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Short Review

Metabolic zonation of the liver: The oxygen gradient revisited

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ABSTRACT

The liver has a multitude of functions which are necessary to maintain whole body homeostasis. This requires that various metabolic pathways can run in parallel in the most efficient manner and that futile cycles are kept to a minimum. To a large extent this is achieved due to a functional specialization of the liver parenchyma known as metabolic zonation which is often lost in liver diseases. Although this phenomenon is known for about 40 years, the underlying regulatory pathways are not yet fully elucidated. The physiologically occurring oxygen gradient was considered to be crucial for the appearance of zonation; however, a number of reports during the last decade indicating that β-catenin signaling, and the hedgehog (Hh) pathway contribute to metabolic zonation may have shifted this view. In the current review we connect these new observations with the concept that the oxygen gradient within the liver acinus is a regulator of zonation. This is underlined by a number of facts showing that the β-catenin and the Hh pathway can be modulated by the hypoxia signaling system and the hypoxia-inducible transcription factors (HIFs). Altogether, we provide a view by which the dynamic interplay between all these pathways can drive liver zonation and thus contribute to its physiological function.

1. Introduction

The liver is the central metabolic organ responsible for maintaining blood glucose levels, ammonia metabolism, for biotransformation of xenobiotics and endogenous metabolic byproducts of metabolism, as well as for bile synthesis. All these processes require that a number of pathways and enzyme reactions are running in parallel in the most efficient manner. To achieve this, the liver parenchyma displays a functional organization known as metabolic zonation. Although known as a specific phenomenon for years, it is still largely unknown which regulatory mechanism(s) establish this pattern. Research during the last decade has shown that the Wnt/b-catenin pathway plays a major role for determining the zonal pattern in addition to the dynamic factors coming from the blood flow. From the latter, oxygen and reactive oxygen species (ROS) have been shown to be able to contribute to some features of metabolic zonation. The current review aims to connect the new observations and the improved understanding between the regulatory processes that can drive liver zonation and their potential impact on liver diseases.

2. Anatomy, functional units and zonation

At a first glance the macro and micro anatomy of the liver gives a quite uniform impression of tissue composition. However, the liver is heterogeneous in terms of cell types and functional organization (for review see [1]). On the histological level the lobule represents the smallest unit [2]. Classically it is of almost hexagonal shape with a central vein in the middle. The corners of the hexagon are formed by so called portal triads consisting of a branch from the portal vein also called terminal portal vein, and a branch from the hepatic artery also called terminal hepatic arteriole as well as a bile duct (Fig. 1A). In the lobule the parenchymal cells of the liver, the hepatocytes, are connected with each other and are visible as cords. The cords radiate out from the central vein towards the portal triads. In the cords the hepatocyte membranes are interconnected and face blood channels called sinusoids at either side. The sinusoids can almost be considered as tubes wrapped with lines of fenestrated endothelial cells; sinusoids are also populated with resident macrophages, the Kupffer cells. Importantly, there is a small space between the endothelial cell lining and the apical membrane of the hepatocytes called the Space of Disse; it is involved in lymph draining and provides a residence niche for Stellate cells which store fat and vitamin A [2] (Fig. 1B).

While the lobule represents a more structural unit, the hepatic acinus is considered to be the functional unit in terms of blood flow [2]. The acinus can be visualized by connecting two portal triads with a line from which it extends into the direction of the two adjacent central veins. Initially two zones, one around the portal triads, i.e. (the periportal zone), and a second one around the central vein (the perivenous, pericentral, or centrilobular zone) were distinguished. In the meantime, an additional intermediary zone (zone 2) is also considered (for review see [1]) (Fig. 1A). Despite the almost homogenous appearance on the histological level, the liver lobules/acini

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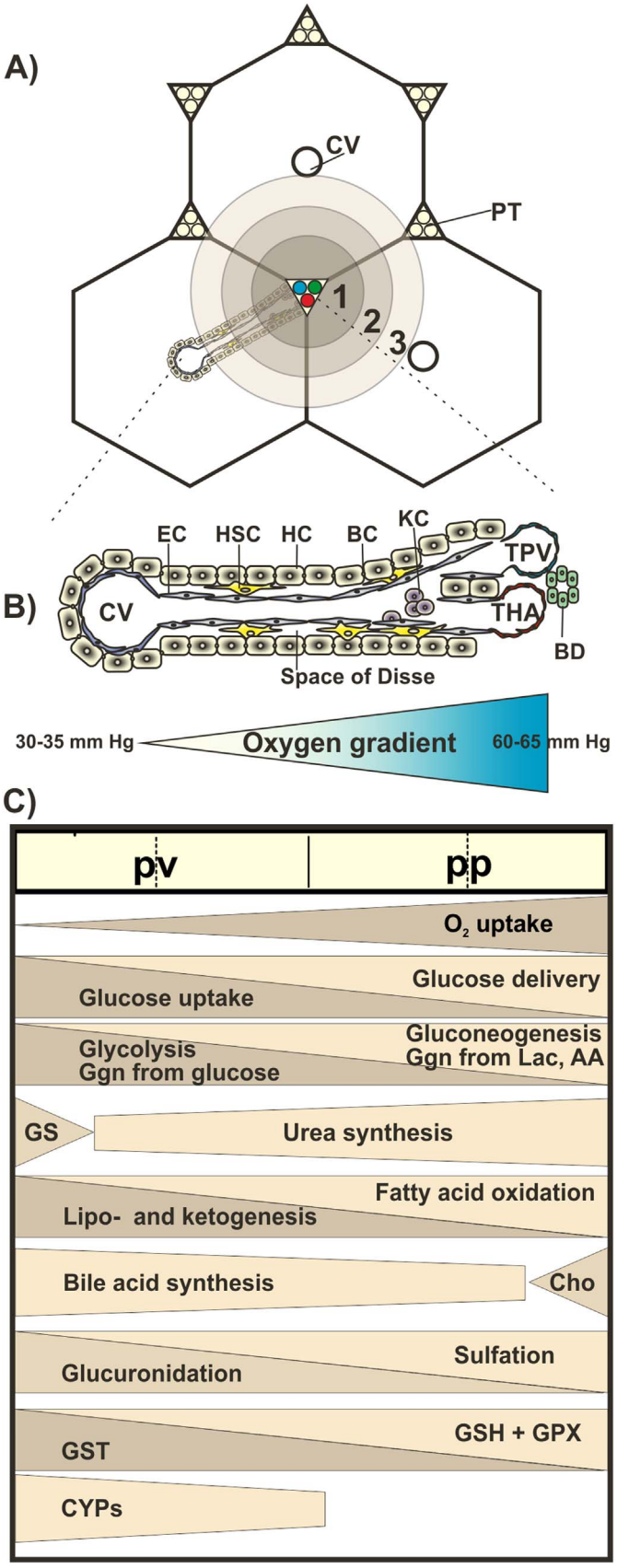

Fig. 1. Liver micro architecture, the oxygen gradient and zonation of metabolism. (A) Classic hexagonal shaped liver lobule with a central vein (CV) in the middle and portal triad (PT) corners with branch from the portal vein also called terminal portal vein (TPV, blue dot), and a branch from the hepatic artery also called terminal hepatic arteriole (THA, red dot) as well as a bile duct (BD, green dot). The acinus extends from a PT into the direction of two adjacent central veins. Three zones can be distinguished. 1, the periportal zone; 2, the intermediary zone; 3, the perivenous, pericentral, or centrilobular zone. (B) Liver sinusoid and oxygen gradient. Hepatocytes (HC), are connected with each other, bile canaliculi (BC) transport the bile formed in HC into the bile duct (BD). Sinusoids are wrapped with fenestrated endothelial cells (EC). HC and EC are separated by the Space of Disse which is the residence niche for hepatic stellate cells (HC). Resident macrophages, the Kupffer cells (KC) are also to be found in the sinusoid. (C) Distribution of major metabolic pathways. pp, periportal; pv, perivenous; AA, amino acids; Cho, cholesterol synthesis; CYP, cytochrome P450 enzymes; Ggn, glycogen; Lac, lactate; GPX, glutathione peroxidase; GS, glutamin synthesis; GST, glutathione transferase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

display an enormous heterogeneity with respect to subcellular [3,4], biochemical and physiological functions [5]. Accordingly, the key enzymes of various pathways and thus the metabolic capacities are found to be preferentially in one or the other zone (Fig. 1C), a pattern which became commonly known as metabolic zonation [6]. The zonation provides several advantages to main organ and whole body homeostasis. For example, it allows that opposing pathways are spatially separated which prevents competition for a common substrate and futile cycles. Further, complementing pathways can be linked, and substrate demanding activities can be carried out at sites with the best substrate provison. Although not all metabolic activities need to be zonated, metabolic zonation has been shown for carbohydrate, amino acid, lipid, ammonia, and xenobiotic metabolisms (covered in many excellent reviews [7–13] (Fig. 1C)). Moreover, the localization and functional activities of nonparenchymal cells also are zonated [1,14].

Zonation is rather dynamic and not static since most gene expression patterns and consequently enzyme distributions change in response to nutrition, drugs, hormones, and other blood borne factors. Although glutamine synthetase was long considered to be an example of static zonation, recent findings showing that its expression area can extend in response to thyroid hormones [15,16] and rspondins (RSPO) [17] changed this view. Accordingly, it can now be also considered to be dynamic, though the signals changing its expression may be scarce.

3. Factors involved in the regulation of zonation

Over the years a number of findings gave rise to different concepts with which the pattern of zonation could be explained. All of them, such as the streaming liver [18], developmental [19], the cell–matrix [20], and the post-differentiation patterning [21] concept have their pros and cons. Up to now, it appears that the post-differentiation pattern concept provides a quite comprehensive view. In this concept, gradients of morphogens such as Wnt, hedgehog, hormones or growth factors such as HGF, and other factors such as oxygen act in concert, in order to restrict gene expression to differentiated hepatocytes located in specific zones of the liver acinus. Thereby, they appear to act in a hierarchical fashion with the gradients of morphogens being basic and the gradients of nutrients, hormones, intrahepatically formed prostanoids, cytokines, and oxygen being modulatory with quite significant impact.

The blood flow and liver metabolism within the acinus, rather than differences in the autonomic nervous system, and biomatrix, appear to be crucial for the generation of the modifier gradients [22,23]. The blood coming from the branches of the portal vein and the hepatic artery in the portal tracts flows as a mixture through the sinusoids to the central vein. Due to metabolism and elimination the composition of blood changes and gradients of substrates, products, hormones, and oxygen are formed. The latter is of particular importance and ranges from about 60–65 mm Hg (84–91 µmol/L) in the periportal blood to about 30–35 mm Hg (42–49 µmol/L) in the perivenous blood [24]. Accordingly, the intracellular pO_2 is about 15 mm Hg lower, i.e. 45–50 mm Hg in periportal cells and 15–20 mm Hg in perivenous cells (for review see [22,23]) (Fig. 1B). This goes in line with differences in the number and structure of mitochondria [3,4,25] as well oxidative capacities in periportal and perivenous zones [26,27].

Since oxygen constitutes the basis for formation of reactive oxygen species (ROS), it is plausible that also an intra-acinar redox gradient exists. Indeed, redox images obtained from perfused livers showed a gradient curve that decreased sigmoidally from the periportal to the pericentral region [28,29].

The importance of oxygen as a regulator of carbohydrate metabolism has long been known from studies in perfused rat livers [30,31] and cultured primary hepatocytes [32–34]. While the role of oxygen for amino acid, ammonia, and lipid metabolism has been less studied, a recent mathematical model points to a role of oxygen in lipid metabolism and stresses the role of insufficient oxygen supply for the development of steatosis [13,35].

In vivo evidence for the role of oxygen as a modulator of zonation came from the observation that in livers of mice transgenic for the human erythrpoietin (EPO) gene, human EPO mRNA was detected only in the less aerobic perivenous hepatocytes [36].

4. Hypoxia-inducible transcription factors (HIFs)

The zonal distribution of key enzymes is mainly controlled by a zonated gene expression pattern which is to a large extent the result of differential transcription factor action. Important transcription factors mediating the regulation of gene expression in response to changes in the oxygen availability are hypoxia-inducible factors (HIFs). To date three HIF transcription factors (HIF-1α, HIF-2α, and HIF-3α) are known (for review see [37]). They act in a heterodimeric complex where the beta subunit is represented by an ARNT protein. The heterodimer binds to DNA areas known as hypoxia responsive elements (HREs) in target genes thereby increasing or decreasing their transcription [38]. HIF-1α and HIF-2α have overlapping but also distinct target genes (for review see [37]) and it has been suggested that HIF-1α is responsible for an acute response to low pO_2, whereas HIF-2α responds to states with more chronic hypoxia [39]. Much less is known about HIF-3; several splice variants have been detected which are even supposed to have an inhibitory function [40–42].

In line with the oxygen gradient in liver, all HIFαs were found with higher levels in the less aerobic perivenous zone [43]. The importance of HIF transcription factors for structural maintenance of the liver is exemplified in studies from mice with hepatocyte-specific HIF-1α deficiency. Those mice displayed an extension of hepatic lobules, an enhanced lobular oxygen consumption and an increased content of mtDNA [44]. Further, HIFs regulate major metabolic liver functions, and in particular studies in which either ARNT or the two HIFα subunits were deleted supported this. Liver-specific ablation of the common beta HIF subunit (ARNT) in mice increased fed insulin levels, gluconeogenesis, lipogenesis, and decreased ketone bodies [45]. Deletion of HIF-1α was shown to impair gluconeogenesis during liver regeneration [46] whereas HIF-2α was crucial for hepatic insulin signaling [47] and accordingly its constitutive activation in mouse liver resulted in development of severe hepatic steatosis associated with impaired fatty acid beta-oxidation, decreased lipogenic gene expression, and increased lipid storage capacity [48].

The abundance of HIFs in response to oxygen is primarily regulated on the level of protein degradation. Under normoxia HIFα subunits become hydroxylated at specific proline residues by prolyl hydroxylase domain-containing proteins (PHDs, encoded by the *egln* genes); four of them are known [49]. Hydroxylated HIFs are then bound by the von Hippel-Lindau (VHL) protein which serves as an E3 substrate recognition component of a multiprotein ubiquitin ligase complex that ubiquitylates HIFs and thus marks them for proteasomal degradation [50,51].

Under hypoxia, PHD activity is reduced; hence HIFs are stabilized, transported to the nucleus where they, together with their beta-subunits, bind to HREs of target genes. Extended periods of hypoxia, activate PHD2 and PHD3 expression via a HIF-1-dependent feedback cycle that leads to a rehydroxylation and degradation of at least HIF-1α [52]. In line with the HIF-dependent PHD induction are findings from liver showing that the PHDs showed a zonal distribution pattern with stronger expression in the less aerobic areas around central veins [53].

With respect to transcriptional activation of HIFs, it is noteworthy to mention another oxygen-sensitive hydroxylase, called factor inhibiting HIF (FIH) [54]. FIH acts as an asparagin hydroxylase which under normoxia hydroxylates a critical asparagine residue in the C-terminal transactivation domain of HIF-1α and HIF-2α, and thereby prevents binding of the coactivator CBP/p300 [21,55]. Importantly, PHDs have a high Km (Km ~230–250 μM – ~21% O_2) for oxygen, whereas the Km of FIH is with about 90 μM (~8%) lower [56,57]. This has impact for their action on HIFs with the effect that the activity of the activity of the N- terminal transactivation domain and the C-terminal transactivation domain of HIFs can be regulated in a biphasic manner [58]. In this way, moderate hypoxia such as 90 μM (~8%) would lead to strong effect on PHD activity but not on FIH, whereas more severe hypoxia such as ~1% would inhibit both, PHDs and FIH; however, no zonation pattern has been so far described for FIH.

Despite the fact that there are a number of common aspects with respect to PHDs and HIFs, there are also more specific differences in the effect of PHD function with respect to the HIF isoforms. For example, liver specific PHD2 deletion has been shown to induce HIF-1α, whereas PHD3 deletion promoted primarily HIF-2α induction which is in line with findings from mice where simultaneous genetic inactivation of PHD1, PHD2, and PHD3 in mice reactivates hepatic expression of the HIF-2 target gene erythropoietin and stimulates red blood cell synthesis [59]. With respect to metabolism it was found that PHD2 inhibition improves glucose and lipid metabolism as well as protects against obesity and metabolic dysfunction [60].

Apart from oxygen, a number of signaling substances, stress inducers and signaling pathways have been shown to be able to regulate HIFs under normoxia, i.e. in the presence of oxygen and ROS; the interested reader is referred to a number of reviews covering that topic in more detail [61–66]. In brief, heavy metals, hormones such as insulin, growth and coagulation factors such as HGF, IGF, thrombin, cytokines such as TNFα, lipopolysaccharides (LPS), and mechanical stress have been shown to contribute to that regulation by involving one or more intracellular signaling pathways such as the phosphatidylinositol-3-kinase (PI3K) protein kinase B/Akt, the mTOR, p70S6K1, and RAS/RAF/ERK1/2 as well as mitochondrial metabolites such as the PHD/FIH substrate α-ketoglutarate [65].

5. HIF, PHDs, redox and zonation

ROS have been shown to be important signaling molecules in a number of the above mentioned pathways and they also play an important role in HIF signaling (for review see [66]). Both HIF-1α and HIF-2α can be modified by ROS in a direct and indirect manner. Direct regulation requires presence of redox factor-1 (Ref-[1]) and affects transactivation of HIF-1α at Cys800 and of HIF-2α at Cys848 [67] as well as recruitment of coactivators such as steroid receptor coactivator-1 and transcription intermediary factor 2 [68]. Another direct redox effect is oxidation of the Cys present in the DNA-binding domain of HIF-2α, but not HIF-1α [69]. The indirect effects of ROS are mediated via regulation of PHDs, FIH, redox-sensitive kinases, and phosphatases (for review see [66,70]). As mentioned the PHDs hydroxylate HIF-1α and HIF-2α at critical proline residues thereby inducing HIF degradation under normoxia. The PHDs belong to a family of oxygen, Fe^{2+}, 2-oxoglutarate, and ascorbate dependent dioxygenases which need a radical cycling system to regenerate the iron after each catalytic cycle [71]. Even though ascorbate is a key agent in the regeneration of iron, glutathione could substitute it in mice deficient in vitamin C synthesis, pointing to the importance of thiol oxidation/reduction cycles [72]. In line, a pair of cysteine residues in one of the PHDs was described to modulate its redox sensitivity, again highlighting the potential of thiol oxidation in regulating PHD activity,

though in endothelial cells subjected to hypoxia, no variation in PHD cysteine oxidation was observed [73].

Both, HIF-1α and HIF-2α could be prevented from degradation by ROS that were generated by NOX4 [74] or at the Qo site of mitochondrial complex III [75,76]. From the ROS formed, hydrogen peroxide seems to be of major importance for HIF regulation since overexpression of glutathione peroxidase or catalase, but not superoxide dismutase 1 or 2, prevented the hypoxic stabilization of HIF-1α [75–77]. Together, ROS appear to constitute an important link for HIF regulation especially in connection with altered mitochondrial activity and abundance [78]. In this respect it is important to note that HIF-1 reduces ROS production under hypoxia via a subunit switch in cytochrome c oxidase from the COX4-1 to COX4-2 regulatory subunit that at the same time increases efficiency of complex IV [79]. HIF-1 also induces pyruvate dehydrogenase kinase 1 and 4 with the effect of blocking pyruvate entry into mitochondria [80]. Moreover, HIF-1 contributes to mitochondrial autophagy under hypoxia via expression of BNIP3 [81] and miRNA-210 [82], which blocks assembly of Fe/S clusters that are required for oxidative phosphorylation and therefore prevent increased ROS formation and cell death. All these mentioned findings with respect to HIF and regulation of mitochondrial metabolism are very much in line with the zonal differences in the number and structure of mitochondria as well oxidative capacities [3,25–27] in periportal and perivenous zones, respectively.

Moreover, the recent findings that autophagy in liver is regulated by a PI3K-protein kinase B/Akt-FOXO3 glutamine synthetase expression network are also in agreement with the above mentioned findings [83]. The FOXO3 glutamine-synthetase-dependent autophagy occurs primarily in the perivenous zone [84] in which FOXO3 appeared to be primarily expressed and active. This feature goes nicely along with findings showing that FoxO3a transcription can be upregulated by HIF-1α in MEFs and NIH3T3 fibroblasts [85] and by the PHD inhibitor dimethyloxalyl glycine in a mouse glomerular microvascular endothelial cell line [86]. Moreover, prolylhydroxylation of FOXO3 by PHD1 (*egln2*) destabilized it by inhibiting its interaction with one of the ubiquitin-specific protease family members (USP9x) [87].

As mentioned above, HIFα signaling is known to undergo a crosstalk with both PI3K/Akt and RAS/RAF/ERK1/2 cascades where ROS act as activators [88–90]. Further, p38 MAPKs and the p38 upstream kinases MKK3 and MKK6 [91] were shown to be involved in the induction of HIF-1α by thrombin [92] and chromium (VI) [91]. In addition, these substances can also induce HIF-1α mRNA levels in several cell types [93–96]. By comparing the expression patterns of periportal and perivenous hepatocytes of liver tumors with activating Ha-RAS mutations it was proposed that growth factor signals such as that of HGF acting via the RAS/RAF/ERK1/2 pathway are important for liver zonation [97]. Although HGF acts primarily as mitogen for hepatocytes during liver regeneration [98–100] with higher activity in the periportal zone than in the perivenous zone [101], it is known that HGF is able to stimulate HIF activity [102] via the redox-sensitive transcription factor NFκB [94].

In line, HIF-1α is a direct target gene of NFκB [103–107], which was also found to be inducible by hypoxia [108] and ROS generated in response to various stimuli like e.g. ethanol [109]. Thus, hypoxia and ROS regulate also NFκB–dependent HIF-1α transcription [104,107], that could have an impact on zonation. Interestingly, NFκB expression and nuclear distribution of NFκB displayed a zonal pattern. In rat liver the overall NFκB p65 subunit expression was higher in hepatocytes of the periportal area. However, and more importantly, NF-κB p65 displayed a predominant nuclear localization in hepatocytes of the perivenous area [110], in line with the findings of a higher HIFα mRNA expression in the same area [43].

Another redox regulated transcription factor which regulates the HIF system is Nrf2 (Nfe2l2; nuclear factor (erythroid-derived 2)-like 2) [111]. Nrf2 is known to contribute to intermediary and xenobiotic metabolism, bile production, as well as liver regeneration, and carcinogenesis [112–118]. Livers deficient in Nrf2 are reduced in size and two thirds of Nrf2-disrupted mice display an impaired vascularization with the appearance of a congenital intrahepatic shunt that directly connects the portal vein to the inferior vena cava. This congenital intrahepatic shunt reduced centrilobular hypoxia and decreased perivenous Cyp2e1 expression while phosphoenolpyruvate carboxykinase, normally confined to the periportal zone, exhibited both a periportal and perivenous expression pattern [119].

Further, proper vascular development and morphogenesis of hepatic sinusoids was shown to be dependent on vascular endothelial growth factor (VEGF) [120], another hypoxia-inducible gene [121]. Thereby, an intercellular crosstalk is also important and lack of VEGF from liver epithelial lineages during midgestational development disturbed zonal endothelial and hepatocyte cell differentiation as well as formation of a three-dimensional vascular and zonal architecture [120].

In vivo, these signaling pathways do not act per se alone and thus it is easy to envision that HIFs are regulated by interconnected signaling which may, with time, and under certain physiological or pathological conditions, induce or prolong HIFα abundance. For example, ischemia due to an acute reduction in blood flow stabilizes HIF-1α [122]. Further, ROS generated during reperfusion can inactivate the PHDs, leading also to HIFα accumulation. Consequently, the following remodeling processes again alter oxygen and nutrient supply which also have an impact on HIF levels and signaling. Thus, it is likely that small changes in tissue pO_2 or activation of non-hypoxic pathways in the less aerobic perivenous zone of the liver (i.e. ~4–8% O_2), becomes highly significant for liver function.

6. Beta-catenin and zonation

Interesting seminal discoveries have shown that the Wnt/β-catenin pathway is an important driver of hepatic zonation [123,124]. Similar to HIFs, β-catenin abundance is regulated post-translationally and in the absence of Wnt signals, β-catenin resides in a multiprotein complex together with glycogen synthase kinase-3 (GSK3), adenoma polyposis coli (APC), CK1, Axin, and the protein Dishevelled (DVL). Within this destruction complex, β-catenin becomes phosphorylated by GSK3 which is the prerequisite for recruitment of the ubiquitin ligase β-TRCP that ubiquitylates β-catenin; thus, marking it for degradation by the proteasome [125,126]. In the canonical Wnt pathway, binding of Wnt ligands to its receptor, Frizzled, and co-receptors, such as the low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6 [127,128], destroys the destruction complex. As a consequence, β-catenin becomes stabilized and is transported into the nucleus where it acts as a coactivator for the transcription of Wnt target genes by binding to transcription factors from the T-cell factor (TCF) and lymphoid enhancer factor (Lef) family [129].

Stabilized and active β-catenin is found in perivenous hepatocytes, whereas the negative regulator of β-catenin, APC, is predominantely localized in periportal hepatocytes [123]. Importantly, genetic ablation of APC activates the β-catenin pathway also in the periportal zone. Reciprocally, inhibition of β-catenin signaling in the liver acinus leads to a periportal phenotype in the perivenous hepatocytes [123].

Experiments with fetal rodent hepatocyte cultures supported the role of the Wnt/β-catenin system for metabolic zonation. When these hepatocytes were differentiated in culture to mature hepatocytes only periportal gene expression markers could be detected. However, when the cells were differentiated in the presence of a β-catenin activator this induced a reversible expression of perivenous marker genes [130,131].

Recent findings have indicated that proteins from the Rspondin (Rspo) family are important Wnt pathway activators [132]. Accordingly, conditional deletion of Rspo3 in mice abrogates proper perivenous zonation. Further, overexpression of another Rspo member, Rspo1, induced expression of perivenous marker genes periportally indicating that Rspo members may be important angiocrine signals

modulating the β-catenin-dependent liver zonation [133].

Rspondins possess their own receptors, the LGRs, which together with Rspo prevent clearance of frizzled receptors from the membrane. As a consequence, Wnt signaling is promoted [134]. The LGR system has been shown to be of importance for stem cell renewal, a process also evidently necessary for liver regeneration. In particular LGR5, but not LGR4, is expressed at high levels in damage-activated liver stem cells [135] and with a zonated expression pattern; LGR5 mRNA was exclusively found in perivenous hepatocytes [136], the area showing the lowest oxygen content but an active Wnt/β-catenin signaling in adult livers. In line with these observations are current findings indicating that deletion of LGR4/5 in mouse liver deregulated the expression of periportal and perivenous marker genes such as glutamine synthetase and reduced liver weight [17]. Further, the LGR ligand Rspo3 shows a highly restricted expression pattern in the liver acinus and can be detected only in the endothelial cells of the central vein. Interestingly our own preliminary findings from hepatocytes and HepG2 cells cultured under hypoxia indicated that LGR5 mRNA is induced by hypoxia whereas Rspo´s are not, which indicates that the LGR-β-catenin connection can be controlled by the oxygen gradient in the liver acinus.

7. Hypoxia, HIFs, and β-catenin signaling: a new liaison

Although an interplay between hypoxia, HIFs, and β-catenin signaling was not yet directly shown for liver zonation, there are several reports showing an interrelation between β-catenin and hypoxia signaling. In particular it was found that lower oxygenated adult brain areas exhibit an enhanced Wnt/β-catenin signaling in-vivo. Exposure of mice to chronic hypoxia (10% O_2 for 6–72 h) stimulated the activation of Wnt/β-catenin signaling, and activated neurogenic cell proliferation in the subgranular zone of the hippocampal dentate gyrus. Concomitant with exposure to 10% O_2, HIF-1α and β-catenin levels were increased and Dvl3 phosphorylation as well as transcription of Wnt target genes in the hippocampus was stimulated [137]. Further, hypoxia increases β-catenin signaling in cultured neonatal hippocampal stem cells [138] and embryonic stem cells (ESCs) [139]. This induction was shown to be mediated by the HIF system since deletion of the *hif-1α* gene and the HIF-beta encoding gene *arnt* diminished expression of Wnt/β-catenin target genes including Dkk-4, Lef-1 and Tcf-1 under hypoxia [139]. Further, it was found that upon exposure of cells to hypoxia, HIF-1α can directly bind to the Lef1 and TCF1 gene promoters [139] and in consequence promote the transcriptional activity of β-catenin.

In β-catenin-deficient mouse liver HIF-1α signaling was reduced and affected by the cellular redox balance indicating a role of β-catenin as coactivator of HIF-1α signaling [140]. Indeed, HIF-1α was found to directly interact with β-catenin, thereby competing with TCF-4. DNA–protein interaction analyses revealed that the HIF-1α-β-catenin interaction occurs at HIF-1 target gene promoters [141]. Thus, these results suggest that β-catenin promotes HIF-1-mediated transcription, adaptation to hypoxia, and cell survival.

The expression of perivenous genes was also associated with the interaction of β-catenin with LEF1 and hepatocyte nuclear factor 4-α (HNF4α) that could also be detected with higher levels in the perivenous area [142]. HNF4α is known to act together with HIFs as transcriptional activator for hypoxia-dependent erythropoietin expression [143] which can be detected perivenously during fetal development. Despite the perivenous predominance of HNF4α, it appeared to act as repressor of perivenous β-catenin regulated genes such as glutamine synthetase (GS) and cytochrome P450 2e1 which in the absence of HNF4α appeared in the periportal zone [144]. Interestingly, deficiency of Cited2, another coactivator of HNF4α and also a HIF target gene [145], lead to a disorganized sinusoidal architecture, as well as impaired lipid metabolism and hepatic gluconeogenesis [146] suggesting that hypoxia mediated feedback mechanisms exist which may be controlled at the level of coactivator recruitment.

Further, in response to hypoxia β-catenin was shown to move from the plasma membrane to the cytoplasm where it binds and stabilizes the zink finger containing snail superfamily member SNAI2 and carbonic anhydrase 9 (CA9) mRNAs, in cooperation with the mRNA stabilizing protein HuR, a process which is important for the onset of cancer stem cell features [147]. These findings link hypoxia signaling with the regulation of RNA abundance which is largely affected by the microRNA pathway. Dicer, the key endoribonuclease that processes precursor microRNAs into mature microRNAs was found to be involved in the regulation of zonation; lack of Dicer led to a loss of the periportal pattern and to a diffuse expression of phosphoenolpyruvate carboxykinase, E-cadherin, arginase 1, and carbamoyl phosphate synthetase-1 within the entire acinus [148]. Although this pattern was similar to that seen upon loss of β-catenin, the authors did not find down-regulation of Dicer1 or any microRNAs in β-catenin-deficient liver and suggested involvement of an indirect mechanism. Interestingly, Dicer1 was found to be inducible by hypoxia and to be necessary for the function of HIFs and full expression of HIF target genes [149] from which some are eventually β-catenin repressors.

While the above mentioned findings indicate the links between hypoxia and β-catenin regulation, it is also of utmost importance that the expression of the negative β-catenin regulator APC is suppressed by hypoxia and HIF [150]. Upon exposure of several cell lines to hypoxia (1% O_2) APC levels were decreased. This decrease was found to be transcriptionally mediated via HIF-1α since depletion of HIF-1α with siRNA restored the APC levels. Further analyses identified a functional hypoxia-responsive element in the APC promoter. Reciprocally, APC was able to mediate a repression of HIF-1α; a process which, in addition to wildtype APC, required low levels of β-catenin and NFκB activity [151]. Importantly, in this context the action of APC appears to involve several cellular compartments such as the nucleus, and mitochondria [152–154]. The latter are being found at higher number in the periportal zone and constitute major sites of ROS production [3,4,25]. Both, β-catenin and HIF-1α signaling are modulated by ROS [155] and in particular superoxide and H_2O_2 are considered to serve as messengers in this regulation [65]. Hence, changes in the oxygen availability and consequently in superoxide production may have a major influence on β-catenin and HIF-1α signaling as well as on zonation. Indeed, hepatocyte-specific deletion of manganese superoxide dismutase (MnSOD; *sod2*) which generates H_2O_2, caused a disruption of the zonal gene expression [156]. Further, HIF-1α as well as β-catenin were absent in hepatocyte-specific MnSOD-deficient mice which were also more prone to chemically-induced carcinogenesis [157].

Together, these findings are in line with the view that the low oxygen content in perivenous hepatocytes leads to induced HIF function which mediates APC repression and consequently stabilization and nuclear localization of β-catenin. Vice versa, loss of HIF function in the more oxygenated and ROS enriched periportal zone allows full APC expression with the result that β-catenin signaling is suppressed.

8. Hedgehog signaling

Recently it was proposed that Hedgehog (Hh) signaling, that is in particular important during development and regeneration, has also a determining role for metabolic zonation [158]. In adult liver, Hh signaling is low in hepatocytes whereas hepatic stellate cells and cholangiocytes are Hh positive [159,160]. In particular Hh signaling becomes activated upon certain states of liver damage as seen in non-alcoholic fatty liver disease (NAFLD), liver cirrhosis, and hepatocellular carcinoma (HCC) as well as after partial hepatectomy [161,162]. The known Hedgehog ligands (Sonic-Hh, Indian-Hh, and Desert-Hh) exert their effects after binding to Patched (PTCH1, -2) receptors on the surface of Hh-responsive cells. In the canonical Hh pathway this relieves the inhibitory actions of PTCH's on the signaling co-receptor

Smoothened (SMO) which then promotes the nuclear localization and activation of the glioma-associated oncogene transcription factors GLI1, GLI2, and GLI3 [163].

Mouse liver displayed a perivenous zonation of IHh [164] and conditional hepatocyte-specific deletion of SMO resulted in periportal lipid accumulation and up-regulation of key lipogenic transcription factors such as SREBP and PPARs and enzymes such as FASN [165]. The latter displayed a reversed zonation in the SMO deleted mice; instead of being perivenous [8,166] like in the control mice, FASN was detected in the periportal zone of the SMO knockouts. Further, from the Gli transcription factors especially Gli3 appeared to be responsible for the observed effects, in particular in the SMO-deletion mediated development of steatosis [165]. Importantly, cholesterol biosynthesis, glycogen content, and glycolysis were not altered in hepatocyte-specific SMO deleted mice suggesting that the major role of the Hh pathway consist in, but is not limited, to regulate lipid metabolism and its zonation.

Interestingly, the regulation of IGF1 and IGFBP1 which were found to be expressed in the periportal and perivenous area, respectively [167], was also affected in the same SMO knockout model; Deletion of SMO decreased IGF1 but increased IGFBP1 expression [168].

9. Hypoxia, HIFs, and hedgehog signaling: another liaison

Although the findings with respect to lipid metabolism as well as IGF1 and IGFBP1 expression in the hepatocyte specific SMO-deficient mice support a role of Hh signaling for metabolic zonation, they correlate also well with the role of oxygen in the regulation of zonation.

With respect to fatty acid synthesis it has been shown that FASN can be induced by hypoxia [169]. Thereby, hypoxic induction of FASN appears to be an indirect HIF effect involving first HIF-dependent up-regulation of SREBP1 and then an action of SREBP1 on the promoter of FASN [169]. By contrast and similar to APC, HIF-1 inhibited β-oxidation by suppressing expression of medium- and long-chain acyl-CoA dehydrogenases (MCAD and LCAD) [170] which is in line with the old findings that under starvation, oxidation of FAs is more pronounced in periportal hepatocytes.

The regulation of IGF1 expression by oxygen is supported from findings showing that serum IGF-1 levels were lower in cyanotic than in acyanotic congenital heart disease patients [171,172] which fits with the reduced perivenous IGF1 appearance. The role of oxygen, ROS, and the HIF system was much more explored with respect to IGFBP1 expression in primary rat hepatocytes where perivenous oxygen tensions enhanced IGFBP-1 expression. Experiments with the iron chelator desferrioxamine and H_2O_2 supported the concept that ROS and the HIF system are involved [173]. Interestingly and in line with experiments from zebrafish [174], the induction of IGFBP1 appeared to be mediated by HIF-3α and HIF-2α, and to a lesser extent by HIF-1α. The participation of the HIF system was further supported by experiments targeting the HIF proline hydroxylases [173].

Although these findings suggest that the Hh and HIF pathways may act in a separate manner, ample evidence exists for a connection between the HIF pathways with the Hh signaling.

The interconnection between the Hh and hypoxia response pathway became first evident by findings showing that hypoxia can induce expression of the Hh ligand SHh, the pathway activity marker Patched1, and subsequently a systemic Hh response in adult mice [175]. Interestingly, the Hh response followed the accumulation of HIF-1α and various HIF inhibitory approaches revealed that lack of HIF-1α blunted hypoxia-dependent Hh activation [175]. Further, it was shown that also SMO can be transcriptionally induced in response to hypoxia in various cell models [176,177]. In addition, hypoxia was also able to act on GLI1 transcription factors without SMO via PI3K or ERK1/2 signaling. Furthermore, the hypoxia effects on Hh signaling were not limited to HIF-1α but also shown to involve HIF-2 α, depending on the cell type [178].

Remarkably and similar to the connection between HIF and β-catenin pathways, there appears to exist also a feed-back regulation between the HIF and the Hh system. In particular, the SHh-GLI1 pathway was able to upregulate HIF-2α levels under normoxic conditions [178]. Predominantly this crosstalk appears to be of importance during liver damage, where cholangiocytes and myofibroblasts secrete Hh ligands in order to promote survival and proliferation of both cell types [159,160]. Upon liver damage, hepatic stellate cells become activated myofibroblasts via an Epithelial-to-Mesenchymal-Transition (EMT) and the Hh pathway was shown to be a major regulator of the stellate cell to myofibroblast transition [160]. During this transition a metabolic switch occurs which is in favor of aerobic glycolysis and GlI transcription factors and HIF-1α appeared to be involved in this switch. In mice with different types of liver injury genetical or pharmacological inhibition of SMO, attenuated HIF-1α expression and suppressed glycolytic gene expression. Reciprocally, activating SMO up-regulated HIF-1α mRNA expression, and chromatin immunoprecipitation assays (ChIP) identified that GLI proteins interact with the *hif-1a* promoter [179].

Moreover, the Hh pathway undergoes also a cross talk with the Wnt/β-catenin signaling pathway. This crosstalk appears to be mutual and even complex with the involvement of common components but different functional aspects of the GLI transcription factors and tissue specificity. In adult tongue epithelium dominant activation of β-catenin lead to a significant up-regulation of Shh which then diminished β-catenin signaling [180]. Vice versa, IHh is a target of Wnt/β-catenin, and in liver it has been shown that the perivenous area expressing IHh is extended upon activation of β-catenin signaling [164]. However, these regulations may further divide at the level of GLI or TCF transcription factors. In quail embryos GLI2 and GLI3 were shown to be regulated by Wnt/β-catenin signaling whereas GLI1 activation in somites was shown to be controlled by SHh signaling [181]. In mouse chondrocytes activation of hedgehog signaling selectively inhibited β-catenin-induced FGF18 expression. The selectivity was shown to be due to the Hh mediated induction of a dominant negative isoform of TCF7L2 (dnTCF7L2) in so called interzone progenitor cells [182]. Moreover, Wnt/β-catenin signaling can have repressive effects on SHh signaling in a number of cancer cells [183]. For example SHh signaling was high during differentiation of gastric cancer cells, whereas Wnt signaling was decreased during differentiation [184]. Altogether, it is obvious that an intricate interplay between Wnt and SHh signaling occurs in which oxygen availability has an important role, although the detailed mechanisms remain still largely unclear, hence more investigations are required to further clarify the role of the Wnt/Hh crosstalk for metabolic zonation in liver.

10. Conclusion

The multitude of functions including being the major metabolic organ puts the liver into an important strategic position for maintaining whole body homeostasis. Metabolic zonation of the liver is an important feature that helps to achieve this. Research during the past decade provided considerable information into the complex underlying networks involved in liver zonation. In particular, these studies have established a major role for β-catenin signaling, and the involvement of the Hh pathway in metabolic zonation. These findings are still well in accordance with the “old” concept that the oxygen gradient within the liver acinus is a regulator of zonation. This is underlined by a number of facts showing that β-catenin signaling and the Hh pathway can be modulated by the HIF system. Given the zonation of non-parenchymal cells such as bile duct cells and hepatic stellate cells which are both predominantely found in the portal tract and periportal area, respectively, they may well be secretors of Hh signals. The Hh signals in turn could, by leaving a gradient, spread into the perivenous direction. In the oxygen rich periportal area Hh signaling could, at least in a large part, inhibit β-catenin signaling. The low oxygen content in the perivenous zone would, via the HIF system, activate β-catenin, and

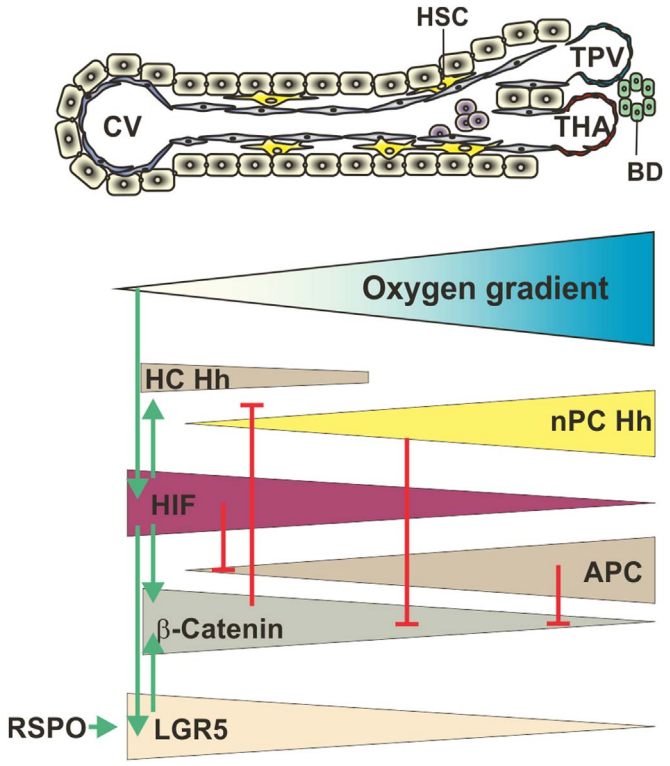

Fig. 2. Impact of the oxygen gradient on β-catenin and hedgehog signaling and metabolic zonation. Non-parenchymal cells such as bile duct cells and hepatic stellate cells are more abundant in the oxygen rich periportal area and secrete Hh signals (nPC Hh) and inhibit β-catenin signaling. The low oxygen content in the perivenous zone activates the HIF system, induces LGR5 expression and activates β-catenin as well as suppresses expression of the negative β-catenin regulator APC. Rspondins secreted from the central vein endothelial cells, activate β-catenin via LGR5 in perivenous hepatocytes. To maintain homeostasis, hypoxia activates expression of Hh components in hepatocytes (HC Hh) to feedback inhibit β-catenin.

LGR5 expression as well as suppress APC expression. These, together with Rspondins secreted from the central vein endothelial cells, may lead to an active β-catenin/TCF genetic program in perivenous hepatocytes. To keep balance and to maintain homeostasis, hypoxia activates expression of Hh components IHh, SMO and likely GLI1 at the same time (Fig. 2).

The view that the oxygen gradient is an organizing principle for tissue structure and organization by mediating a critical balance between several regulatory pathways such as β-catenin and hedgehog may be of critical importance for physiological and pathological as well as developmental processes in liver and in general. Nonetheless, the picture of liver zonation does not yet have the highest resolution. In order to improve it, more comprehensive mechanistic analyses are necessary. This may include experiments designed to unravel the complex interplay between the oxygen gradient, ROS, and the different liver cell types. At the cellular level this encompasses investigations with respect to the role of long non-coding RNAs, miRNAs, and different epigenetic modifications. Thereby, novel techniques and improved animal models will help to solve conflicting data which may exist due to technical limitations in older studies. Finally, this will lead to a much clearer picture about the role of the oxygen gradient and ROS action in liver, and improve our understanding of several diseases associated with hypoxia and ROS such as ischemia/reperfusion injuries, NAFLD, NASH, and hepatocellular carcinoma.

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