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REVIEW

The Hepatic Central Vein: Anatomical and Molecular Links to Perivenous Liver Biology

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ABSTRACT

The hepatic central vein, the initial branch of the hepatic vein, occupies the central position of the classic liver lobule. Endothelial cells of the central vein contain organelles showing activities for protein production and secretion. The endothelium is supported by a thin wall, which contains a collagenous matrix and few myofibroblasts. The ultrastructure of perivenous hepatocytes differs from that of periportal hepatocytes. Perivenous hepatocytes stably express glutamine synthetase serving as a reliable immunohistochemical cell marker. Central veins are a primary source of Wnt2, Wnt9b and R-spondin3. These angiocrines participate in the cellular-molecular circuitry of the Wnt/ β -catenin and R-spondin-LGR4/5-ZNRF3/RNF43 signaling

modules associated with perivenous hepatocytes. These signaling pathways regulate hepatic metabolic zonation and perivenous gene expression. The structures of the molecules of these pathways have been elucidated. Expression levels of glutamine synthetase and cytochrome P4502E1 are indicators of β -catenin activity in liver injury and regeneration. The production, transport and secretion of lipidated Wnts in Wnt-producing cells and their passage in the extracellular matrix towards responder cells have been described. The modes of Wnts and R-spondin3 release from central vein endothelial cells and their transit in the venular wall towards perivenous hepatocytes are unknown. GS⁺, Axin2⁺, Tbx3⁺, HNF4 α ⁺ and CPS⁻ perivenous hepatocytes were shown to exhibit stem cell properties, subserving homeostatic liver renewal. However, the existence of perivenous stem cells in the liver needs further investigations for validation. The hepatic central vein constitutes an anatomical niche of perivenous hepatocytes and provides a molecular link to perivenous hepatic biology.

Key words: Hepatic central vein; Glutamine synthetase; Wnt/ β -catenin signaling; R-spondin-LGR4/5-ZNRF3/RNF43 module; Cytochrome P-4502E1; Perivenous hepatocyte stem cells

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INTRODUCTION

Recent advances have indicated that the hepatic central vein provides a source of angiocrines Wnt2, Wnt9b^{1,2,3,4} and R-spondin (RSPO)^{3,5} that activate β -catenin signaling, leading to perivenous metabolic zonation and gene expression in the liver. In addition, the central vein has been considered as an anatomical niche of perivenous hepatic stem cells that subserve homeostatic hepatocyte renewal¹. These discoveries have disclosed an emerging role of the central vein in the mediation of liver biology and physiology. This article will cover the structure of the central vein and perivenous hepatocytes. The central vein as a primary source of Wnts and RSPO3 is presented. The participation of Wnts and RSPO3 in the cellular-molecular circuitry

of the β -catenin pathway and RSPO-LGR4/5-ZNRF3/RNF43 module is schematized. This leads to an overview of the signaling pathways of Wnt/ β -catenin and RSPO-LGR4/5-ZNRF3/RNF43, structures of the molecules of these pathways and their involvement in perivenous diverse cellular processes. The roles of glutamine synthetase (GS) and cytochrome P-450E1 (CYP2E1) in centrilobular liver injury and regeneration are summarized. The cellular transport and secretion of lipid-modified Wnt proteins require the Wntless protein in Wnt-producing cells, and secreted Wnts are carried on exosomes across the extracellular matrix to reach responder cells. The modes of Wnt2, Wnt9b and RSPO3 release from the central vein endothelium and their transit in the wall towards perivenous hepatocytes are unknown. The final part of the review will discuss the report implicating that the central vein constitutes an anatomical niche of perivenous axis inhibition protein 2 (Axin2)⁺ hepatocyte stem cells that replace lobular hepatocytes during homeostatic renewal.

HEPATIC CENTRAL VEIN AND CLASSIC LIVER LOBULE

The central vein is so named because of its central position in the classic liver lobule^[6]. It is also called terminal hepatic venule^[7], as it is the terminal branch of hepatic veins. The term centrilobular vein has occasionally been used^[8,9]. In humans, the central veins typically have a cross-sectional diameter of 25 to 150 μm with a mean of 75 μm ^[7,8,10].

The classic liver lobule, the first type of lobule to be described by Kiernan^[11], is a hexagon with a central vein at the center and portal tracts at each of the corners. In 3-dimensions--as illustrated in standard textbooks of Histology--the lobule presents as a hexagonal cylinder with its longitudinal axis 2 mm long and its diameter 0.7 mm wide^[6]. The ratio of the cylinder length to its diameter is approximately 3^[12]. As the central vein occupies the longitudinal axis of the lobule, its length is in the order of 2 mm. The lobular cylinder diameter actually shows differences between species. It is ~ 0.8 mm in the liver of humans^[13], ~ 0.7 mm of rats and ~ 0.4 mm of mice^[14]. Even the number of liver lobules differs among species: An adult human liver contains around 1-1.5 millions lobules (Liver Sieve Research Group Live Structure at University of Otago, Christchurch, NZ; 15), which are more than 20 times and 250 times as many lobules as a rat liver and a mouse liver, respectively^[12,16].

ANATOMICAL RELATIONSHIP BETWEEN HEPATIC CENTRAL VEINS, SINUSOIDS AND SUBLOBULAR VEINS: DRAINING AND BRANCHING PATTERNS

Hepatocytes radiate from the central vein toward the periphery of the classic lobule like the spokes of a wheel, forming anastomosing plates of liver cells, one-cell thick. The spaces between the plates are occupied by hepatic sinusoids--the liver capillaries. Because there is only one central vein in each lobule, it receives blood from every sinusoid of that lobule. Hepatic sinusoids draining into hepatic veins appear to differ among mammalian species. In humans and dogs, hepatic sinusoids empty solely into the central veins, whereas in rats, sinusoids drain into the central veins and the larger sublobular veins as well^[16,17,18]. Livers with these two draining patterns reflect poor and good drainage, respectively. Sinusoidal draining patterns in baboons, rabbits and sheep appear to be intermediate with openings seen in both central veins and sublobular veins, although the number

of sinusoids emptying into the larger vessels gradually decreases with increasing diameter of the draining vessels^[8,16]. The diameter of the central vein increases as it travels through the lobule. As the central vein (< 150 μm in diameter) leaves the lobule, it terminates in the sublobular vein (> 500 μm in diameter)^[16]. Multiple sublobular veins converge to form larger collecting veins that in turn form the right, middle and left hepatic veins. Branching patterns of the central veins also differ between humans and rats. In humans, a single central vein empties directly into the sublobular vein, whereas in rats, two to four central veins enter the sublobular vein^[16].

MORPHOLOGY OF CENTRAL VEIN ENDOTHELIUM AND ADVENTITIA OF VENULAR WALL

The central vein is a thin-walled vessel with numerous sinusoidal inlets draining the sinusoidal blood (Figure 1A). The openings are 5-20 μm wide, unlike the endothelial fenestrae of the sinusoids that are ~ 100 nm in diameter^[19]. The endothelium expresses Factor VIII-related antigen (Figure 1B), which is a systemic vascular phenotypic marker^[20], and is also immunoreactive for CD31 and CD34. The endothelium lining the lumen of the vein is attenuated, ranging from 1.5 μm in thickness to as thin as 0.04 μm thick, assessed by electron microscopy. As shown in Figure 2, the cytoplasm of endothelial cells contains vesicles for pinocytosis or exocytosis, coated vesicles for receptor-mediated endocytosis, and vacuoles for phagocytosis. Small channels of rough endoplasmic reticulum (RER) and scattered free ribosomes are present, while the Golgi apparatus appears small. Mitochondria, lysosomes and dense bodies are occasionally observed. The complement of organelles of the endothelium reflects that while the central vein is active in endocytosis and secretion, it is also involved in the synthesis of proteins such as Wnt2, Wnt9b and RSPO3.

The endothelium of the central vein is supported by an adventitia making up the wall. The wall thickness varies with mammalian species. In humans, the wall does not exceed 4 μm in width^[7]; in baboons, the wall does not exceed 8 μm ^[21]. As the venular diameter becomes greater, the wall thickness increases from 2.9 ± 1.5 μm in vein with a 25 μm in diameter to 8.9 ± 0.8 μm in vein with a diameter of 150 μm ^[8]. No data on the wall thickness are available for mice and rats; the wall is barely discernible at the light microscopic level.

The adventitia of the central vein wall contains types I, III, V and VI collagen (Figure 3A-D). Collagen V is a fibrillar collagen, regulating fibrillogenesis of collagen I and III, while collagen VI is a filamentous collagen and a connecting protein, linking fibrillar collagens to provide strength to the matrix scaffold of the vein^[22,23]. The collagenous matrix is further supported by the presence fibronectin, an adhesive connective tissue glycoprotein, which binds collagens. Among the collagen fibers are a few myofibroblasts, the dominant fibrogenic cells producing the fibrous matrix. Myofibroblasts are identified by immunostaining for α -smooth muscle actin^[24], as shown in Figure 2D, and by electron microscopy^[7]. The basement membrane collagen IV is regularly present, but laminin, another principal basement membrane protein is not detectable (Figure 3E and F), which may account for the lack of a morphologically identifiable continuous basement membrane beneath the venular endothelium^[25]. Electron microscopy revealed no structural contacts between the central vein and the first layer of perivenous (pericentral) hepatocytes surrounding the vein.

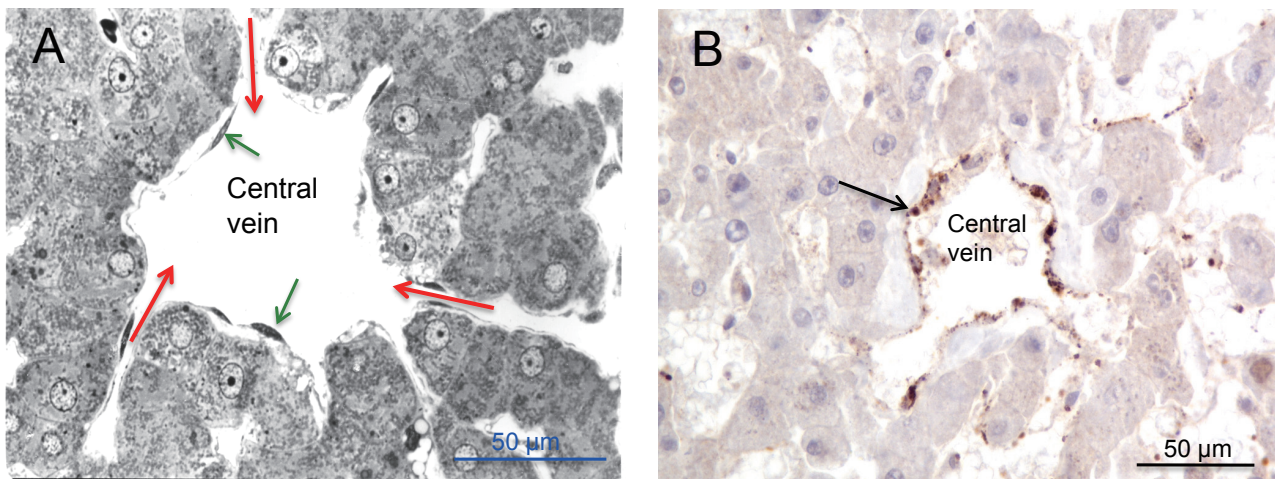


Figure 1 Hepatic central veins. A: One μm -thick plastic section, toluidine blue stain; human liver. Red arrows indicate sinusoidal inlets through the venular openings. Green arrows mark nuclei of endothelial cells. The lightly stained area between the endothelium and perivenous hepatocytes is the wall adventitia, 3-4 μm thick. B: Immunoperoxidase staining of central vein for Factor VIII-related antigen. The endothelial cell cytoplasm is clearly marked by immune deposits of Factor VIII-related antigen (brown). (From Mak et al., 2014; open access journal).

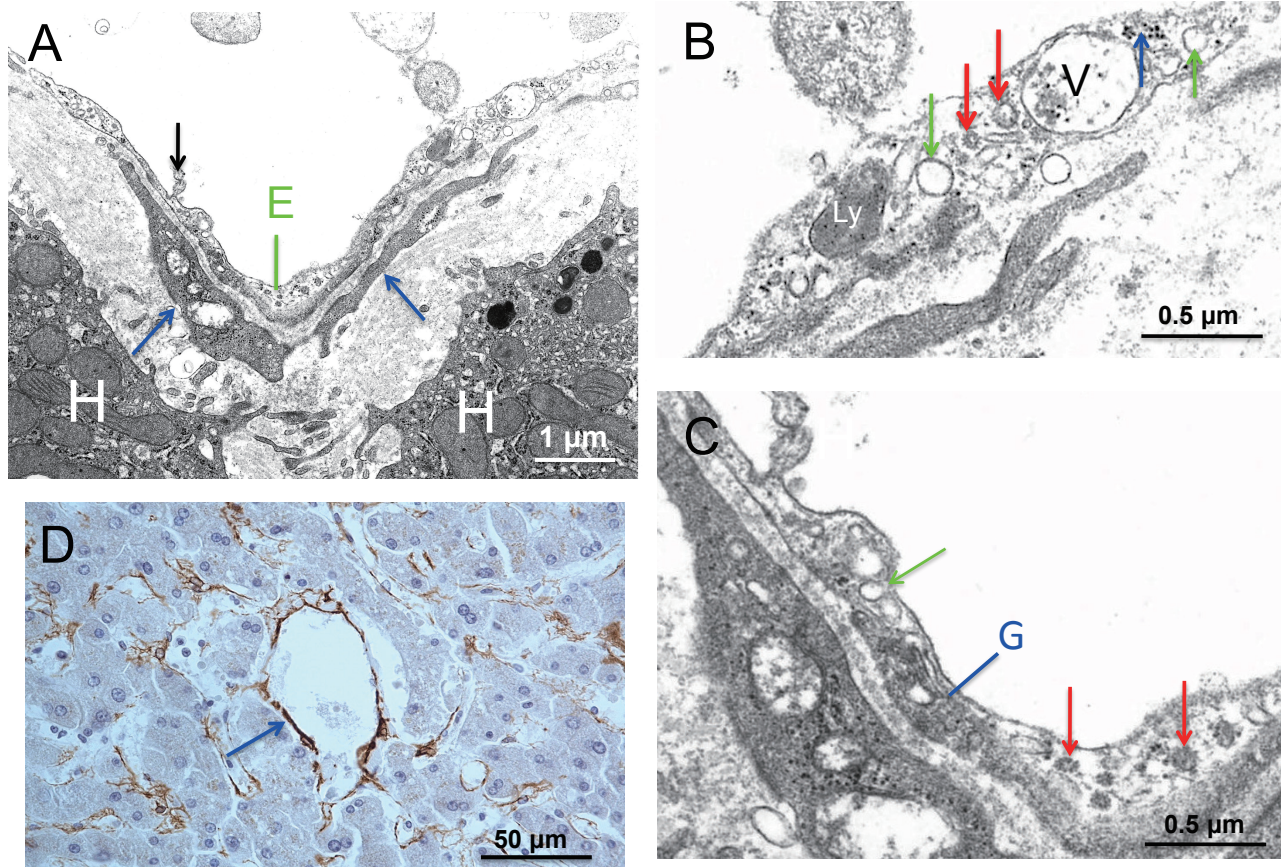


Figure 2 Central vein endothelium and its adventitia; human liver. A, B and C are electron micrographs. A: The endothelium (E) lining the lumen of the vein is as thin as 0.1 - 0.6 μm . The nucleus and endothelial openings are not included in this image. Cytoplasmic blebs (black arrow) are seen on the luminal surface of the vein. The adventitia of the venular wall between the endothelium and perivenous hepatocytes (H) is less than 2 μm in width and contains a collagenous matrix and a myofibroblast cell process (blue arrows). B: Higher magnification of the endothelial cell cytoplasm. It shows a collection of vesicular structures including smooth-surfaced vesicles (green arrows), coated vesicles (red arrows), and a large vacuole (V) containing cell debris. A lysosome (Ly) is also present. Blue arrow marks a cisterna of RER. Free ribosomes are scattered in the cytoplasm. C: A Golgi apparatus (G) is discernible, showing a few cisternae and associated vesicles. Numerous coated vesicles (two of them are marked by red arrows) are present. Green arrow marks a smooth-surfaced pinocytotic vesicle, showing its opening into the lumen of the vein. The basal cytoplasm contains electron dense filamentous materials. D: Immunoperoxidase staining for α -smooth muscle actin. Positively stained myofibroblasts are discernable in the wall of a central vein (arrow). Hepatic stellate cells in the lobular parenchyma are also stained for α -smooth muscle actin.

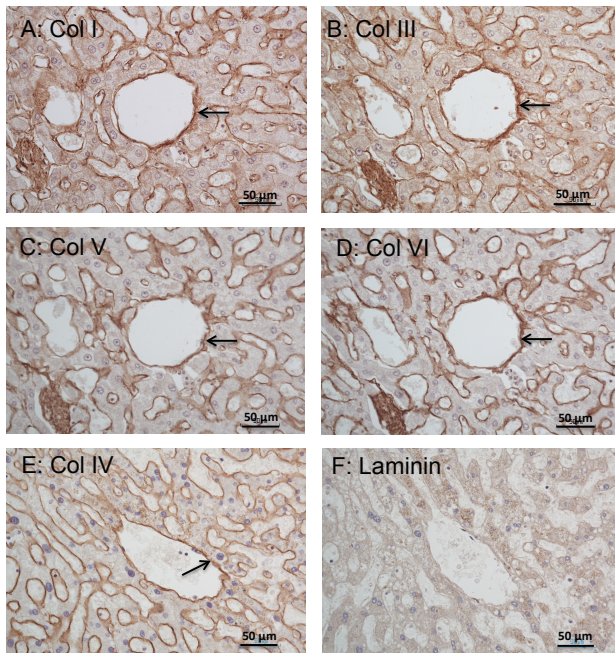


Figure 3 Immunoperoxidase staining for collagen types in adventitia of hepatic central vein; human cadaveric liver. A-D: are consecutive liver sections (5-µm thick), demonstrating codistribution of collagens I, III, V and VI in the wall of a central vein (arrow). Also note perisinusoidal staining for the collagens. E: Collagen IV immunostaining (arrow) is discernible around the lumen of a central vein F: Consecutive section of (E) showing absence of laminin in the same vein.

ULTRASTRUCTURE OF PERIVENOUS HEPATOCYTES

Applying quantitative stereology at the electron microscopic level, Loud^[26] has compared the composition of organelles of perivenous hepatocytes with that of periportal and midlobular hepatocytes of normal adult rat liver. The morphometric analysis revealed that the fine structure of perivenous cells is significantly different from that of other lobular cells, reflecting the specialized functions of perivenous cells, elaborated below:

Because of the great energy demands of hepatocytes, each cell contains more than a thousand mitochondria. Perivenous cells from cell layers 1-4 nearest the central vein have nearly 1.5 times as many mitochondria than periportal hepatocytes occupying the first 4 cell layers adjacent the portal tract--1600 mitochondria per perivenous cell vs 1060 mitochondria per periportal cells. Perivenous hepatocyte mitochondria are smaller in size than periportal hepatocyte mitochondria.

Smooth endoplasmic reticulum (SER) of hepatocytes is specialized in metabolism of xenobiotics--alcohol, drugs, carcinogens, mutagens, pesticides and anaesthetics^[27]. Cells in the perivenous area are richer in SER than periportal cells, which reflects the higher capacity for activating and/or detoxifying xenobiotic compounds in the centrilobular parenchyma. Moreover, alcohol consumption or phenobarbital administration induces SER proliferation specifically in perivenous hepatocytes.

The number of peroxisomes is nearly 2 times higher in perivenous cells (810 per cell) compared to periportal cells (440 per cell), which accounts for the greater anti-oxidative function of perivenous cells. Perivenous hepatocytes exhibit diffuse glycogen deposits while cells in the periportal cells show large clumps of β -particles surrounded by SER. The number of these particles varies with dietary state of the

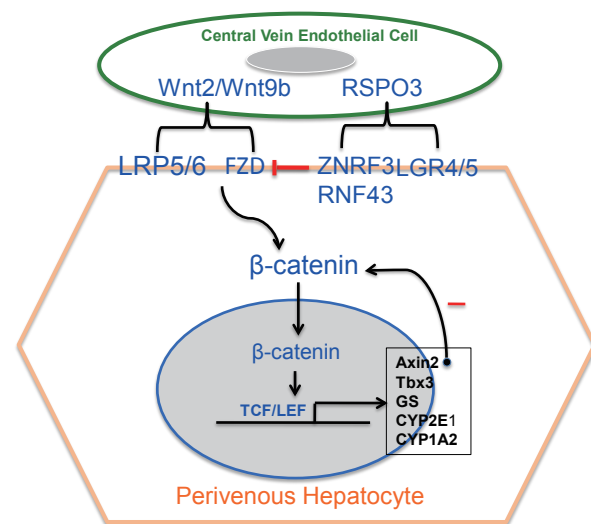


Figure 4 Schematic view of central vein-derived Wnt2, Wnt9b and RSPO3 participating in the cellular/molecular circuitry of signaling pathways of Wnt/ β -catenin and RSPO-LGR-ZNRF3/RNF43 module in the liver. In this circuitry, the cellular compartments are central vein endothelial cell and perivenous hepatocyte; the venular endothelium provides the molecular components consisting of Wnt2, Wnt9b and RSPO3. Wnt2 or Wnt9b binds to its receptor Frizzled (FZD) and co-receptors LRP5 or LRP6 localized on the cell membrane of perivenous hepatocyte. Binding of Wnts ligand to its receptors initiates signaling cascade that activates β -catenin in the cell cytoplasm; this signaling constitutes the canonical Wnt/ β -catenin pathway. β -catenin is translocated into the nucleus where it binds to TCF/LEF transcription factors and mediates the transcription of Wnt-responsive genes Axin2, among others. Axin2 provides a negative feedback inhibition loop, modulating β -catenin levels after a Wnt signal. The Wnt/ β -catenin pathway is enhanced by RSPO3. ZNRF3/RNF43 proteins are localized on the perivenous cell membranes and normally facilitate membrane clearance of FZD-LRPs from the cell surface via their ubiquitin ligase activities, leading to down-regulation of β -catenin activity. RSPO3 interacts with ZNRF3/RNF43, resulting in inhibition of the ligase activities and thus allowing persistence of FZD-LRPs receptors on the cell surface.

individual as well. They are more abundant subsequent to eating than after fasting.

The amounts of RER, Golgi complex, lysosomes and endosomes are similar in all parenchymal cells. The structure of midlobular hepatocytes is more similar to that of periportal cells or intermediate between perivenous cells and periportal cells.

GLUTAMINE SYNTHETASE (GS) AS BIOMARKER OF PERIVENOUS HEPATOCYTES

GS, an enzyme involved in the detoxification of hepatic ammonia^[28,29], is an accurate and reliable immunohistochemical marker of hepatocytes that normally reside in cell layers 1-3 surrounding the central vein of the liver of humans^[30,31,32] or rats^[12,16,33,34], and 1-2 cell layers around the central vein in mice^[1,3,35]. These GS-positive hepatocytes have been referred to in various publications as perivenous hepatocytes or pericentral hepatocytes. Topographically, in the case of human liver, GS-positive cell layers 1-3 have been regarded as a subcompartment of the centrilobular zone or the proximal part of that zone^[36,37,38,39], because the human centrilobular zone comprises 7-8 cell thick layers. In the case of mouse liver, cell layers 1-3 surrounding the central vein make up the entire centrilobular zone and are simply designated as perivenous or pericentral cells. GS serves as the Axin2 surrogate immunohistological marker of perivenous hepatocytes^[1], because no suitably sensitive Axin2 antibodies are available for immunohistochemistry.

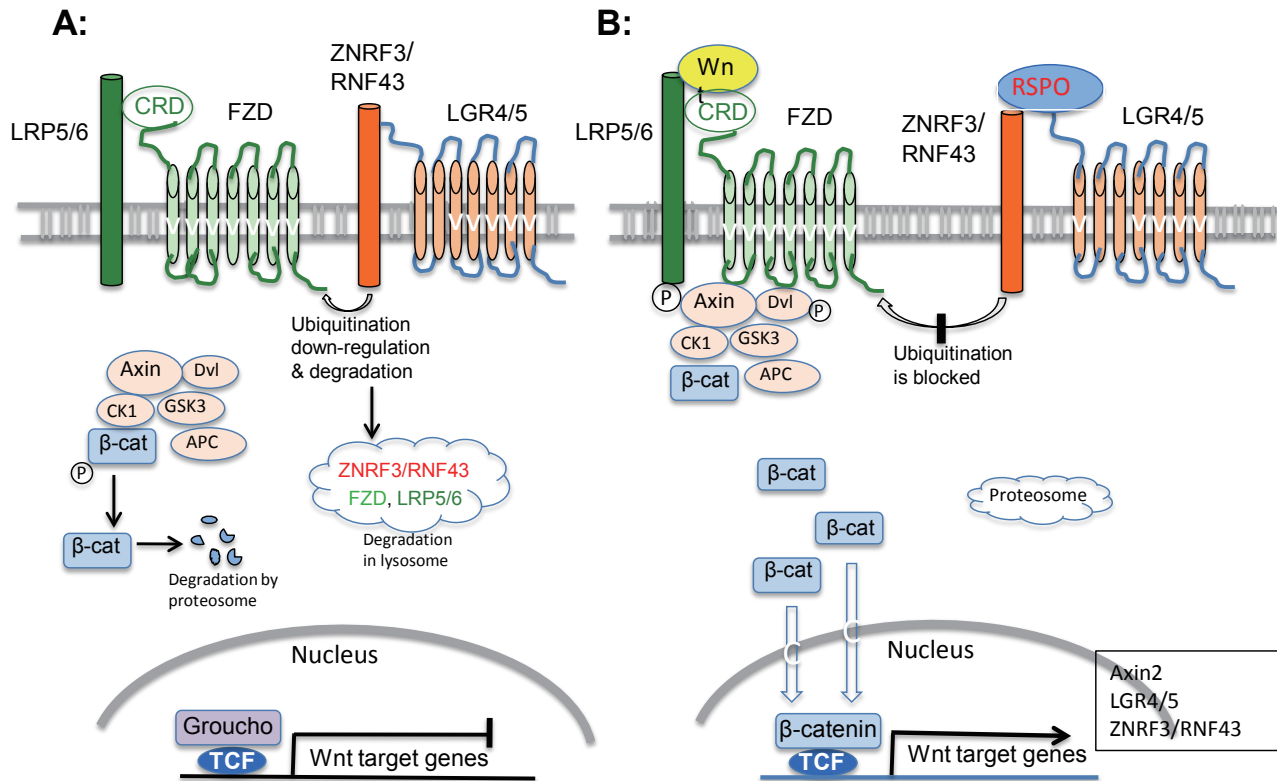


Figure 5 Schematic overview of canonical Wnt/ β -catenin signaling pathway and RSPO-LGR4/5-ZNRF3/RNF43 module. This is a composite scheme illustrating interactions between the Wnt/ β -catenin signaling and RSPO-LGR4/5-ZNRF3/RNF43 module in the absence of Wnt and RSPO (Panel A), and in the presence of Wnt and RSPO (Panel B). In Wnt off condition and absence of RSPO, β -catenin is sequestered in the destruction complex consisting of Axin, Dvl, APC, CK1 and GSK3 in the cytoplasm. Axin provides a scaffold for the components of the destruction complex. β -catenin is phosphorylated by CK1 and GSK3 and then ubiquitinated for degradation in proteasomes in the cytoplasm without reaching the nucleus. ZNRF3/RNF43 ubiquitinate FZD and its co-receptors LRP5/6 leading to their degradation in lysosomes. Wnt on and in the presence of RSPO: Wnt binds to FZD-LRP5/6 receptors on the cell surface. Wnt interacts with the CRD domain of FZD while LRP5/6 is phosphorylated by CK1 and GSK3. Axin and Dvl are recruited to the cell surface to interact with FZD and LRP5/6. The destruction complex disintegrates and β -catenin is stabilized, available for translocation into the nucleus to mediate transcription of Wnt-responsive genes: Axin2, LGR4/5, ZNRF3/RNF43. RSPO enhance Wnt signals by interacting with LGR4/5 and simultaneously with ZNRF3/RNF43 on the cell surface. The interaction blocks the ubiquitination of FZD by the ligase activity of ZNRF3/RNF43, thereby allowing persistence of FZD on the cell surface for reception of Wnt signaling.

CENTRAL VEIN ENDOTHELIUM-DERIVED WNT2, WNT9b AND RSPO3 PARTICIPATE IN THE CELLULAR-MOLECULAR CIRCUITRY OF WNT/ β -CATENIN PATHWAY AND RSPO-LGR4/5-ZNRF3/RNF43 MODULE THAT DIRECT PERIVENOUS GENE EXPRESSION AND ZONATION

In the mouse, mRNAs of Wnt2 and Wnt9b^[1,2,3,4] and of RSPO3^[5]—detected by *in situ* hybridization—are localized in the endothelium of the central vein, which is considered to be a primary source of the angiocrines. Central vein-derived Wnt2, Wnt9b and RSPO3 participate in the cellular-molecular circuitry^[3] of the Wnt/ β -catenin signaling and RSPO-LGR4/5-ZNRF3/RNF43 module that direct perivenous gene expression and zonation (Figure 4). The circuitry starts by the binding of secreted Wnt2 or Wnt9b to its primary receptor Frizzled and co-receptors low density lipoprotein receptor-related protein (LRP) 5 or LRP6 localized on the cell surface of perivenous hepatocytes, which leads to activation of β -catenin in the hepatocyte cytoplasm. RSPO3 derived from the central vein interacts with the transmembrane receptors leucine-rich repeat-containing G protein-coupled receptors (LGR) 4 or LGR5 and Zinc and Ring finger 3 and its paralog Ring finger 43 (ZNRF3 and RNF43) proteins localized

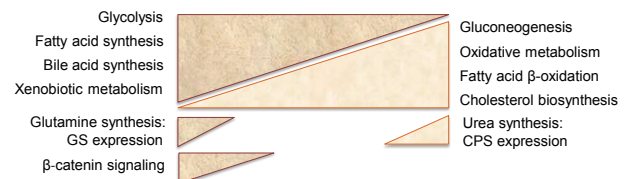


Figure 6 Schematic representation of hepatic metabolic zonation. The centro-portal axis of the classic liver lobule is zoned into centrilobular, midlobular and periportal zones. Centrilobular hepatocytes are mainly involved in glycolysis, fatty acid synthesis and bile acid synthesis and xenobiotic metabolism. Periportal hepatocytes are involved in oxidative energy metabolism, gluconeogenesis, fatty acid β -oxidation and cholesterol biosynthesis. While metabolic zonation is dynamic for most functions, glutamine synthesis with expression of GS associated with ammonia detoxification is strictly compartmentalized in periportal hepatocytes. Correspondingly, urea synthesis with expression of CPS is strictly periportal zoned. This scheme also includes the centrilobular distribution of β -catenin, the master regulator of hepatic zonation.

on perivenous hepatocyte cell surface—though LGR4 and ZNRF3 are also expressed in midlobular and periportal hepatocytes^[35]. The interaction blocks the ubiquitin ligase activities of ZNRF3/RNF43 that normally cause removal of Frizzled and LRP5/6 from the cell surface. Acting in concert, this cellular-molecular circuitry orchestrates the β -catenin pathway to direct perivenous gene expression of Axin2, GS and Tbx3 (a pluripotency factor and an early liver progenitor biomarker), as well as to maintain centrilobular metabolic zonation of GS, CYP2E1 and CYP1A2^[1,5,35]. Moreover, the induction of Axin2

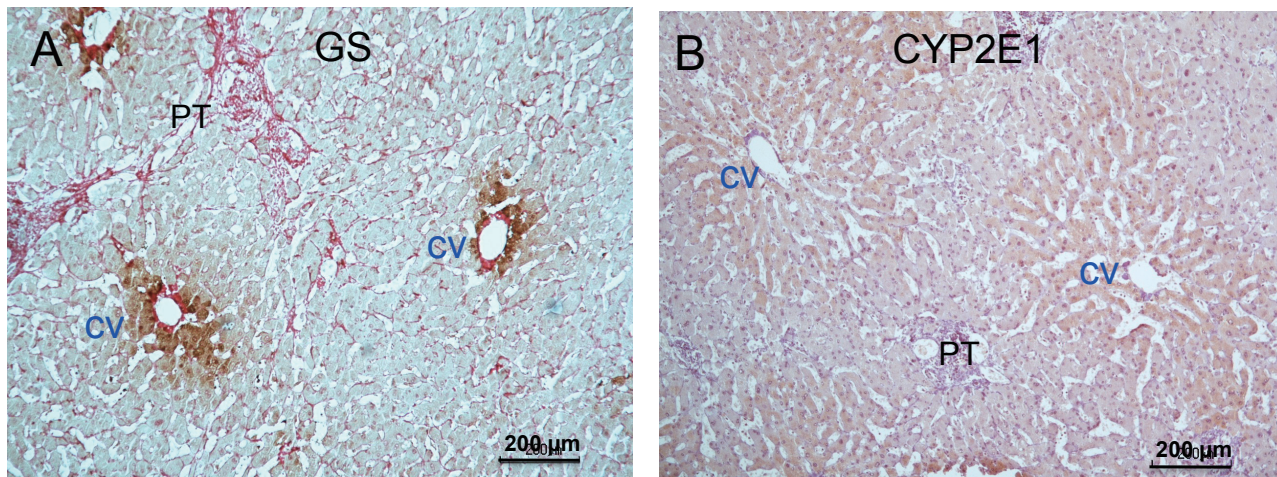


Figure 7 Immunoperoxidase staining for GS and CYP2E1 in liver lobules demonstrating hepatic metabolic zonation; human cadaveric liver. A: GS staining is observed in perivenous hepatocytes arranged in 1-3 layers around the central veins. Post-stained with Sirius red for collagens. B: CYP2E1 staining—in a consecutive section—shows a wider lobular distribution than GS, occurring in the centrilobular and midlobular areas. Hematoxylin counterstained. CV, central vein; PT, portal tract.

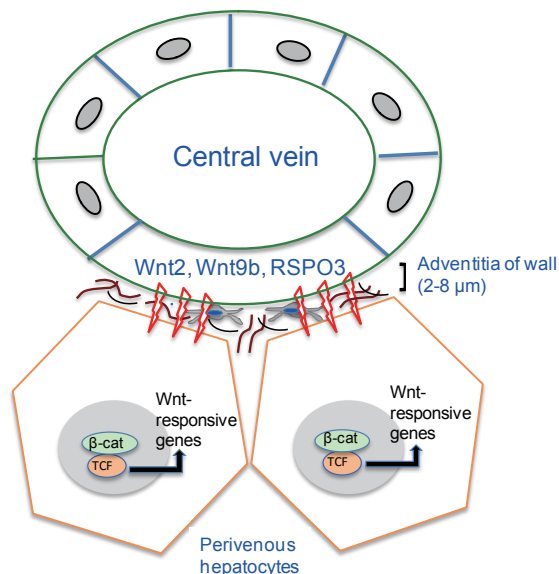


Figure 8 Illustrating the anatomical relationship between hepatic central vein, perivenous hepatocytes, and endothelial secretion of Wnt2, Wnt9b and RSPO3. The angiocrines are released at the basal cell surface of endothelial cells into the adventitial matrix of the wall to reach responder perivenous hepatocytes. In a normal vein, the wall thickness is 2 - 8 μm . The central vein and its adventitia constitute an anatomical niche of perivenous hepatocytes. Collagen fibrils are illustrated in brown, and fibronectin is depicted in black. Myofibroblasts are shown in grey.

may provide a negative feedback for down-regulating β -catenin to normal levels upon transduction of a Wnt signal^[40,41]. The cellular-molecular circuitry highlights in particular that the hepatic central vein and its angiocrines provide anatomical and molecular links to the Wnt/ β -catenin signaling in perivenous diverse cellular processes.

OVERVIEW OF CANONICAL WNT/ β -CATENIN SIGNALING PATHWAY AND RSPO-LGR4/5-ZNRF3/RNF43 MODULE

This section highlights the complexities and interplays of the Wnt/ β -catenin pathway and RSPO-LGR4/5-ZNRF3/RNF43 module with the aid of the schema in Figure 5. This will serve as an introduction to the discussion on biological properties of the main players of these pathways and their involvement in hepatic biology.

Table 1 Centrilobular distribution of proteins, enzymes and synthetic activities.

Molecular cell biomarkers	Axin2, GS, Tbx3, HNF4 α , LGR4/5, ZNRF3, RNF43
Ammonia metabolism	Glutamine synthesis, GS, GDH, OAT
Bile acid synthesis	
Carbohydrate metabolism	Glycolysis
Fatty acid synthesis	
Oxidative metabolism	NADPH-cytochrome P450 reductase, NADPH
Xenobiotic metabolism	Alcohol, Acetaminophen, CCl ₄ , CYP1A2, CYP2A1, CYP2B, CYP2C, CYP2E1, CYP3A1, CYP7A1

GS: Glutamine synthetase; Tbx3: Pluripotency factor and an early liver progenitor biomarker; HNF4 α : Hepatocyte fate determination transcription factor; LGR: Leucine-rich repeat-containing G protein-coupled receptor; ZNRF3 and RNF43: Zinc and Ring finger 3 and its paralog Ring finger 43; GDH: Glutamine dehydrogenase; OAT: Ornithine aminotransferase; NADPH: Nicotinamide adenine-dinucleotide-phosphate dehydrogenase.

Wnt/ β -catenin Pathway

The canonical Wnt/ β -catenin pathway is centered on regulation of the activity of its effector β -catenin^[42,43,44,45,46]. In the absence of a Wnt signal (Figure 5, panel A), β -catenin is sequestered in the cytoplasm and bound to a multi-protein destruction complex consisting of Axin, APC gene product, CK1 (Casein kinase1), GSK3 β (Glycogen synthetase kinase) and E3-ubiquitin ligases. CK1 and GSK3 β sequentially phosphorylate the serine and threonine residues in the amino terminal region of β -catenin. Axin acts as a scaffold protein interacting with β -catenin and promoting its phosphorylation. The phosphorylated β -catenin is then ubiquitinated and degraded in the proteasome. As a result of this continuous degradation, β -catenin is prevented from accumulating in the cytoplasm and, therefore, from reaching the nucleus. In the absence of nuclear β -catenin, the transcriptional repressor Groucho binds the DNA-bound transcription factors T-cell factor/lymphoid enhancer factor (TCF/LEF)--collectively referred to as TCF--thereby blocking the transcription of Wnt-target genes^[42,47].

The canonical pathway is activated by Wnt ligand binding to its primary receptor Frizzled and co-receptors LRP5/6 on the cell surface membranes (Figure 5, panel B). The binding with recruitment of Axin leads to phosphorylation of LRP. Dishevelled (Dvl), a cytosolic phosphoprotein, is also phosphorylated and recruited to the cell surface. The recruitment causes an inactivation



Mak KM. Hepatic central vein

of the β -catenin destruction complex. Consequently, β -catenin is hypo-phosphorylated and accumulates in the cytoplasm, making it available for translocation into the nucleus, where it displaces Groucho from its binding to TCF and forms a complex with TCF to mediate expression of Wnt target genes, including Axin2, LGR4/5 and ZNRF3/RNF43^[40,41,48,49], indicated in Figure 5.

RSPO-LGR4/5-ZNRF3/RNF43 Module

Wnt signals can be enhanced by the presence of RSPO proteins--known as Wnt-enhancer^[44,48,49,50]. RSPO, acting through receptors LGR 4 or LGR5, inhibits the transmembrane E3 ubiquitin ligases ZNRF3 and RNF43 that ordinarily ubiquitinate cell surface Frizzled receptor for proteosomal degradation; these interactions are schematized in Figure 5. By antagonizing ZNRF3/RNF43 activity, RSPO stabilizes the Frizzled protein, enhancing Wnt signal strength. However, the relative contributions of Wnt ligand-Frizzled-LRP5/6 pathway and RSPO-LGR4/5-ZNRF3/RNF43 module to β -catenin signaling in physiological conditions remain to be evaluated.

OVERVIEW OF BIOLOGICAL PROPERTIES OF WNTS, β -CATENIN, FRIZZLEDS, LRP, RSPO, LGR AND ZNRF3/RNF43

Wnts

Wnt is an acronym that stands for “Wingless/integrated”. The first member of the Wnt pathway was first identified in the fruit fly *Drosophila melanogaster* as the product of the Wingless gene (*wg*), a mutation that led to the frequent loss of the wing^[51]. Subsequently, it was discovered that the mouse mammary oncogene *int-1* is an ortholog of the *Drosophila* Wingless gene^[52,53]. The first member of the Wnt pathway is now called Wnt-1 (from *Wg* and *int-1*). Members of the Wnt gene family are defined by their sequence similarity to the mouse Wnt-1 and *Drosophila* Wingless. Nineteen Wnt genes have been identified in the human and mouse genomes^[47]. Wnt proteins are 40 kDa in size containing ~350-400 cysteine residues in length^[54]. Wnts are glycoproteins, lipid-modified and hydrophobic, and may share the following features characteristic of secretory proteins, namely a signal peptide, several potential N-glycosylation sites and 22 conserved cysteine residues engaged in intramolecular disulfide linkages, conferring a secondary globular structure. The N-terminus is dominated by palmitoleic acid, while the C-terminus contains the conserved amino acids^[54]. Wnt ligands bind to its primary receptor Frizzled and co-receptors LRP5 or LRP6 on the cell surface of responder cells to initiate intracellular signal transduction leading to activation of β -catenin-dependent signaling in the “canonical pathway”. Secretion of lipidated Wnt proteins requires cooperation of Wntless proteins in Wnt-producing cells--to be discussed in section below on “MODES OF WNT SECRETION AND REQUIREMENT FOR WNTLESS (WIS) PROTEIN”.

β -catenin

In Latin, the definition of catena is chains. This reflects the linking of E-cadherins localized in the intercellular adherens junction. β -catenin encoded by the human gene, *CTNNA1*, is a subunit of the cell surface cadherin protein complex and a multifunctional protein. β -catenin is a central player in the canonical Wnt signaling pathway, acting as an intracellular transducer to mediate transcription of Wnt responsive genes by interacting with transcription factors TCF/LEF in the nucleus (Figure 4). A second major role of β -catenin is its participation in cell-cell adhesion in epithelial tissues by interacting with E-cadherin, a transmembrane component of the intercellular

adhesion junctions, as well as binding to α -catenin that in turn interacts with the cytoplasmic cytoskeletal actin^[43].

Frizzleds

Frizzleds are cognate and principal receptors of Wnts. Frizzled proteins have seven-pass transmembrane (7TM) domains and a large extracellular cysteine-rich domain (CRD), which forms a hydrophobic pocket at the N-terminus^[54]. The Wnt ligand engages the Frizzled CRD through its palmitoleate moiety at the N-terminus, while the C-terminus of Wnt makes contact with the CRD as well. Wnt binding induces a dimerization of Frizzleds with transmembrane coreceptors LRP5 or LRP6 for propagation of Wnt signals intracellularly, leading to the stabilization of β -catenin-dependent signaling.

LRP5 and LRP6 (low-density lipoprotein receptor-related protein)

LRP5 and LRP6 are a subclass of the low-density lipoprotein receptor family. The peptide structure contains a large extracellular domain, a single pass transmembrane domain and a relatively short cytoplasmic domain. They act as a co-receptor of Frizzled receptor for binding of Wnt ligands, initiating the transduction Wnt signals and are therefore required for the canonical β -catenin dependent pathway^[55,56]. When Wnt binds Frizzled and LRP5/6, Axin is recruited from the degradation complex to the cell surface, thereby disassembling the complex and preventing β -catenin destruction (Figure 5).

RSPO-LGR4/5-ZNRF3/RNF43 module

RSPO proteins are a group of 4 secreted angiocrines (RSPO1-4) participating in the activation and regulation of Wnt signaling pathway. All RSPO contain two furin repeats at the N-terminal domain, a thrombospondin domain, and a positively charged C-terminal tail^[57,58,59]. The RSPO furin domains interact with the extracellular domains of LGR 4/5 receptors and ZNRF3/RNF43^[59,60,61]. The interaction results in an inhibition of the activity of E3 ubiquitin ligases ZNRF3/RNF43 that continuously down-regulate Frizzled and LRP5/6 receptors on the cell surface^[59,62,63]. Thus, RSPO induces membrane clearance of ZNRF3/RNF43 from the cell surface, thereby stabilizing Frizzled and LRP5/6 complex on the cell surface and amplifying Wnt/ β -catenin signal duration and strength. Accordingly, RSPO are agonists of Wnt ligands. They are also called enhancer or coactivator of Wnts.

LGR4 and LGR5 (leucine-rich repeat-containing G protein-coupled receptors)

LGRs are transmembrane receptors with a large N-terminal extracellular domain composed of 17 leucine-rich repeats (LRR) and a Hinge region; the latter connects the LRR domain to a seven transmembrane domain (7TM)^[59]. LGR4 and LGR5 are receptors for RSPO. LGRs interact with RSPO through their extracellular domains and the complex formation of LGRs/RSPO counteracts and thereby neutralizes the activity of ZNRF3/RNF43 that functions to removing Frizzled receptors from the cell surface membranes^[49]. LGRs bind RSPO with high affinity and are essential for signal enhancement of low doses of Wnts^[60,61].

ZNRF3/RNF43 (Zinc and Ring finger 3 and its paralog Ring finger 43)

ZNRF3/RNF43 are single-pass transmembrane proteins of Ring type E3 ubiquitin ligases encoded by the Wnt target genes^[59,62,63]. These proteins have an extracellular protease-associated domain and a cytoplasmic RING domain separated by a transmembrane domain. ZNRF3/RNF43 interact with the Wnt Frizzled receptor and their

ligase activities promote turnover of Frizzled and its co-receptor LRP5/6 through ubiquitination. Consequently, ZNRF3/RNF43 are negative regulators of Wnt signaling by promoting clearance of Frizzled and LRP receptors on the cell surface^[48,62].

INVOLVEMENT OF β -CATENIN, FRIZZLED, LRP, RSPO, LGR AND ZNRF3/RNF43 IN HEPATIC BIOLOGY

Hepatic β -catenin

Liver-specific conditional β -catenin knockout (LKO) mice had defects in β -catenin responsive gene expression in the liver^[64]. Expression levels of GS, CYP2E1 and CYP1A2 in perivenous hepatocytes were almost completely abrogated. These mice also had impaired liver regeneration^[65,66]. Transgenic mice over-expressing active β -catenin in the liver exhibited increased expression of GS, glutamine transporter GLT-1 and ornithine aminotransferase (OAT); the latter provides glutamate as the second substrate required by GS to form glutamine^[67]. Liver tumors in mice with activating β -catenin mutations showed a high level of β -catenin-dependent GS expression and also increased levels of CYP450 isoforms, particularly CYP2E1 and CYP1A2^[68]. However, β -catenin LKO mice were protected from liver injury caused by APAP overdose due to absent CYP2E1 and CYP1A2^[64,69]. Therefore, β -catenin is required for hepatic metabolism of ammonia, xenobiotics and drugs, as well as liver regeneration.

Hepatic Frizzleds

There are 19 Wnts and 10 Frizzled genes identified in the mammals, and in the normal mouse liver, a total of 11 Wnts and 8 Frizzled genes were identified in association with hepatocytes, Kupffer cells, hepatic stellate cells, sinusoidal endothelial cells, and biliary epithelial cells^[70]. Thus most Wnt and Frizzled genes are expressed in the liver and may participate in the canonical and noncanonical pathways mediating liver biology and physiology. However, which one/more of the 8 Frizzled receptors are normally transducing the Wnt signal in perivenous hepatocytes is not known.

Hepatic LRP5 and LRP6

In the liver, binding of Wnts to hepatocyte surface initiates signals to activating β -catenin that in turn translocates to the nucleus to bind TCF/LEF transcription factors. The binding results in hepatic gene expression in the centrilobular parenchyma, in particular GS, CYP2E1, CYP1A2 and Cyclin-D1. To determine the role of LRP5/6 in Wnt-dependent β -catenin signaling, liver-specific LRP5/6 double LKO mice were generated to disrupt upstream Wnt signaling while β -catenin expression is intact^[71]. Deleting both LRP5 and LRP6 has been shown to prevent any compensation with assurance of complete abrogation of Wnt signaling^[56]. It was found that LRP-LKO mice has defective hepatic zonation and delay in liver regeneration following partial hepatectomy, which suggests that β -catenin is primarily regulated by Wnt ligands in mouse liver homeostasis and during regeneration. LRP-LKO also showed significantly lower serum AST and ALT after a high dose of APAP administration compared to controls^[71]. Thus, LRP-LKO mouse phenocopies β -catenin-LKO mouse in mediating perivenous gene expression and hepatic regeneration^[64,65,71].

Hepatic RSPO

The potential role of hepatic RSPO in the regulation of pericentral gene expression in association with the canonical β -catenin pathway has been investigated^[5]. Mice with conditional deletion of RSPO3

almost completely lost the expression of β -catenin targets GS, CYP2E1, Axin2 and LGR5 in the pericentral area, demonstrating a requirement for RSPO3 in the maintenance of pericentral gene expression. Other centrally expressed genes OAT and CYP7a1 were also significantly reduced, while the periportal genes CYP2E1 and Hsd17 were upregulated, reflecting a profound effect of RSPO3 in the establishment of hepatic metabolic zonation. Furthermore, ectopic expression of RSPO1 in hepatocytes induced an up-regulation of Axin2 and LGR5 pericentrally and an expansion of GS expression from the pericentral into the midlobular parenchyma, validating that RSPO1 is sufficient to induce Wnt/ β -catenin when the protein is ectopically expressed in hepatocytes.

Regarding the cellular site of RSPO in the liver, it was found that RSPO3 mRNA is specifically expressed in endothelial cells of the central vein of mice; hence it is an angiocrine^[5]. The endothelial residence of RSPO3 likely confers a close range of action between RSPO3 and responder hepatocytes in the perivenous area. Incidentally, Wnt2 and Wnt9b mRNA were also found to localize in the central vein endothelium^[1,2,3,4]. However, expression of central vein endothelial-specific Wnt2 and Wnt9b was not affected by RSPO3, implicating that the RSPO-induced effects are not due to loss of the Wnt ligands but due to Wnt/ β -catenin modulation.

RSPO appears to be capable of controlling liver size and liver regeneration^[55]. Inducible overexpression of the extracellular domain of ZNRF3 in mice blocked RSPO-induced accumulation of Frizzled proteins and compromised the Wnt/ β -catenin activity gradient in the liver. These mice also had a striking reduction in the expression of metabolic enzymes, notably GS and CYP2E1. Conversely, in mice with combined inducible ZNRF3 and RNF43 deletion, marked increases in the expression of GS and CYP2E1 were observed. Injections of recombinant RSPO1 in these ZNRF3 and RNF43 deleted mice led to greater cell proliferation, increased expression of GS and CYP2E1, and expansion of Wnt/ β -catenin signaling into the periportal area. However, RSPO1 injections did not increase the expression of metabolic enzymes in the absence of LGR in LGR4/5 double knockout mice. Thus, RSPO plays a role in the control of the Wnt/ β -catenin pathway downstream of LGR4/5 to regulate metabolic liver zonation. Furthermore, recombinant RSPO1 injections increased liver size and improved liver regeneration following partial hepatectomy, whereas double knocked out LGR4/5 caused the opposite effects, producing smaller livers.

Hepatic LGR4/5

To determine whether LGR4 and LGR5 receptors are essential for hepatic metabolic zonation, liver-specific LGR4 or LGR5 single KO and LGR4/5 double KO mice were generated^[55]. Single LGR4 KO and LGR4/5 double KO abrogated Wnt signaling with almost complete loss of expression of Wnt target metabolic enzymes, notably GS and CYP2E1. Moreover, metabolic hepatic zonation was deranged, with centrally expressed metabolic genes now showing lower expression, while genes expressed periportal being upregulated. These mice also had reduced liver weight; however, LGR5 single KO mice did not show such changes. Hence, LGR4/5 represent upstream effectors of the β -catenin signaling activity.

Hepatic ZNRF3/RNF43

Conditionally combined deletion of ZNRF3/RNF43 in mice resulted in marked increases in GS and CYP2E1 expression in the liver^[55]. GS expression was progressively expanded along the centro-portal gradient, while CYP2E1 was even extended into the periportal parenchyma, implicating deregulation of β -catenin-dependent



Mak KM. Hepatic central vein

signaling and derangement of hepatic metabolic zonation. The findings support a role for ZNRF3/RNF43 as a component of the RSPO-LGR4/5-ZNRF3/RNF43 module that controls hepatic metabolic zonation and perivenous metabolic gene expression--discussed below.

HEPATIC METABOLIC ZONATION - Regulation by β -Catenin-Dependent Signaling and RSPO-LGR4/5-ZNRF3/RNF43 Module

Hepatic metabolic zonation reflects the heterogeneity of gene expression and functions of hepatocytes along the centro-portal axis of the liver lobule. The zonation endows metabolic homeostasis and proper functioning of the liver under various physiological conditions. The discovery of metabolic zonation in the liver^[72] leading to the establishment of the Wnt/ β -catenin pathway as the master regulator of hepatic zonation has been comprehensively reviewed in a number of articles^[36,37,38,39,73,74]. More recent studies revealed that the Wnt/ β -catenin-dependent metabolic zonation is modulated by RSPO5^[5], which signals through the LGR4/5-ZNRF3/RNF43 module^[35]. The key features of metabolic zonation in the liver are illustrated (Figure 6). Notably, glycolysis, glutamine synthesis and bile acid biosynthesis predominate in the centrilobular zone, while the opposing metabolic functions of gluconeogenesis, urea synthesis and cholesterol biosynthesis are most prominent in the periportal zone. Some functions show stable zonation, in particular ammonia detoxification in which expression of GS is restricted to a few layers of perivenous hepatocytes around the central vein, while the urea synthesis enzyme carbamyl phosphate synthetase (CPS) is restricted to periportal hepatocytes around the portal tract. On the other hand, carbohydrate metabolism represents an example of dynamic zonation along the centro-portal axis and can shift in response to nutritional or hormonal state. The centrilobular zone is more actively involved in xenobiotic metabolism than the periportal zone, although several CYP450 isoforms are distributed equally along the centro-portal axis^[75]. Significantly, CYP2E1 and CYP1A2 that are involved in ethanol, APAP and CCl₄ metabolism are zoned in the centrilobular parenchyma. Figure 7 illustrates the metabolic zonation of GS and CYP2E1. Table 1 catalogs the centrilobular metabolic zonation of mRNA, proteins, enzymes and synthetic activity of the liver.

GS AND CYP450 ARE INDICATORS OF CENTRILOBULAR β -CATENIN ACTIVITY

Expression levels of GS along with CYP2E1 and CYP1A2 have been regularly used as indicators of β -catenin activity in hepatic homeostasis, injury caused by hepato-noxious agents and regeneration following partial hepatectomies^[1,3,5,35,64, 65,69,71]. These are reviewed in the following sections:

CENTRILOBULAR INJURY: EFFECTS OF HEPATO-NOXIOUS AGENTS

Carbon tetrachloride (CCl₄) Intoxication and Histogenesis of GS Expression

CCl₄ is a hepatic toxin that specifically inflicts centrilobular parenchymal necrosis around the central veins. CCl₄ is metabolized by CYP2E1^[27]--expressed in centrilobular hepatocytes--and becomes toxic to perivenous hepatocytes. After a single non-lethal dose, hepatocyte regeneration appeared in 6-8 days, and the livers of treated animals were completely back to the pretreatment state in about 2-3 weeks. Accordingly, CCl₄ treatment in rodents has been used to assess the histogenesis of GS-expressing cells in the perivenous area during

hepatic regeneration. As evaluated by GS immunohistochemistry, CCl₄ administration to rats abolished the characteristic 1-3 cell layers formation of GS positive cells around the central veins^[33]. Signs of reappearance of GS staining began at 3 days following the treatment with sporadic appearance of GS positive cells among negative cells in the first layer of perivenous cells. Thereafter, GS staining appeared more intense, yet only the first layer of perivenous hepatocytes was positive for GS. It is thought that the newly formed GS expressing cells are derived from GS negative cells that are induced to express GS if they come into contact with the border of central veins.

CCl₄ treatment completely abolished the transcription of GS^[76]. Repair of liver damage was associated with invasion of inflammatory cells and macrophages that removed dead cells to allow re-growth. Remarkably, only when hepatocytes resumed physical contacts with central veins were they able to restore the pretreatment GS transcription levels, which occurred at 2-3 weeks after the treatment, while hepatocytes that were separated from central veins by inflammatory cells were GS negative. Similar findings were observed for OAT. Since both the central veins and collecting veins carry venous blood, it is thought that it is not blood-borne elements that trigger the histogenesis of GS expressing cells around the central veins or along the wall of collecting veins. Hence, it is proposed that some extracellular signals in the local environment/niche of the central vein interacting with perivenous hepatocytes or contacts between central vein endothelial cells and hepatocytes to trigger expression of GS. Along this line is the speculation that endothelial cells of the central vein produce Wnt proteins that activate β -catenin in adjacent perivenous hepatocytes^[38,77], which actually is consistent with the revelation that the hepatic central vein is a source of Wnt2 and Wnt9b^[1,2,3,4]--mentioned above.

CCl₄ Induction of Cirrhosis and Loss of GS Expression

Chronic treatment of CCl₄ induces histogenesis of cirrhosis in rodents. In rats with cirrhosis, the normal association of GS-expressing hepatocytes with the central vein was lost^[34]. Instead, GS positive hepatocytes were rare in the nodular parenchyma but were scattered in the periphery of the nodules or localized within the fibrous septa. CPS, the first and rate-limiting enzyme in the urea cycle^[29], was distributed in the periportal and midlobular parenchyma, and was absent in the perivenous area. In cirrhosis, CPS immunoreactivity appeared to lose its zonal gradient and was uniformly distributed in the nodular parenchyma. In these animals, decreased GS contributed to hyperammonemia in cirrhosis. Loss of normal perivenous GS was also observed in cirrhosis of humans, which was thought to be due to a disrupted endothelium-hepatocyte contact in the local environment of the central vein (32); the speculation actually reflects the emerging role of the hepatic central vein in constituting an anatomical niche of perivenous hepatocyte stem cells^[1]--to be discussed in section below on "CENTRAL VEIN ENDOTHELIUM CONSTITUTES AN ANATOMICAL PERIVENOUS HEPATOCYTE STEM CELL NICHE".

Centrilobular Injury by Acetaminophen (APAP): Involvement of CYP450

APAP is a widely used over-the-counter analgesic, which is known to cause hepatotoxicity when ingested in large quantities in both humans and animals. The toxicity manifests as cellular necrosis and is localized in the centrilobular parenchyma around the central veins. Bioactivation of APAP is mediated by microsomal CYP2E1 to reactive intermediate N-acetyl-p-benzoquinone imine that covalently binds cellular proteins and depletes glutathione, thereby impairing

cellular functions and cell death^[78]. Alcohol consumption has been recognized to exacerbate APAP hepatotoxicity in both humans and animals in doses of APAP not generally regarded as harmful—2.6-5 gm/24 hr. Alcohol also causes induction of CYP2E1 predominantly in the centrilobular hepatocytes. The up-regulation of CYP2E1 is reflected by an extension of immunostaining reaction of CYP2E1 from the centrilobular to midlobular parenchyma^[27,79,80]. Thus, elevated microsomal CYP2E1 levels explain not only the increase of APAP bioactivation after prolonged ethanol ingestion, but also the enhanced susceptibility to APAP toxicity in the centrilobular parenchyma. This is confirmed by studies using perivenous hepatocytes isolated by digitonin/collagenase perfusion^[81]. Furthermore, CYP1A2, another P450 isozyme of predominantly centrilobular distribution, is also responsible for catalyzing APAP to its toxic metabolite^[78].

Centrilobular Injury by APAP and Liver Regeneration: Involvement of β -catenin-Responsive Targets GS, Cyclin-D1 and CYP450

The link between APAP toxicity and β -catenin activity was evaluated^[69]. Mice were given a dose of APAP that was nonlethal and yet sufficient to induce liver regeneration. Centrilobular parenchyma necrosis developed around the central veins. APAP treatment led to stabilization and activation of β -catenin for 1-12 hours, which was accompanied by increased expression of β -catenin target gene GS in pericentral hepatocytes. Levels of Cyclin-D1, another β -catenin target gene, were also increased. Baseline values were eventually restored at 24 and 48 hours. Liver regeneration was manifested by increased immunostaining of cell proliferation marker PCNA in the liver lobules, although not preferentially distributed to the centrilobular parenchyma. β -catenin knockout mice demonstrated resistance to APAP-induced centrilobular necrosis, which was associated with an almost complete loss of CYP2E1 and CYP1A2 expression^[64]. Moreover, retrospective studies of liver samples from APAP overdosed patients with acute failure revealed a positive correlation between β -catenin activation, parenchyma cell proliferation and spontaneous regeneration^[69]. In line with this, β -catenin signaling was shown to transcriptionally regulate CYP2E1 and CYP1A2 in primary human hepatocytes^[82] and HepaRG cells^[83]. Incidentally, CYP2E1 induction also occurred in isolated human hepatocytes exposed to high doses of APAP.

MODES OF WNT SECRETION AND REQUIREMENT FOR WNTLESS (WIS) PROTEIN

Wnt proteins are glycoproteins synthesized in the endoplasmic reticulum and are post-translationally lipidated by acylation of the palmitoleic acid using Porcupine palmitoyl-transferase^[84]. The palmitoylated Wnt proteins are then bound by the carrier protein Wntless (WIs)^[85], also called Evenness interrupted (Evi)^[86], for cellular transport from the Golgi apparatus to the cell surface for release into the extracellular space, as shown in a variety of Wnt-secreting cells^[49,50,85,86]. WIs protein is a multi-pass transmembrane G-protein-coupled receptor that binds the lipid-modified Wnts for cellular transport of Wnts in Wnt-secreting cells.

Being lipid-modified, Wnts are quite hydrophobic and thus are not likely to diffuse as soluble forms through the extracellular matrix to reach target cells. Rather, Wnts are secreted while attaching to WIs tethering to exocytic vesicles (exosomes) into the extracellular space and act as cellular signals targeting responder cells, elucidated in *Drosophila* cells and human cell lines^[87]. Whether Wnts act as short-range cues or long-range signals seem to be an unsettled issue^[49,50].

The hydrophobicity of Wnts has hampered the determination of Wnt signal activation and the functional importance of Frizzled subtypes, as well as the use of Wnt proteins as therapeutic agents. To that end, non-lipidated surrogate Wnt agonists that are water-soluble Frizzled-LRP5/LRP6 heterodimerizers have been generated^[88]. Wnt agonists phenocopy the canonical Wnt/ β -catenin signaling. The surrogate can be systemically expressed and exhibits Wnt activity in mouse liver, regulating metabolic liver zonation, stimulating hepatocyte proliferation and causing liver enlargement.

ENODOTHELIAL WNTLESS (WIS) IN PERIVENOUS GENE EXPRESSION AND LIVER INJURY

Deletion of WIs specifically in central vein endothelial cells resulted in a loss of Wnt signaling in perivenous hepatocytes with a concomitant decreased expression of β -catenin-responsive Axin2 and GS genes^[1]. Further, the Wnt/ β -catenin signaling pathway plays a role in localized liver tissue repair following acute injury by a single injection of CCL₄ in mice^[4]. Acute CCL₄ injury also led to increased expression of Wnt2 and Wnt9b in central vein endothelial cells. Another two studies showed that endothelial cell-WIs-KO mice lack β -catenin-dependent GS, CYP2E1 and CYP1A2 expression in the liver^[3,89]. Also, these mice are resistant to liver injury induced by APAP^[3]. These data are consistent with the role of endothelial cell-derived Wnt signals in the maintenance of β -catenin activity in liver biology and physiology.

SECRETION AND TRANSPORT OF WNTS AND RSPO3 IN CENTRAL VEIN

Regarding endothelial secretion of Wnts and RSPO3 in the liver, the morphological basis for their constitutive release from the central vein endothelial cells is presently unknown. It is unlikely that these angiocrines are released at the endothelial apical surface into the venous circulation. Rather, it is more likely that Wnts and RSPO3 are released at the basal cell surface into the adventitial tissue of the venular wall, depicted in Figure 8. In that case, it is conceivable that secreted Wnts and RSPO3 ferry across the collagenous matrix interposed between the endothelium and perivenous hepatocytes. No extracellular vesicular transport of Wnts or RSPO3 by exosomes across the venular wall to contacting perivenous hepatocytes has been reported. Notwithstanding, the hydrophobic properties of Wnts might constraint their range of actions on the β -catenin activity to 3 layers of hepatocytes immediately surrounding the central vein^[3], even if the molecules did reach across the adventitial matrix. It can be estimated that such a distance is ~ 60 μ m long from the wall of the vein—3 perivenous hepatocyte wide, each ~20 μ m in diameter. It is possible that Wnts first bind to hepatocytes residing in the first layer of the perivenous compartment and subsequently spread radially deeper to other perivenous hepatocytes via cell-to-cell contacts by a paracrine mechanism. Obviously, morphological-functional correlates are needed to understand the synthesis and secretion of Wnts and RSPO3 in hepatic central vein endothelial cells.

CENTRAL VEIN CONSTITUTES AN ANATOMICAL PERIVENOUS HEPATOCYTE STEM CELL NICHE

In the mouse a single layer of hepatocytes surrounding the central



Mak KM. Hepatic central vein

vein was found to selectively express the transcript of Axin2 mRNA^[1,4,89]. These perivenous hepatocytes are two-thirds diploid and one-third tetraploid, whereas other lobular hepatocytes are mostly polyploidy^[1]. They are capable of self-renewal and have a greater proliferative activity compared to other hepatocytes. Thus they are considered as hepatocyte stem cells, subserving homeostatic hepatocyte renewal associated with physiological turnover of the liver parenchyma. Following the fate of Axin2 cells by lineage tracing disclosed that their descendants move away from the perivenous area towards the portal region, replacing lobular uninjured or senescent hepatocytes. As Axin2-tagged cells leave the perivenous compartment and stream along the centro-portal axis into the midlobular parenchyma, they lose Axin2, Tbx3 and GS, but retain HNF4 α (hepatocyte fate determination transcription factor), which has a panlobular distribution^[90]. In the process, the cells acquire CPS, a phenotypic marker of periportal hepatocytes^[29]. It was estimated that after one year, on average, descendants of Axin2 cells replenish ~40% of hepatocytes in the liver under normal conditions.

Furthermore, conditional deletion of Wntless protein specifically in central vein endothelial cells of mice resulted in a loss of Wnt signaling in hepatocytes around the central vein^[1]. Because the central vein provides a source of Wnt signals that maintain perivenous Axin2⁺ hepatocytes in a local environment, the endothelium and its associated adventitial tissue of the wall is considered to constitute an anatomical niche of perivenous hepatocyte stem cells. This proposal of Wang et al^[1] is corroborated by the existence of a population of central vein-associated Axin2 progenitors in conditions of localized liver regeneration after acute injury produced by 20-30% resection of a liver lobe in mice^[91]—rather than the traditional 70% hepatectomy.

The role of perivenous Axin2 cells as hepatocyte stem cells has not been corroborated by a study in which Lgr5⁺ perivenous hepatocytes did not show any higher rate of proliferation compared to cells in other lobular zones, nor the descendants of Lgr5⁺ perivenous cells extensively re-populated the mouse liver in normal homeostasis or during liver regeneration following a 70% hepatectomy^[36]; notwithstanding Lgr5 has been shown to mark damage-induced liver stem or progenitor cells nearby the portal vein^[48,92]. Accordingly, the contribution of perivenous Axin2⁺ hepatocyte stem cells to homeostatic cell renewal in the adult liver deserves further research using additional mouse models and controls.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The Wingless gene was discovered 43 years ago^[51], and the first member of the Wnt pathway named Wnt-1 was identified some 37-32 years ago^[52,53]. Since then 19 Wnt ligands have been identified in humans and mice^[47]. Also, 11 Wnt ligands were found expressed by various cell types in mouse liver^[70]. Hepatic metabolic zonation was described 41 years ago^[72]. Some 28 years later, the canonical Wnt/ β -catenin pathway was recognized as the master regulator of hepatic metabolic zonation in the mouse^[37]. It was not until as recent as 4 years ago that the hepatic central vein was recognized as a source of Wnt2, Wnt9b, and RSPO3^[1,5]. These angiocrines activate the Wnt/ β -catenin pathway and RSPO-LGR4/5-ZNRF3/RNF43 module^[35] that regulate perivenous gene expression and maintain centrilobular metabolic zonation. Most provocatively, the hepatic central vein is considered as a component of an anatomical niche of perivenous hepatocyte stem cells that subserve homeostatic renewal of hepatocytes. The phenomenon, however, still needs validation using additional mouse models and controls. Notwithstanding, little

attention has been given to the morphological-functional correlates of the hepatic central vein in the mouse or rat, let alone in the human, either in normal or diseased liver.

Therefore, in this review, we have described the structure of the central vein of human liver. In rat liver, perivenous hepatocytes exhibit fine structural differences from periportal hepatocytes^[26]. The richer SER and peroxisome contents of perivenous cells reflect their greater activities in xenobiotic metabolism and anti-oxidative function, respectively. Similar analysis of perivenous and periportal hepatocytes in human livers is necessary.

The cellular transport and secretion of Wnts in Wnt-producing cells and the passage of these molecules in the extracellular space towards responder cells have been described^[85,86,87]. Whether the same transport mechanisms can *a priori* be applied to the situation of the hepatic central vein is not known; this awaits investigation. This is intriguing in the scenario of fibrosis of the central vein considering the thickening of the wall with increased collagenous matrix between the endothelium and perivenous hepatocytes. Moreover, fibrogenesis of the central vein is coincident with the spreading of fibrous tissue into the perivenous parenchyma, producing fibrosis of the perisinusoidal space of Disse—particularly prominent in alcoholic liver disease^[7,21,93,94,95]. The development of these pathologies may alter the local environment of perivenous hepatocytes, which in turn could affect the transit and/or strength of endothelium-derived Wnt signals. Similarly, centrilobular fibrosis is a feature of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). β -catenin activity might increase hepatic gluconeogenesis and insulin resistance, often associated with NASH (reviewed in 46). But, it is not clear what factors regulate Wnt/ β -catenin signaling in the pathogenesis of NASH. Surprisingly, there are limited advances in our understanding the role of Wnt/ β -catenin in the pathogenesis of alcoholic liver disease, NAFLD and NAHS, the most prevalent chronic liver disease worldwide.

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