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Pivotal roles of glycogen synthase-3 in hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, and represents the second most frequently cancer and third most common cause of death from cancer worldwide. At advanced stage, HCC is a highly aggressive tumor with a poor prognosis and with very limited response to common therapies. Therefore, there is still the need for new effective and well-tolerated therapeutic strategies. Molecular-targeted therapies hold promise for HCC treatment. One promising molecular target is the multi-functional serine/threonine kinase glycogen synthase kinase 3 (GSK-3). The roles of GSK-3β in HCC remain controversial, several studies suggested a possible role of GSK-3β as a tumor suppressor gene in HCC, whereas, other studies indicate that GSK-3β is a potential therapeutic target for this neoplasia. In this review, we will focus on the different roles that GSK-3 plays in HCC and its interaction with signaling pathways implicated in the pathogenesis of HCC, such as Insulin-like Growth Factor (IGF), Notch, Wnt/β-catenin, Hedgehog (HH), and TGF-β pathways. In addition, the pivotal roles of GSK3 in epithelial-mesenchymal transition (EMT), invasion and metastasis will be also discussed.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, and represents the second solid neoplasm and third most common cause of death from cancer worldwide (Torre et al., 2015).

The incidence of this disease is increasing in the economically developed regions, such as Japan and the United States (Thomas and Zhu, 2005), but the largest number of cases are recorded in sub-Saharan Africa, and also in Korea and China where there is about 50% of cases worldwide (El-Serag and Rudolph, 2007), with recent studies reporting an incidence of more of 780,000 new cases annually, and with about 745,000 deaths/year. The most important risk factors for HCC development are infection with hepatitis B or C viruses, cirrhosis due to alcohol abuse and non-alcoholic steatohepatitis.

To date, the therapeutic approach used in the early stages of the disease is surgery, with which it is possible to remove most of the damaged tissue, but unfortunately this type of approach is not applicable to all patients. Over 80% of patients with diagnosis of HCC are indeed at an already advanced stage of the disease, and for this reason are not good candidates for surgical resection or transplantation. In fact, at advanced stage HCC is a highly aggressive tumor with a poor prognosis and with very limited response to common therapies.

However, treatment options for patients with advanced HCC have improved considerably thanks to the use of new therapeutic agents that target some of the molecular pathways involved in hepatocarcinogenesis (Cervello et al., 2012). A new era began with the approval by the Food and Drug Administration (FDA) for treatment of advanced HCC patients with sorafenib, an oral multikinase inhibitor, which inhibits Raf kinase, as well as platelet-derived growth factor receptor-β (PDGFR-β), vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3, c-Kit, and Flt-3 (Llovet et al., 2008; Cervello et al., 2012).

However, the side-effects associated with sorafenib therapy, as well as the modest clinical benefit observed after treatment with the drug, indicate the need to investigate other new treatment options to improve the effectiveness and safety of drugs available today. Therefore, there is still the need to develop new strategies to prevent and treat this disease.

The new anti-cancer therapies are principally based on pharmacological approaches, including small molecule inhibitors which target a specific molecule(s) involved in the key signal transduction pathways implicated in HCC pathogenesis (Cervello et al., 2012).

One promising molecular target is the multifunctional serine/threonine kinase glycogen synthase kinase 3 (GSK-3). In this review, we will focus on the different roles that GSK-3 plays in HCC and its interaction with signaling pathways implicated in the pathogenesis of HCC, such as Insulin-like Growth Factor (IGF), Notch, Wnt/β-catenin, HH, and TGF-β pathways. In addition, the pivotal role of GSK-3 in EMT, invasion and metastasis will be also discussed.

2. GSK-3 family of kinases: GSK-3α and GSK-3β

GSK-3 was initially identified as a key mediator in insulin-dependent glycogen metabolism. It is now recognized that GSK-3 is an important factor involved in different signaling pathways controlling cell differentiation, cell proliferation and survival, transcription and protein synthesis (Phukan et al., 2010; Patel and Woodgett, 2017). Due to its multifunctional roles GSK-3 has become an emerging therapeutic target for many diseases, such as diabetes, neurological disorder and cancer (Phukan et al., 2010; Amar et al., 2011; Fitzgerald et al., 2015; McCubrey et al., 2016).

GSK-3 is a peculiar multifunctional serine/threonine kinase ubiquitously found in all mammalian tissues (Lau et al., 1999; Yao et al., 2002). In humans, there are two GSK isoforms, GSK-3α and GSK-3β, encoded by two separate genes present on chromosome 19 and 3, respectively (Fig. 1A). Each gene comprises 11 exons and share 98% of sequence identity within their kinase domain, whereas they diverge in their N- and C-terminal region. GSK-3α and GSK-3β have a bi-lobal architecture, consisting of a large C-terminal globular domain, responsible for the kinase activity, and a small N-terminal lobe responsible for ATP binding. In addition, a glycine-rich domain (73% glycine) is present only in the N-terminal α isoform (Fig. 1A). However, although the two isoforms share a high degree of similarity and in many cases have overlapping functions, they are not redundant and functionally identical.

GSK-3α is a 51 kDa protein whereas GSK-3β is a 47 kDa protein, both are constitutively active in resting cells.

Active GSKs are phosphorylated at tyrosine 279 (Y279) (GSK-3α) and at tyrosine 216 (Y216) (GSK-3β). Phosphorylation of GSK-3α and GSK-3β at their site-specific tyrosine residues is mediated by kinases, such as proline-rich tyrosine kinase 2 (PYK2) and FYN2 (Hartigan et al., 2001; Lesort et al., 1999) (Fig. 1B). Upon phosphorylation at Tyr216, GSK-3β activity increases ~5-fold (Dajani et al., 2003; Hughes et al., 1993).

GSK-3α and GSK-3β are inhibited by different extracellular signals through phosphorylation at serine 21 (S21) and serine 9 (S9), respectively, by other kinases, such as AKT/protein kinase B (PKB), p70S6K, p90RSK (Stambolic and Woodgett, 1994), protein kinase A (PKA, also known as cAMP-dependent protein kinase) and protein kinase C (PKC) (Fig. 1B). Both GSK-3

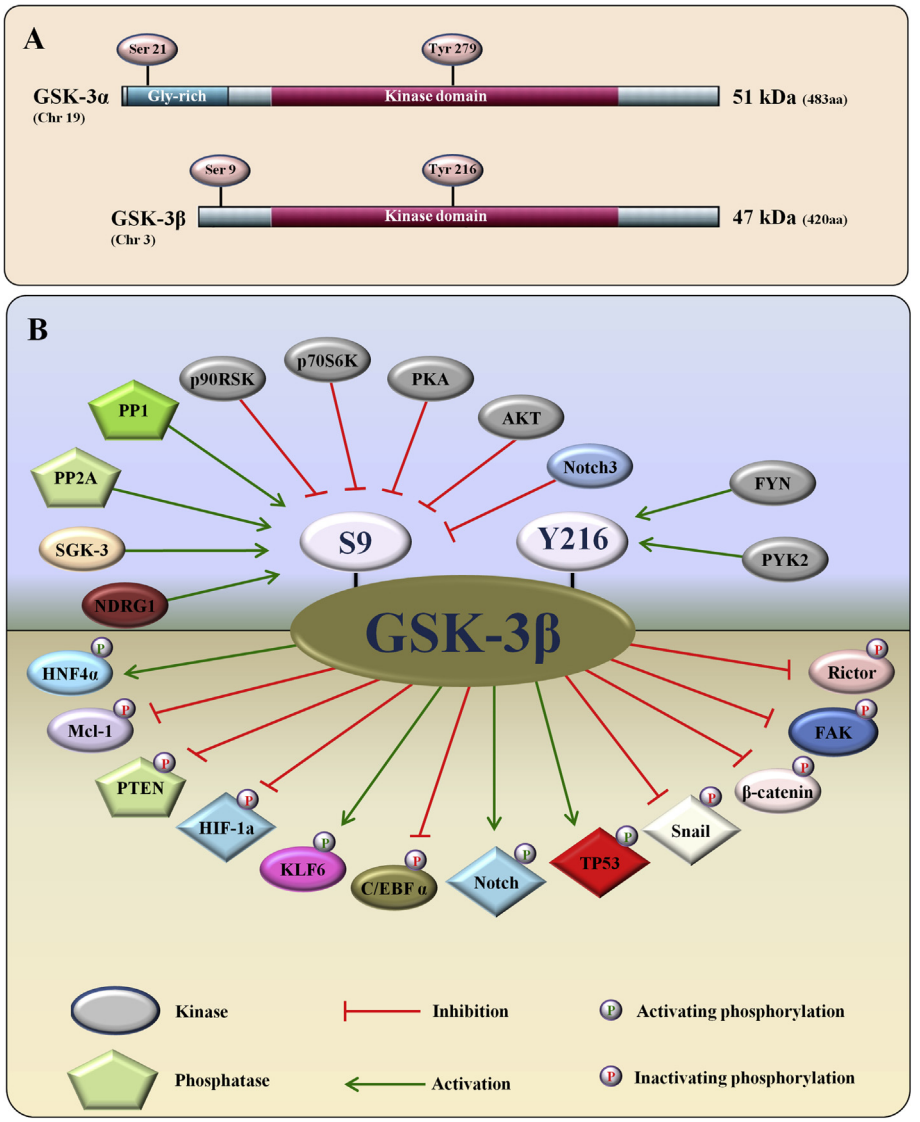


Fig. 1. A) Comparison of structural and functional domains of GSK-3α and GSK-3β. B) Top side, regulation of GSK-3 activity by kinases and phosphatases; Bottom side, examples of some of the proteins phosphorylated by GSK-3.

isoforms preferentially phosphorylate proteins pre-phosphorylated by other kinases (primed substrates). Over 80 proteins have been proposed to be phosphorylated by GSKs, including transcription factors, catalytic enzymes and structural proteins, such as Mcl-1, Snail, PTEN, TP53, C/EBFα (Domoto et al., 2016; Sutherland, 2011) (Fig. 1B). GSK could be dephosphorylated by the action of two phosphatases, such as protein phosphatase 1 (PP1) and PP2A, with higher action of PP1 on GSK-3β and PP2A on GSK-3α (Hernández et al., 2010) (Fig. 1B). The function and signaling pathways elicited by GSK-3β have been much better studied. This review will focus on the action of GSK-3β.

3. GSK-3 inhibitors

As mentioned before, GSK-3 has become an emerging therapeutic target for many diseases, as a result, many efforts have been made in the discovery and development of highly selective inhibitors able to modulate GSK-3 activity.

Multiple inhibitors have been described and many others are in the early phase of discovery. One of the first GSK-3 inhibitors discovered was the cation lithium (Johnson and Amdisen, 1983), and in the early 1970s, lithium was approved by the Food and Drug Administration (FDA) to treat manic depressive patients. Lithium is used in the range of millimolar, however, other metal anions are more potent inhibitors of GSK-3 than lithium, working at micromolar concentration range, such as copper, beryllium, mercury, and zinc (Ilouz et al., 2002; Ryves et al., 2002). Many small molecules pharmacological inhibitors

of GSK are of synthetic origin, whereas others have natural origins (Eldar-Finkelman and Martinez, 2011). GSK-3 inhibitors could be divided into three main categories: i) non-ATP-competitive inhibitors; ii) ATP-competitive inhibitors; iii) substrate competitive inhibitors. In Table 1, a list of some GSK-3 inhibitors is presented, their inhibitory potency and structure, as well as some references and clinical trials, related to cancer treatment, in which they have been tested.

As reported in Table 1, some GSK-3 inhibitors are or have been evaluated are in clinical trials, however, no clinical trials, have been done or are ongoing in patients with HCC. The only data available on GSK-3 inhibitor in HCC are, as yet, on pre-clinical studies, nevertheless, due to their well-tolerated profile in patients with other cancer types (such as leukemia and pancreatic cancer) they provide hope for HCC treatment.

4. Implication of GSK-3 in multiple signaling pathways

GSK-3 is a common pivotal component involved in many pathways, including those mediated by the Insulin/Insulin receptor (IR), Insulin-like growth factor (IGF)/IGF receptor (IGFR), Wnt/β-catenin, HH and TGF-β signaling pathways. Once activated, these pathways will ultimately induce cell proliferation, apoptosis resistance, stimulation of angiogenesis, EMT, invasiveness and metastasis. In recent years, new discoveries have been made about the role of many components of signaling pathways involved in aberrant signaling, uncontrolled cell proliferation and sensitivity/resistance to targeted therapies in HCC (Cervello et al., 2012).

5. GSK-3 and IGF system and HCC

The Insulin-like Growth Factor (IGF) system regulates several cell processes, such as metabolism, proliferation, differentiation, motility and apoptosis (Moeini et al., 2012). The IGF system consists of few components: 1) ligands (insulin, IGF-I and IGF-II); 2) receptors [insulin receptor (IR), IGF-I and IGF-II receptors (IGF-1R and IGF-2R, respectively)]; 3) IGF high affinity binding proteins (IGFBPs) (Enguita-Germán and Fortes, 2014) (Fig. 2).

Insulin is a hormone consisting of two polypeptide chains, A and B, linked by two disulphide bridges. It has an essential role in glucose metabolism, as well as in protein and lipid metabolism (Sowers, 1990). Beta-cells of pancreatic islets produce insulin. Through its receptor, insulin receptor (IR), insulin exerts its function of maintaining glucose levels constant in the blood. Insulin can also bind to Insulin-like Growth Factor-1 receptor (IGF-1R), although with low affinity (Varewijck and Janssen, 2012). In the liver, insulin plays an important role during liver regeneration by stimulating normal hepatocytes proliferation (Michalopoulos and DeFrances, 1997).

IGF-I is produced and secreted from hepatocytes following growth hormone (GH) induction. IGF-I acts through its receptor IGF-1R and has a function similar to that of insulin, promoting anabolic processes including the synthesis of glycogen and proteins (LeRoith and Roberts, 1993).

IGF-II is mostly produced from liver cells and its production is independent of GH stimulation. Unfortunately, the molecular mechanism responsible for regulation of IGF-II expression is currently unknown (LeRoith and Roberts, 1993).

IR and IGF-1R are composed of two α and two β subunits (Fig. 2). The α subunits are located outside the cell membrane while the β subunits are transmembrane. In both receptors, the tyrosine kinase domains are present on the cytoplasmic side of β subunits.

Upon ligand binding to either the IGF-1R or IR, they undergo conformational changes which lead to auto-phosphorylation on the β subunits recruiting specific proteins, the adaptor proteins insulin receptor substrate 1 (IRS-1) and Shc (Lee and Pilch, 1994; Alison et al., 1999; Menting et al., 2013). Phosphorylated Shc binds to the adaptor protein growth factor receptor-bound protein 2 (Grb2) which forms a complex with Ras-guanine exchange factor Son of sevenless (Sos). These events activate several pathways downstream. PI3K/AKT pathway is among the main pathway involved in the IGF system signaling, which promotes cell survival and apoptosis inhibition. The activation of AKT by insulin signaling pathway induces glycogen synthesis through inhibition of GSK-3, and thus resulting in activation of glycogen synthase. In addition, insulin stimulates glucose uptake through translocation of glucose transporter protein (Glut) from cytoplasm compartment to the plasma membrane (Cheatham, 2000) (Fig. 2).

Another pathway activated by IGF-1R is the Ras/Raf/mitogen-extracellular activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, which is associated with cell differentiation, proliferation and migration (Sebolt-Leopold, 2000).

The importance of IGF axis in hepatocarcinogenesis is based on clinical and preclinical studies (Scharf et al., 2001). Aberrant activation of the IGF axis in HCC primarily occurs via IGF- II, which is overexpressed in 16–40% of human HCC cases (Whittaker et al., 2010; Morace et al., 2012). Hepatitis virus infection can also promote IGF-II expression. More, IGF-II protein overexpression has been found in tissues of HBV- and HCV-induced HCC than in virus negative and normal tissues (D'Arville et al., 1991; Saber et al., 2017). Thus, there is clearly a relationship between IGF-II overexpression and hepatocarcinogenesis (Nussbaum et al., 2008).

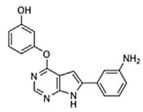
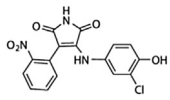
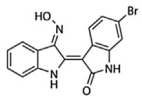
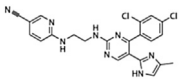
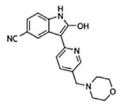
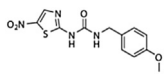
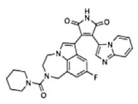
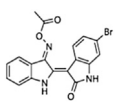
IGF-I has a controversial role in HCC. Unlike other types of cancer, where it is widely considered to be pro-oncogenic, in HCC it may play an anti-oncogenic role. Patients with a higher expression of IGF-I in the adjacent liver to tumor have a significantly lower overall survival (OS), suggesting that increased secretion of IGF-I by adjacent hepatocytes may lead to tumor development with aggressive biology (Chun et al., 2014). Otherwise, IGF-I messenger expression is lower in HCC tissue as compared with the adjacent non-tumorous tissue (Luo et al., 2005). In addition, low serum IGF-I levels correlate with

advanced clinicopathologic parameters and very low OS (Kaseb et al., 2011). On the other hand, low serum levels of IGF-I are associated significantly with early recurrence in patients with HCC who underwent liver resection (Yao et al., 2017). Therefore, the role of IGF-I in HCC should be further clarified.

Up-regulation of IGF-1R has been observed in different types of tumors (Pollak, 2012), including HCC (Zeng et al., 2010; Tovar et al., 2010), however the underlying mechanisms remain poorly defined.

MicroRNAs (miRNAs) seem to play a key role in regulation of IGF-1R. miRNAs are small non-coding RNA of 22–24 nucleotides in length (Acunzo et al., 2015). The most common mechanism of microRNAs is to repress protein translation by

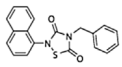
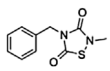
Table 1
GSK inhibitors.

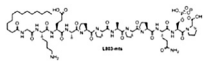
A: ATP competitive				
Inhibitor	Inhibition potency (IC_{50}) GSK-3α	Inhibition potency (IC_{50}) GSK-3β	Structure	Ref/ Clinical Trials
SB216763	34.3 nM	34.3 nM		To et al., 2017
TWS119	–	30 nM		Sun et al., 2016
SB415286	78 nM	78 nM		Parameswaran et al., 2016 Alexeev et al., 2014
BIO	5 nM	5 nM		Grassilli et al., 2014
CHIR-99021	10 nM	6.7 nM		Li et al., 2017 Koo et al., 2015 Marchand et al., 2015
AZD1080	6.9 nM	31 nM		Chen et al., 2016a Chen et al., 2016b
AR-A014418	–	104 nM		Ito et al., 2016 Kunnimalaiyaan et al., 2015 Carter et al., 2014 Madhunapantula et al., 2013
LY2090314	1.5 nM	0.9 nM		NCT01287520 NCT01632306 NCT01214603
BIO-acetoxime	10 nM	10 nM		Duffy et al., 2014

(*continued on next page*)

Table 1 (*continued*)

A: ATP competitive				
Inhibitor	Inhibition potency (IC_{50}) GSK-3α	Inhibition potency (IC_{50}) GSK-3β	Structure	Ref/ Clinical Trials
Indirubin	–	0.6 μM		Cheng and Merz, 2016

B: Non-ATP competitive				
Inhibitor	**Inhibition potency (IC_{50}) GSK-3α**	**Inhibition potency (IC_{50}) GSK-3β**	**Structure**	
Tideglusib (NP-12, NP031112)	–	60 nM		Zhou et al., 2016 Mathuram et al., 2016
TDZD-8 (NP01139)	–	2 μM		Wiese et al., 2016 Fu et al., 2014

C: Substrate competitive				
Inhibitor	**Inhibition potency (IC_{50}) GSK-3α**	**Inhibition potency (IC_{50}) GSK-3β**	**Structure**	
L803-mts (peptides)	–	40 μM		Sun et al., 2016 Zhu et al., 2011

NCT01287520: this study is to determine a recommended Phase II dose and dosing regimen of LY2090314 in combination with pemetrexed and carboplatin in patients with advanced/metastatic cancer.
NCT01632306: purpose of this Phase I/II study is to test how well LY2090314 works in combination with different chemotherapies in treating participants with metastatic pancreatic cancer.
NCT01214603: a Phase II study of LY2090314 in participants with acute leukemia.

binding to complementary sequences in the 3' untranslated region (UTR) of target messenger RNAs (RNAs), or by inducing mRNA degradation (Hayes et al., 2014). Accumulating evidence indicates that miRNAs play important roles in the development and progression of HCC (Shen et al., 2016). In normal liver tissue, IGF-1R expression is downregulated by miRNAs (Zheng et al., 2010). One of these miRNAs is the liver-specific miR-122, expressed at very low levels in HCC (Tsai et al.,2009). However, during hepatocarcinogenesis due to dietary exposure to aflatoxin B1 (AFB1), a genotoxic hepatocarcinogen, a novel mechanism of regulation of IGF1-R by miR-122 expression has been reported (Zeng et al., 2010). Exposure to AFB1 increased the levels of IGF-1R protein by AKT activation, which in turn inhibited GSK-3β activity. Inhibition of GSK-3β resulted in decreased levels of miR-122 due to reduced activity of CCAAT/enhancer-binding protein alpha (C/EBPα), a transactivator for miR-122 transcription (Zeng et al., 2010). On the contrary, other miRNAs inhibits HCC cell growth by directly targeting IGF-1R (Xiao et al., 2016; Youness et al., 2016). Thus, through the repression of IGF-1R, an essential component of the IGF-axis, these miRNAs act as tumor suppressors in HCC.

As for insulin receptor, it has been suggested it has a pro-tumorigenic effect. IR binds also IGF-II, which, as previously reported, is overexpressed in HCC (Frasca et al., 1999).

The expression of other downstream components in the IGF axis has been studied in HCC. Overexpression of IRS-1 has been described in 80% of human HCC tissues and its expression is correlated with tumor growth (Tanaka and Wands, 1996; Boissan et al., 2005). IRS-1 expression is also regulated by miRNAs (Lai et al., 2016; Wang et al., 2014). Similarly, to IRS-1, IRS-2 is also upregulated in HCC patients (Boissan et al., 2005). Furthermore, IRS-1 and -2 are found to be co-expressed in 80% of HCC (Boissan et al., 2005).

In physiological conditions, expression of IGF-2R contributes to maintaining IGF-II levels under control through lysosomal degradation. IGF2-R is downregulated in certain cancers, including HCC. Loss of IGF2-R expression in HCC results from diverse alterations such as mutations and/or loss of heterozygosity (LOH) (Oka et al., 2002; Hanafusa et al., 2002; Jang et al., 2008). Thus, many consider IGF-2R to function as a tumor suppressor in HCC.

IGFBPs are expressed by hepatocytes and other liver cells. The IGFBP family comprises six members: IGFBP-1 to -6. IGFBPs have a high affinity for IGF-I and IGF-II. Therefore, by sequestering the bioavailable IGFs, they exert a protective effect towards IGFs-induced cell proliferation (Pollak, 2008). IGFBPs are frequently found downregulated in cancers, including HCC. IGFBP1 has been shown to be downregulated in 36.7% HCC tissues compared to the adjacent non-cancerous liver tissues, and its low expression is significantly associated with poor survival of HCC patients (Dai et al., 2014).

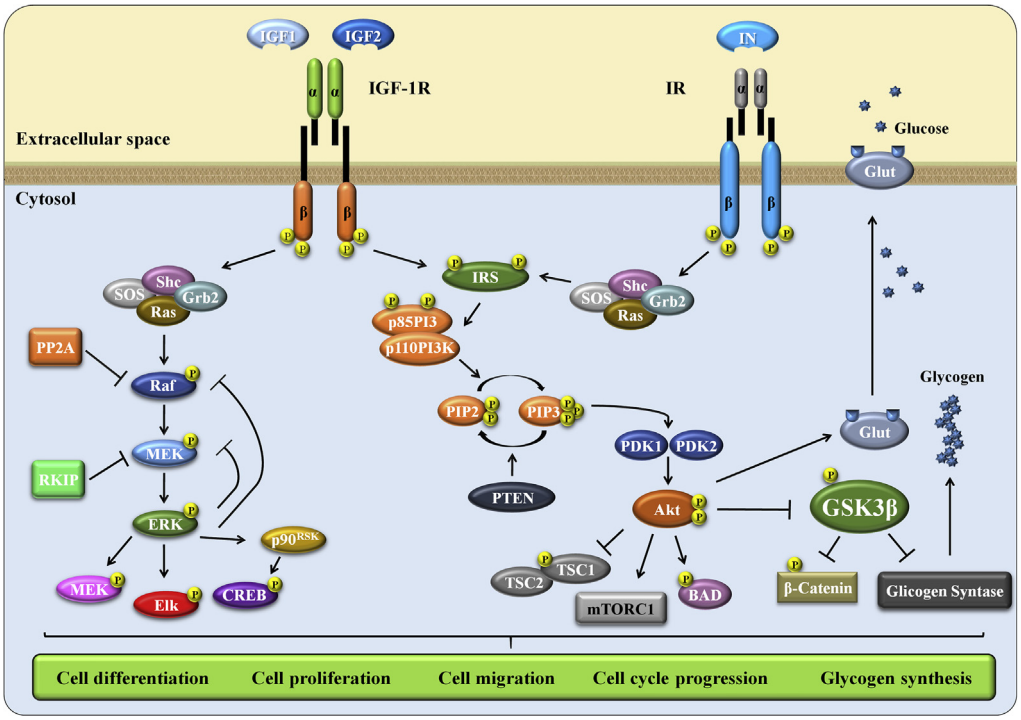


Fig. 2. Schematic overview of IGF/IGFR and IN/IR signaling pathways. Binding of ligands leads to activation of two main signaling pathways: IRS-1/PI3K/Akt/mTOR and Ras/Raf/MEK/ERK pathways. Activation of these pathways regulate several cellular processes including cellular differentiation, proliferation and migration, cell cycle progression and glycogen synthesis.

IGFB3 is also found downregulated in 75% of HCC, with 33% of cases associated with promoter hypermethylation (Hanafusa et al., 2002). In summary, abnormal activation of the IGF signaling contributes to HCC growth and development.

6. GSK-3 and Wnt/β-catenin signaling and HCC

The Wnt signal transduction pathway is composed of proteins that transmit signals through binding to cell surface receptors. In humans, the Wnt family comprises 19 members of secreted and glycosylated proteins. Wnt proteins act in autocrine and paracrine manners to activate Wnt signaling, mainly controlling gene transcription, by binding to Frizzled (Fz) receptors. In humans, 10 family members of the Fz protein receptors have been identified (Nusse, 2005; He et al., 2004) Most of the Wnts and Fzs proteins are expressed in the liver. Two main Wnt signaling pathways have been well characterized: non-canonical pathway (β-catenin-independent) and canonical pathway (β-catenin-dependent) (Fig. 3).

In the “non-canonical pathway”, Wnts may signal essentially in two ways, through the involvement of the small GTPase family members RhoA and Rac1 (Katoh, 2005), or through mobilization of intracellular Ca^{2+} (Saneyoshi et al., 2002; Kühl et al., 2000). The non-canonical Wnt signaling, which involves RhoA and Rac1, is important in organ morphogenesis and in the polarized cell movement by controlling cytoskeletal rearrangement (Yang, 2012; Toyama et al., 2010). Calmodulin kinases and PKC are involved in the control of cell proliferation and migration, cell fates, cell functions and developmental process of different organs in the non-canonical Wnt/Ca^{2+} pathway, through the transcriptional factor NF-AT, (Kühl et al., 2000).

The “canonical pathway” is much better characterized and mainly controls cell proliferation and differentiation. It is activated by binding of Wnt proteins to Fz receptors. One of the proteins most involved in this process is β-catenin. This protein accomplishes many additional non Wnt-mediated essential functions, such as maintaining epithelial integrity by stabilizing the interaction of the adhesion molecule E-cadherin with the cytoskeleton and with α-catenin (Huber and Weis, 2001; Oloumi et al., 2004).

In humans, under physiological conditions, in the absence of the binding between Wnt and the corresponding Fz receptor (Fig. 3), β-catenin interacts with a protein complex formed by several proteins including adenomatous polyposis coli (APC), Axin, Casein Kinase 1 (CK-1) and GSK-3β. Upon assembly of this complex, GSK-3 β phosphorylates β-catenin at N-terminal serine and threonine residues, resulting in β-catenin recognition by β-transducin repeat-containing protein (β-TrCP), an E3 ubiquitin ligase subunit, with its subsequent ubiquitination and degradation by the proteasome (Gattinoni et al., 2010).

In the presence of Wnt ligands (Fig. 3), Wnts bind to their receptors Fz, and low-density-lipoprotein-related protein5/6 (LRP5/6) are phosphorylated and Dishevelled (Dvl) is activated, as consequence β-catenin will not be degraded due to

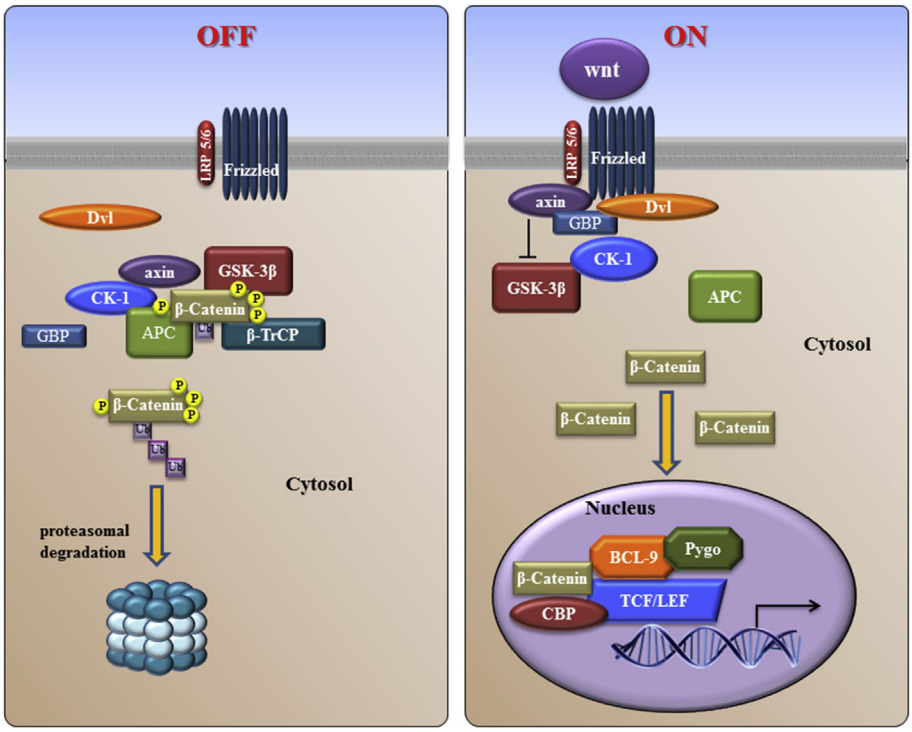


Fig. 3. A simplified overview of canonical Wnt/β-catenin signaling pathway. Left side, in the absence of Wnt, β-catenin is ubiquitinated and targeted for degradation by a protein complex that consists of APC, axin, and GSK-3β, CK-1 and β-TrCP. Right side, in the presence of Wnt, Wnt binds the receptor complex, which consist of Fz receptor and LRP5/6, and Dvl is activated. FRAT/GBP competes with Axin for binding to GSK-3β, as consequence β-catenin is not degraded due to inactivation of the "destruction complex". Thus, β-catenin translocates to the nucleus and interacts with TCF/LEF and other cofactors, and promotes transcription of target genes.

inactivation of the "destruction complex". Frequently rearranged in advanced T-cell lymphomas (FRAT)/GSK-3 binding protein (GBP) competes with Axin for binding to GSK-3β, thus preventing the phosphorylation and degradation of β-catenin. Therefore, β-catenin accumulated, enters the nucleus where it acts as a transcriptional factor, by binding to lymphoid enhancing factor (LEF)/T-cell factor (TCF) transcription factors, and thus regulating transcription of genes which are implicated in cell proliferation, apoptosis, angiogenesis and invasion, such as *c-myc*, *cyclin D1*, *survivin* and *MMP7* (Behrens, 2000).

An increase in the activity of Wnt/β-catenin pathway is often found in some types of cancers. It has been shown that activation of this pathway may be an early event in hepatocarcinogenesis, and has been also associated with an aggressive phenotype of HCC, being correlated to survival, proliferation, migration and invasion mechanisms. All this suggests that components of this signal cascade can be considered as possible targets for potential therapeutic approach in HCC treatment (Vilchez et al., 2016).

Point mutations are found in exon 3 of *CTNNB1* gene (encoding β-catenin) in HCC. These mutations are associated with an alteration of normal Wnt-mediated signal transduction. Most of the reported mutations in the *CTNNB1* gene are found in the Ser37, Thr41 and Ser45 phosphorylation sites located in the N-terminus domain of the protein, and impair GSK-3β-mediated phosphorylation of the protein. It has also been shown that 20–40% of HCC cells have the mutations of *CTNNB1* gene that leads to the accumulation of β-catenin in the nucleus (Nishida et al., 2007). However, other mutations in Wnt pathway components can also affect normal signaling and lead to the accumulation of β-catenin in the nucleus (Lee et al., 2006), such as in the case with alterations in Axin1, as they can result in activation of Wnt/β-catenin signaling (Satoh et al., 2000).

7. GSK-3 and Hedgehog signaling and HCC

The evolutionarily conserved HH signaling pathway is implicated in development and differentiation of tissues and organs during embryogenesis. Aberrant activation of the HH pathway in adult tissues can lead to the development of several types of cancer (Evangelista et al., 2006).

In the mammalian canonical pathway, HH signaling is initiated by a family of ligands including Desert hedgehog (DHH), Indian hedgehog (IHH), and Sonic hedgehog (SHH) which interact with the 12-span transmembrane protein cell surface receptor Patched (PTCH) and to co-receptors cell adhesion molecule-related/down-regulated by oncogenes (CDON) and Brother of CDON (BOC) (Heretsch et al., 2010) (Fig. 4).

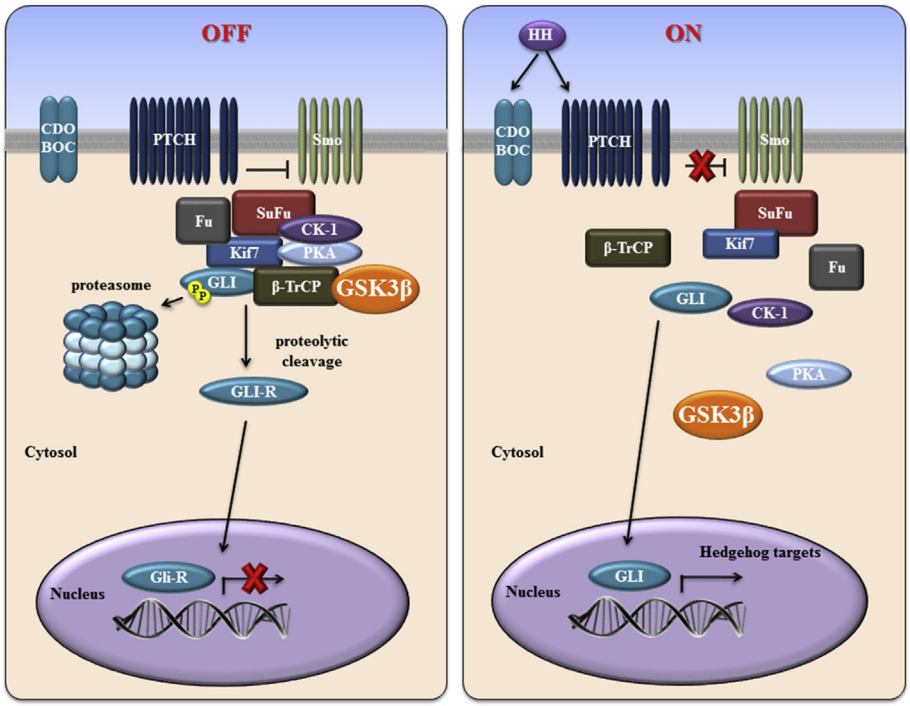


Fig. 4. Schematic overview of Hedgehog signaling pathway. Left side, in the absence of HH ligand, PTCH inhibits Smo allowing GLI to form a complex with Kif7 and SuFu, promoting GLI phosphorylation by PKA, CK-1, and GSK-3β. Upon phosphorylation, GLI proteins are processed into transcriptional repressors (GLI-R) or are targeted to proteasome. Right side, HH ligand binding to PTCH and to co-receptors CDON and BOC and relieves repression of Smo, triggering its interaction with Kif7. This facilitates the release of GLI from SuFu/Fu complex, bypassing proteolytic cleavage. Full-length GLI factors lead to the expression of HH target genes.

In the absence of a HH protein, PTCH acts as a negative regulator of the HH pathway, inhibiting the G protein-coupled receptor Smoothened (Smo). When a HH protein binds to PTCH, Smo is activated. As a result, glioma-associated oncogene homolog (GLI) proteins are cleaved into activated forms, which are released from the kinesin-family protein (Kif7), Fused (Fu), a serine-threonine kinase, and suppressor of fused (SuFu) complex. Activated GLI thereby translocate into the nucleus and regulate the transcription of target genes (Fig. 4). There are three members of GLI family found in humans, including GLI1, GLI2 and GLI3 (Kinzler et al., 1987; Corbit et al., 2005; Huangfu and Anderosn, 2005; Kasper et al., 2006a). GLI1 and GLI2 act as transcriptional activators, whereas GLI3 acts as a transcriptional repressor of HH signaling pathway. Several GLI target genes have been identified, such as *N-myc* (Kenney et al., 2003), *CCND1* (Kasper et al., 2006b), *CCND2* (Yoon et al., 2002), *BCL2* (Kasper et al., 2006b), *SNAI1* and *TWIST2* (Li et al., 2007; Zheng et al., 2012).

In the absence of a HH, GLI proteins form a complex in the cytoplasm with KiF7, Fu and SuFu, promoting GLI phosphorylation on serine and threonine residues in its C-terminal cytoplasmic tail by cAMP-dependent Protein Kinase (PKA), casein kinase 1 (CK-1), and GSK-3β. Upon phosphorylation, GLI proteins are processed into transcriptional repressors or are targeted to proteasome for degradation, through the β-transducin repeat containing E3 ubiquitin protein ligase (β-TrCP), a member of F-box protein family (Fig. 4).

HH ligands levels are increased in the liver of patients with chronic hepatitis, liver cirrhosis and HCC (Pereira et al., 2010).

Many studies have demonstrated the aberrant activation of HH signaling in HCC (Sicklick et al., 2006; Zheng et al., 2010; Lu et al., 2012; Huang et al., 2006; Patil et al., 2006; Zheng et al., 2012).

As mentioned before, hepatitis virus infections are major risk factors for HCC development. HBV-encoded protein HBx is one of the four proteins encoded by the HBV genome. Inhibition of HH signaling has been found to eliminate the ability of HBx to promote cell migration, anchorage-independent growth *in vitro* and tumor development in two animal models of HCC. Therefore, it seems that activation of HH signaling is necessary for HBx to accelerate hepatocarcinogenesis (Arzumanyan et al., 2012).

Infection with HCV has been reported to stimulate cultured hepatocytes to produce HH ligands (Pereira et al., 2010).

In a recent study, conducted on 26 patients with HCC of different etiology, SHH, PTCH and GLI1 have been detected in all HCC tissues analyzed (Dugum et al., 2016). Positive expression of SHH has been reported to be remarkably stronger in liver tissues with metastasis than in non-metastatic HCC. Recently, expression of SHH has been reported to promote liver cirrhosis and liver cancer development induced by other oncogenes in a transgenic mouse model (Chung et al., 2016a). *In vitro* results have confirmed the correlation between HH signaling activation and cell invasion and migration, since treatment with KAAD-

cyclopamine (KAAD-cyc), a specific inhibitor of the HH pathway, decreased the localization of GLI1 in the nucleus and inhibited the invasion and migration of HCC cells (Lu et al., 2012).

GLI1 seems to promote the proliferation, viability, colony formation, migration and invasion of HCC cells, while *GLI1* gene silencing produces opposite results (Zheng et al., 2012; Chen et al., 2014). In addition, expression of PTCH-1 mRNA and GLI1 mRNA in HCC tissues may be associated with the rapid recurrence of HCC tumors after surgery (Jeng et al., 2013).

Activation of HH signaling pathway in HCC may also result through the down-regulation of HH-interacting protein (HHIP) (Tada et al., 2008). Down-regulation of HHIP transcription was observed in most HCC tissue samples examined, due to LOH at the HHIP locus and/or DNA hypermethylation of the *HHIP* gene.

8. GSK-3 and Notch signaling and HCC

Notch signaling is a highly evolutionarily conserved pathway involved in a variety of essential cellular processes, including cell fate, cell polarity and tissue homeostasis (Artavanis-Tsakonas et al., 1999; Lai, 2004).

In humans, the Notch system components include four large single pass transmembrane Notch receptors (Notch-1, -2, -3, -4), and two types of transmembrane ligands, Delta-like (DLL1, 3, and 4) and Jagged (Jag1 and Jag2), which are expressed on neighboring cells (also referred as signaling-sending cells) (Kopan and Ilagan, 2009) (Fig. 5). Notch receptors are composed of an extracelluar domain (Notch extracellular domain, NECD), rich in Epidermal Growth Factor (EGF)-like repeats, a transmembrane domain and an intracellular domain (Notch intracellular domain, NICD).

Upon ligand binding, Notch receptors on the "signal-receiving" cell is activated by cleavage events. First, the NECD is cleaved by the protease TACE (Tumor necrosis factor (TNF)-α ADAM converting enzyme). Thereafter, the Notch fragment, in the signaling-receiving cell, is cleaved by γ-secretase, with the release of NICD fragment for the transmembrane domain. NICD released in the cytoplasm is translocated to the nucleus where it associates with to CSL (CBF1/Suppressor of Hairless/Lag1; also called RBP-Jk) to form a transcriptional complex, together with other transcriptional co-activators, to activate transcription of Notch responsive genes, such as *Hairy Enhancer of Split* (HES), *HES-related proteins* (HEY), *CDKN1A* (p21), *CCDN1* (Cyclin D1), and *MYC* (c-myc) (Jarriault et al., 1995; Previs et al., 2015; Ronchini and Capobianco, 2001) (Fig. 5). GSK-3 is also an important component of Notch signaling pathway, however, its role is controversial. Some studies have shown that GSK-3 phosphorylates NICD, and as result Notch is stabilized and activated via inhibition of proteasomal degradation (Foltz et al., 2002; Song et al., 2008). In addition, GSK-3 activity has been reported to enhance nuclear localization and transcriptional activity by phosphorylation of two S/T-P-S/T domains (379TP381T, 383SP385T) in Notch1 intracellular domain (Han et al., 2012). On the other hand, other studies have reported that GSK-3 phosphorylates and decreases Notch protein levels and down-regulates its transcriptional activity (Espinosa et al., 2003; Jin et al., 2009; Kunnimalaiyaan et al., 2015).

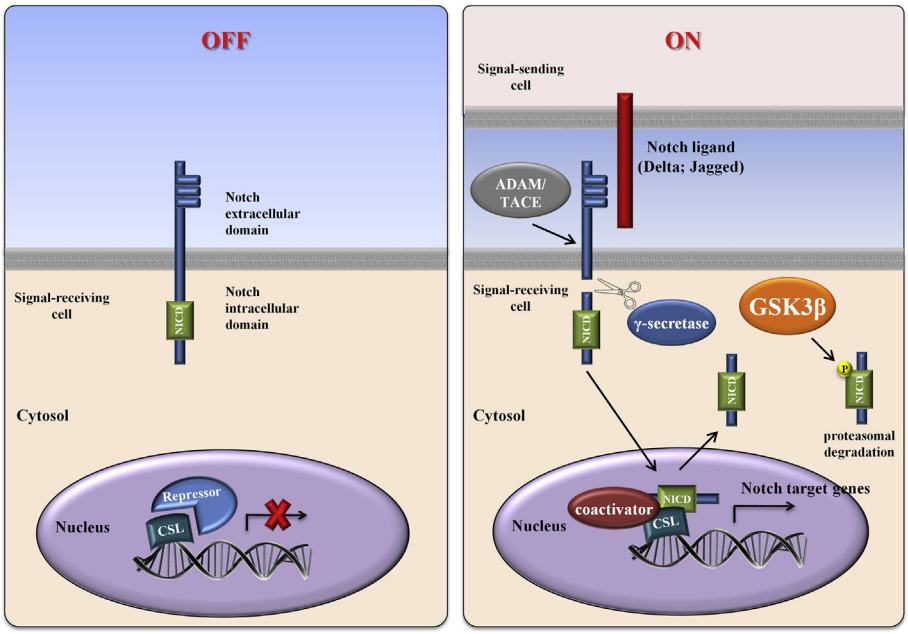


Fig. 5. Schematic overview of Notch signaling pathway. Left side, in the absence of ligand, the Notch receptor, composed of NECD and NICD domains, is preserved. Right side, following the binding of Notch to a ligand (Jagged/Delta), the NECD domain is cleaved by TACE and the NICD domain is cleaved by γ-secretase. The cleaved intracellular domain translocates to the nucleus to form a heterocomplex with the transcription factor CSL and coactivators, thereby inducing the transcription of Notch target genes. GSK-3β can phosphorylate NICD promoting its degradation.

Recent studies suggest that Notch activation is associated with HCC development and progression (Morell et al., 2013; Morell and Strazzabosco, 2014). Activation of Notch signaling pathway has been reported to promote HCC proliferation, invasion, stemness and resistance to conventional cancer therapy as well as to targeted therapy (Lim et al., 2011; Giovannini et al., 2009, 2013, 2016, 2017; Zhang et al., 2015). Virus infection with HBV activates Notch signaling pathway during hepatocarcinogenesis. It has been reported that Jagged1 is highly expressed in HCC tissues compared with adjacent non-tumor liver, and its expression was found to be closely related with HBx protein expression in HCC tissues (Gao et al., 2007). Taken together, these data indicate that Notch signaling plays an oncogenic role in HCC.

9. GSK-3 and TGF-β signaling and HCC

TGF-β signaling pathway has pleiotropic functions being involved in the control of tissue and organ development, extracellular matrix production, cellular proliferation, differentiation, motility, survival and apoptosis (Cohen, 2003).

The TGF-β superfamily of ligands consist of about approximately 40 secreted polypeptide growth factors that comprise TGFs, activins, nodals and bone morphogenetic proteins (BMPs). Signaling is initiated following binding of the ligand to a dimeric TGF-β receptor type II (TGF-βRII), which contains an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic serine-threonine kinase domain (Fig. 6). Following binding of the ligand TGF-βRII is autophosphorylated and recruits and then transphosphorylates a type I receptor dimer, TGF-βRI, forming a hetero-tetrameric complex at the cell surface.

Two main TGF-β signaling pathways have been well characterized: the non-canonical pathway (SMAD-independent) and the canonical pathway (SMAD-dependent) (Zhang, 2009; Moustakas et al., 2001) (Fig. 6). The non-canonical pathway includes various branches of the mitogen-activated protein kinase (MAPK) pathway (i.e., Raf/MEK/ERK, MKK4/JNK and MKK3/6/p38), phosphatidylinositol-3-kinase (PI3K)/AKT pathway, and Rho-like GTPase signaling pathway (Fig. 6).

In the canonical pathway, once activated by the TGFβRII, the TGFβRI phosphorylates SMAD. After phosphorylation, receptor-activated SMADs (R-SMADs: SMAD1, SMAD2, SMAD3, SMAD5, SMAD8) form homomeric and heteromeric complexes with other activated SMAD proteins. Thereafter, activated SMADs form complexes with the common mediator SMAD (Co-SMADs: SMAD4), which are imported to the nucleus and regulate the transcription of target genes. Other SMADs are inhibitory SMADs (I-SMADs: SMAD6 and SMAD7).

Many studies have demonstrated that TGF-β modulates the expression of numerous genes relevant to tumor growth and development (Massague, 2008). In cancer, the TGF-β pathway may have dichotomous function, with both pro- and anti-tumor activities (Tian et al., 2011; Jakowlew, 2006; Drabsch and ten Dijke, 2012). The anti-tumor activity is exerted by cell cycle arrest, induction of apoptosis and autophagy, whereas, tumor progression is mediated by promoting angiogenesis, cell

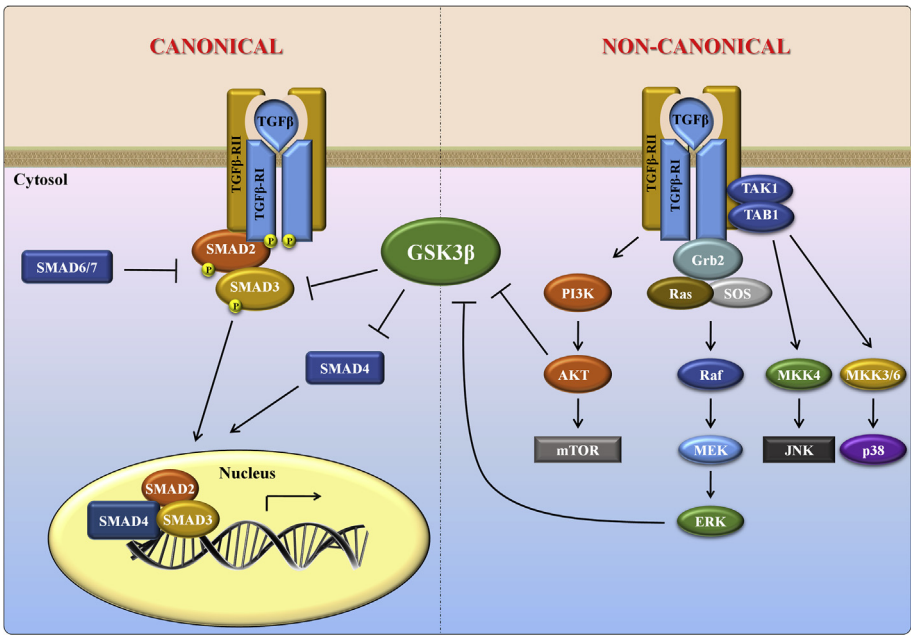


Fig. 6. A simplified overview of canonical and non-canonical TGF-β signaling pathways.
Left side, after binding to TGFβ ligand, the signal starts with assembling of heteromeric complexes (TGF-βRI and TGF-βRII). Activated TGF-βRI phosphorylates the downstream effectors, SMAD2 and SMAD3, and induces the canonical SMAD pathways. GSK-3 acts as a negative regulator of canonical TGF-β signaling. Right side, in the non-canonical pathway, the activated TGF-β receptor complex transmits a signal through other factors, such as MAPK pathway (ERK, JNK and $p38^{MAPK}$) or PI3K/AKT.

motility, invasion, EMT, and cell stemness (Tian et al., 2011; Jakowlew, 2006; Drabsch and ten Dijke, 2012). GSK-3 acts as a negative regulator of TGF-β signaling (Hua et al., 2010; Guo et al., 2008).

As demonstrated by Hua et al. GSK-3β physically and functionally interacts with SMAD3 and thus inhibits SMAD3 nuclear translocation (Hua et al., 2010). Accordingly, Guo et al. reported that GSK-3β can negatively modulate TGF-β/SMAD3 signaling by promoting SMAD3 degradation after phosphorylation of SMAD3 at Thr66 (Guo et al., 2008).

The dichotomous functions of TGF-β signaling pathway have also observed during hepatocarcinogenesis. In early steps of hepatocarcinogenesis, TGF-β exhibits tumor-suppressive properties whereas in late-stage, together with activation of other pathways, it promotes tumor progression by stimulating EMT, cell invasion and thus, cancer metastasis (Neuzillet et al., 2014).

In hepatocytes, insights into the molecular mechanisms underlying the various TGFβ-induced EMT process and GSK-3 activity have been reported (Marchetti et al., 2008). TGF-β, through a Src-dependent pathway, activates ERK5, can then phosphorylate GSK-3β on Ser9, thus inhibiting its activity. Inhibition of GSK-3β results in protein stabilization of Snail, a zinc-finger transcription factor controlling expression of genes involved in EMT. For example, Snail represses E-cadherin transcription (Cano et al., 2000), and therefore promote the tumor-associated EMT process. GSK-3 phosphorylates Snail and therefore promotes its nuclear export, resulting in its cytoplasmic translocation and increased E-cadherin expression.

Recently, a different mechanism implicating GSK-3β in TGF-β-induced EMT program has been reported (Cozzolino et al., 2013). TGF-β, through inhibition of GSK-3 kinase activity, hampers phosphorylation of the tumor suppressor hepatocyte nuclear factor 4 alpha (HNF4α), a transcription factor controlling expression of EMT master genes such as *SNAI1*, resulting in its functional inactivation, contributing to EMT progression.

10. GSK-3 as therapeutic target in HCC

The roles of GSK-3β in HCC remain controversial, several studies suggested a possible role of GSK-3β as a tumor suppressor gene in HCC, however, other studies indicate that GSK-3β is a potential therapeutic target for this neoplasia.

A study, performed on HCC patients, demonstrated that low GSK-3β protein expression were related to HCC progression (Huang et al., 2014). GSK-3β expression was found to be significantly lower in HCC tissues than that in normal liver tissues and surrounding tumor tissues. Furthermore, reduced expression of GSK-3β was significantly correlated with advanced clinicopathological characteristics and poor prognosis of HCC patients. The levels of GSK-3β expression were also correlated with vascular invasion, histological grade and TNM classification, suggesting its possible involvement in process of HCC metastasis (Huang et al., 2014). However, in this study, the GSK-3 activation/inhibition state was not analyzed.

A recent study addresses this matter analyzing in paired HCC tissue and nontumoral tissue the expression of the inactive form of GSK-3β, *i.e.,* p-Ser9-GSK-3β. Over-expression of p-Ser9-GSK-3β was observed in 50% HCC tissues, whereas, normal hepatocytes in nontumoral tissue showed no detectable p-Ser9-GSK-3β expression. Patients with over-expression of p-Ser9-GSK-3β had higher recurrence and lower overall survival rates, than those with low p-Ser9-GSK-3β expression, and therefore GSK-3β could be a prognostic factor in patients with HCC. Similar results have reported by Ban and co-workers (Ban et al., 2003), in a study performed in 23 HCC specimens from Asian patients. Overexpression of phospho-GSK-3β was found in 52% of HCC tissues and 26% of the adjacent non-tumor tissues, however, no correlation between the expression of phospho-GSK-3β and clinicopathological parameters was observed.

As mentioned earlier, infection with either HBV or HCV are well known risk factors for HCC. Several studies suggest that the Wnt/β-catenin pathway is a common target for HBV and HCV in human HCC. The mechanisms responsible for activation of this pathway has been to some extent clarified.

A mechanism for GSK-3β inactivation after infection with HBV has been described recently (Ding et al., 2005). Numerous studies have reported the contribution of HBx in hepatocarcinogenesis induced by HBV. HBx activates many signaling pathways, such as the Ras/Raf/MEK/ERK and PI3K/AKT signaling pathways in hepatocytes. Interestingly, cross-talk between the Ras/Raf/MEK/ERK, PI3K/AKT and Wnt/β-catenin signaling has been observed in hepatoma cells (Ding et al., 2005; Chung et al., 2016b). HBx activates ERK1/2 and as a consequence, it associates and phosphorylated GSK-3β at Thr43. This is required to prime the subsequent GSK-3 phosphorylation at Ser9 by p90RSK kinase resulting in inactivation of GSK-3β. In turn, inactivation of GSK-3β leads to β-catenin stabilization and activation of signaling. This mechanism has been reported to be activated by the signal transduction of different growth factors, including IGF1 and TGF-β, and is responsible for up-regulation of β-catenin in HCC and in other cancer types (Ding et al., 2005).

Through a different mechanism, ERK-mediated inactivation of GSK-3β of HBx has been reported to stabilize and increase cyclin D1 nuclear accumulation during HCC development and progression (Chen et al., 2015). Cyclin D1 level is regulated precisely during S-phase, it rapidly declines because of phosphorylation at Thr-286 by GSK-3β (Diehl et al., 1998). This promotes its nuclear export and subsequent degradation by the 26S proteasome. Therefore, HBx through ERK1/2 activation, might facilitate cell proliferation and malignant transformation at least by two different molecular mechanisms: i) GSK-3β inactivation and consequently β-catenin stabilization, which in turn actives *CCND1* gene transcription (Ding et al., 2005); ii) GSK-3β inactivation and inhibition of cyclin D1 phosphorylation at Thr286 (Chen et al., 2015).

In addition, HBx through PI3K/AKT/GSK-3β, promotes EMT in hepatoma and hepatic cells due to stabilization of Snail protein (Liu et al., 2012). It has been reported that HBx activates the PI3K/AKT pathway thus promoting inactivation of GSK-3β by its phosphorylation at Ser9. This prevents Snail phosphorylation and therefore inhibiting its degradation by ubiquitin/proteasome system. Snail stabilization facilitates tumor invasion and metastasis during HBV-induced HCC progression (Liu et al., 2012).

Among the four HCV gene products, (namely the structural protein HCV core, the non-structural proteins NS3, NS4B and NS5A) the HCV core protein and NS5A have been reported to contribute to HCC carcinogenesis. HCV core protein increases and stabilizes β-catenin levels in hepatoma cells through inactivation of GSK-3β by phosphorylation at S9. This results in β-catenin stabilization and activation of the canonical Wnt/β-catenin signaling pathway, with an increase in transcription of β-catenin target genes, such as *c-Myc*, *cyclin D1*, WNT1-inducible-signaling pathway protein 2 (*WISP2*) and connective tissue growth factor (*CTGF*), and thus increases HCC cell proliferation (Liu et al., 2011). Thus, this study highlight that GSK-3β/β-catenin signaling plays an important role in HCV-associated carcinogenesis.

The HCV non-structural protein NS5A also contributes to EMT during HCV-induced HCC. HCV infection of hepatocytes leads to an increase in the expression and secretion of Osteopontin (OPN) (Iqbal et al., 2013). OPN has been linked to tumor progression and metastasis in a variety of cancers, including HCC (Rangaswami et al., 2006; Cao et al., 2015). In HCC, increased expression levels of OPN is associated with poor prognosis (Sun et al., 2010), and its expression promotes EMT and metastasis (Sun et al., 2008; Dong et al., 2016). Following HCV infection, secreted OPN binds to cell surface receptors, integrin αVβ3 and CD44, and activates a signaling cascade which promotes AKT activation and inhibition of GSK-3β by its phosphorylation on Ser9, followed by β-catenin stabilization and thus activation of β-catenin-mediated signaling cascade, ultimately leading to EMT and HCC tumor progression (Iqbal et al., 2013).

The involvement of dysregulation of GSK-3β phosphorylation in hepatocarcinogenesis has been studied *in vitro* using human HCC cell lines of different origins (Desbois-Mouthon et al., 2002). It was reported that only some HCC cell lines expressed high basal levels of p-Ser9-GSK-3β, due to an autocrine signaling loop involving the IGF-1R-dependent pathway. Because of the persistent phosphorylation of GSK-3β, β-catenin was stabilized and thus contributed to HCC cell growth.

Elevation of β-catenin levels and activation of its downstream target *cyclin D*1, has been reported in HCC because of GSK-3β association with N-Myc downstream regulated gene 1 (NDRG1), a stress response protein (Lu et al., 2015). NDRG1 has been observed to be up-regulated in HCC tissues compared to adjacent non-tumor liver tissues, and it has also been proposed as a new marker of HCC prognosis and recurrence (Chua et al., 2007; Cheng et al., 2011). Over-expression of NDRG1 in HCC patient samples is correlated positively with p-Ser9-GSK-3β expression levels, and nuclear β-catenin expression (Lu et al., 2015). NDRG1 directly interacts with GSK-3β and competitively disrupts binding of GSK-3β to β-catenin, thus preventing its proteasomal degradation and enhancing its accumulation in the nucleus (Lu et al., 2015).

Aberrant activation Wnt/β-catenin signaling because of reduced activity of GSK-3β, *i.e.,* increased levels of p-Ser9-GSK-3β, has been reported by physical interaction between the RNA binding motif on the Y chromosome (RBMY), a novel oncofetal protein, and GSK-3β (Chua et al., 2015). Increased expression of RBMY is significantly observed in cytoplasm and nucleus in HCC tissues and RBMY and is significantly correlated with poorer prognosis (Chua et al., 2015). The RBMY/GSK-3β interaction competitively inhibited the association of GSK-3β with APC or Axin-2. This resulted in β-catenin stabilization and translocation to the nucleus and activation Wnt/β-catenin which facilitated hepatic stemness and hepatocarcinogenesis.

GSK-3β/β-catenin signaling is also involved in hepatocarcinogenesis induced by lack of the tumor suppressor Matrilin-2 (Matn2) (Fullár et al., 2014). Matn2 is a member of non-collagenous glycoprotein family implicated in the assembly of extracellular matrix (ECM), which is expressed in the liver progenitor oval cells and deposited in the basement membrane zone around the tubules formed by these cells, suggesting an important role for the protein in liver regeneration (Szabo et al., 2007). In absence of Matn2, liver tumor development is favored by β-catenin signaling activation which occurs due to the phosphorylation and inactivation of GSK-3β (Fullár et al., 2014). GSK-3β is phosphorylated, and thereby inhibited, by phosphorylation at the Thr43 residue by ERK1/2 due to activation of EGFR signaling pathway (Fullár et al., 2014).

GSK-3β/β-catenin signaling plays also a key role in the Sirtuin 2 (SIRT2)-mediated HCC tumorigenesis (Chen et al., 2013). SIRT2 is a member of sirtuin family, which is upregulated in about 50% of HCC patients and is positively correlated with poor patient prognosis (Chen et al., 2013). The authors demonstrated that SIRT2 regulated activation of AKT by its deacetylation, and therefore, activated AKT phosphorylated and inactivated GSK-3β (Chen et al., 2013). As a result, β-catenin-signaling cascade was enhanced and promoted HCC cell migration and EMT.

In another scenario, induction of EMT due to Snail stabilization is induced in HCC patients which have amplification and overexpression of the novel oncogene Maelstrom (MAEL) (Liu et al., 2014). MAEL expression enhanced AKT activity and a subsequent increase in p-Ser9-GSK-3β, which resulted in Snail stabilization and promoted HCC cell migration and tumor metastasis (Liu et al., 2014).

Together these results highlighted the possible role of GSK-3β as a tumor suppressor gene in HCC, and consequently loss of GSK-3β expression and/or inhibition of its activity may contribute to HCC development. However, other studies have reported that inhibition of GSK-3 affect cancer cell survival and proliferation in a manner not related to Wnt/β-catenin signaling.

As reported previously, expression of SIRT2 may promote HCC metastasis and invasion through the AKT/GSK-3β/β-catenin signaling pathway. On the contrary, recently it has been reported that another member of the sirtuin family SIRT3 functions as a tumor suppressor in HCC via the GSK-3β/Bax signaling pathway (Song et al., 2016). The role played by SIRT3 in carcinogenesis has been controversial. Downregulation of SIRT3 expression is frequently observed in clinical HCC specimens and negatively correlated with tumor differentiation, size, and poor prognosis, suggesting that SIRT3 might play a role as tumor suppressor in HCC (Zhang et al., 2012; Song et al., 2016). Mechanistically, SIRT3 increases the total abundance of GSK-3β, whereas it reduces the p-GSK-3β levels. In addition, SIRT3 directly associates with GSK-3β and efficiently deacetylates it at Lys205 resulting in its activation. The acetylation of GSK-3β at Lys205 site by SIRT1 has been also reported (Monteserin-Garcia et al., 2013). Activation of GSK-3 induces expression and mitochondrial translocation of the pro-apoptotic protein BCL2-

associated X protein (Bax) to promote apoptosis of HCC cells (Song et al., 2016). Therefore, this study suggested that SIRT3/GSK-3β/Bax signaling pathway might play a pivotal role in the suppression of HCC growth.

Moreover, a therapeutic effect of suppression of GSK-3β activity was observed by modulation of human telomerase reverse transcriptase (hTERT) and telomerase (Mai et al., 2009). In HCC cells expressing high levels of GSK-3β and p-GSK-3β Tyr216 (active) and low levels of p-GSK-3βSer9 (inactive), treatment with the GSK-3β inhibitors SB-216763 and AR-A014418, decreased cell viability and proliferation and increased apoptosis through reduction of hTERT expression and telomerase activity (Mai et al., 2009).

Lithium is a well-known inhibitor of GSK-3 activity. Treatment of cancer cells with lithium leads to β-catenin accumulation and activation of Wnt/β-catenin signaling. However, many HCC cell lines are responsive to lithium-mediated growth inhibition in a manner independent of its effect on Wnt/β-catenin signaling (Erdal et al., 2005). In HCC cells, lithium, in addition to activate the Wnt/β-catenin pathway, induces depletion of PKB/AKT and cyclin E proteins. This results in stimulation of cell cycle arrest and overcomes any potential stimulating effect on cell proliferation mediated by Wnt/β-catenin pathway (Erdal et al., 2005).

In a different context, GSK-3β inhibitors, lithium and SB-415286, have been reported to overcome the intrinsic resistance of HCC cells to TRAIL-induced apoptosis (Beurel et al., 2009). The GSK-3β inhibitors sensitized HCC cells, but not normal hepatocytes, to TRAIL-induced apoptosis by a complex mechanism which involved the activation of apoptotic signals (stabilization of TP53 and activation of caspase-3 and -8), as well as protective signals (activation of JNK) (Beurel et al., 2009). Therefore, GSK-3 inhibitors, in association with TRAIL, may open a new therapeutic opportunity in HCC.

11. Conclusions

The roles of GSK-3β in HCC remain controversial. Several studies have suggested possible roles of GSK-3β as a tumor suppressor gene in HCC, whereas, other studies have indicated that GSK-3β is a potential therapeutic target for this cancer. Caution must be taken before using GSK-3 inhibitors as antitumor agents in HCC. Therefore, the clinical relevance of GSK-3 as a target in HCC and its therapeutic potential as a target for therapy remain to be elucidated. Although, there are no clinical data available on GSK-3 inhibitor in HCC, ongoing or completed clinical trials in patients with other diseases, including different cancer types, leaves promise for HCC treatment. However, it should be noted that only a sub-group of HCC patients might receive a beneficial effect by treatment.

Combination therapy of GSK-3 inhibitors with either conventional cytotoxic drugs, or other inhibitors which targets specific molecules, may also be a key approach for improving the effectiveness and usefulness of new therapies for HCC treatment.

Conflicts of interest

The authors declare that they have no conflicts of interest with publication of this manuscript.

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