Figure 2A and B).

As expected, scavenger cell markers such as GS, GLT1 and ornithine aminotransferase (OAT), were detected in higher abundances in GS⁺ hepatocytes (Figure 2C, left panel). Moreover, proteins characteristic for periportal hepatocytes such as mitochondrial ornithine carbamoyl-transferase (OTC), argininosuccinate synthase (ASS1) and phosphoenolpyruvate carboxykinase (PCK1) were only barely detected in this cell population (Figure 2C, left panel). Interestingly, heat shock protein 25 (HSP25), basal transcription factor 3 (BTF3) and RNase 4 were higher abundant in GS⁺ HCs compared to GS⁻ mouse hepatocytes. Further proteins showing higher abundances in GS⁺ scavenger hepatocytes, are shown in Figure 2B and Table 1.

In contrast to GS⁺ hepatocytes, GS⁻ hepatocytes showed significantly higher abundances of GLS2, PCK1 and urea cycle enzymes such as CPS1, OTC, ASS1 and arginase 1 (ARG1), whereas the amounts of GS⁺ scavenger cell markers were very low (Figure 2C, left panel).

Proteome profiles of GS⁺ and GS⁻ hepatocytes slightly differed between mouse and rat (Supplementary Figure 2). For instance, glutathione S-transferase alpha-5 (GSTA5) and scavenger receptor class B member 1 (SCARB1) were detected in higher abundances in rat but not in mouse GS⁺ hepatocytes (Figure 2C, right panel and Supplementary Figure 2).

Protein network and gene ontology (GO) biological terms analyses revealed an enrichment of proteins related to 115 and 173 biological processes in mouse and rat GS⁺

Figure 2: Proteome analysis of GS expressing scavenger hepatocytes and GS negative hepatocytes in mouse and rat. Hepatocyte subpopulations were isolated from mouse and rat livers and analyzed by mass spectrometry as described in materials and methods (*n* = 4, respectively). (A) Pie charts illustrating the number of proteins differentially abundant in GS⁺ or GS⁻ hepatocytes (HCs) from mice (left panel) or rat (right panel). (B) Volcano plots illustrating differentially abundant proteins in scavenger cells (GS⁺ hepatocytes) compared to periportal hepatocytes (GS⁻ hepatocytes) in mice (left panel) or rat (right panel). Proteins significantly higher abundant in GS⁺ hepatocytes are labeled in green and proteins higher abundant in GS⁻ hepatocytes are represented in red. The fold change represents the difference of the means of the respective $\log_2$ LFQ intensity values. (C) Bar chart indicating fold changes of selected proteins in GS⁺ scavenger cells (upper panel) or GS⁻ hepatocytes (lower panel) in mice (left) and rat (right), respectively.

Table 1: Selection of proteins with significantly distinct protein abundances in GS⁺ compared to GS⁻ hepatocytes in mouse and rat.

A)	Gene name	Protein name	Fold change (GS ⁺ vs. GS ⁻ HCs)
Murine GS⁺ HCs vs. GS⁻ HCs	Glul	Glutamine synthetase	272.30
	Ighg/Igh-1a	Ig gamma-2A chain C region, A allele/Ig gamma-2A chain C region. membrane bound form	99.74
	Gstm2	Glutathione S-transferase Mu 2	48.75
	Cyp2c37	Cytochrome P450 2C37	35.61
	N/A	Ig heavy chain V region M511/Ig heavy chain V region HPCM6/Ig heavy chain V region H8/Ig heavy chain V regions TEPC 15/S107/HPCM1/HPCM2/HPCM3/Ig heavy chain V region M603/Ig heavy chain V region HPCG14/Ig heavy chain V region HPCG8/Ig heavy chain V region HPCG13/Ig heavy chain V region M167	31.94
	Blvrb	Flavin reductase (NADPH)	26.43
	Oat	Ornithine aminotransferase, mitochondrial	22.96
	Cyp2a5	Cytochrome P450 2A5	11.99
	Gstm3	Glutathione S-transferase Mu 3	11.55
	Aldh3a2	Aldehyde dehydrogenase	11.51
	Hspb1	Heat shock protein beta-1	10.47
	Btf3	Transcription factor BTF3	10.18
	Ces2c	Acylcarnitine hydrolase	9.71
	Slc1a2/GLT1	Excitatory amino acid transporter 2/Amino acid transporter (GLT1)	8.87
	Slc22a1	Solute carrier family 22 member 1	7.98
B)	Gene name	Protein name	Fold change (GS ⁺ vs. GS ⁻ HCs)
Rat GS⁺ HCs vs. GS⁻ HCs	Glul	Glutamine synthetase	3964.71
	Aox3	Aldehyde oxidase	34.25
	Gclm	Glutamate-cysteine ligase regulatory subunit	13.00
	N/A	Urinary protein 2	12.38
	Slc22a1	Solute carrier family 22 member 1	10.87
	Oat	Ornithine aminotransferase, mitochondrial	10.12
	Sult1e1	Estrogen sulfotransferase, isoform 1	10.03
	N/A	Urinary protein 1	8.02
	Ces1c	Carboxylic ester hydrolase	7.91
	Cd36	Platelet glycoprotein 4	6.62
	Fabp7	Fatty acid-binding protein, brain	6.22
	Gsta5	Glutathione S-transferase alpha-5	6.17
	Cyp3a1	Cytochrome P450 3A1	5.82
	Rab6a	Ras-related protein Rab-6A	5.07
	Ste	Estrogen sulfotransferase, isoform 3	4.68
C)	Gene name	Protein name	Fold change (GS ⁻ vs. GS ⁺ HCs)
Murine GS⁻ HCs vs. GS⁺ HCs	Hal	Histidine ammonia-lyase	43.34
	Aldh1b1	Aldehyde dehydrogenase X, mitochondrial	23.22
	Rp126	60S ribosomal protein L26	22.13
	Gldc	Glycine dehydrogenase (decarboxylating), mitochondrial	19.49
	Hsd17b13	17-Beta-hydroxysteroid dehydrogenase 13	18.86
	Amdhd1	Probable imidazolonepropionase	16.09
	Sds	L-serine dehydratase/L-threonine deaminase	15.50
	Sfxn1	Sideroflexin-1	14.51
	Gls2	Glutaminase liver isoform, mitochondrial	14.45
	Hsd17b6	17-beta-hydroxysteroid dehydrogenase type 6	14.20
	Cyp2f2	Cytochrome P450 2F2	11.52
	Glb1l2	Beta-galactosidase/Beta-galactosidase-1-like protein 2	9.15
	Hsd17b13	17-beta-hydroxysteroid dehydrogenase 13	8.59
	Eppk1	Epiplakin	7.59
	Arg1	Arginase-1	7.50

Table 1: (continued)

D)	Gene name	Protein name	Fold change (GS ⁻ vs. GS ⁺ HCs)
Rat GS ⁻ HCs vs. GS ⁺ HCs	Gls2	Glutaminase liver isoform, mitochondrial	25.61
	Gldc	Glycine cleavage system P protein	21.64
	Hsd17b13	17-beta-hydroxysteroid dehydrogenase 13	16.60
	Agxt	Serine-pyruvate aminotransferase, mitochondrial	13.85
	Aldh1b1	Aldehyde dehydrogenase X, mitochondrial	12.81
	Uroc1	Imidazolonepropionate hydrolase	9.11
	Srd5a1	3-oxo-5-alpha-steroid 4-dehydrogenase 1	9.09
	Ugt2b37	UDP-glucuronosyltransferase	9.03
	Mtn5	NADH-ubiquinone oxidoreductase chain 5	8.21
	Cyp17a1	Steroid 17-alpha-hydroxylase/17,20 lyase	7.45
	Rpl3	60S ribosomal protein L3	6.32
	Sfxn1	Sideroflexin-1	5.57
	Fads1	Fatty acid desaturase 1	5.54
	Ugt2b15	UDP-glucuronosyltransferase	5.44
	Sult2a1/St2a1	Sulfotransferase; Bile salt sulfotransferase	5.41

(A–B) Proteins showing higher abundances in GS⁺ scavenger cells (GS⁺ HCs) compared to GS⁻ hepatocytes (GS⁻ HCs) in (A) mouse and (B) rat. (C–D) Selection of proteins significantly higher abundant in GS⁻ HCs vs. GS⁺ HCs in (C) mouse and (D) rat. Fold changes represent the differences of the means of the respective log₂ LFQ intensity values.

scavenger cells compared to GS⁻ hepatocytes, respectively (Supplementary Figure 3 and Supplementary Table 2). Some of these categories such as “xenobiotic metabolic process”, “response to drug” and “glutathione metabolic process” are well established in perivenous hepatocytes (Jungermann 1988).

To further validate the mass spectrometry data, we performed immunofluorescence analyses on mouse liver sections which allow for the investigation of both, the spatial protein distribution as well as the heterogeneity of protein expression within hepatocyte subpopulations on liver sections. As depicted in Figure 3A, heat shock protein 25 (HSP25) and basic transcription factor 3 (BTF3) immunofluorescence was high in the majority of GS⁺ scavenger cells. However, in a subset of GS⁺ hepatocytes and in GS⁻ cells, the BTF3 and HSP25 immunofluorescence intensities were very weak or only barely detectable. Roughly 68.1% of the GS⁺ hepatocytes showed high staining intensities of HSP25, while 56.6% of GS⁺ hepatocytes were strongly positive for BTF3.

These data show that protein expression differs among GS⁺ hepatocytes which is indicative for the existence of scavenger cell subpopulations.

Characterization of the cellular proteome of periportal hepatocytes in mouse liver

In following experiments, we aimed to characterize the proteome of periportal GLS2⁺ hepatocytes in relation to GS⁺ hepatocytes in mouse liver. Due to antibody incompatibilities,

scavenger cells were labeled in these experiments not with antibodies directed against GS, but with antibodies detecting an extracellular epitope of GLT1⁺. Periportal hepatocytes were labeled with antibodies directed against E-cadherin and GLS2. As shown by immunofluorescence analysis on liver sections, virtually all E-cadherin expressing periportal hepatocytes coexpressed GLS2 (Figure 4A) suggesting the suitability of E-cadherin as an epitope for the purification of GLS2⁺ periportal hepatocytes. Moreover, in isolated primary mouse hepatocytes, a strong colocalization of GLS2 and E-cadherin was observed, while GLT1⁺ hepatocytes did not coexpress E-cadherin (Figure 4A, lower panel and Supplementary Figure 4). Next, GLT1⁺ and E-cadherin⁺ hepatocytes were isolated by FACS sorting (Supplementary Figure 5) and their proteome profile was analyzed by mass spectrometry and bioinformatic analyses (Figure 4B and C). As shown in Figure 4D, GS was present in higher amounts in the fraction of GLT1⁺ hepatocytes but only barely detectable in the fraction of E-cadherin expressing hepatocytes. Of note, abundances of perivenous scavenger cell marker such as GS and GLT1 were lower in GLT1⁺ sorted perivenous hepatocytes compared to GS⁺-sorted perivenous hepatocytes. This may be explained by the different experimental set-up (lower signal intensity of GLT-staining) or by a lower purity of the GLT1⁺ and E-Cad⁺ sorted cells due to technical limitations. Conversely, GLS2 was only weakly detectable in the fraction of GLT1⁺ hepatocytes but highly abundant in the fraction of E-cadherin⁺ hepatocytes (Figure 4D). These data indicate a strong enrichment of GS-expressing perivenous scavenger hepatocytes and GLS2-expressing periportal hepatocytes in FACS sorted fractions of GLT1⁺ and E-cadherin⁺ hepatocytes, respectively.

Figure 3: Validation of proteome analysis by immunofluorescence analysis in mouse liver sections.

(A) Analyses of glutamine synthetase (GS), heat shock protein 25 (HSP25) and basic transcription factor 3 (BTF3) by immunofluorescence and fluorescence microscopy. Cell nuclei were counterstained with Hoechst 34580. (B) Proportions of scavenger cells showing high or low levels of HSP25 (left) or BTF3 (right), respectively.

As shown by proteome analysis the abundances of 77 proteins were lower and of 48 proteins were higher in E-cadherin⁺ hepatocytes when compared to GLT1⁺ hepatocytes. Besides proteins well-known to be expressed in periportal hepatocytes, the proteome analysis revealed a number of proteins which have not yet been recognized to be highly abundant in E-cadherin⁺ hepatocytes compared to GLT1⁺ scavenger cells. Among these proteins were mitochondrial glycine dehydrogenase (GLDC), sideroflexin-1 or mitochondrial aldehyde dehydrogenase X (Table 2). Protein network and GO biological terms analyses revealed an enrichment of proteins related to 107 biological processes in mouse E-Cad⁺ hepatocytes compared to GLT1⁺ hepatocytes, respectively (Supplementary Figure 3 and Supplementary Table 2). Proteins that were enriched in E-Cad⁺ hepatocytes relate to biological processes already established in these cells such as “organonitrogen compound metabolic process” or “urea cycle” (Supplementary Figure 3 and Supplementary Table 2).

The results of the two independent proteome analyses partially overlapped (Figure 4E). For instance, 48 proteins were concordantly more abundant in both, GS⁺ hepatocytes

(compared to GS⁻ hepatocytes) and GLT1⁺ hepatocytes (when compared to E-cadherin⁺ hepatocytes). Moreover, 25 proteins were identified as being higher abundant in GS⁻ periportal hepatocytes (compared to GS⁺ hepatocytes) as well as in E-cadherin⁺ hepatocytes (compared to GLT1⁺ hepatocytes) (Figure 4E). Moreover, the comparison of enriched GO terms revealed that 69 biological process were enriched in both, GS⁺ and GLT1⁺ hepatocytes in mouse (Supplementary Figure 6). The comparison of GS⁻ hepatocytes and E-Cad⁺ periportal hepatocytes showed that 80 terms were likewise enriched in these hepatocytes (Supplementary Figure 6). Interestingly, 103 additional biological processes were found enriched only in GS⁻ hepatocytes, which may be dedicated to the fact that the GS⁻ subpopulation comprises periportal (GLS2⁺) as well as midzonal hepatocytes.

The differences of the proteome and GO analyses resulting from the two different approaches may be explained by the fact that the GS⁺ perivenous hepatocytes were compared to the periportal plus mid-zone hepatocytes, while perivenous GLT1⁺ hepatocytes were directly compared to periportal E-cadherin⁺ cells.

Figure 4: Proteome analysis of GLT1⁺ scavenger hepatocytes and E-cadherin⁺ periportal hepatocytes in mouse. E-cadherin⁺ and GLT1⁺ hepatocytes were isolated from mouse livers and analyzed by mass spectrometry as described in Materials and methods ($n = 3$). (A) Immunofluorescence analysis of E-cadherin (E-Cad), glutamate/aspartate transporter II (GLT1) and glutaminase 2 (GLS2) in mouse liver sections or in isolated mouse hepatocytes. Cell nuclei were counterstained with Hoechst 34580. (B) Pie charts illustrating the number of proteins differentially abundant in mice in GLT1⁺ or E-Cad⁺ hepatocytes (HCs). (C) Volcano plots illustrating differentially abundant proteins in GLT1-expressing scavenger cells (GLT1⁺ HCs) compared to periportal E-cadherin⁺ hepatocytes (E-Cad⁺ HCs) from mice. Proteins significantly higher abundant in GLT1⁺ hepatocytes are labeled in green and proteins with higher abundances in periportal E-Cad⁺ hepatocytes are represented in red. The fold change represents the difference of the means of the respective log₂ LFQ intensity values. (D) Bar chart indicating fold changes of selected proteins in GLT1⁺ scavenger cells (upper panel) or E-Cad⁺ periportal hepatocytes (lower panel) in mice. (E) Venn diagram indicating the overlap of proteins higher abundant in perivenous (GS⁺ and GLT1⁺) hepatocytes (left panel) or in GS⁻ and E-Cad⁺ hepatocytes (right panel).

Effects of dietary protein load on levels and distribution of key proteins involved in ammonium metabolism in mouse liver

With the aim to investigate whether levels and the distribution of proteins involved in ammonia metabolism change in response to the dietary nitrogen load, mice were fed with standard chow or with low or high protein diet for two weeks (Supplementary Figure 7). While protein levels of the scavenger cell marker GS remained unchanged, levels of RhBG were elevated in mice fed with high protein diet compared to low protein or standard diet (Figure 5A). HSP25 protein levels were substantially decreased in low protein fed mice and tended to be increased in livers of animals receiving a high protein diet (Figure 5A).

In periportal hepatocytes, GLS2 protein levels were elevated after high protein diet (Figure 5, Supplementary Figure 8). In order to quantify GLS2⁺ hepatocytes after high

protein diet, individual hepatocytes were discriminated on liver slices by staining polymerized actin filaments with phalloidin-TRITC and GLS2⁺ hepatocytes were counted. Here we found, that the amount of GLS2⁺ hepatocytes was significantly increased in mice fed with a high protein diet ($59.2 \pm 1.5\%$) compared to those fed with a low protein diet ($50.9 \pm 2.4\%$) (Figure 5C). This was reflected by an enlargement of the area comprising GLS2⁺ HCs.

These data show that the dietary protein intake can not only alter the expression of proteins related to ammonium metabolism, as already shown decades ago for urea cycle enzymes by Schimke (Schimke 1962), but also their sub-acinar distribution in mouse liver. This especially holds for GLS2, which provides ammonium ions for urea synthesis. Thus, the increase of the GLS2⁺ zone at the expense of the GLS2⁻ and GS⁻ mid-zone suggests that more hepatocytes become recruited for urea synthesis in response to high protein diet.

Table 2: Selection of proteins with significantly distinct protein abundances in murine GLT1⁺ compared to E-Cad⁺ hepatocytes.

A)	Gene name	Protein name	Fold change (GLT1 ⁺ HCs vs. E-Cad ⁺ HCs)
GLT1⁺ HCs vs. E-Cad⁺ HCs	Glul	Glutamine synthetase	44.90
	Oat	Ornithine aminotransferase, mitochondrial	32.97
	Cyp2c37	Cytochrome P450 2C37	21.92
	Rgn	Regucalcin	17.96
	Aldh3a2	Aldehyde dehydrogenase	16.40
	Cyp2a4/Cyp2a5	Cytochrome P450 2A4/Cytochrome P450 2A5	14.80
	Rab11a/Rab11b	Ras-related protein Rab-11A/Ras-related protein Rab-11B	11.66
	Ces2c	Acylcamitine hydrolase	11.51
	Ugtla2/Ugt1a5/Ugtla7c/	UDP-glucuronosyltransferase 1-2/	10.52
	Ugt1a8	UDP-glucuronosyltransferase 1-7C	
	S1c22a1	Solute carrier family 22 member 1	10.42
	Cyp2c67	Cytochrome P450, family 2, subfamily c, polypeptide 67	10.16
	Gulo	L-gulonolactone oxidase	9.52
	Cyp2c54	Cytochrome P450 2C54	8.32
	Cyp2e1	Cytochrome P450 2E1	8.27
	Cyp2c50	Cytochrome P450 2C50	7.90
	Cyp2c29	Cytochrome P450 2C29	7.81
	Cyp1a2	Cytochrome P450 1A2	7.41
	Pex14	Peroxisomal membrane protein PEX14	5.77
	Aldh1a1	Retinal dehydrogenase 1	5.74
	Hpd	4-hydroxyphenylpyruvate dioxygenase	5.59
	Cyp2c40	Cytochrome P450 2C40	5.12
B)	Gene name	Protein name	Fold change (E-Cad ⁺ HCs vs. GLT1 ⁺ HCs)
E-Cad⁺ HCs vs. GLT1⁺ HCs	Hsd17b13	17-beta-hydroxysteroid dehydrogenase 13	26.25
	Gls2	Glutaminase liver isoform, mitochondrial	14.42
	Gldc	Glycine dehydrogenase (decarboxylating), mitochondrial	13.36
	Hsd17b13	17-beta-hydroxysteroid dehydrogenase 13	12.80
	Sfxnl	Sideroflexin-1	9.07
	Aldh1b1	Aldehyde dehydrogenase X, mitochondrial	7.49
	Cyp2f2	Cytochrome P450 2F2	6.80
	My16	Myosin light polypeptide 6	5.38
	H2afv/H2afz	Histone H2A	5.18
	Tmem256	Transmembrane protein 256	5.06

(A) Proteins showing higher abundances in GLT1⁺ hepatocytes (GLT1⁺ HCs) compared to E-Cad⁺ hepatocytes (E-Cad⁺ HCs). (B) Selection of proteins significantly higher abundant in E-Cad⁺ HCs vs. GLT1⁺ HCs. Fold changes represent the differences of the means of the respective log₂ LFQ intensities.

Discussion

This is the first study which characterizes the proteome profile of GS⁺ scavenger cells in comparison to GS⁻ hepatocytes from mouse and rat livers. In recent studies, single-cell sequencing was applied to establish a detailed gene landscape across the liver acinus in spatially defined areas (Ben-Moshe et al. 2019; Halpern et al. 2017). However, in these approaches, the defined areas were not specifically confined to GS⁺ or to GS⁻ cells and cell heterogeneities within defined areas were not taken into account. We therefore performed mass spectrometry and

immunofluorescence analyses on isolated scavenger cells to characterize their proteome profile.

With this approach, we identified not only well-established scavenger cell specific proteins such as RhBG or GLT1 but also proteins hitherto not known to be highly abundant in the GS⁺ scavenger hepatocytes such as BTF3 and HSP25. Importantly, BTF3 and HSP25 protein levels were not evenly distributed among the GS⁺ scavenger cells when investigated by immunofluorescence analyses, suggesting a functional heterogeneity of GS⁺ hepatocytes.

BTF3 was shown to modulate the expression of genes participating in the regulation of apoptosis and cell cycle

Figure 5: Effects of dietary protein content on the expression of RhBG, GS, HSP25, GLS2, GLT1 and CPS1 in mouse liver.

C57BL/6J mice at the age of eight-weeks were fed *ad libitum* with control, low protein or high protein diet for 14 days as described in Materials and methods ($n = 3$). Animals were sacrificed, livers were dissected and snap-frozen in liquid nitrogen. (A) Immunofluorescence analyses of glutamine synthetase (GS) and glutaminase 2 (GLS2), carbamoylphosphate synthetase 1 (CPS1), ammonium transporter Rh type B (RhBG), heat shock protein 25 (HSP25) or glutamate transporter (GLT1). Cell nuclei were counterstained with Hoechst 34580. (B) Western Blot analyses of GS and GLS2 protein lysates prepared from livers of mice fed with diets with different protein content. Data represent the average $\pm$ standard error of the mean of three animals per condition ($n = 3$). *Statistically significantly different. (C) Relative quantification of GLS2 positive hepatocytes in livers from mice fed with low or high protein diet. Liver sections were stained with phalloidin-TRITC (red) to identify individual cells and for GLS2 (green) as indicated. The number of GLS2 positive and GLS2 negative hepatocytes was evaluated for three animals per condition in 5–10 distinct positions in each liver (left panel). Bar chart indicates percentage of GLS2 positive hepatocytes in mouse liver (right panel).

(Chen et al. 2019; Jeon et al. 2016; Liu et al. 2013; Zhang et al. 2019). BTF3 is strongly expressed in diverse tumor tissues such as human prostate and gastric cancer (Chen et al. 2019; Liu et al. 2013; Symes et al. 2013). Hu and colleagues demonstrated that BTF3 triggers undifferentiated, stem cell-like properties in prostate cancer cells, thereby emphasizing the role of BTF3 in tumor development and progression (Hu et al. 2019). The fact that high BTF3 protein levels were only found in roughly 56.6% of GS⁺ hepatocytes

may point to the existence of a scavenger cell subpopulation with stem cell-like properties which may contribute to the regeneration of the damaged liver. In this regard it should be noted that axin⁺ and GS⁺ perivenous hepatocytes were suggested to fuel homeostatic renewal of the liver (Wang et al. 2015).

Heat shock protein HSP25 was also highly abundant in GS⁺ hepatocytes (Figure 3). HSP25 acts as a chaperone and antioxidant thereby protecting cancer cells from oxidative

stress (Cheng et al. 2015; Vidyasagar et al. 2012). The exact role played in GS⁺ hepatocytes, however, remains unclear.

Using bioinformatics analyses, we identified detailed protein networks as well as biological processes showing a characteristic heterogeneity in hepatocyte subpopulations isolated from both, rat and mouse (Supplementary Figures 3 and 6). For instance, processes significantly enriched in GS⁺ perivenous hepatocytes from rat and mouse and GLT1⁺ perivenous hepatocytes isolated from mouse showed an enrichment for proteins associated to “xenobiotic catabolic processes” or “glutathione metabolic processes”. While periportal E-Cad⁺ hepatocytes and GS[−] hepatocytes were enriched for processes related to “urea synthesis” and “glutamine metabolic process”. Altogether, these data fit well into the established functions of hepatocyte subpopulations, while providing more detail into the spatial distribution of individual proteins. Despite the significant enrichment of perivenous hepatocyte markers in GS⁺ and GLT1⁺ hepatocyte preparations as well as periportal hepatocyte markers in GS[−] and E-Cad⁺ hepatocyte preparations which was achieved by our FACS sorting approaches, we cannot exclude that the results are limited due to contaminating cells. This is reflected by the detection of trace amounts of GS protein in the fraction of GS[−] cells or by lower abundances of GS and GLT1 in GLT1⁺ hepatocytes (Figure 4) compared to GS⁺ hepatocytes (Figure 2). Therefore, future studies applying single-cell sequencing might be required to study the hepatic scavenger cell heterogeneity in detail.

Our study further revealed that the zonation of ammonium metabolism-related proteins is highly conserved across mice and rat. Moreover, the levels and spatial distribution of ammonium metabolism-related proteins are dynamically adapted in response to the protein content of the diet in mice (Figure 5). GLS2 protein levels increased and the area of GLS2 positive hepatocytes strongly expanded in mice fed with high protein diet. In contrast, the area comprising GS positive cells and GS expression remained unchanged. This indicates that the liver adapts to the higher demand for ammonium detoxification by enhancing the capacity of the urea cycle through upregulation not only of CPS1, but also by a zonal expansion of GLS2 expression, thus suggesting that more hepatocytes become engaged in urea synthesis.

The present study also revealed the expression of proteins not suggested before to be enriched in periportal hepatocytes, such as GLDC, sideroflexin-1 or mitochondrial aldehyde dehydrogenase X. However, the precise roles of these proteins in periportal hepatocytes are currently unknown and remain to be established. The comprehensive proteome data of the present study may open up new

perspectives and warrant future research on the functional impact of metabolic zonation of the liver.

Materials and methods

Animals and treatments

All animal experiments were reviewed and approved by the appropriate authorities and were performed in accordance with the German animal protection law (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection, reference number Az. 81-02.04.2016.A289). In order to study the effect of protein load on the liver zonation, eight-weeks old male C57BL/6J mice ($n = 3$) were fed *ad libitum* with either low protein diet (9 kJ% protein, Ssniff, Soest, Germany), high protein diet (49 kJ% protein, Ssniff) or standard chow (26 kJ% protein, Ssniff) for two weeks. All animals had free access to water and food. Detailed compositions of all diets are given in the supplement (Supplementary Figure 7). At the end of the treatment, mouse livers were briefly perfused with physiological saline to remove residual blood, dissected, shock frozen and analyzed by Western blot and immunofluorescence analyses.

Hepatocyte isolation, staining and sorting

Hepatocytes were isolated from eight-weeks old C57BL/6J mice using a two-step collagenase perfusion method. Briefly, livers were perfused via the portal vein with Ca²⁺-free HEPES buffer (HANKS buffer) for 3 min, followed by perfusion with HANKS buffer supplemented with 5 mM CaCl₂ and 0.4 mg/ml type II collagenase for 15 min at 42 °C. After digestion with collagenase, the liver was dissected and carefully disrupted with forceps in Krebs-Henseleit-buffer. The resulting cell suspension was filtrated through a filter with a diameter of 70 µm. The isolated single hepatocytes were immediately fixed with 4% formaldehyde for 10 min at room temperature (RT) and permeabilized with 0.3% (v/v) Tween-20 in PBS. Thereafter, hepatocytes were incubated in blocking reagent (3% bovine serum albumin (BSA) in PBS) for 30 min. Cells were stained for the intracellular marker glutamine synthetase (GS) using the mouse anti-glutamine synthetase antibody (#610518, BD Bioscience, Heidelberg, Germany) at a concentration of 1:100 in blocking reagent for 1 h at RT. For the labeling of extracellular markers anti-glutamate transporter 1 (#ab41621, Abcam, Cambridge, UK) or E-cadherin (#610182, BD Biosciences), isolated hepatocytes were treated with blocking solution 3% BSA (15 min) without prior fixation and permeabilization. Cells were then stained with fluorophore-conjugated secondary antibodies (1:100 1 h, RT) and cell nuclei were counterstained with Hoechst 34580 (1:5000, Thermo Fisher Scientific, Schwerte, Germany). Hepatocytes were separated and collected by using FACS Aria III (BD Biosciences). A 2.0 neutral density filter in front of the forward scatter detector was used to decrease the FSC signal. The sorting of primary hepatocytes was performed using a 100 µm Nozzle. The collected Hoechst⁺-labeled hepatocytes were divided in GS positive (GS⁺) and GS negative (GS[−]) hepatocytes or GLT1 (GLT1⁺) and E-cadherin (E-Cad⁺) positive hepatocytes, respectively. For proteome analysis, 500,000 cells of each fraction were collected ($n = 3-4$).

Mass spectrometric analysis of GS positive and negative cells

In order to characterize the similarities and differences between different hepatocyte populations, quantitative mass spectrometry was conducted essentially as described (Grube et al. 2018). Briefly, cell lysates were prepared and analyzed from three to four independent replicates per group. Five μg of protein per sample were prepared by in-gel digestion with trypsin after reduction and alkylation with iodoacetamide. Finally, 500 ng of resulting peptides were separated using a 2 h gradient via an Ultimate 3000 rapid separation liquid chromatograph system on C18 material. Separated peptides were sprayed by an electrospray ionization nano source directly into a Q Exactive plus mass spectrometer operated in data-dependent positive mode. First, survey scans were recorded with a resolution of 70,000 (or 140,000 for the comparison of GLT1⁺ and E-Cad⁺ cells) and subsequently up to ten 2- and 3-fold charged precursors were selected by the quadrupole (2 m/z isolation window), fragmented by higher-energy collisional dissociation and analyzed at a resolution of 17,500. For the analysis of GS⁺ and GS⁻ hepatocytes, spectra were further processed for peptide and protein identification as well as precursor spectra-based quantification with MaxQuant version 1.6.1.0 (Max Planck Institute for Biochemistry, Planegg, Germany) using standard parameters if not stated otherwise. For the three different experiments, different versions of MaxQuant and protein datasets downloaded from the UniProt knowledgebase were used: MaxQuant 1.6.1.0 and 52548 *Mus musculus* sequences (UP000000589, 18th January 2018) for the comparison of mouse GS⁺ and GS⁻ cells, MaxQuant 1.6.3.4 and 29951 *Rattus norvegicus* sequences (UP000002494, 10th April 2019) for the comparison of rat GS⁺ and GS⁻ cells and MaxQuant 1.6.10.43 and 55192 *Mus musculus* sequences (UP000000589, 7th June 2019) for the comparison of mouse GLT1⁺ and E-Cad⁺ cells. Carbamidomethylation at cysteines was considered as fixed and protein N-terminal acetylation as well as methionine oxidation as variable modifications. The 'match between runs' function was enabled as well as label-free quantification, peptides and proteins were accepted at a false discovery rate of 1%. Quantitative data (LFQ intensities) was further processed with Perseus version 1.6.1.1 (Max Planck Institute for Biochemistry, Planegg, Germany) for GS⁺ and GS⁻ hepatocytes or with Perseus 1.6.6.0 (Max Planck Institute for Biochemistry) for GLT1⁺ and E-Cad⁺ hepatocytes. Here, only proteins were considered showing at least two different peptides and three valid intensity values in at least one group. To reveal differences between the groups, the significant analysis of microarrays method ($S_0 = 0.8$ for GS⁺ and GS⁻ hepatocytes or $S_0 = 0.6$ for GLT1⁺ and E-Cad⁺ hepatocytes, FDR = 5%; (Tusher et al. 2001)) based on Student's t -tests was applied to $\log_2$ -transformed values. This method includes permutations of repeated measurements to estimate the false discovery rate and, therefore, accounts for repeated measurements in this case several hundred/thousands of proteins. Before analysis, missing values were filled in with values drawn from a down-shifted normal distribution (width 0.3, downshift 1.8 standard deviations). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2019) partner repository (dataset ID: PXD023561).

Proteins showing significant differences in the aforementioned comparisons were separately analyzed for higher and lower abundant proteins by STRING v11 (Szklarczyk et al. 2019). Here, a network analysis was carried out for categorical enrichment and network analysis. Only high confidence interactions were considered from

textmining, experiments, databases and co-expression. Respective proteins have been further analyzed by STRING for enriched gene ontology biological process terms. Only terms were reported which were associated with a Benjamini-Hochberg corrected p -value < 0.05.

Western blot

For Western blot analysis, liver tissue was homogenized using a pistol and thoroughly lysed in ice-cold lysis buffer (10 mM Tris pH 7.4, 1% (v/v) Triton X-100, 0.5% (v/v) NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 0.2 mM PMSF). Cell debris was removed from soluble proteins by centrifugation at 20,000 g at 4 °C for 10 min. Protein concentrations of liver homogenates were determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). Equal amounts of protein mixed with gel loading buffer were utilized for polyacrylamide gel electrophoresis on 10–15% SDS polyacrylamide gels as described before (Görg et al. 2019; Qvartskhava et al. 2015). After electrophoresis, proteins were transferred on nitrocellulose membranes by semi-dry blotting technique. Membranes were incubated in 5% BSA in Tris-buffered saline containing 0.01% (v/v) Tween-20 (TBST) for 30 min. Subsequently, membranes were incubated with anti-GAPDH antibody (1:5000, #H86504M, Biodesign International, Saco, USA) for 1 h at RT, while incubation with antibodies directed against GS (1:1000, #610518, BD Bioscience), GLS2 (1:1000, #ab93434, Abcam) were conducted at 4 °C RT overnight. Membranes were then washed three times with TBST and incubated with horseradish peroxidase (HRP)-coupled secondary antibodies at a concentration of 1:10,000, for 1 h at RT (HRP-coupled goat-anti mouse antibody [#170-6516, Bio-Rad Laboratories]; HRP-coupled goat-anti rabbit antibody [#P0448, Dako, Biozol, Echig, Germany]; HRP-coupled mouse-anti rabbit antibody [#sc2357, Santa Cruz, Heidelberg, Germany]). Unbound antibodies were removed by washing the blots three times with TBST. HRP activity was detected using ECL Western Blotting Substrate (Promega, Walldorf, Germany) and images were acquired using ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories). Densitometric analysis was performed using Image Lab software (Bio-Rad Laboratories). Relative protein levels of a given protein were quantified by analyzing the raw signal intensities from the digitally acquired pictures and normalizing to GAPDH levels for each sample.

Immunofluorescence staining

Cryosections (7 μm) of mouse and rat livers were prepared using the Leica Cryostat CM1950 (Leica Biosystems, Wetzlar, Germany). Sections were fixed with ice-cold 100% methanol for 10 min and washed three times with PBS. Sections were blocked with 5% BSA in PBS for 30 min and incubated with primary antibody solutions at 4 °C overnight. Anti-GS (#610518, BD Bioscience) and anti-RhBG (#ab106801, Abcam) antibodies were used at a concentration of 1:500, while anti-GLS2 (#ab93434, Abcam), anti-CPS1 (#ab3682, Abcam), anti-GLT1 (#ab41621, Abcam), anti-E-Cad (#610182, BD Biosciences), anti-HSP25 (#ADI-SPA-801-F, Enzo Life Sciences, Lörrach, Germany), and anti-BTF3 (#ab203517, Abcam) were used at 1:200 in blocking buffer. Following three washing steps with PBS, sections were incubated with fluorochrome-coupled secondary antibodies (1:500) and Hoechst 34580 (1:20,000) for counterstaining of the nuclei for 1 h at RT. Sections were mounted with Fluoromount-G (Thermo Fisher Scientific) and immunofluorescence analysis was performed using the epifluorescence microscope Observer.Z1 (ZEISS, Ober-

kochen, Germany) or confocal laser scanning microscopy (LSM510 and LSM880, ZEISS).

Quantification of GLS2⁺ and GLS2[−] hepatocytes in the liver

GLS2⁺ and GLS2[−] hepatocytes were quantified in fixed liver slices costained with anti-GLS2 antibodies anti-GLS2 (#ab93434, 1:200, Abcam) and phalloidin-TRITC (#P1951, 1:200, Sigma-Aldrich). Per liver slice 5–10 immunofluorescence pictures were acquired using an epifluorescence microscope (dx25, ObserverZ.1, ZEISS). Individual hepatocytes were discriminated by membranous f-actin staining. Numbers of hepatocytes along the acinus were determined in each picture straight from the centers of the portal to the central vein. The number of GLS2⁺ hepatocytes surrounding one portal vein was determined in triplicates, averaged and given relative to the total number of hepatocytes.

Statistical analysis

Data are presented as means $\pm$ standard error of the mean (SEM). Statistical differences between the groups were determined using Student's *t*-test or one way analysis of variance (ANOVA) followed by Tukey's multiple comparison *post hoc* test (GraphPad Prism V5.01, La Jolla, USA) as appropriate. A *p*-value ≤ 0.05 was considered significant.

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