Wnt ligand and receptor patterning in the liver

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ABSTRACT
In the liver, the tight spatiotemporal regulation of Wnt/β-catenin signaling is required to establish and maintain a metabolic form of tissue polarity termed zonation. In this review, we discuss the latest technologies applied in the study of liver zonation and provide a summary of the Wnt ligand and receptor expression patterns in the hepatic lobule. We further discuss the mechanisms by which Wnt instructive cues might be spatially confined and propagated along the central vein-portal triad axis.

Introduction

Mammals rely on the liver for the detoxification of xenobiotics, the metabolism of lipids, the biosynthesis of plasma proteins and the modulation of glucose levels [1]. The efficient coupling of these diverse processes is accomplished by the spatial organization of physiological tasks in different metabolic zones of the liver lobule zonation. Disturbances in this 'zonation' are common in liver pathologies including fatty liver disease, cirrhosis, hepatitis and hepatocellular carcinoma [2]. Liver zonation may be an exemplar of how physiological tasks can be spatially-separated amongst cells of the same apparent cell type and how functional polarity is maintained in the structural subunits of organs.

Hepatocyte zonal specialization is closely linked to the architecture of the fundamental structural unit of the liver, the hepatic lobule (Fig.1A). In this unit, hepatocytes are organized into cords between a hepatic central vein and the portal triad, at which point blood flows in from the periphery of the lobule towards the central vein. Gradients of oxygen, nutrients and growth factors in combination with local cell-cell interactions generate a complex microenvironment along the porto/venous axis that shapes the transcriptional program of these cells. Although many molecular cascades have been suggested to regulate zonation, the best data to date relates to the role of Wnt pathway and it is estimated that ≈30% of zonated genes are under the control of the transcription factor β-catenin, the main downstream effector of the canonical pathway [3-7].

Several liver resident cell types are source of Wnt ligands and Wnt pathway modulators [8-13]. Deletion of Wls, a membrane protein dedicated to the secretion of Wnt proteins, from hepatocytes and cholangiocytes (Alb-Cre; $Wls^{fl/fl}$), liver-resident Kupffer (Lyz2-Cre; $Wls^{fl/fl}$) and stellate cells (Lrat-Cre; $Wls^{fl/fl}$) in the developing liver did not alter liver zonation [11-13]. By contrast, Wnt cues of angiocrine origin are essential for zonation to occur as depletion of Wls in the endothelial compartment during embryogenesis (Lyve1-Cre; $Wls^{fl/fl}$ and Stab2-Cre; $Wls^{fl/fl}$) or adulthood (VE-Cadherin-CreERT2; $Wls^{fl/fl}$) prevents the establishment and maintenance of zonated targets including RhBg, Cyp2e1, Cyp1a2, Axin2, and GS [8-10].

Endothelial cells in the hepatic lobule include (1) liver sinusoidal endothelial cells (LSECs) that line the sinusoids and (2) vascular endothelial cells comprising endothelial cells from the central vein, the hepatic artery and the portal vein. *In situ* hybridization (ISH) and RT-qPCR

experiments showed that the endothelial cells of the central vein express Wnt2 and Wnt9b (Fig.1B) [14] [8] [9]. Central vein endothelial cells were also shown to express Rspo3, an indirect activator of Wnt signaling (Fig.1B) [14]. Rspo ligands bind Lgr4/5 receptors, inactivating the Rnf43/Znrf43 ubiquitin ligases that would otherwise degrade Fzd and Lrp receptors at the cell surface (Fig.1B) [15]. Rocha et al., (2015) showed that loss of Rspo3 in embryos (from E16.5) or in the adult mouse, using a tamoxifen-inducible pan cre-recombinase (cCAG-CreERT2: $Rspo3^{fl/fl}$), abolished pericentral GS protein levels without altering Wnt2 and Wnt9b gene expression. These results suggest that angiocrine expression of both Wnt and Rspo ligands is critical for zonation [14].

The centrolobular location of the central vein places these endothelial cells as the putative source of the Wnt/β-catenin pathway activation gradient that spreads out from the center of the lobule (Fig.1A) [4-6,8]. However, recent 'profiling' studies have identified additionally complexity in the patterns of Wnt ligand and receptor expression across the liver lobule, including expression outside the central vein domain during homeostasis [7,16-19]. These potential Wnt ligand/receptors interactions constitute an additionally layer of complexity to the spatiotemporal control of liver physiology that hasn't yet been fully explored. In this review, the latest technical approaches used to profile liver gene expression are reviewed and the patterns of Wnt ligands and receptors revealed are discussed in relation to hepatic function.

Molecular profiling techniques to decipher liver zonation

Laser capture microdissection (LCM) is a technique that allows the molecular profiling of small tissue areas with the guidance of histological features. In the context of the liver, McEnerney et al (2017) and Brosch et al (2018) used this technique to assign human mRNA expression profiles to the pericentral, midlobular and perivenous regions of the lobule (Fig.2A) [18,19]. Additional strategies have been developed to profile gene and protein expression patterns in the liver with a higher resolution than the 3 zones. In 2017, Bahar Halpern and colleagues generated the first transcriptomic map of murine hepatocytes lobule with a near single-cell resolution (layers 1-9) using single cell RNAseq (scRNAseq) spatial reconstruction (Fig.2B) [7]. scRNAseq spatial reconstruction is a technical approach in which a panel of well-characterized 'landmark' zonated genes is used to infer the zonal location of single cell-sequenced hepatocytes. A similar transcriptomic zonal resolution has been achieved by "spatial sorting", a technique described in a recent preprint from Ben-Moshe and colleagues. Spatial sorting of hepatocytes is based on the differential cell surface expression of perivenous (CD73) and periportal markers (E-cadherin) allowed the separation of hepatocytes into 8 overlapping compartments by flow-cytometry (Fig.2C) [16].

Molecular profiling has also been extended to endothelial cells. Liver sinusoidal endothelial cells (LSECs) have been reported to express zonally-restricted genes [10,20]. In 2018, Bahar Halpern and colleagues embraced the challenge to uncover the zonal transcriptomics of LSECs [17]. As a panel of zonal landmark genes was not available for LSECs, Bahar Halpern and colleagues developed a technique termed paired-single cell RNAseq (pcRNAseq). pcRNAseq uses the zonal identity of hepatocytes to infer and map the spatial location of LSECs by sorting hepatocyte-endothelial cell doublets for the endothelial marker CD31 following incomplete liver tissue digestion (Fig.2D). Using this technique, Bahar Halpern identified a panel of 140 LSECs zonated genes that allowed the subsequent clustering of individually sequenced LSECs

into 4 zones. It should be noted however, that while CD31+ is a well-accepted vascular endothelial cell marker, its expression levels in LSECs is contentious [20-24].

In this review, the gene expression patterns of Wnt ligands and receptors from these studies have been combined and integrated in Fig.3 [7,16-18]. For an analysis of the wider implications of differential gene expression patterns in other signaling cascades, see the excellent review of Ben-Moshe and colleagues [25].

Zonal profiling reveal asymmetries in Wnt pathway upstream components

The reconstructed LSEC and hepatocytes zonation profiles by Bahar Halpern revealed a high degree of complexity in the patterns of Wnt-related ligands and receptors (Fig.3A) [7,17]. Technically, the approach was validated by the observation that Rspo3 was expressed in layer 1 endothelial cells, possibly corresponding to the central vein [14]. However, Wnt2 and Wnt9b were also expressed in LSECs layers 2 and 3 (Fig.3A). A number of additional Wnt ligands were shown to be expressed in LSECs including Wnt4, Wnt5a, Wnt5b, Wnt7b, Wnt8b and Wnt11, although only Wnt11 appeared to have the same pattern of pericentral expression as observed for Wnt2 and Wnt9b (Fig.3A) [7]. Wnt2b, Wnt3, Wnt9a and Wnt10a that were previously reported to be expressed by RT-qPCR in LSECs were not detected by this approach (Fig.3A) [26].

As previously reported, receptors for Rspo (Lgr4/5) and Wnt ligands (Fzd and Lrp5/6) were expressed in hepatocytes but their distribution was complex [7,12,14,26,27]. In accordance to Planas-Paz et al. (2016) studies, Lgr4 was ubiquitous expressed across the lobule while Lgr5 expression was restricted to layer1 hepatocytes [7,27]. Expression of the E3 ubiquitin ligases Rnf43 and Znfr3 was also found restricted to pericentral hepatocytes (Fig.3A) [7]. The physiological importance of the Rspo3-Lgr4/5-Rnf43/Znrf3 module has been demonstrated by the finding that deletion of Rnf43 and Znrf43 induced the expansion of pericentral GS and Cyp2e1 staining (Fig.3A) [27]. Sole deletion of Lgr4 caused a dramatic decline in GS and Cyp2e1 staining while a function for Lgr5 in maintaining pericentral Wnt target expression was only revealed following the loss of Lgr4 [27]. Thus, non-pericentrally expressed receptors might still crucial for zonation to occur.

In the human liver, the expression of the ligands WNT2, WNT11 and RSPO3 was enriched in pericentral domains that included both hepatocytes and endothelial cells, mirroring the patterns observed in murine LSECs (Compare Fig1.A and C) [7,18,19]. By contrast, WNT4 and DKK3 showed opposite polarities of expression to that in the mouse while RSPO2 was only detected in human samples (Fig. 3.A, C). In total, only 1/3 of the 313 zonated genes Brosch and colleagues identified had the same zonal distribution as those identified by Bahar Halpern et al (2017) [7,18]. These apparent discrepancies might result from interspecies differences, but could also be related to differences in technical approach as Brosch approach captures microenvironment transcriptomics rather than pure hepatocytes gene expression patterns.

A high level of overlap was detected when comparing hepatocyte zonation patterns based on the ‘scRNAseq spatial reconstruction’ and the ‘spatial sorting’ approaches, with the exception of layer 1 hepatocytes in which the spatial sorting approach failed to detect expression of some receptors (compare Fig.3A and B) [7,16]. However, data from the sorting approach (Fig.3B) might need to be interpreted with caution as analyses were restricted to 4n cells.

Hepatocytes surrounding the central vein are predominantly 2n and therefore this population might have not been accurately represented in Ben-Moshe approach [8,16].

The expression of Fzd receptors in liver endothelial cells has been previously reported and was confirmed by the scRNAseq studies (Fig.3A) [17,26]. This raised the possibility that Wnt-β-catenin signalling regulates function within LSECs. The fact that multiple Wnt ligands including Wnt2, Wnt4, Wnt5a, Wnt5b Wnt9a, Wnt9b and Wnt11 were expressed in the hepatocytes suggested the possibility that hepatocytes might signal in reverse to endothelial cells [7,26]. This reverse signaling however, is not expected to feedback to hepatocyte zonation as early deletion of Wls in hepatocytes (Alb-Cre; $Wls^{fl/fl}$) did not appear to alter zonation [7,11,12]. The physiological processes that the Wnt/Rspo/Fzd/Lgr/Lrp receptor pairs regulate remain unclear.

Loss of Wls during hepatocyte, cholangiocyte (Alb-Cre; $Wls^{fl/fl}$) or Kupffer cell development (Lyz2-Cre; $Wls^{fl/fl}$) delayed liver regeneration after partial hepatectomy, without causing obvious alterations in homeostatic liver zonation [11,12]. By contrast, Wnt signaling is activated and required for recovery from injury to both periportal and perivenous domains [9,28-30]. As part of the response to injury, Wnt and Rspo ligands are also induced, but the relative contributions of endogenous and induced ligands have not been determined [9,28-31]. It is also currently unknown whether hepatocyte-expressed Wnts could be involved in signaling to cholangiocyte, stellate or Kupffer cells.

Mechanisms of Wnt signaling propagation and decay

A consideration of the different mechanisms by which Wnt signals are propagated can aid understanding of the means by which Wnt activation is fine tuned in the liver.

Ligand diffusion, transcytosis and cytoneme projection.

The direct contact between Rspo-/Wnt-producing central vein endothelial cells can account for the activation of Wnt targets in the first layer of hepatocytes (juxtacrine signaling), but does not of itself explain how Wnt-dependent patterning extends across subsequent layers. A simple ligand 'diffusion model' might not be sufficient to explain how a concentration gradient is established, as diffusion of hydrophobic Wnt-ligands would have to occur against the direction of blood-flow (Fig4.A). However, alternative Wnt diffusion-independent mechanisms have been proposed in other organisms including the transcytosis of Wnts and via the projection of specialized philopodia termed cytonemes (Fig4.A) [32].

Local Wnt presentation model

Bahar Halpern studies reveal that Rspo3 expression was restricted to centrally-located endothelial cells but Wnt2 and Wn9b expression extended to the first two zonal 'layers' of LSECs on the pericentral-periportal axis (Fig.3A) [7,17]. While central vein endothelial cells might constitute a strong 'juxtracrine' Wnt/Rspo niche for layer1 hepatocytes, Wnt signaling in hepatocyte layers2 to 4 might be supported by juxtacrine Wnt signaling from pericentral LSECs which express Wnt2, Wnt4, Wnt5b and Wnt9b (Fig.3A, Fig.4B) [17]. It has been shown that Wnt9b synergizes with Wnt2 and Wnt4 to activate B-catenin [33]. Wls KO experiments in the endothelial cell compartment performed by Leibing (2018) and Preziosi (2018) abolished Wnt secretion in both the central vein and LSECs altering hepatocyte zonation

[9,10]. The contribution of pericentral LSECs to the Wnt-zonation patterning needs to be determined.

Cell memory model

In mammalian tissues such as the intestine, Wnt activation gradients are thought to, in part, depend on the inheritance of a Wnt-activated cell state. When stem cells close to the Wnt source divide, it has been suggested that they inherit membrane-associated Wnt ligands and a Wnt-activated state during the hours that follow cell division [15]. Results from fate mapping of the hepatocyte population lining the central (described as Axin2+) has been used to suggest that pericentral (high Wnt) hepatocytes self-renew to replace more distally-located hepatocytes (Fig.4C) [8]. However, this model may not be consistent with the low homeostatic rates at which hepatocytes are replacement by cells originating at the central vein which is much smaller than that in the intestinal crypt [8,15].

Opposing signals model

Benhamouche et al (2006) and Planas-Paz et al (2016) noted that hepatocyte-specific deletion of the downstream Wnt negative regulator APC (TTR-CreERT2; $APC^{fl/fl}$) did not cause the immediate ectopic expression of pericentral markers in periportal areas [6,27]. Instead, histological spreading of pericentral markers (Axin2, GS, Rnase4, RhBg, Glt1 and Lect2) occurs in a step-wise manner across the lobule in a pericentral to periportal direction. This observation is not consistent with the fast responses typically associated with Wnt pathway activation, suggesting that hepatocytes are not simply poised to respond to instructive Wnt signals but are instead subjected to spatial-temporal inhibitory mechanisms that actively repress Wnt propagation (Fig.4D). A range of potential Wnt inhibitors and pathways have been implicated in dampening Wnt signaling. Most directly, it has been shown that Wnt pathway inhibitors such as DKK3 and sFRP5 are expressed periportally (Fig.3A, Fig.4D) [18,34,35]. Nine percent (298 genes out of 3,496 genes) of zonated genes appear to be modulated by Ras-dependent mechanisms and expression of EGFR was highly enriched in periportal hepatocytes [36,37]. While no genetic model has yet proven that EGF signaling represses the pericentral signature, a recent organoid protocol showed that EGF-based media induced expression of periportal genes [38]. Other systemic factors may similarly regulate Wnt pathway activity and zonation. For example, glucagon may repress Wnt-driven zonation since its loss led to an enlargement of the GS pericentral area while thyroid hormones had the opposite effect [36]. The comprehensive LCM-based analysis of Brosch et al (2018) also implicated Notch/Notum signaling as potential Wnt negative regulator in the portal area [18]. Finally, a polarized gradient of E-cadherin expression that is highest at the portal triad may negatively regulate β-catenin transcriptional activity by binding β-catenin at the cell surface [10,14,39].

Wnt signaling appears to actively antagonize the expansion of the periportal signature as abolition of Wnt signaling by loss of Wls triggered the progressive histological invasion of periportal markers such as E-cadherin and Arg1 into the pericentral areas instead of just the leading to the loss of central marker expression [10,14].

Final remarks and conclusions

Pericentral Wnt morphogen gradients control functional hepatocyte specification and mammals seem to have developed strategies to repress Wnt signaling in periportal areas.

Stringent regulation of Wnt signaling in the hepatocellular compartment is achieved by the local production of Wnt ligands and Wnt pathway modulators. In this end, the latest liver zonation studies driven by single cell sequencing, LCM and spatial sorting might provide the sufficient spatial resolution to uncover possible Wnt ligand-receptors specificities that haven't been explored yet *in vivo*. Spatial confinement of such pairs might add an additional layer of spatiotemporal control to the system. Further studies will be needed to evaluate the contribution of such ligand/receptors pairs to both metabolic zonation and liver regeneration.

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Conflict of interest
Nothing declared.

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Figures

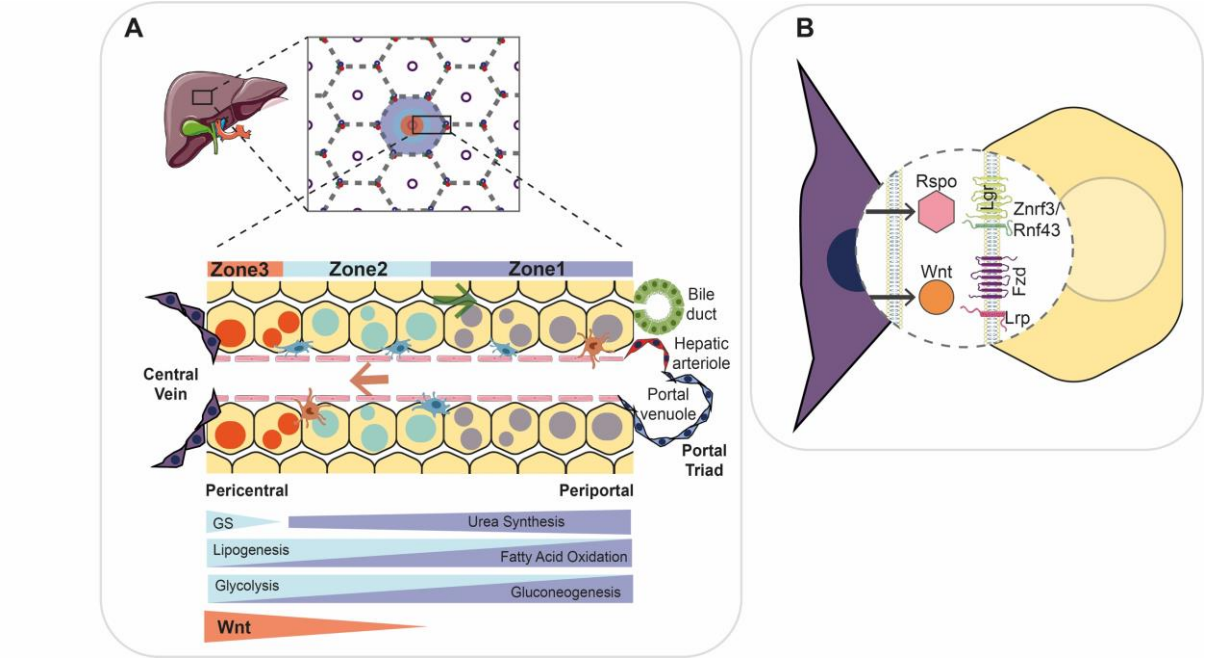


Figure1. The structure of the liver lobule. (A) Structure of the zonated liver lobule. The liver is organized in polygonal lobules. Blood flows from the portal venuole and hepatic arteriole through sinusoids to the central vein. 14 to 25 hepatocytes escorted by liver sinusoidal endothelial cells (LSECs) are arranged in branching cords. Between the sinusoidal endothelial cells and the hepatocytes, a space (space of Disse) host other specialized cell types including phagocytic cells (Kupffer cells), stellate cells and occasionally, myofibroblasts. Bile produced by the hepatocytes is secreted into bile canaliculi and transported to bile ducts formed by cholangiocytes. Green and red arrows indicate the direction of the bile and blood, respectively. Hepatocytes have traditionally been described as having three different metabolic zones (zone1-3**). (B)** Endothelial cells from the central vein are source of Wnt and Rspo ligands that activate Wnt/b-catenin signaling in hepatocytes.

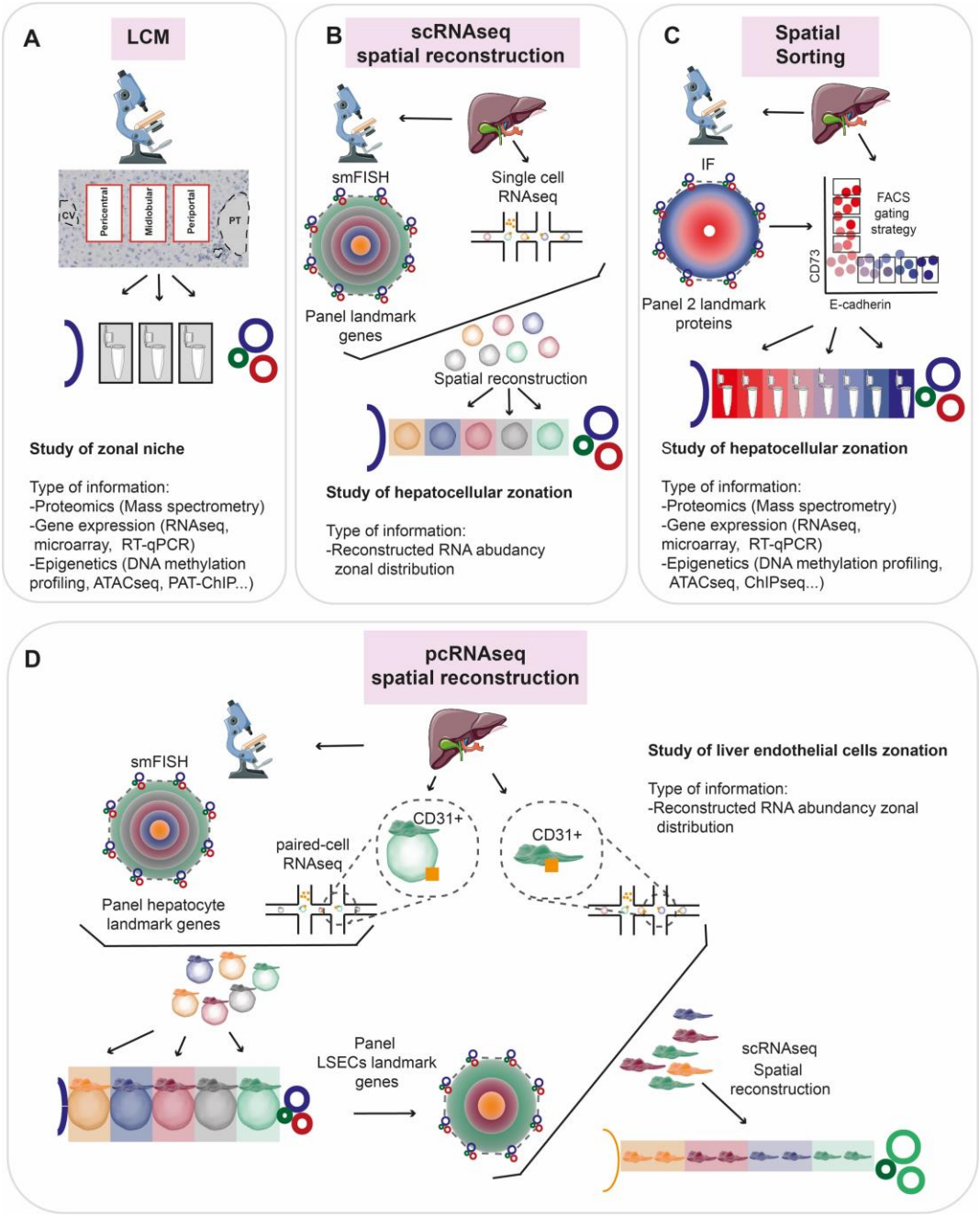
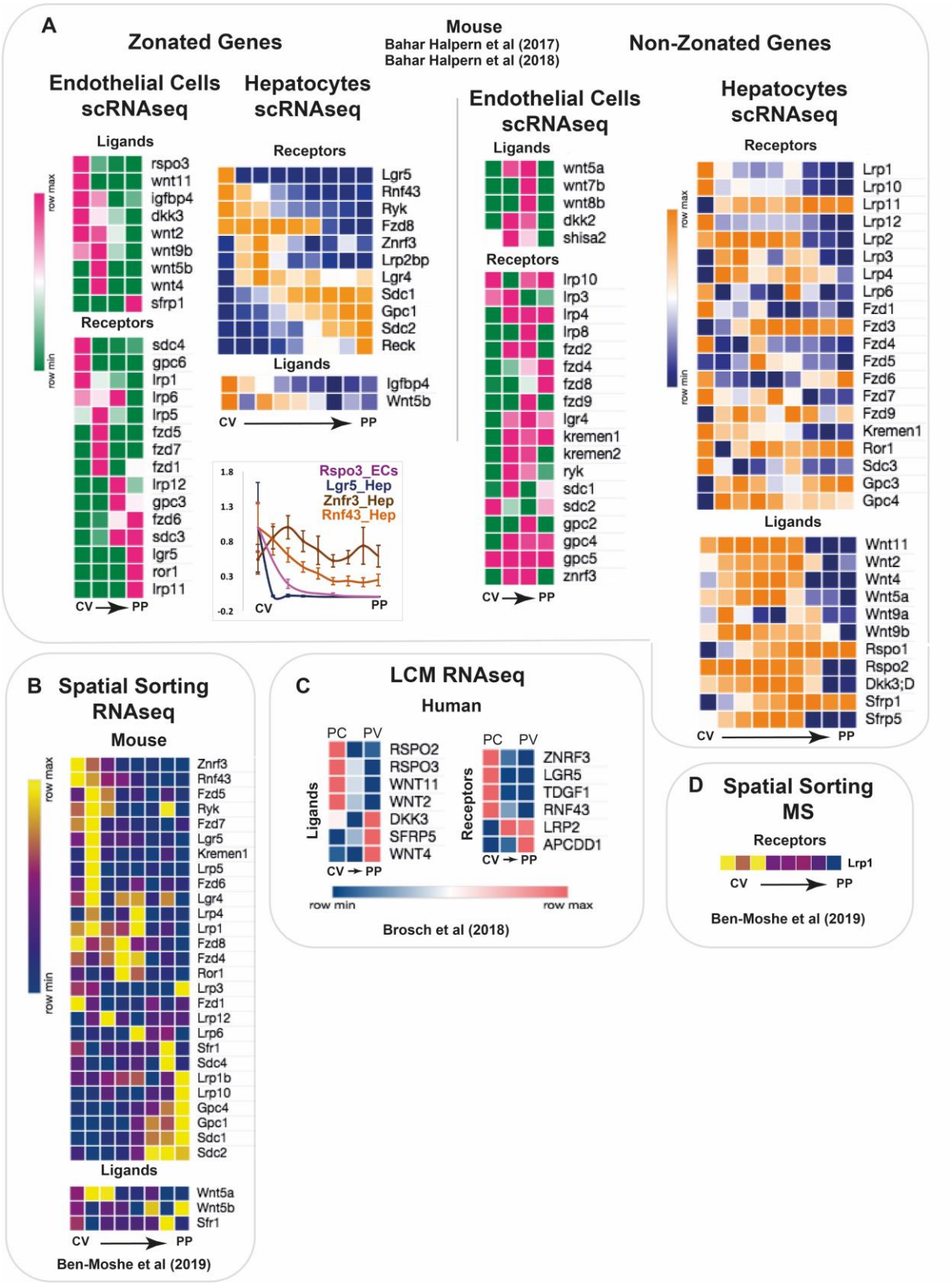


Figure2. Strategies for the study of liver zonation (A) Laser capture microdissection (LCM) allowed the molecular profiling of small liver tissue areas from pericentral, midlobular and perivenous areas that aimed to exclude portal triad, connective tissue and central vein cells [18,19]. Figure adapted from [19]. **(B)** Single cell RNAseq Spatial (scRNAseq) Reconstruction approach of Bahar Halpern et al. (2017) [7]. A panel of 6 landmark genes (Glul, Cyp2e1, Ass1, Asl, Alb and Cyp2f2) with differential zonal mRNA expression validated by smFISH was used to infer the location of individual hepatocytes in 9-zones in the lobule. **(C)** Spatial Sorting strategy from Ben-Moshe et al. (2019) based on the differential surface expression of two landmark proteins (periportal E-cadherin and pericentral CD73) for the 8-way zonal separation of hepatocytes by FACS[16]. **(D)** Paired single-cell RNAseq (pcRNAseq) developed by Bahar Halpern et al. (2018) was based on the isolation and sequencing of hepatocyte-endothelial pairs as cell doublets by flow cytometry[17]. Such pairs were identified by the doublet size (FSC-A and SSC-A parameters) and surface levels of CD31. The location of the associated LSECs was inferred using the hepatocyte RNA complement as guide. This generated a panel of 140 LSECs landmark genes were used to map-back the location of the individually sequenced endothelial cells to 4 different zones.


A
Mouse
Bahar Halpern et al (2017)
Bahar Halpern et al (2018)
Zonated Genes
Non-Zonated Genes
Endothelial Cells scRNAseq
Hepatocytes scRNAseq
Endothelial Cells scRNAseq
Hepatocytes scRNAseq
Ligands
Receptors
row max
row min
rspo3
wnt11
igfbp4
dkk3
wnt2
wnt9b
wnt5b
wnt4
sfrp1
sdc4
gpc6
lrp1
lrp6
lrp5
fzd5
fzd7
fzd1
lrp12
gpc3
fzd6
sdc3
lgr5
ror1
lrp11
CV
PP
Lgr5
Rnf43
Ryk
Fzd8
Znrf3
Lrp2bp
Lgr4
Sdc1
Gpc1
Sdc2
Reck
Igfbp4
Wnt5b
Rspo3_ECs
Lgr5_Hep
Znfr3_Hep
Rnf43_Hep
1.8
1.3
0.8
0.3
-0.2
wnt5a
wnt7b
wnt8b
dkk2
shisa2
lrp10
lrp3
lrp4
lrp8
fzd2
fzd4
fzd8
fzd9
lgr4
kremen1
kremen2
ryk
sdc1
sdc2
gpc2
gpc4
gpc5
znrf3
Lrp1
Lrp10
Lrp11
Lrp12
Lrp2
Lrp3
Lrp4
Lrp6
Fzd1
Fzd3
Fzd4
Fzd5
Fzd6
Fzd7
Fzd9
Kremen1
Ror1
Sdc3
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Wnt4
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Wnt9a
Wnt9b
Rspo1
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Dkk3;D
Sfrp1
Sfrp5
B Spatial Sorting RNAseq
Mouse
Znrf3
Rnf43
Fzd5
Ryk
Fzd7
Lgr5
Kremen1
Lrp5
Fzd6
Lgr4
Lrp4
Lrp1
Fzd8
Fzd4
Ror1
Lrp3
Fzd1
Lrp12
Lrp6
Sfr1
Sdc4
Lrp1b
Lrp10
Gpc4
Gpc1
Sdc1
Sdc2
Wnt5a
Wnt5b
Sfr1
Ben-Moshe et al (2019)
C LCM RNAseq
Human
PC
PV
RSPO2
RSPO3
WNT11
WNT2
DKK3
SFRP5
WNT4
ZNRF3
LGR5
TDGF1
RNF43
LRP2
APCDD1
row min
row max
Brosch et al (2018)
D Spatial Sorting MS
Receptors
Lrp1
Ben-Moshe et al (2019)

Figure3. Distribution of Wnt pathway receptors along the hepatic lobule. (A) Hepatocyte and liver endothelial transcriptomic profiles were obtained by hepatocyte and LSECs scRNAseq together with hepatocyte-endothelial cell pcRNAseq, respectively[7,17] Hierarchical clustering was applied to the patterns of expression. Zonated genes were defined as those with either (1) significant differential expression between layer 1 and 9 when divided by the mean over all layers (monotonically zonated) or (2) where expression levels between layer 3 and 7 was 10% higher than in layer 1 and 9 (non-monotonically zonated) based on the authors' statistical criteria[7]. An example of the expression profiles of 3-hepatocyte monotonically zonated receptors (Lgr5, Znfr3 and Rnf43) and one monotonically zonated LSEC ligand (Rspo3) is shown with a normalized Y-axis against an X-axis representing the position on the central vein (CV)- periportal (PP) axis. The LSEC Rspo3 profile was 'stretched' to match the 9-layer hepatocyte template. **(B)** Heat maps with relative RNAseq expression values from spatially sorted murine hepatocytes[16]. (C) Relative zonal gene expression of human liver samples. Tissue from 3 regions (zone 1 to 3) containing both hepatocytes and endothelial cells was obtained by LCM and analyzed by RNAseq. Central vein and portal triad connective tissue were excluded from the analysis [18]. (D) Relative protein distribution obtained by mass spectrometry from Spatially sorted murine hepatocytes [18]. Heat maps generation and hierarchical clustering were performed using the Broad Institute online tool Morpheus. (https://software.broadinstitute.org/morpheus/). Supplementary table shows a list with the genes included in the analysis.

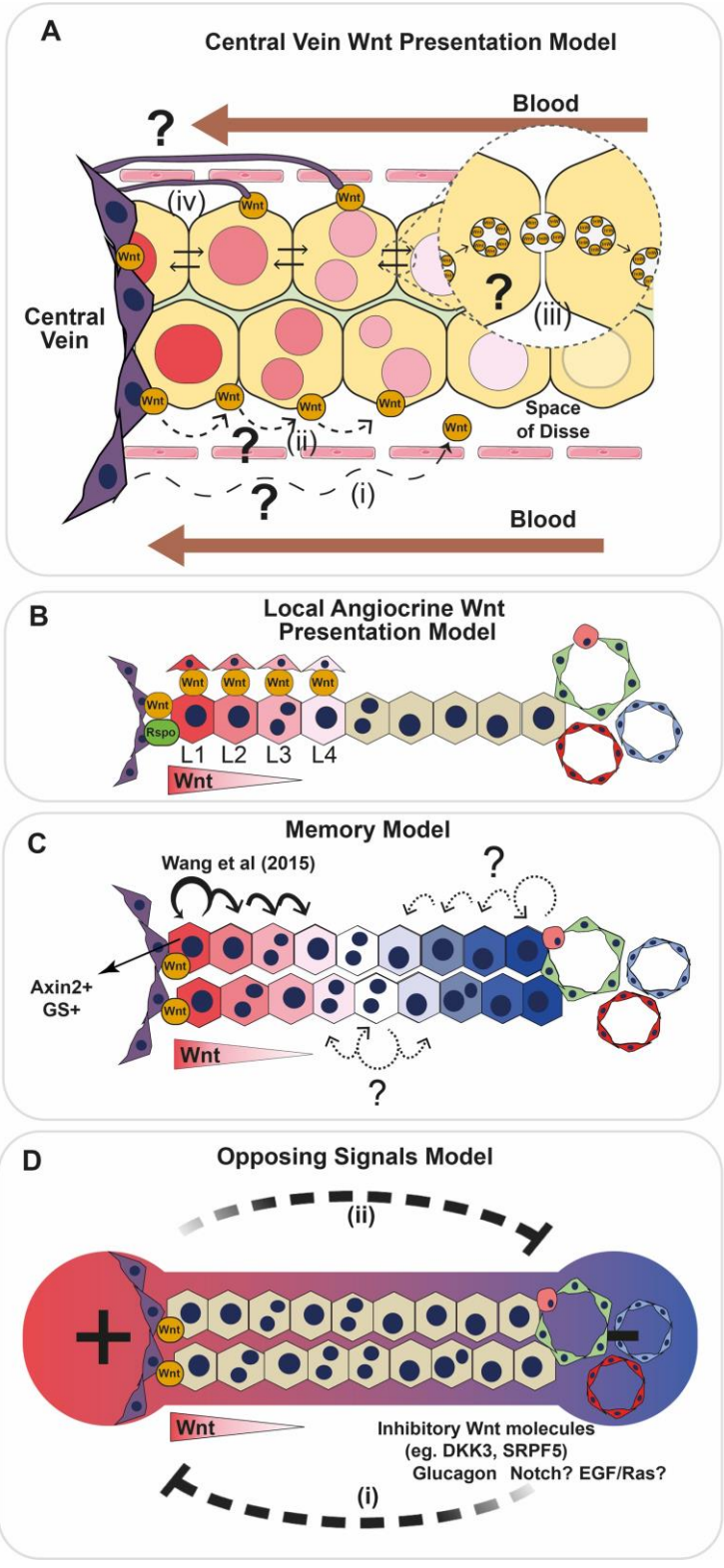

Figure4. Mechanisms for Wnt signalling propagation and decay. (A) Central vein endothelial cells provide local Wnt/Rspo signals sensed by layer 1 hepatocytes in a juxtacrine manner. Transmission of Wnt to hepatocytes of layers 2 to 4 might occur through **(A, i)** the diffusion of Wnt-instructive signals. The soluble diffusion of Wnt ligands bound to carrier proteins or in exosomes is unlikely as aqueous phase material would have to travel up to 6 hepatocyte layers against the direction of blood flow. Wnt ligands may alternatively **(A,ii)** diffuse within cell surface heparin sulfate proteoglycans or in association with the lipid bilayer of LSECs or hepatocytes. **(A,iii)** Wnt could potentially be transmitted through transcytosis or may be projected from secreting cells to hepatocytes via **(A,iv)** cytonemes which can transmit signals over distances of up to 150um in other biological systems[32]. **(B)** Wnt and Rspo from the central vein are presented to layer 1 (L1) hepatocytes. Moderate Wnt activation in L2 to L4 hepatocytes is sustained by juxtacrine signaling from pericentral LSECs. **(C)** Hepatocytes lining the central vein (Axin2+) self-renew giving rise to daughters that inherit a Wnt-ON state of pathway activation that subsequently decays during migration away from the central vein. Sixty to seventy percent of the Wnt-OFF portal parenchyma was not replenished by hepatocytes that were suggested to have originated adjacent to the central vein (Axin2+)[8]. **(D)** Hepatocytes are subjected to periportal-derived inhibitory mechanisms that repress Wnt signaling. Wnt signaling may also actively antagonize the expansion of the periportal signature.