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Review

Wnt signaling in liver regeneration, disease, and cancer

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The liver exhibits the highest recovery rate from acute injuries. However, in chronic liver disease, the long-term loss of hepatocytes often leads to adverse consequences such as fibrosis, cirrhosis, and liver cancer. The Wnt signaling plays a pivotal role in both liver regeneration and tumorigenesis. Therefore, manipulating the Wnt signaling has become an attractive approach to treating liver disease, including cancer. Nonetheless, given the crucial roles of Wnt signaling in physiological processes, blocking Wnt signaling can also cause several adverse effects. Recent studies have identified cancer-specific regulators of Wnt signaling, which would overcome the limitation of Wnt signaling target approaches. In this review, we discussed the role of Wnt signaling in liver regeneration, precancerous lesion, and liver cancer. Furthermore, we summarized the basic and clinical approaches of Wnt signaling blockade and proposed the therapeutic prospects of cancer-specific Wnt signaling blockade for liver cancer treatment. **Clin Mol Hepatol 2022 Jul 4. [Epub ahead of print]**

Keywords: WNT signaling pathway; Liver regeneration; Liver diseases; Hepatocellular carcinoma

INTRODUCTION

Liver regeneration has been extensively studied.[1-3] *In vivo* studies have shown that partial hepatectomy or chemical injury activates extracellular and intracellular signaling pathways, leading to liver regeneration. Hepatocyte loss during chronic liver diseases triggers compensatory proliferation of the surviving hepatocytes.[4-6] Apart from liver regeneration in physiological conditions, genotoxic risk factors might lead them to convert to neoplasia. Hepatitis virus, alcohol abuse, non-alcoholic fatty liver disease (NAFLD), and aflatoxin-B1 exposure are also the main etiological factors to induce the development of precancerous lesions in the liver. Liver cancer is one of the top 10 lethal cancers worldwide. Its estimated

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death rate in 2021 is 6% in males and 4% in females.[7] Liver cancer consists of hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), hepatoblastoma (HB), and several other rare tumors (angiosarcoma, intraductal papillary neoplasm of the bile duct, and mucinous cystic neoplasm). HCC is the most common primary liver cancer frequently developed with chronic liver disease, such as cirrhosis caused by hepatitis virus infection.[8]

Among various signaling pathways associated with liver biology,[9-12] Wnt signaling is involved in all stages of liver disease progression, from liver injury to inflammation, fibrosis, cirrhosis, and tumorigenesis. Several Wnt ligands are secreted by various hepatic cells, including hepatocytes, stellate cells, Kupffer cells, biliary epithelial cells, and sinusoidal endothelial cells.[13-16] Based on the oncogenic roles of Wnt signaling in cancer, several components and regulators of Wnt signaling have been proposed as the druggable targets to improve the current therapeutic efficacy in the liver cancer treatment.[17]

Herein, we review the roles of Wnt signaling in liver regeneration and liver tumorigenesis and the therapeutic targets of Wnt signaling in liver cancer treatment.

Wnt SIGNALING

Wnt signaling is evolutionarily conserved and orchestrates various cellular processes, including cell proliferation, differentiation, migration, polarity, stemness, and lineage plasticity.[18,19] Consequently, Wnt signaling plays a pivotal role in organogenesis, tissue homeostasis, tissue regeneration, and tumorigenesis.[20-25] The Wnt signaling is triggered by the binding of the Wnt ligands to the frizzed (FZD) receptors. The mammals have 19 Wnt ligands and 10 FZD receptors,[26] resulting in the complexity and specificity in Wnt signaling activation. Based on the involvement of β-catenin, a key component of Wnt signaling, Wnt signaling is generally classified into canonical (β-catenin-mediated) and non-canonical (β-catenin-independent) Wnt signaling (Fig. 1). In the canonical Wnt/β-catenin pathway, the protein destruction complex (casein kinase 1 [CK1], glycogen synthase kinase 3 [GSK3], adenomatous polyposis coli [APC], and axis inhibition proteins [AXINs]) targets the β-catenin protein for degradation via CKI1 and GSK3-mediated sequential phosphorylation at the N-terminus (Ser-45, Thr-41, Ser-37, and Ser-33) of β-catenin followed by β-TrCP, an E3 ligase, recruitment. Conversely, binding of the canonical Wnt ligands to the FZD receptors and LRP5/6 co-receptors activates dishevelled (DVL), which inhibits the protein destruction complex. As a result, β-catenin protein is stabilized and translocated into the nucleus to transactivate the canonical Wnt target genes by replacing the co-repressors associated with the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) with the co-activators. Non-canonical Wnt signaling pathways include the planar cell polarity pathway (involved in c-Jun N-terminal kinase [JNK] activation, small GTPase activation, and cytoskeletal rearrangement), and the Wnt/Ca^{2+} pathway (activating phospholipase C [PLC] and protein kinase C [PKC]).[18,19]

Wnt SIGNALING IN LIVER REGENERATION

Upon partial hepatectomy or acute liver injury, the number of hepatocytes is drastically reduced. Various signaling pathways (epidermal growth factor [EGF], hepatocyte growth factor [HGF], Wnt/β-catenin, and Notch) stimulate the hepatocytes in the G0 phase to proliferate, compensating tissue loss and restoring the physiological functions of the liver.[27-29] During liver regeneration, endothelial cells under shear stress

Abbreviations:

AFB1, aflatoxin type B1; ALD, alcoholic liver disease; APC, adenomatous polyposis coli; AXIN, axis inhibition protein; CCA, cholangiocarcinoma; CCl_4, carbon tetrachloride; CEBPA, CCAAT enhancer-binding protein α; CK1, casein kinase 1; CREBBP/CBP, CREB binding protein; CV, central vein; DDC, 1,4-dihydro-2,4,6-trimethyl-pyridine-3,5-di carboxylate; DEN, diethylnitrosamine; DKK1, Dickkopf 1; DVL, dishevelled; E2F1, E2F transcription factor 1; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; EtOH, ethanol; FZD, frizzed; GPC3, glypican-3; GSK3, glycogen synthase kinase 3; HB, hepatoblastoma; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HGF, hepatocyte growth factor; Ig, immunoglobulin; IGF1, insulin-like growth factor 1; JNK, c-Jun N-terminal kinase; KO, knock-out; LGR5, leucine-containing repeat G-protein-coupled receptor 5; LOF, loss-of-function; LRP6, lipoprotein receptor-related protein 6; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; MMP7, matrix metallopeptidase; mTOR, mammalian target of rapamycin; MVBs, multivesicular bodies; NAFLD, non-alcoholic fatty liver disease; NCOA2, nuclear receptor coactivator 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; peg-IFN, pegylated-Interferon-α2a; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PORCN, porcupine; PPARG, peroxisome proliferator-activated receptor γ; RanBP3, Ran-binding protein 3; ROS, reactive oxygen species; SAL, salinomycin; SFRP1, secreted frizzled-related protein 1; sFZD7, secreted FZD7; SREBF, sterol regulatory element binding transcription factor; TCF/LEF, T-cell factor/lymphoid enhancer-binding factor; TMEM9, transmembrane protein 9; TNKS, tankyrase; VEGF, vascular endothelial growth factor; WF1, Wnt inhibitory factor 1; WISP1, Wnt1-inducible-signaling pathway protein 1

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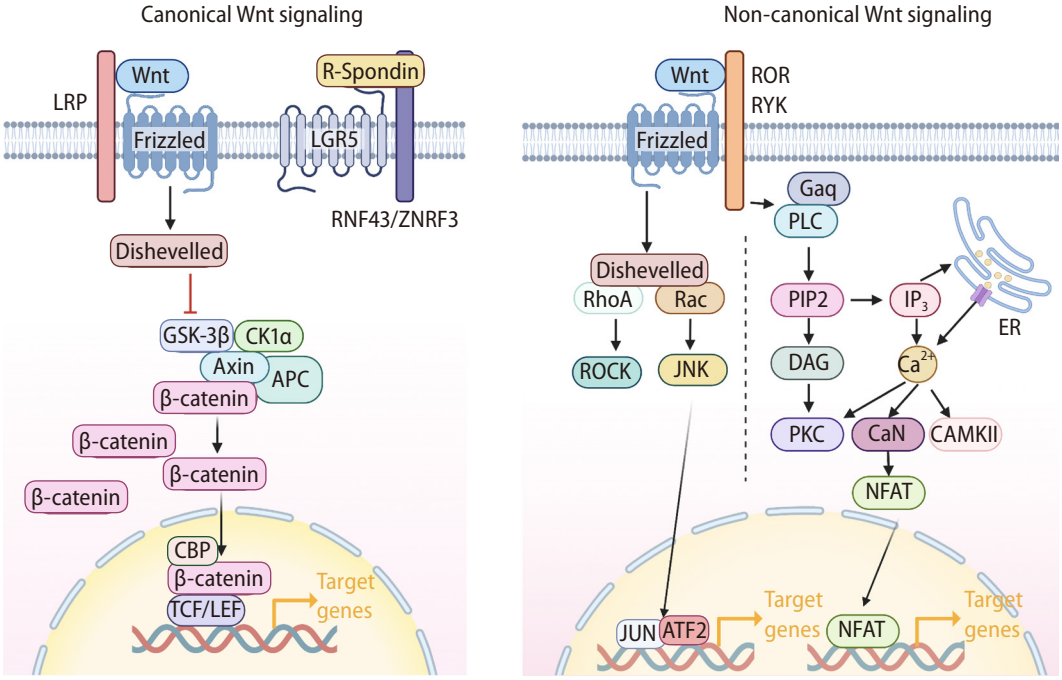

Figure 1. Wnt signaling. Illustration of canonical and non-canonical Wnt signaling. The hallmark of the canonical Wnt/β-catenin pathway is the stabilization and nuclear translocation of β-catenin. In the absence of Wnt ligands, cytoplasmic β-catenin is degraded by the destruction complex (Axin, APC, GSK3β, and CK1α). Upon Wnt ligand binding to Frizzled receptors (FZDs) and LRP, the destruction complex is inhibited, β-catenin protein is stabilized in the cytosol and translocated into the nucleus. Nuclear β-catenin then recruits transcriptional coactivator CREBBP to transactivate target genes in conjunction with TCF/LEF transcription factors. Additionally, FZDs are ubiquitinated by ZNRF3 and RNF43 E3 ligases, which are inhibited by R-spondin binding to LGR5, increasing the cells' sensitivity to Wnt ligands. In Wnt/PCP signaling, Wnt ligands bind to FZDs or their co-receptors (ROR and RYK) to trigger a cascade reaction, involving the small GTPases RhoA and Ras-related C3 botulinum toxin substrate (Rac), then activating Rho-associated protein kinases (ROCKs) and JUN N-terminal kinases (JNK), respectively. These lead to cytoskeletal rearrangements and/or transcriptional responses such as ATF2. In Wnt/Ca^{2+} signaling, the activation of phospholipase C (PLC) triggers the release of Ca^{2+} from the endoplasmic reticulum (ER), which promotes the transcription of nuclear factor of activated T cells (NFAT) through several intermediate steps. Created with BioRender.com. LRP, lipoprotein receptor-related protein; LGR5, leucine-containing repeat G-protein-coupled receptor 5; RNF43, ring finger protein 43; ZNRF3, zinc and ring finger 3; GSK-3β, glycogen synthase kinase 3β; CK1α, casein kinase 1α; APC, adenomatous polyposis coli; CBP, CREB binding protein; TCF/LEF, T-cell factor/lymphoid enhancer-binding factor; ROR, receptor tyrosine kinase-like orphan receptor; RYK, receptor tyrosine kinase; ARF2, activating transcription factor 2; PIP2, phosphatidylinositol 4,5-biphosphate; DAG, diacylglycerol; PKC, protein kinase C.

produce Wnts to activate Wnt/β-catenin signaling in hepatocytes. Additionally, the organ precisely senses the size of the regenerating liver and adjusts its size to 100%.[2]

Several animal models (rat, mouse, and zebrafish) were utilized for liver regeneration study.[30-33] The partial hepatectomy is the classic strategy to create the murine liver regeneration model.[30] Carbon tetrachloride (CCl_4) is a frequently used chemical to induce liver injury in rats and mice.[34] Meanwhile, several dietary-induced liver injury models are also commonly used.[35] Biliary injury and regeneration can be induced by the 1,4-dihydro-2,4,6-trimethyl-pyridine-3,5-dicarboxylate (DDC) diet.[35] Besides murine models, zebrafish emerged as a potent model for drug screening of liver generation.[32,33] Partial hepatectomy, drug-induced liver injury, and nitroreductase-mediated hepatocyte ablation were employed to establish the zebrafish liver injury model.[32,33,36,37]

Transient activation of the canonical Wnt signaling is indispensable for liver regeneration (Fig. 2).[13,15,27] In rat models, overexpressed Wnt1 and nuclear β-catenin are predominantly accumulated in remaining parenchymal cells after 70% partial hepatectomy. The level of β-catenin increased within 5 minutes after hepatectomy, accompanied by its nuclear translocation and subsequent target gene expression for hepatocyte proliferation.[27] Significantly, genetic ablation of β-catenin/Ctnnb1 impairs liver regeneration of mice from partial hepatectomy.[38] The liver-specific *Ctnnb1* knock-out

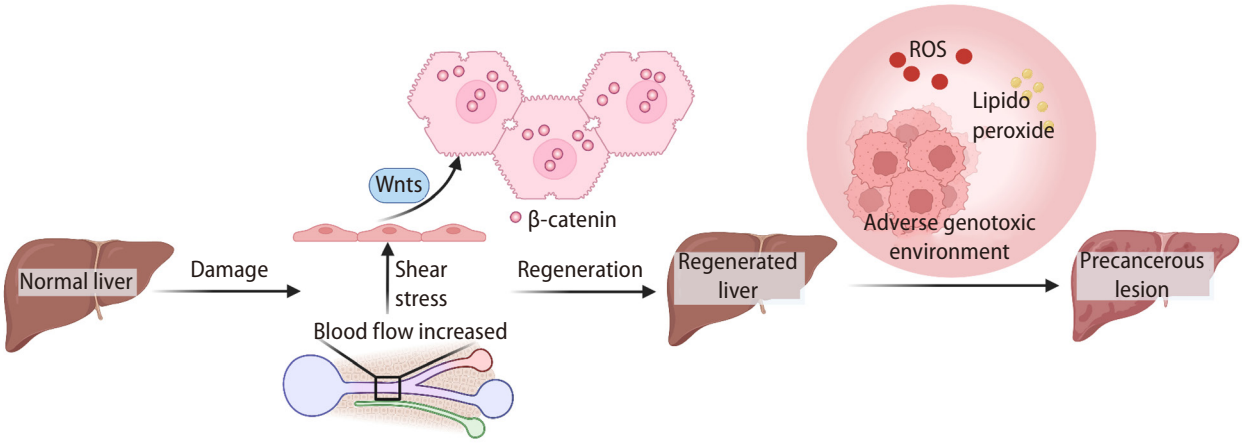

Figure 2. Wnt signaling in liver regeneration. In normal liver, most hepatocytes are polyploid with random chromosomal deletions. Upon liver injury, the increased narrow portal vein pressure stimulates the initiating signals for liver regeneration. The activation of Wnt signaling is crucial in liver regeneration. Moreover, in chronic liver injury, the ROS and lipid peroxide are the risk factors damaging the reproducing hepatocytes, leading to precancerous lesion development. Created with BioRender.com. ROS, reactive oxygen species.

(KO) delayed DNA synthesis and hepatocyte proliferation in mice after partial hepatectomy. Conversely, activation of Wnt/β-catenin signaling accelerates liver regeneration in the zebrafish model.[39] It was also shown that liver damage upregulated leucine-containing repeat G-protein-coupled receptor 5 (LGR5) and AXIN2 in the hepatocytes.[40] LGR5 is a marker of actively dividing stem and progenitor cells in Wnt-driven self-renewing tissues.[41] LGR5 interacts with FZD and lipoprotein receptor-related protein 6 (LRP6) to enhance phosphorylation of LRP6, which in turn enhances the Wnt/β-catenin signaling.[42] While Lgr5 is not expressed in healthy adult livers, after liver damage, Lgr5+ cells appear near the bile ducts, consistent with strong activation of the Wnt signaling.[41] AXIN2 is another Wnt downstream target gene transactivated by β-catenin.[43] Like AXIN1, AXIN2 combined with other destruction complex components degrades β-catenin, serving as a negative feedback regulator of the Wnt signaling.[44]

Other than core components of Wnt signaling, additional regulators of Wnt signaling were implicated in liver regeneration. Recently, our group identified the transmembrane protein 9 (TMEM9) gene as an amplifier of Wnt/β-catenin signaling. TMEM9 is a type I transmembrane protein primarily localized in lysosomes and multivesicular bodies (MVBs). While the ablation of TMEM9 inhibits the activity of the Wnt/β-catenin signaling, β-catenin transactivates *TMEM9*, leading to hyperactivation of Wnt/β-catenin signaling.[45] Interestingly, TMEM9 is highly expressed in hepatocytes around the central vein (CV) of regenerating liver.[46] TMEM9 hyperactivates Wnt/β-catenin signaling to promote liver regeneration through lysosomal degradation of APC protein.[46] *Tmem9* KO impairs CCl4-induced liver regeneration with downregulation of Wnt/β-catenin signaling.[46]

In addition to the role of Wnt/β-catenin signaling in regeneration, sustained activation of the Wnt signaling is associated with the progression of chronic liver diseases and liver tumorigenesis (Fig. 2). Additionally, reactive oxygen species (ROS) and lipid peroxide are the risk factors for the development of the precancerous lesion in the liver.[47,48] However, the crosstalk between Wnt signaling and ROS has not been fully revealed in the liver. It was reported that β-catenin can be further stabilized by ROS.[49] Meanwhile, lipid peroxidation products mainly generated by ROS activate the canonical Wnt pathway through oxidative stress.[50] Therefore, it is likely the potential crosstalk between Wnt signaling and ROS might contribute to liver cancer development.

Accumulating evidence suggests that many chronic liver diseases contribute to liver cancer development, described below.

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Wnt SIGNALING IN PRECANCEROUS LIVER LESION

Hepatitis virus

Globally distributed hepatitis B virus (HBV) and hepatitis C virus (HCV) are the crucial triggers of HCC initiation. Both HBV and HCV can induce chronic infections and are essential pathogenic factors in cirrhosis and liver cancer (Fig. 3).[51,52] The epidemiological data show that more than 70% of patients with liver cancer have HBV infection, 10–20% have HCV infection, and a significant proportion of patients have both HBV and HCV infection.[53-55]

After infection, the DNA of HBV is integrated into the host genome, inducing genomic instability and transactivation of cancer-related genes, which culminates in the formation of early cancer cell clones. Mechanistically, HBV contributes to HCC development through direct and indirect means.[56] Direct mechanisms include virus mutations, HBV DNA integration, growth regulatory genes activation by HBV-encoded proteins.[57] Indirect mechanisms include the activation of cellular oncogenes associated with HBV DNA integration, genetic instability induced by viral integration or the regulatory protein HBx, and the development of liver disease mediated by immune enhancement due to viral proteins.[58]

Both hepatitis B virus surface antigen (HBsAg) and HBx modulate the expressions of genes involved in Wnt signaling activation. HBsAg activates the transcription factor LEF1 of the Wnt signaling.[59] The X protein encoded by the hepatitis B virus has a vital role in stimulating viral gene expression and replication, critical for maintaining chronic carrier status. HBx, a 17 kDa multifunctional protein, upregulates the expression of Wnt ligands (WNT1 and WNT3), the receptor (FZD2 and FZD7), a component of the destruction complex (GSK3β), E-cadherin, and Wnt1-inducible-signaling pathway protein 1 (WISP1), a suppressor of Wnt antagonists (secreted frizzled-related protein 1 [SFRP1] and SFRP5). On the other hand, the Wnt signaling key components (β-catenin and AXIN1) are highly mutated in HBV-associated HCC. Loss-of-function (LOF) mutations of the *AXIN1* are observed in HBV-HCC patients. In HBV and/or HCV-associated HCC patients, the most frequent mutation in the *CTNNB1* gene is enriched in the exon 3 encoding the N-terminal phosphorylation sites.[60-62] These aberrantly controlled genes in Wnt signaling subsequently promote and lead to the development of HCC.[63-65]

The oncogenic mechanism of HCV in liver cancer is mainly mediated by Wnt/β-catenin signaling hyperactivation via the core protein and two nonstructural proteins, NS3 and NS5A.[66] The core protein (HCV core antigen) is a significant component of HCV. It regulates hepatocyte transcription and promotes Wnt/β-catenin signaling by upregulating Wnt ligands (WNT1 and WNT3A), FZD receptors, and LRP5/6.[67,68] Additionally, at the early stage of HCV infection, the secreted Wnt antagonists, SFRP2 and Dickkopf 1 (DKK1), are downregulated by their promoter hypermethylation.[69,70] HCV core protein also promotes hypermethylation of the *CDH1* gene promoter region,[71] destabilizing the cadherin-catenin-actin complex for β-catenin release and activation.[72] NS5A stabilizes β-catenin via activating phosphoinositide 3-kinase (PI3K)/AKT, leading to GSK3β inactivation followed by inhibiting the

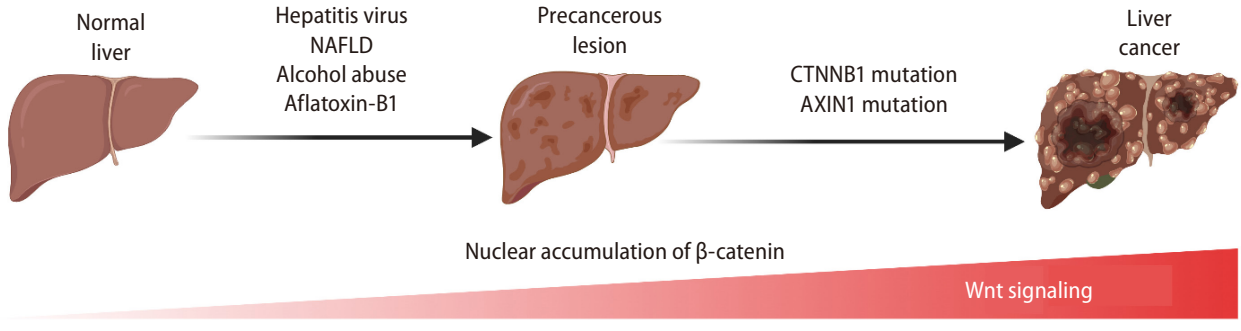

Figure 3. Wnt signaling in liver cancer. Dynamic activation of β-catenin and Wnt signaling-related gene mutations from risk factor exposure to final liver cancer. With the precancerous lesions induced by hepatitis virus, NAFLD, alcohol assumption, or aflatoxin-B1, genetic and epigenetic alteration (e.g., mutations in the CTNNB1 or AXIN1 genes) lead to the accumulation and nuclear translocation of β-catenin, resulting in initiating liver cancer development. Created with BioRender.com. NAFLD, non-alcoholic fatty liver disease.

protein-destruction complex-mediated β-catenin degradation for Wnt target gene activation. At the early stage of viral infection, HCV-activated Wnt/β-catenin signaling also promotes liver fibrosis by enhancing the activation and survival of hepatic stellate cells.[17,73,74]

Alcohol abuse

Alcohol is a well-known risk factor for liver cancer. Alcoholic liver disease (ALD) is a chronic liver disease caused by long-term alcohol consumption (Fig. 3). ALD is characterized by the fatty liver at the beginning, then progressed to alcoholic hepatitis, liver fibrosis, and cirrhosis, which is pathologically associated with the precancerous lesions of HCC. *In vivo*, ethanol (EtOH) is metabolized into the reactive metabolite acetaldehyde, promoting liver tumorigenesis. Mice administered with the chemical carcinogen, diethylnitrosamine (DEN), for 7 weeks and the subsequent EtOH feeding for 16 weeks exhibited the increased total number of cancer foci and liver tumors.[75] Also, these tumors showed a 3- to 4-fold increase in the expression of proliferation markers and an increased expression of β-catenin, compared to non-tumor hepatocytes.[75] In a rat model of chronic liver disease, EtOH-treated liver was accompanied by the increased proliferation of hepatocytes, depletion of retinol and retinoic acid storage, augmented expression of phospho-GSK3β at the cell membrane, significant upregulation of soluble Wnt ligands (Wnt2 and Wnt7a), accumulation of nuclear β-catenin, and upregulation of β-catenin target genes (cyclin D1/CCND1, c-Myc/MYC, WISP1, and matrix metallopeptidase [MMP7]). These data suggest that long-term EtOH consumption activates the Wnt/β-catenin signaling and increases hepatocyte proliferation, promoting liver tumorigenesis.[75] Additionally, ROS accumulation, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein (MCP)-1 upregulation, and activation of extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) signaling also contribute to EtOH-induced liver tumorigenesis.[76-80]

NAFLD

The increasing prevalence of NAFLD was caused by an over-nourished lifestyle.[81,82] NAFLD is characterized by fat accumulation in the liver, evolving to end-stage liver diseases such as cirrhosis and HCC (Fig. 3).[83] The main risk factors of NAFLD include central obesity, overnutrition, insulin resistance, and metabolic syndrome.[84] In severe NAFLD, many tissue repair-related genes (TMEM204, FGFR2, matrix molecules, and matrix remodeling factors) were hypomethylated at their promoters and overexpressed. Conversely, genes in specific metabolic pathways (lipid metabolism, cytochrome P450 family, multidrug resistance, and fatty acid anabolic pathways) were hypermethylated and silenced.[85] Hyperinsulinemia is one of the risk factors of NAFLD.[86] SOX17 plays a vital role in regulating insulin secretion. *Sox17* KO mice display high susceptibility to high-fat diet-induced hyperglycemia and diabetes.[87] SOX17 directly interacts with the TCF/LEF transcription factor to repress the transcription of Wnt signaling target genes. The methylation of the *SOX17* promoter is a frequent event in human cancers. Epigenetic silencing of *SOX17* contributes to the aberrant activation of Wnt/β-catenin signaling,[88] accelerating progression from NAFLD to HCC. Besides, β-catenin inhibits the expression of CCAAT enhancer-binding protein α (CEBPA) and peroxisome proliferator-activated receptor γ (PPARG), which in turn inhibits the preadipocyte differentiation.[89] As the co-receptor of the Wnt/β-catenin signaling, LRP6 induces lipid accumulation in the liver via insulin-like growth factor 1 (IGF1)/AKT/mammalian target of rapamycin (mTOR)/sterol regulatory element binding transcription factor (SREBF) 1/2 signaling. Intriguingly, inhibiting the non-canonical Wnt signaling reduces lipid accumulation and inflammation.[90] Therefore, while reducing the effects of NAFLD risk factors, inhibition of the Wnt signaling is also essential for attenuating the development of NAFLD and preventing the initiation of HCC.

Aflatoxin-B1 exposure

Among the aflatoxins, aflatoxin type B1 (AFB1) primarily targets the liver as a highly potent hepatotoxin and hepatocarcinogen (Fig. 3). AFB1 impairs DNA repair processes, resulting in severe DNA mutagenesis, and also inhibits DNA and RNA metabolism. This pathological event ultimately leads to excessive liver lipid accumulation, liver enlargement, bile duct epithelial hyperplasia, and liver cancer. The potency of aflatoxin to cause liver cancer is significantly enhanced in the presence of HBV infection. Under chronic HBV infection, cytochrome P450s could metabolize inactive AFB1 to muta-

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genic AFB1-8,9-epoxide. Also, the infection leads to hepatocyte necrosis and regeneration, producing oxygen and nitrogen reactive species and increasing the incidence of AFB1-induced mutagenesis.[91] Clinical studies have shown that *CTNNB1* mutations are present in approximately one-quarter of HCC in areas with low aflatoxin B1 exposure. Interestingly, these *CTNNB1* mutations were similar to those previously reported in the human HCC.[92]

Wnt SIGNALING IN LIVER CANCER

HCC

HCC is a common and fatal malignancy worldwide.[93] Regardless of the risk factors mentioned above, aberrant hyperactivation of Wnt/β-catenin signaling is observed in 95% of HCCs.[94] The most common genetic mutations of the Wnt signaling in HCC are the gain-of-function mutations in the *CTNNB1* gene encoding β-catenin,[61,95] which is somewhat distinct from colorectal cancer where Wnt/β-catenin signaling hyperactivation is mainly driven by the *APC* gene inactivation.[96] Missense mutations of *CTNNB1* exon 3 were observed in 18.1% of HCC cases. Missense mutations at codons 32, 33, 38, or 45 of the *CTNNB1* gene lead to the unphosphorylation of the N-terminus of β-catenin for its stabilization, nuclear translocation, and target gene transactivation.[60] Secondly, the LOF mutations in the *AXIN1* gene were observed in 5–19% of HCC cases.[97] *CTNNB1* and *AXIN1* mutations occur in patients with advanced HCC (Fig. 3).[98-100] Importantly, hyperactivation of the Wnt signaling is considered a hallmark of advanced HCC.[101] It should also be noted that mutations in the *CTNNB1* and *AXIN1* genes lead to different HCC subtypes accompanied by distinct clinical and pathological features. *CTNNB1* mutations are associated with less aggressive HCC, including chromosomally stable and highly differentiated tumors,[102] with a better prognosis.[95] In contrast, *AXIN1* mutations occur more frequently in more aggressive HCC tumors characterized by hypodifferentiated tumor cells and chromatin instability.[102] Consistently, the HCC tumors with *CTNNB1* mutations or *AXIN1* mutations showed different target gene expression.[61,95,103]

CCA

CCA is ranked as the second most common hepatobiliary cancer after HCC. CCA originates mainly from differentiated bile duct epithelial cells.[104] CCA is often diagnosed at an advanced stage with a poor prognosis. Current chemotherapy has not improved the survival rate of unresectable CCA patients. Clinical and preclinical studies have shown that activation of the Wnt/β-catenin signaling occurs throughout the initiation and progression of CCA. Wnt ligands (WNT2, WNT7b, and WNT10A) and TCF4 are upregulated in CCA, accompanied by nuclear translocation of β-catenin.[105,106] The progression of epithelial-mesenchymal transition (EMT) was observed in CCA, represented by the disrupted epithelial cell-cell junctions and mesenchymal characteristics.[107-109] Wnt/β-catenin signaling is one of the critical pathways promoting the EMT transition.[110,111] In CCA cells, suppression of Wnt/β-catenin signaling increased E-cadherin and downregulated vimentin,[112,113] suggesting that the Wnt/β-catenin signaling is associated with EMT during CCA tumorigenesis. β-catenin interacts with E-cadherin to form the cadherin-catenin-actin complex, maintaining epithelial cell adhesion, cytoskeleton, and integrity. During CCA development, the decreased E-cadherin releases β-catenin, resulting in β-catenin accumulation and nuclear translocation.[111] Then, β-catenin activates the transcription of twist, snails, and ZEB1 to induce the EMT process in CCA cells.[111]

HB

HB is a rare malignant tumor found in infants and children.[114] The preclinical and clinical studies showed the hyperactivation of Wnt/β-catenin signaling in HB. In HB cases, β-catenin was found to be increased in the cytoplasm and nucleus of the tumor cells.[115,116] While *CTNNB1* mutations are limited in the exon 3 in embryonal HB, the *CTNNB1* mutations in fetal HB encompass exon 3 and 4.[117] Meanwhile, missense, deletion, or insertion mutations in the *AXIN1* gene were detected in 8% of HB cases.[118]

MANIPULATING Wnt SIGNALING

Porcupine (PORCN)

PORCN is a membranous protein mainly localized in the endoplasmic reticulum. PORCN mediates the palmitoylation of Wnt ligands, an essential process for Wnt ligands secretion and ligand-frizzled receptor binding.[119-121] Genetic and pharmacological blockade of PORCN reduces palmitoylation and inhibits the secretion of Wnt ligands, suppressing Wnt signaling.[122] The clinical trials showed promising results of PORCN inhibitors in HCC treatment. ETC159, CGX1321, and RXC004 have entered phase I clinical trials, and IWP12 is still in the preclinical studies (Fig. 4).[123] In mouse models, *Porcn* KO induces embryonic lethality.[124,125] *Porcn* inhibition could cause adverse effects on bone homeostasis.[126]

Wnt ligands

In physiological conditions, Wnt signaling is activated by binding of secreted Wnt ligands to LRP5/6 coreceptors and FZD receptors.[127] Thus, targeting Wnt ligands by chemicals or

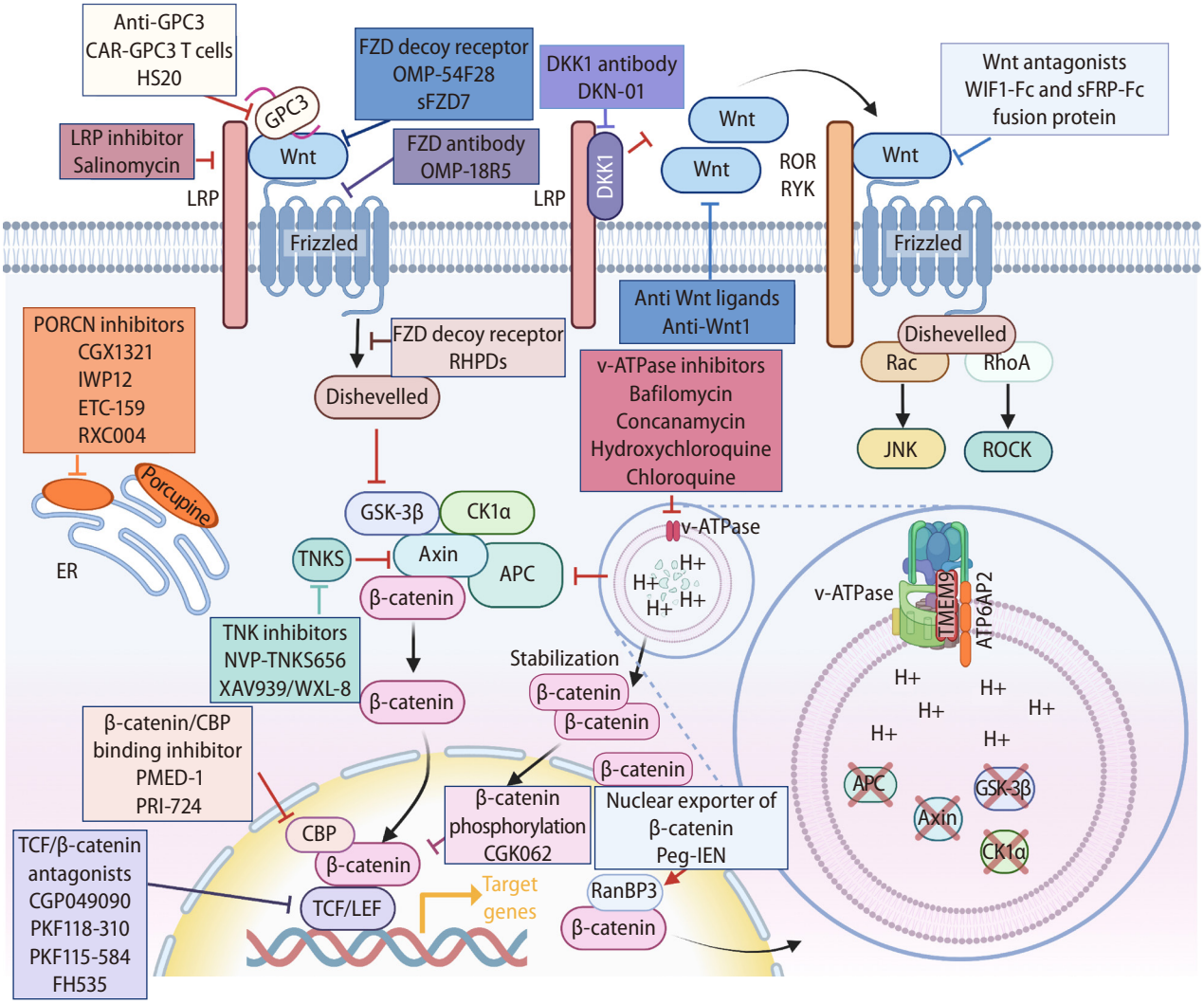

Figure 4. Manipulating Wnt signaling. Illustration of components and processes of Wnt signal transduction as druggable targets for liver cancer treatment. See the text for detail. Created with BioRender.com. GPC3, glypican-3; LRP, lipoprotein receptor-related protein; FZD, frizzed; DKK1, Dickkopf 1; ROR, receptor tyrosine kinase-like orphan receptor; RYK, receptor tyrosine kinase; PORCN, porcupine; ER, endoplasmic reticulum; GSK-3β, glycogen synthase kinase 3β; CK1α, casein kinase 1α; APC, adenomatous polyposis coli; TCF, T-cell factor; CBP, CREB binding protein; LEF, lymphoid enhancer-binding factor; peg-IFN, pegylated-Interferon-α2a; RanBP3, Ran-binding protein 3.

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neutralizing antibodies efficiently inhibits Wnt signaling. Based on the high expression of WNT1 in human HCC cell lines and tissues. Anti-WNT1 neutralizing antibody showed its growth inhibitory effect on HCC cell lines but not on normal hepatocytes, with reduced β-catenin's transcriptional activity (Fig. 4).[128]

Wnt antagonists

SFRPs, WIFs, and DKKs are the secreted Wnt signaling antagonists.[129,130] SFRP-1 and Wnt inhibitory factor 1 (WIF1) inhibit Wnt signaling by directly binding to Wnt ligands.[131] The fusion proteins WIF1-Fc and SFRP1-Fc were constructed by adding the Fc fragment of human immunoglobulin (Ig) G1 to WIF1 and SFRP1, respectively (Fig. 4).[132] The fusion proteins exert potent anti-tumor activity by downregulating E2F transcription factor 1 (E2F1), cyclin D1, and c-Myc, increasing apoptosis of HCC cells and impairing tumor vascularization. DKK1 was initially considered a β-catenin-dependent tumor suppressor.[130,133] Several studies have shown that DKK1 promotes tumor cell proliferation, which may be due to DKK1-induced endocytosis of LRP and subsequent activation of the Wnt/PCP signaling pathway.[134,135] DKN-01 is a humanized monoclonal antibody targeting DKK1 in phase I/II clinical trial for HCC (Fig. 4). Phase I investigated the safety of DKN-01 as a single agent and in combination with sorafenib to treat HCC. Phase II explores the anti-tumor activity and safety of DKN-01 in patients with advanced HCC.

FZD receptors

The FZD receptors are promising therapeutic targets for HCC. The anti-FZD antibody can effectively reduce the HCC tumor growth by blocking the activation of FZD receptors on the Wnt signaling.[136] FZD decoy receptor OMP-54F28 (ipafricept) is a recombinant fusion protein that binds to a human IgG1 Fc fragment of FZD8,[137,138] which acts synergistically with chemotherapeutic agents (Fig. 4).[139] A phase 1b dose-escalation clinical trial evaluated the safety, tolerability, and pharmacokinetics of OMP-54F28 when combined with sorafenib. Secreted FZD7 (sFZD7) is the extracellular domain of FZD7, expressed and purified from *Escherichia coli*. sFZD7 binding to WNT3 decreased the transcriptional activity of β-catenin/TCF4 and inhibited the growth of HepG2, Hep40, and Huh7.[140] In combination with doxorubicin, sFZD7 inhibited the expression of c-Myc/MYC, Cyclin D1/CCND1, and Survivin/BIRC5, reduced the phosphorylation levels of AKT and ERK1/2, inhibited the growth of Huh7 xenograft tumors, and acted as a chemosensitizer.[140] OMP-54F28 is entering phase I clinical trials, while sFZD7 remains in preclinical studies (Fig. 4).

FZD antibody OMP-18R5 (vantictumab) is a monoclonal antibody directly binding to FZD receptors, which blocks the binding of Wnt ligands to FZD 1, 2, 5, 7, and 8,[141] which inhibits β-catenin-mediated transactivation (Fig. 4). In patient-derived xenograft models, OMP-18R5 combined with chemotherapeutic agents synergistically inhibited the development of several cancers.[141,142] However, like PORCN inhibitors, OMP-18R5 has the same risk of impairing bone homeostasis.[143] In a dose-escalation clinical trial of OMP-18R5, one patient developed bone degeneration, controllable with zoledronic acid. The skeletal toxicity appeared to be manageable and reversible.[144]

LRP co-receptors

Salinomycin (SAL), isolated from *Streptomyces albus,* is a monocarboxylic polyether ionophore antibiotic.[145,146] SAL blocks Wnt-induced LRP phosphorylation and leads to LRP protein degradation, destabilizing the Wnt/FZD/LRP complex and inhibiting the Wnt/β-catenin signaling (Fig. 4).[147] SAL effectively inhibits β-catenin expression in HepG2/C3a cell line.[148] SAL also inhibits the migration and invasiveness of liver cancer stem cells through the Wnt/β-catenin signaling suppression.[149]

Tankyrase (TNKS)

TNKS mediates PARsylation and subsequent degradation of AXIN via the ubiquitin-proteasome pathway, which in turn disrupts the β-catenin destruction complex.[150] Subsequently, the released β-catenin enters the nucleus to transactivate Wnt target genes.[151,152] TNKS is overexpressed in many cancers, including HCC, gastric cancer, and colorectal cancer.[153-155] The TNKS inhibitors XAV939, WXL-8, and NVP-TNKS656, attenuated Wnt/β-catenin signaling and inhibited the growth of HCC cells (Fig. 4).[155-157] Moreover, TNKS inhibitors also suppressed HCC metastasis and invasion.[157] However, there are no relevant clinical trials for TNKS inhibitors in HCC.

Nuclear export of β-catenin

As shown in Table 1 and Supplementary Table 1, pegylated-Interferon-α2a (peg-IFN), the first-line therapy for the HCV-infected,[158] attenuates the recurrence of HCC (Fig. 4).[159] Mechanistically, peg-IFN upregulates the expression of Ran-binding protein 3 (RanBP3),[160] which enhances the nuclear export of β-catenin.[160] Thus, it is likely that peg-IFN-induced β-catenin nuclear export is a mechanism delaying HCC and improving survival in HCV patients.

β-catenin-mediated gene transactivation

The small molecule ICG-001 inhibits the interaction between β-catenin and CREB binding protein (CREBBP/CBP) for suppression of β-catenin-mediated gene transactivation (Fig. 4).[161] A phase Ib/IIa clinical trial of the ICG-001 derivative, PRI-724, targeting HCC has been terminated.[162] Similar to ICG-

Table 1. Targeting Wnt signaling in liver cancers

Agent	Target	Phase	Trial identifier	Type
DKN-01	DKK1	Phase I/II	NCT03645980	Protein
OMP-18R5	FZD1, 2, 5, 7, and 8	Phase I	NCT01345201	Protein
sFZD7	FZD7	Preclinical	NA	Protein
RHPDs	FZD7	Preclinical	NA	Protein
OMP-54F28	FZD8	Phase I	NCT02069145	Protein
Salinomycin	LRP5/6	Preclinical	NA	Natural compounds
CGX1321	PORCN	Phase I	NCT03507998	Small molecule inhibitors
IWP12	PORCN	Preclinical	NA	Small molecule inhibitors
ETC-159	PORCN	Phase I	NCT02521844	Small molecule inhibitors
RXC004	PORCN	Phase I	NCT03447470	Small molecule inhibitors
NVP-TNKS656	Tankyrase	Preclinical	NA	Small molecule inhibitors
XAV939/WXL-8	Tankyrase	Preclinical	NA	Small molecule inhibitors
CGP049090	TCF/β-catenin	Preclinical	NA	Natural compounds
PKF118-310	TCF/β-catenin	Preclinical	NA	Natural compounds
PKF115-584	TCF/β-catenin	Preclinical	NA	Natural compounds
FH535	TCF/β-catenin	Preclinical	NA	Small molecule inhibitors
Peg-IFN	TCF/β-catenin	Phase II	NCT00610389	Protein
WIF1-Fc and sFRP-Fc	Wnt ligands	Preclinical	NA	Protein
Anti-Wnt1	Wnt1	Preclinical	NA	Protein
CGK062	β-catenin phosphorylation	Preclinical	NA	Small molecule inhibitors
PMED-1	β-catenin/CBP	Preclinical	NA	Small molecule inhibitors
PRI-724	β-catenin/CBP	Phase I/II	NCT01302405	Small molecule inhibitors
Hydroxychloroquine	v-ATPase	Phase II	NCT03037437	Small molecule inhibitors
Chloroquine	v-ATPase	Preclinical	NA	Small molecule inhibitors
Bafilomycin	v-ATPase	Preclinical	NA	Small molecule inhibitors
Concanamycin	v-ATPase	Preclinical	NA	Small molecule inhibitors
CAR-GPC3 T cell	GPC3	Phase I	NCT02932956	Cells
Anti-GPC3 antibody	GPC3	Phase II	NCT01507168	Protein
CIK with anti-GPC3	GPC3	Phase II	NCT03146637	Cells

DKK1, Dickkopf 1; FZD, frizzed; NA, not available; PORCN, porcupine; TCF, T-cell factor; peg-IFN, pegylated-Interferon-α2a; CBP, CREB binding protein; v-ATPase, vacuolar-type ATPase.

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001, PMED-1 disrupts β-catenin-CREBBP interaction and suppresses β-catenin target gene activation.[163] PMED-1 inhibits HCC cell proliferation but not normal human hepatocytes.[163]

PKF118-310, PKF115-584, and CGP049090 are small-molecule inhibitors targeting the β-catenin-TCF complex (Fig. 4).[164] These antagonists displayed the dose-dependent cytotoxicity in HepG2, Hep40, and Huh7 cell lines, with reduced cytotoxicity (10%) to normal hepatocytes. PKF118-310, PKF115-584, and CGP049090 downregulated β-catenin target genes (*MYC*, *CCND1*, and *Survivin*/*BIRC5*) and inhibited the growth of HepG2 xenografts.[164,165] Similar to the mechanism of PKF118-310, PKF115-584, and CGP049090, FH535 inhibits β-catenin-mediated gene transactivation by interrupting the recruitment of nuclear receptor coactivator 2 (NCOA2)/GRIP1 to the β-catenin transcriptional complex.[166] It was shown that FH535 inhibits HCC cell proliferation by reducing cancer cell stemness.[165]

β-catenin phosphorylation

CGK062 promotes PKCα-mediated phosphorylation of β-catenin at Ser33/Ser37, which degrades β-catenin by the proteasome (Fig. 4).[167] Consistently, CGK062 inhibited the expression of β-catenin target genes (*CCND1*, *MYC*, and *AXIN2*) and suppressed the growth of Wnt/β-catenin-activated HCC cells.[167]

Cancer-specific targeting of Wnt signaling

Given the pivotal role of Wnt signaling in the homeostasis and regeneration of multiple organs,[168-170] broad-spectrum Wnt signaling inhibitors cause detrimental effects on the normal cells and organs. Therefore, cancer-specific Wnt signaling regulators may be attractive for Wnt signaling blockade therapy. TMEM9, an amplifier of Wnt/β-catenin signaling, promotes lysosomal protein degradation via v-ATPase, resulting in APC downregulation.[46] TMEM9 is highly expressed in liver regeneration and HCC. Genetic ablation of *TMEM9* inhibits HCC tumorigenesis with downregulation of Wnt/β-catenin signaling.[46] Similarly, v-ATPase inhibitors, bafilomycin and concanamycin,[171,172] also inhibit Wnt/β-catenin signaling without toxicity to normal cells and animals (Fig. 4, Table 1).[45,46] Thus, molecular targeting of the TMEM9-v-ATPase axis can be used as cancer-specific Wnt/β-catenin blockade.

Glypican-3 (GPC3) is a proteoglycan binding to the FZD receptor and stimulates Wnt ligands-FZD interaction, resulting in the Wnt signaling activation (Fig. 4).[173] GPC-3 is specifically expressed in HCC but not in normal human liver tissue.[174] The ectopic expression of GPC3 promotes the proliferation of HCC cells.[175] HS20 (an anti-GPC3 monoclonal antibody) suppresses Wnt/β-catenin signaling via inhibiting the interaction of Wnt3a with the GPC3.[176] In xenograft mouse models, HS20 inhibited HCC progression without apparent concomitant toxicity.[176] To date, including CAR-GPC3 T cells or anti-GPC3 antibodies, 33 clinical trials related to GPC3 for HCC treatment were registered (https://clinicaltrials.gov/) (Table 1, Supplementary Table 1).

Concluding remarks

Wnt signaling activation plays a pivotal role in liver regeneration, metabolic zonation, liver diseases, and liver cancer. Aberrantly hyperactivated Wnt signaling promotes liver tumorigenesis and progression, often in conjunction with liver diseases. Although direct targeting of Wnt signaling sounds attractive as cancer therapy, given the crucial roles of Wnt signaling in tissue homeostasis and regeneration, severe adverse effects from Wnt blockade are inevitable. Nonetheless, an in-depth understanding of the biology of Wnt signaling in liver cancer and exploring cancer-specific Wnt signaling regulators are expected to identify molecular targets specific to liver cancer, which may overcome the current limitations of Wnt signaling inhibitors, and further improve therapeutic strategies of liver cancer treatment.

Authors' contribution

G.Z. and J.I.P. wrote the manuscript.

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Conflicts of Interest

The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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Supplementary Table 1. Additional clinical trials related with peg-IFN, hydroxychloroquine and CAR-GPC3 T cell in liver cancers

Agent	Target	Phase	Trial identifier	Type
Peg-IFN	TCF/β-catenin	Phase II	NCT00610389	Protein
Peg-IFN	TCF/β-catenin	Phase III	NCT01821963	Protein
Hydroxychloroquine	v-ATPase	Phase II	NCT03037437	Small molecule inhibitors
Hydroxychloroquine	v-ATPase	Phase I/II	NCT02013778	Small molecule inhibitors
Hydroxychloroquine	v-ATPase	Phase I	NCT04873895	Small molecule inhibitors
CAR-GPC3 T cell	GPC3	Phase I	NCT02932956	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT04093648	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT05003895	Cells
CAR-GPC3 T cell	GPC3	Phase I/II	NCT04864054	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT04951141	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT04842812	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT04377932	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT04715191	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT05103631	Cells
CAR-GPC3 T cell	GPC3	Phase I/II	NCT02715362	Cells
CAR-GPC3 T cell	GPC3	NA	NCT03146234	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT03198546	Cells
CAR-GPC3 T cell	GPC3	Phase I/II	NCT03130712	Cells
CAR-GPC3 T cell	GPC3	NA	NCT05047510	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT04506983	Cells
CAR-GPC3 T cell	GPC3	Phase I/II	NCT05120271	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT03884751	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT02395250	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT03980288	Cells
CAR-GPC3 T cell	GPC3	Phase I/II	NCT02723942	Cells
CAR-GPC3 T cell	GPC3	Phase I/II	NCT03084380	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT02905188	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT04121273	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT05070156	Cells
CAR-GPC3 T cell	GPC3	NA	NCT03302403	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT04756648	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT04973098	Cells
CAR-GPC3 T cell	GPC3	Phase I/II	NCT02959151	Cells
CAR-GPC3 T cell	GPC3	Phase I	NCT05155189	Cells

peg-IFN, pegylated-Interferon-α2a; CRA-GPC3, glypican-3; TCF, T-cell factor; v-ATPase, vacuolar-type ATPase; GPC3, glypican-3; NA, not available.