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Regulation of amphiregulin gene expression by β-catenin signaling in human hepatocellular carcinoma cells: a novel crosstalk between FGF19 and the EGFR system.

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A chi ogni giorno mi sta,
o mi è stato,
vicino.

"La ciencia será siempre una búsqueda, jamás un descubrimiento real.
Es un viaje, nunca una llegada"
Karl Popper

ABBREVATIONS

AB: acid box

ADAM: A Disintegrin and Metalloproteinase

AKT: Protein Kinase B (PKB)

ANG II: Angiotensin II

APC: Adenomatous Polyposis Coli

AR: Amphiregulin

BRCA1: Breast Cancer 1

BSA: Bovin Serum Albumin

BTC: Betacellulin

cDNA: complementary DNA

CK1: Casein kinase 1

COX-2: Cyclooxygenase-2

CRE: cAMP response element

CREB: CRE binding protein

CTNNB1: human β-catenin gene

CYP7A1: Cholesterol 7a-hydroxylase

DEPC: Diethyl pyrocarbonate

DKKs: Dickkpf proteins

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

dNTP: Desoxinucleotides triphosphates

DSH: Dishevelled

DTT: Dithiothreiol

DUSP: Dual Specifity Phosphatase

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

ELISA: Enzyme-Linked ImmunoSorbent Assay

EPG: Epigen

EPR: Epiregulin

FBS: Fetal Bovine Serum

FGF: Fibloblast Growth Factor

FGFR: Fibloblast Growth Factor Receptor

FRS2: FGF Substrate 2

Frs2a: Fibroblast growth factor Receptor substrate 2

Fz: Frizzled

Gab1: Grb2-associated binding protein 1

GEO: Gene Expression Omnibus

GPCR: G Protein-Coupled Receptor

GRB2: Growth factorRreceptor-Bound 2

GS: Glutamine Synthetase

GSK3β: Glycogen Synthase Kinase 3 beta

HB-EGF: Heparin-binding Growth Factor

HBV: Hepatitis B viral

HCC: Hepatocellular Carcinoma

HCV: Hepatitis C viral

HER: Human Epidermal Receptor

HGF: Hepatocyte Growth Factor

HS: Heparin Sulfate

HSP70: Hot Shot Protein 70

HSPGs: Heparan sulfate proteoglycans

Ig: Immunoglobuline

IGF: Isulin-like Growth Factor

IGF1R: Isulin-like Growth Factor 1 Receptor

IL-1b: Interleukin-1b

INM: Inner Nuclear Membrane

INTγ: Interferon γ

KL: Klotho

KLB: β-Klotho

LPA: Lysophosphatidic acid

LRPs: Low-density lipoprotein receptor related proteins

MAPK: Mitogen-Activated Protein Kinase

MMPs: Metalloproteasas

mRNA: Messenger RNA

NFAT: Nuclear Factor of Ativated T cells

NLK: Nemo-like kinase

NRGs : Neuregulins

NSCLC: Non-Small-Cell Lung Cancer

PCP: Planar Cell Polarity

PCR: Polymerase Chain Reaction

PDGFR: Platelet-derived growth factor receptors

PGE2: Prostaglandine 2

PI3K: Phosphatidylinositol 3-kinase

PKC: Protein Kinase C

PLCγ: Phospholipase C γ

PMA: Phorbol ester-12-Myristate-13-Acetate

qPCR: Real Time PCR

RNA: Ribonucleic acid

ROK: Rho-associated protein

ROS: Reactive oxygen species

SDS: Sodium Dodecyl Sulfate

Sef: Similar Expression to FGF

Ser: Serine

sFRP: Secreted Frizzled-related protein

Shp2: Src homology region 2 domain containing phosphatase 2

SOS: Son of Sevenless

STAT: Signal Transducers and Activators of Transcription

TACE: Tumor necrosis factor-α-converting enzyme

TBEs: Tcf-binding elements

TCF/LEF: T cell factor/lymphoid enhancer factor

TE: Tris-EDTA

TGFα: Transforming Growth Factor α

Thr: Threonine

TKIs: Tyrosine kinase inhibitors

TMB: 3,3',5,5'-tetramethylbenzidine

TNF-α: Tumor Necrosis Factor α

VEGF: Vascular Endothelial Growth Factor

WIF-1: Wnt Inhibitory Factor-1

ΔNTcf4: Dominant negative Tcf4 variant

ABSTRACT

Hepatocellular carcinoma (HCC) is the most prevalent liver tumor and a deadly disease with limited therapeutic options. Dysregulation of cell signalling pathways is a common denominator in tumorigenesis, including hepatocarcinogenesis. The epidermal growth factor receptor (EGFR) signalling system is commonly activated in HCC, and is currently being evaluated as a therapeutic target in combination therapies. We and others have identified a central role for the EGFR ligand amphiregulin (AR) in the proliferation, survival and drug resistance of HCC cells. *AR* expression is frequently up-regulated in HCC tissues and cells through mechanisms not completely known. Here we identify the β-catenin signalling pathway as a novel mechanism leading to transcriptional activation of the *AR* gene in human HCC cells. Activation of β-catenin signalling, or expression of the T41A β-catenin active mutant, led to the induction of *AR* expression involving three specific β-catenin/T cells Factor (Tcf) responsive elements in its proximal promoter. We demonstrate that HCC cell expressing the T41A β-catenin active mutant show enhanced proliferation that is dependent on AR expression and EGFR signalling. We also demonstrate here a novel crosstalk of the EGFR system with fibroblast growth factor 19 (FGF19). FGF19 is a recently identified driver gene in hepatocarcinogenesis and an activator of β-catenin signalling in HCC and colon cancer cells. We show that FGF19 induced *AR* gene expression through the β-catenin pathway in human HCC cells. Importantly, AR up-regulation and EGFR signalling were necessary for the induction of cyclin D1 and cell proliferation by FGF19. Finally, we demonstrate a positive correlation between *FGF19* and *AR* expression in human HCC tissues, therefore validating in clinical samples our experimental observations. These findings identify the AR/EGFR system as a key mediator of FGF19 responses in HCC cells involving β-catenin signalling, and suggest that combined targeting of FGF19 and AR/EGFR may enhance therapeutic efficacy.

INTRODUCTION

1. Hepatocellular Carcinoma (HCC)

1.1. Epidemiology of HCC

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. Globally, it ranks fifth among the most common cancers but it is the third leading cause of cancer death, with an estimate of more than 500000 new cases every year (Ferlay et al. 2000; Parkin et al. 2000). There is a striking geographical difference in the incidence of HCC. Eighty per cent of new cases occur in developing countries. High-incidence areas include the sub-Saharan Africa, east and Southeast Asia, whereas low incidence areas include northern and western Europe and North America. Better control of the risk factors has resulted in a recent decline in HCC in some places such as Taiwan and China (Lee CL et al. 2003a). However, recently there is a trend of rising rates of HCC in developed countries in Europe and North America (El-Serag HB 2004). In Southeast Asia, HCC is the second most common fatal cancer. It has been the second most common fatal cancer and its ranking among the common causes of fatal cancers has not changed since the 1970s. A male predominance is obvious, with a male to female ratio of 2–4:1 (El-Serag and Rudolph 2007).

The risk factors of HCC are well established and include chronic hepatitis B viral (HBV) and hepatitis C viral (HCV) infection (Anzola 2004), cirrhosis (Fattovich et al. 2004) and Aflatoxin B1- contaminated food (Yu and Yuan 2004).

Alcohol abuse leading to cirrhosis increases HCC risk appreciably by promoting liver cirrhosis; only severe, but not moderate, alcohol consumption is related to HCC (Mandayam S et al. 2004). The role of tobacco smoking in the causation of HCC is controversial. Other risk factors include inherited metabolic diseases such as hereditary haemochromatosis (Kowdley 2004), α-1-antitrypsin deficiency and hereditary tyrosinaemia. Obesity and diabetes can lead to non-alcoholic steatohepatitis, which is also

an established risk factor for HCC, most likely via progression of the steatohepatitic disease to cirrhosis and HCC (Calle et al. 2003).

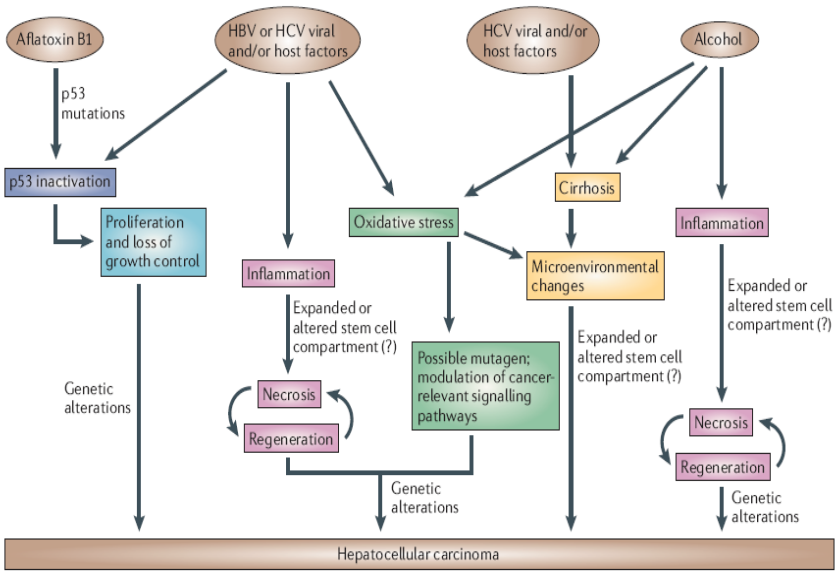


Figure 1: Mechanisms of hepatocarcinogenesis. (From Farazi and DePinho 2006)

1.2 Molecular pathogenesis

Hepatocarcinogenesis is a multistep process that could last for decades and involves the progressive accumulation of different genetic and epigenetic alterations ultimately leading to malignant transformation. Some of the molecular steps involved in the development of HCC have been elucidated in the past few years (Anthony 2002; Pachiadakis et al. 2005; Park et al. 2003; Majumder et al. 2002; Foy et al. 2005; Li et al. 2005). HCCs are phenotypically and genetically very heterogeneous, and this heterogeneity might partially reflect the heterogeneity of aetiological factors that are implicated in the development of HCC, the complexity of hepatocyte functions and the late stage at which HCC usually becomes clinically symptomatic and detectable. The diverse HCC-inducing aetiologies provoke continuous rounds of hepatocyte damage and regeneration, culminating in chronic liver disease (Gale and Foy 2005) (Figure 2). Hyperplastic nodules of regenerating hepatocytes have normal cytological features, and

represent a potential first step towards HCC. These lesions can progress to pre-malignant dysplastic nodules, which have abnormal cytological features including clear cell changes and nuclear crowding, and these lesions are associated with the increased thickening of the trabeculae, which indicates abnormal liver architecture. These dysplastic nodules can evolve to frank HCC which, in addition to all the aforementioned abnormal features, is endowed with the capacity to invade the surrounding fibrous stroma and vessels, and occasionally has metastatic potential (Okuda 2000). Although considerable progress has been made in understanding the sequence of events that lead to other forms of cancer, most notably for colorectal cancer and certain hematopoietic malignancies, the molecular factors that are involved in hepatocellular carcinogenesis are still poorly understood.

Increased turnover of liver cells and the malignant transformation of hepatocytes may occur in response to various stimuli, for example chronic liver injury, cellular regeneration in the context of inflammation, or in response to oxidative DNA damage. These stimuli can result in genetic and epigenetic alterations, such as the activation of cellular oncogenes and the inactivation of tumor suppressor genes, possibly in conjunction with the induction of genomic instability, such as DNA mismatch repair defects and impaired chromosomal segregation. Such defects may lead to the disruption of signaling pathways and could, for example, cause the overexpression of growth factors or growth-factor receptors, which might result in increased resistance of the cell to apoptosis, the stimulation of angiogenesis, hepatocyte proliferation and invasion or metastasis (Anthony 2002; Murakami et al. 2005; Pachiadakis et al. 2005; Park et al. 2003; Majumder et al. 2002; Foy et al. 2005; Li et al. 2005; Hino et al. 2002; Macdonald et al. 2003; Majumder et al. 2001; Moriya et al. 2001). Evidences suggest that HBV and perhaps also HCV might have a direct role in the molecular pathogenesis of HCC through interactions with cellular components. Various viral proteins interact with growth-factor signaling cascades involved in the control of cell proliferation, migration and apoptosis (Kamegaya et al. 2005). In addition, aflatoxins induce mutations in the tumor suppressor gene, *TP53*. This finding suggests a

mechanism by which other environmental factors might contribute to tumor development at the molecular level. A study that used a transgenic mouse model demonstrated that the presence of chronic liver cell injury induced by an abnormal immune response is sufficient to cause HCC, even in the absence of viral transactivation, insertional mutagenesis or genotoxic chemicals (McClain et al. 2002). Furthermore, neutralization of Fas ligand activity by anti-Fas-ligand antibody therapy prevented hepatocyte apoptosis and increased proliferation and liver inflammation, which eventually led to the development of HCC in this rodent model (Hoek and Pastorino 2002) a study in another transgenic mouse model demonstrated that the transcription factor NFκB is essential for promotion of inflammation associated cancer; (Campbell et al. 2005; Comporti et al. 2005) NFκB is, therefore, a potential target for cancer prevention in chronic inflammatory disease. Finally, polymorphisms in enzymes that are responsible for drug metabolism, such as the cytochrome P450 oxidases, N-acetyltransferases and glutathione-*S*-transferase, might also contribute to the genetic susceptibility of an individual to development of HCC (Osna et al. 2005).

Furthermore telomere shortening has been described as a key feature of chronic hyperproliferative liver disease, specifically occurring in the hepatocyte compartment (Kitada et al. 1995). These observations have fueled speculation that telomere shortening associated with chronic liver disease and hepatocyte turnover contribute to the induction of genomic instability that drives human HCC. Another aspect of telomere biology common to HCC is the robust activation of telomerase (correlating with increased *TERT* mRNA levels) in nearly 90% of human HCCs. Telomerase re-activation has been suggested to promote HCC progression (Piao et al 2004; Kobayashi et al. 2001). Collectively, the current data suggest a model wherein telomere shortening drives chromosomal instability and cancer-promoting lesions during early stages of hepatocarcinogenesis, whereas telomerase re-activation is necessary for malignant progression as it restores

chromosomal stability to a level compatible with cancer-cell viability (Farazi and DePinho 2006) (Figure 2).

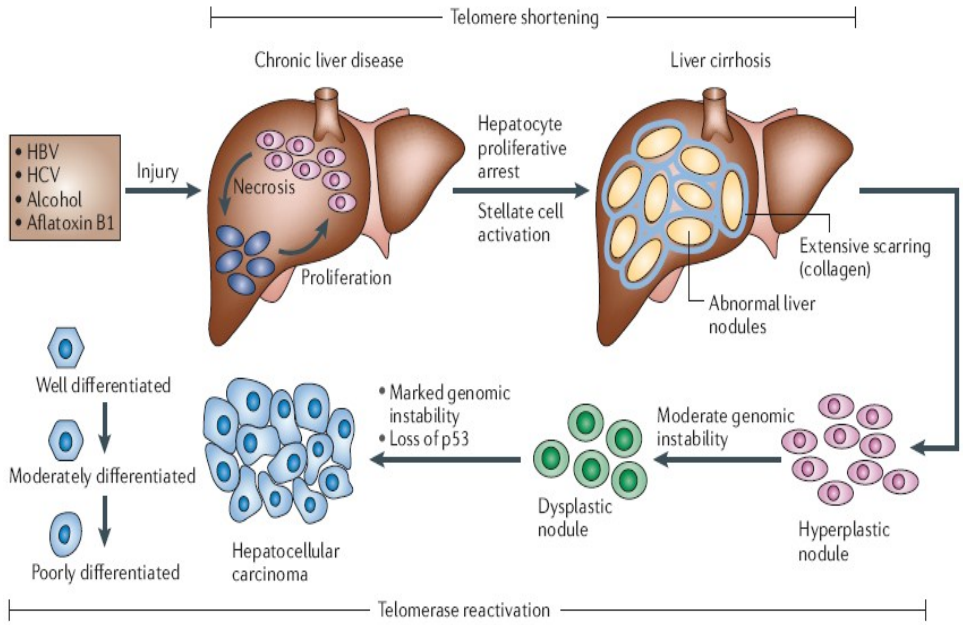


Figure 2: Histopathological progression and molecular features of HCC. Continuous cycles of hepatocyte destructive–regenerative process foster a chronic liver disease condition that culminates in liver cirrhosis. Cirrhosis is characterized by abnormal liver nodule formation surrounded by collagen deposition and scarring of the liver. Subsequently, hyperplastic nodules are observed, followed by dysplastic nodules and ultimately HCC, which can be further classified into well differentiated, moderately differentiated and poorly differentiated tumours, the last of which represents the most malignant form of primary HCC. Telomere shortening is a feature of chronic liver disease and cirrhosis. Telomerase reactivation has been associated with hepatocarcinogenesis. Loss and/or mutation of p53 and genomic instability also characterize hepatocarcinogenesis. p53 loss and/or mutation is shown to occur during progression to HCC, however, there is some evidence that loss and mutation of p53 might also occur in the initial stages of hepatocarcinogenesis. (From Farazi and DePinho 2006)

1.3 Signaling pathways and tumor development

Intact signaling pathways are central to the maintenance and regulation of normal functions of cells, tissue and organs, including regulation of cell apoptosis, proliferation, migration, invasion and angiogenesis. In the case of signaling pathways that involve receptor tyrosine kinases, the activation of the receptor is stimulated by the binding of a ligand, such as a growth factor, to the extracellular domain of the receptor. This binding causes dimerization of the receptor and activation of an intracellular tyrosine kinase by autophosphorylation. Activated tyrosine kinases catalyze the phosphorylation of tyrosine

residues in polypeptides that have downstream effects on apoptosis, cell proliferation, migration and invasion, and angiogenesis (Wilhem et al. 2006).

Many malignancies are caused by a disruption of such signaling pathways, which leads to overactivation of downstream regulatory genes. Such overactivation can, in fact, be caused by four different mechanisms: overexpression of the growth-factor receptor; overexpression of the ligand, which leads to increased activation of the receptor; mutations in the receptor tyrosine kinase that result in its constitutive activation, even in the absence of ligand binding and receptor dimerization; and lastly, the abnormal association of a cellular protein with an intracellular protein tyrosine kinase (Krause and Van Etten 2005).

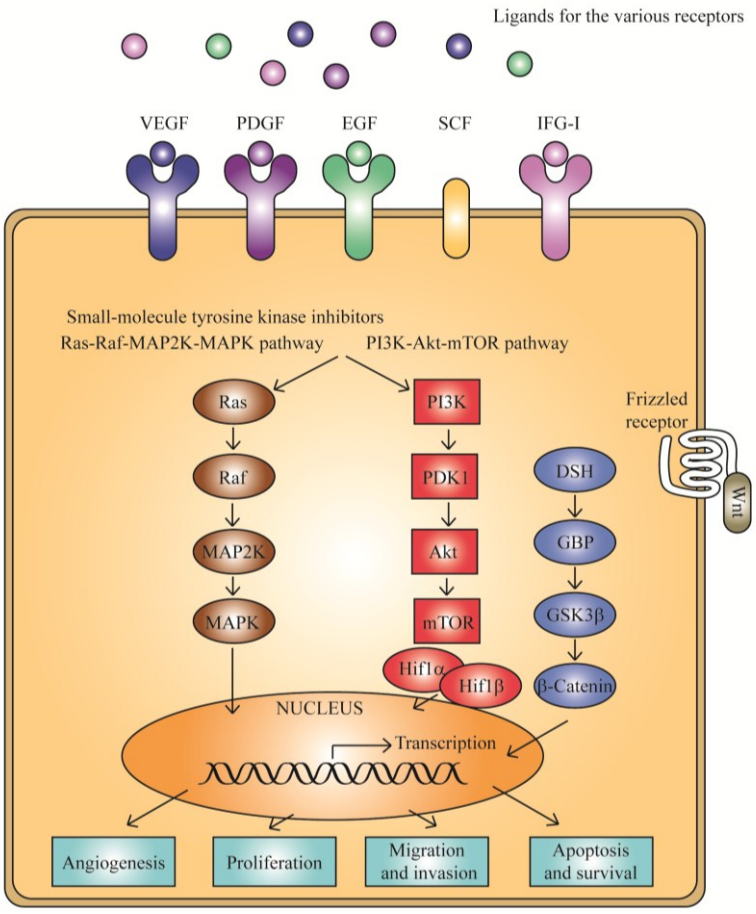


Figure 3: Signaling pathways activated during hepatocarcinogenesis. (Adaptacion from Spangenberg et al. 2009)

The key signal-transduction pathways that have been implicated in hepatocellular carcinogenesis include those mediated by VEGF (Vascular Endothelial Growth Factor), PDGFR (Platelet-derived Growth Factor Receptors), HGF (Hepatocyte Growth Factor), IGF (Insulin-like Growth Factor) and EGFR (Epidermal Growth Factor Receptor), and the ras/raf/MAP2K/MAPK, Wnt/β-catenin and PI3K/AKT/mTOR signaling pathways (Figure 3) (Llovet and Bruix 2008).

2. Epidermal Growth Factor Receptor (EGFR)

2.1 Generality

Human epidermal growth factor receptors (EGFR or HERs), also known as the ErbB family of tyrosine kinase receptors and their ligands (EGFR ligands), are important regulators of cancer cell growth, angiogenesis and metastasis.

There are four receptors in the ErbB family: EGFR (HER1 or ErbB1); HER2 (neu or ErbB2); HER3 (ErbB3); and HER4 (ErbB4). EGFR, HER2 and HER4 have a tyrosine kinase domain, while HER3 lacks the tyrosine kinase domain; thus, HER3 is only able to propagate signals in heterodimerization receptor complexes with other HER members (Kataoka 2009).

The receptors have a molecular mass between 170 and 185 kilodalton and are composed of a large extracellular ligand-binding domain, which has four subdomains (I–IV), followed by a transmembrane domain, a small intracellular juxtamembrane domain preceding the kinase domain, and a C-terminal tail, on which the docking sites for phosphotyrosine-binding effector molecules are found (Citri and Yarden 2006). The tyrosine kinase is highly conserved among the different members of the family, except for ErbB3, in which key amino acids have been substituted, rendering this receptor devoid of tyrosine kinase activity (Normanno and Gullick 2006). In contrast, the extracellular domains are less conserved among the four receptors, suggesting that they have different

specificity in ligand binding (Olayioye et al. 2000; Yarden 2001; Yarden and Sliwkowski 2001).

As shown in Figure 4, ten ligands of EGFR have been reported. These ligands are divided into three different categories. The first group includes epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin (AR) and Epigen (EPG) which bind specifically to the EGFR. The second group includes betacellulin (BTC), heparin-binding growth factor (HB-EGF) and epiregulin (EPR), which show dual specificity by binding both EGFR and ErbB-4. The third group is composed of the neuregulins (NRGs) and can be divided in two subgroups based upon their capacity to bind ErbB-3 and ErbB-4 (NRG-1 and NRG-2) or only ErbB-4 (NRG-3 and NRG-4) (Carraway et al. 1997; Chang et al. 1997; Harari et al. 1999). None of the EGF family of peptides binds ErbB-2.

Binding of ligands to the extracellular domain of ErbB receptors induces the formation of receptor homo- or heterodimers, and subsequent activation of the intrinsic tyrosine kinase domain (Figure 5) (Olayioye et al. 2000), the subsequent autophosphorylation of the EGFR createa docking sites for several signaling proteins such as Shc, Grb7, Grb2, Crk, phospholipase Cg (PLCg) the kinases Src and PI3K, the protein phosphatases SHP1 and SHP2 and the Cbl E3 ubiquitin ligase. Other signaling proteins, such as phospholipase D (PLD) and the STAT 1, 3 and 5 proteins, do not bind to the ErbB receptors through the C-terminal phosphotyrosines, but are also activated upon ligand binding. These interactions trigger intracellular signaling cascades such as the ras/raf/MEK/MAPK pathway (including the activation of ERK and JUN NH2-terminal kinase-JNK), p38 mitogen-activated protein kinase (p38-MAPK), the protein kinase C (PKC) pathway, the PI3K/AKT pathway (which can lead to NF-kB activation) and the STAT pathway (Berasain et al. 2007b). This network is further complicated by several regulatory feedback mechanisms. ErbB receptors are activated by binding to growth factors of the EGF-family that are produced by the same cells that express ErbB receptors (autocrine secretion) or by

surrounding cells (paracrine secretion) (Figure 5) (Olayioye et al. 2000; Yarden and Sliwkowski 2001; Holbro et al. 2003).

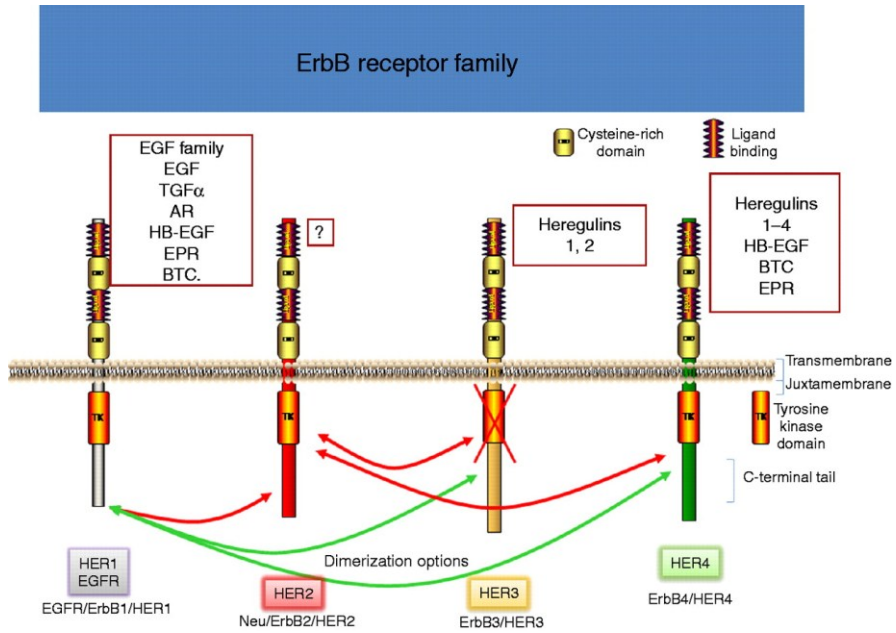


Figure 4: EGFR ligands and their binding specificities for human HER family members. (From Cooper et al. 2011)

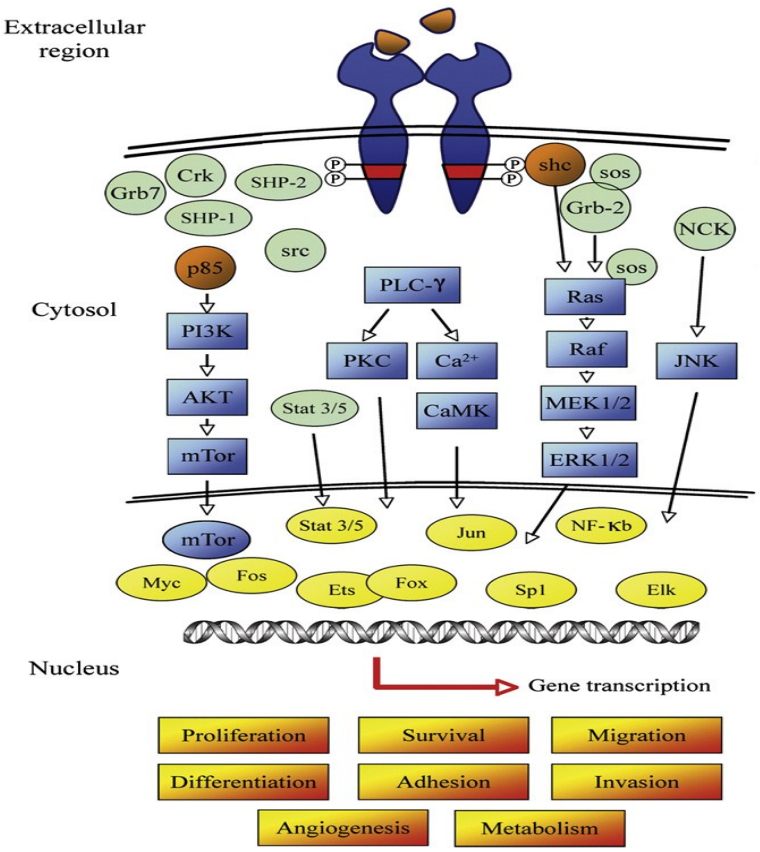


Figure 5: EGFR activation and signaling. (Adapted from Holbro et al 2003)

ErbB proteins are widely expressed in human and animal tissues, notably in cells of epithelial or neuroectodermal origin. Hepatocytes of the mature liver express the highest levels of EGFR of any non transformed cell, suggesting an important role for ErbB signaling in normal liver function (Dunn et al. 1984).

2.2 EGFR transactivation

It has been demonstrated that EGFR activation can be potentiated by the activation of other signaling pathway systems. The mechanism by which, a physiological stimulus can activate the EGFR without a direct interaction is known as EGFR transactivation.

Transactivation of the EGFR by ligands of G protein-coupled receptors (GPCRs) is perhaps the best characterized example of EGFR activation by heterologous ligands (Ohtsu et al. 2006). These include lysophosphatidic acid (LPA), angiotensin II (ANG II), endothelin-I, thrombin, IL-8, and prostaglandins such as PGE2. Different mechanisms have been proposed to mediate ADAM (A Disintegrin and Metalloproteinase) activation by GPCRs.

These mechanisms can involve the phosphorylation and activation of the ADAMs, and are were not completely know, but certainly plays an important role the PKC activation, the intracellular fluctuation of Ca^{2+} and the formation of reactive oxygen species (ROS) (Ohtsu et al. 2006).

The subsequent release of the EGFR transmembrane ligands to the extracellular medium leads to the receptor activation (Berasain et al. 2009).

In addition to GPCRs, the EGFR can be activated by proinflammatory cytokines such as interleukin-1b (IL-1b), interferon γ (INTγ) and TNFα, through a metalloproteinase-dependent release of EGFR ligands (Berasain et al. 2007b; Berasain et al 2009; Pastore et al. 2008). TGF-β1 induces a rapid secretion of a member of the EGF family of growth factors by activation of the metalloprotease TACE/ADAM 17 in normal hepatocyte

(Murillo et al. 2005). Even the TLRs that are classically activated by pathogen-derived ligands and are primarily associated with the innate immune response (chemokine and cytokine release), can also be activated as a result of release of malignant cell contents (such as the hot shock protein 70 or HSP70), which occurs frequently in progressing cancer cells and can activate the ADAMs proteins or the SRC kinase (Shepard et al. 2008).

2.3 Ligands of the EGF family

EGFR signaling is stimulated by members of the EGF family of peptide growth factors, whose roles in stimulating ErbB receptor signaling and coupling to biological responses have been intensively studied (Hynes et al. 2005; Wieduwilt et al. 2008; Riese and Sterne 1998; Wilson et al. 2009).

EGFR agonists include the epidermal growth factor (EGF), transforming growth factor-α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR), and epigen (EPG) (Harris et al. 2003). These agonists are expressed as integral membrane proteins and are cleaved by ectodomain (proteolytic) shedding such as metalloproteinases to release soluble, mature ligands. These metalloproteinases are typically members of the ADAM (a disintegrin and metalloproteinase) family of membraneous proteases (Foley et al. 2010). This process regulates, for example, bioavailability (and therefore bioactivity) of the ligands and signal duration (Massague and Pandiella 1993).

All the ligands are typically ~55 amino acids in length (Stein and Staros, 2000) and share an overall structural homology consisting of a hydrophilic N-terminus (ectodomain) containing the EGF-domain, a hydrophobic transmembrane region, a hydrophilic cytoplasmic C-terminus called cytoplasmatic tail, and additional motifs like an immunoglobulin-like domain, heparin-binding sites and glycosylation sites (Berasain et al. 2007a) (Figure 6).

EGF is the only one that has nine EGF domains, but only the one adjacent to the membrane binds the EGFR. The EGF domain is characterized by a consensus sequence consisting of six spatially conserved cysteine residues (CX7 CX4–5 CX10–13 CXCX8 C) that form three intramolecular disulfide bonds that form a motif crucial for binding members of the HER receptor tyrosine kinase family (Harris et al. 2003).

Among these ligands and receptors, TGF-α, AR, HB-EGF, EGFR (HER1) and HER2 play crucial roles in cancer cell growth, particularly in stomach, colon, breast and lung cancers, as well as melanoma (Miyamoto et al. 2006). No ligands bind to HER2; thus, HER2 has been reported to be an orphan receptor and is the preferred co-receptor for dimer formation with EGFR, HER3 or HER4, and heterodimers consisting of HER2 and other receptors have a greater capacity for translating cell growth signals than homodimers, and act synergistically to promote cellular transformation (Graus-Porta et al. 1997). These ten EGF ligands bind to EGFR, HER3 and HER4, leading to the formation of heterodimers or homodimers, which triggers downstream signal transduction via Ras/ERK, PI3K/AKT or STAT (Ono et al. 2006).

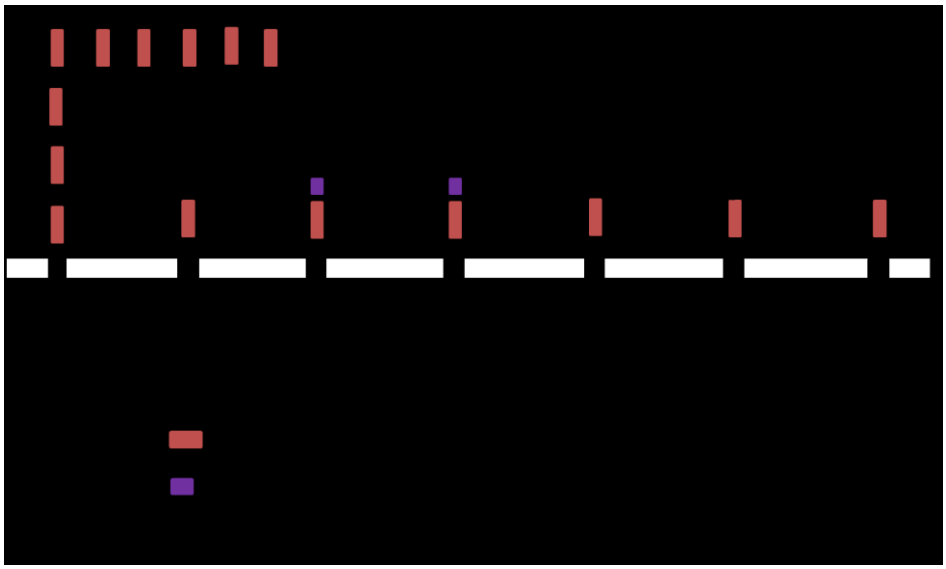


Figure 6: EGF family ligands. (Adaptacion of Harris et al. 2003)

2.4 Amphiregulin

2.4.1 Generality

Amphiregulin (AR) was the third member of the epidermal growth factor (EGF) family discovered. It was isolated from the conditioned media of phorbol ester-12-myristate 13-acetate (PMA) stimulated MCF-7 human breast carcinoma cell (Shoyab et al 1988). AR was initially described as a bifunctional regulator of cell growth; it was originally identified as a factor capable of inhibiting the growth of certain carcinoma cell lines, while stimulating the proliferation of normal cells such as fibroblasts and keratinocytes. Actually, depending on its concentration and the nature of the target cell AR promotes the growth of most cell types, including normal and transformed cells (Berasain et al. 2007a).

The human AR gene (geneID 374) spans about 10Kb of genomic DNA and is located on the p13-q21 regions of chromosome 4. It is composed of six exons, encoding a 1.4kb pre-protein mRNA transcript. The exons vary in size from 112 to 270bp and are interrupted by introns ranging from 1.25 to 2.1kb (Figure 7) (Plowman et al. 1990).

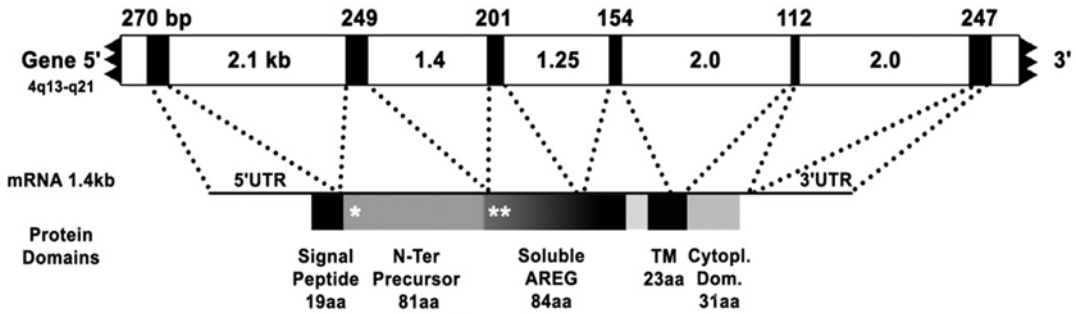


Figure 7: Human AR gene showing the exon organization and protein domains. (Plowman et al. 1990).

The AR protein is synthesized as a 252 amino acid transmembrane glycoprotein, also known as transmembrane precursor or pro-form (pro-AR) (Figure 8). Sequence analysis revealed that AR contains an N-terminal domain of six spatially conserved cysteines and

several other semi-conserved residues that form disulfide bridges giving rise to a three-looped structure, which is involved in binding to the EGFR (Plowman et al. 1990). Besides EGF, this so called EGF domain is shared by other members of the EGF family (Tzhar and Yarden 1998; Harris et al. 2003; Sanderson et al. 2006; Lee et al. 2003c). Outside this EGF-like region these precursors show little sequence homology, yet AR also has an N-terminal extension of 43 amino acids containing glycosylation sites and composed mainly of very basic, hydrophilic residues that are thought to confer heparin-binding properties, and a similar heparin-binding domain is also present in HB-EGF (Higashiyama et al. 1992). Interestingly, the N-terminal extension of pro-AR also contains two consecutive Lys-Arg-Arg-Arg motifs resembling nuclear targeting signals, which may account for its reported association with the nucleus (Modrell et al. 1992). AR precursor (pro-AR), like other growth factors members of the EGF family, share an overall structural homology consisting of a hydrophilic N-terminus (ectodomain) containing the EGF-domain, a hydrophobic transmembrane region and a hydrophilic cytoplasmic C-terminus (cytoplasmatic tail) (Figure 8) (Berasain et al. 2007a). The cytoplasmatic tail of HB-EGF can traslocate from the cell surface to the inner nuclear membrane (INM) where it promotes cell cycle progression (Higashiyana et al. 1991). Recent work shows that the cytoplasmatic tail of AR participates in the regulation of autocrine growth and differentiation of keratinocytes (Stoll et al. 2010). Other works have demonstrated that the proAR translocation into the INM and interaction with lamin A, results in the induction of heterochromatinization and global transcriptional suppression (Isokane et al. 2008).

At the plasma membrane AR precursor is subjected to sequential proteolitic cleavage to release the mature soluble factor in a process known as ‘‘ectodomain shedding’’. Depending on the cell type and microenvironment, AR can be produced in multiple cellular and mature forms using alternative pro-AR cleavage sites and glycosylation motifs, thus impacting the biological activity of AR (Busser et al. 2011). Pro-AR cleavage at two N-terminal sites six amino acids apart gives rise to two major soluble forms of 78 and 84

amino acids (of ~19 and ~21kDa), alternatively pro-AR cleavage can produce a larger 43kDa soluble protein corresponding to the entire extracellular domain. Additionally a 9kDa soluble form composed almost exclusively of the EGF-like domain has been also observed (Brown et al. 1998, Shoyab et al. 1988) (Figure 9).

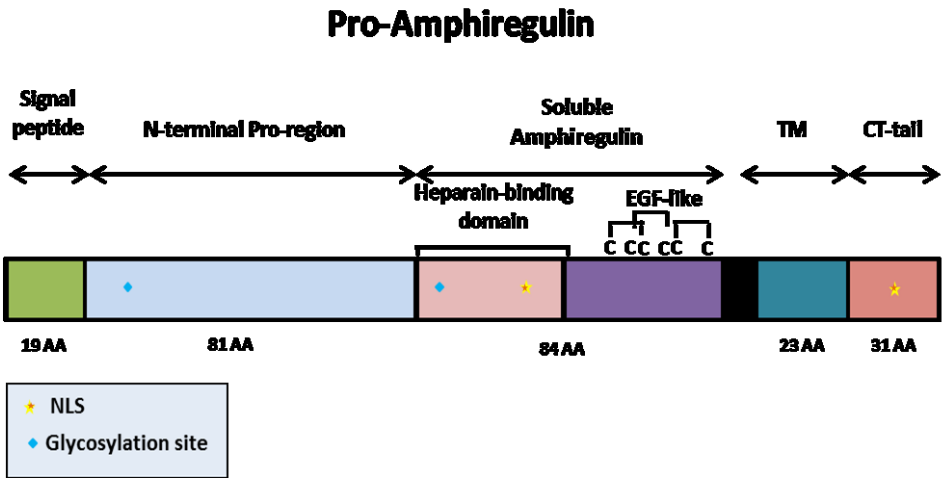


Figure 8: Structure and cellular processing of pro-amphiregulin. (Adaptation of Berasain et al. 2007a)

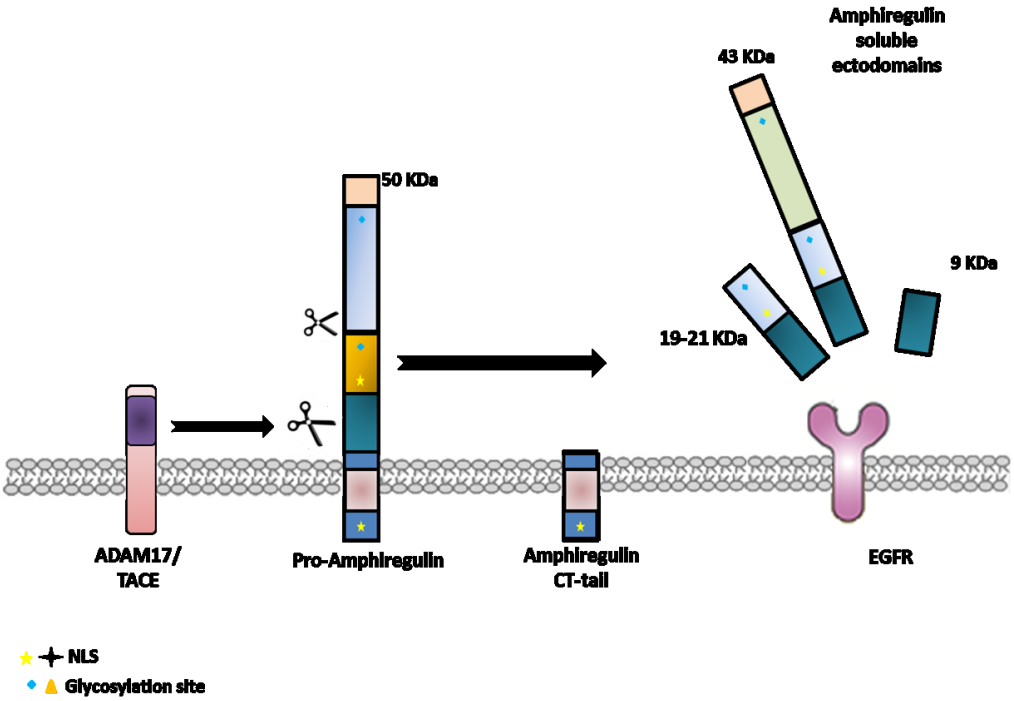


Figure 9: Proteolytic pro-AR processing at the cell surface. (Adaptation of Berasain etal. 2007a)

The proteolytic reaction is mediated mainly by membrane-anchored metalloproteases of the ADAM family (a disintegrin and metalloprotease) (Blobel 2005; Ohtsu et al. 2006). Various members of the ADAM family have been implicated in EGFR ligand cleavage, including ADAM 9, 10, 12, 15, 17, and 19 (Ohtsu et al. 2006). However, ADAM17 (ADAM metallopeptidase domain 17), which is also known as tumour necrosis factor-α (TNF-α)-converting enzyme, or TACE, together with ADAM10, are thought to play a central role (Blobel 2005). ADAM17 can cleave the AR, EREG, TGF-α, and HB-EGF membrane anchored precursors, while ADAM 10 is a key sheddase for EGF, BTC, and can also cleave the HB-HGF transmembrane precursor (Berasain et al. 2009).

The proteolytic activity of ADAMs is therefore crucial for the generation of soluble EGFR ligands and receptor activation. Importantly, the proteolytic activity of ADAMs is in turn subject to regulation by multiple upstream signals, which adds another layer of complexity to the system. In fact, there is a growing list of physiological stimuli that can trigger EGFR signaling through the stimulation of ligand shedding, a process known as EGFR transactivation (Ohtsu et al. 2006; Fisher et al. 2003). Transactivation of the EGFR by ligands of G protein-coupled receptors (GPCRs) is perhaps the best characterized example of EGFR activation by heterologous ligands (Berasain et al. 2009).

2.4.2 Functions of the Amphiregulin

As previously mentioned, historically, this growth factor was named Amphiregulin because it can either induce proliferation and differentiation of fibroblasts in culture or can inhibit the growth of normal epithelial cells and aggressive carcinoma cell lines (Shoyab et al. 1988). In humans AR is constitutive expressed in the normal ovary, testis, and breast tissue, but can also be detected in the placenta, pancreas, heart, colon, lungs, spleen, and kidneys and may be involved in early development or in processes as diverse as gonadogenesis, hematopoiesis, and tissue modeling and repair (Plowman et al.1990).

The physiological role of AR seems to be related to the development of the mammary epithelium (Luetteke et al. 1999; Sternlicht et al. 2005) and with a mitogenic and hepatoprotective effects (Berasain et al. 2005a; Berasain et al. 2005b).

On one side the expression of AR is induced in chronic experimental liver damage and in human liver cirrhosis and is readily detected in rodent liver after partial hepatectomy (Berasain et al. 2005a), participating in the proliferative responses of the hepatocyte. On the other hand, the AR has a hepatoprotective function in different mouse models of acute injury, such as carbon tetrachloride (CCl_4) administration or Fas receptor activation (Berasain et al. 2005b).

The role played by AR in hepatocyte proliferation and cytoprotection seems to be unique because studies in AR knockout mice showed that the effects exerted by AR on liver parenchymal cells could not be compensated by other EGFR ligands, such as TGF-α and HB-EGF, also induced during liver (Berasain et al. 2005b; Berasain et al. 2005a).

2.4.3 AR and EGFR system in the HCC

The EGFR system is considered as an important defense mechanism for the liver during acute tissue injury, however, its chronic stimulation can participate in the neoplastic conversion of the liver (Berasain et al. 2007b). The receptors EGFR/ErbB-1 (Nakopoulou et al. 1994; Kira et al. 1997; Ito et al. 2001b), neu/Her2/ ErbB-2 (Nakopoulou et al. 1994; Ito et al. 2001b), Her3/ErbB-3 (Ito et al. 2001b) and Her4/ErbB-4 (Ito et al. 2001b) are expressed in HCCs; however, staining frequency and intensity of the tumor tissues in comparison to the surrounding non-tumorous liver tissues differ between these studies. It is noteworthy that in contrast to other carcinomas (e.g. breast cancer), amplification of the ErbB2 gene is considered uncommon in human HCCs (Xian et al. 2005).

Overexpression of AR, TGF-α, BTC and HB-EGF has been observed in liver cirrhosis and hepatocellular carcinoma tissues, human HCC cell lines and in experimental models of

acute and chronic liver injury. These factors together with ADAM17 overexpression are believed to contribute to EGFR activation during hepatocarcinogenesis (Berasain et al. 2007b; Berasain et al. 2005a; Schiffer et al. 2005). These findings suggest an enhanced availability of soluble AR from the early stages of hepatocarcinogenesis (Castillo et al. 2006). Interestingly, were observed that AR was able to stimulate its own gene expression in hepatocellular carcinoma cells through the activation of the EGFR, suggesting the existence of a positive feedback loop for AR production (Castillo et al. 2006). A similar response has been reported in other tumor cell types, such as colon cancer cells, where AR expression is induced by the EGFR ligand TGF-α in conjunction with the cyclooxygenase-2-derived prostaglandin E2 (Shao et al. 2003). AR was constitutively expressed and released to the CM of hepatocellular carcinoma cells, on which it exerted promitogenic and antiapoptotic effects through the activation of the EGFR (Castillo et al. 2006).

Depending on the cellular context, AR can promote such diverse effects as self-sufficiency in growth signals, tissue invasion, and evasion of apoptosis, processes all involved in tumor development and progression (Hanahan and Weinberg 2011). AR gene overexpression has been demonstrated in a wide variety of human cancer tissues. However, the genetic or epigenetic alterations responsible for this overexpression remain unknown despite numerous identified stimuli.

AR stimulates cell growth and the apoptosis resistance as well as allowing the cancer cells growth under non adhesive conditions. All these characteristics were lost when gene AR expression was silencied in a specific manner (Castillo et al. 2006). Stably transfected HCC cell clones that overexpress AR show an increase in growth rate, greater capacity to growth in absence of substrate and increase aggressiveness and tumorigenic potential when injected in nude mice (Castillo et al. 2006).

Crosstalk between the Insuline-like growth factor (IGF) pathway and ErbB1 has also been demonstrated in various cellular backgrounds by showing that activation of IGF-1 receptor (IGF1R) leads to the shedding of different ErbB1 ligands including AR, TGFa and

HB-EGF (Adams et al. 2004, Berasain et al. 2007b) and the proliferative effect of IGF-2 in human HCC cell lines requires the EGFR activation through the autocrine/paracrine liberation of the AR (Desbois-Mouthon et al. 2006).

Recent evidence indicates that cyclooxygenase-2 (COX-2) and EGFR are involved in hepatocarcinogenesis. The transactivation of the EGFR was induced by the prostaglandin E2 (PGE2) in human HCC cell line through the activation of the tyrosin kinase c-Src (Han et al. 2006). It has also been demonstrated that PGE2 induces the expression of AR in *in vitro* cultures of hepatocytes (Berasain et al. 2005a), while EGF can induce COX-2 expression in human HCC cells (Han et al. 2006).

3. β-catenin

3.1 Generality

β-catenin is an archetypal member of the armadillo repeat protein family, which includes α-catenin, β-catenin and γ-catenin. β-catenin was the first identified and it plays an integral role in establishing adherents junctions by directly interacting with cadherin (McCrea et al. 1991). It is also a critical transcriptional coactivator in the canonical Wnt signaling pathway that controls cell fate and proliferation when it forms a complex with members of Tcf/LEF-1 transcription factors in the nucleus (Graham et al. 2000).

As β-catenin is involved in various protein–protein interactions that are crucial for embryogenesis, development and tumorigenesis, its protein levels are tightly regulated by a dedicated degradation mechanism (Angers and Moon 2009). In the cadherin–catenin cell adhesion complex, β-catenin can simultaneously interact with cadherin, a cell adhesion molecule, and α-catenin, an actin-binding protein. The formation of a cadherin/β-catenin/α-catenin ternary complex is essential for linking cadherin-mediated cell–cell adhesion with actin dynamics (Meng and Takeichi 2009).

The human β-catenin gene (CTNNB1) is located on chromosome 3p21. The size of the gene is 23.2kb, containing 16 exons (Nollet et al. 1996). The primary sequence of β-catenin is highly conserved from insects to humans, and its critical biological role in vertebrates is especially highlighted by strict conservation (>95% sequence identity) of a 781 residue sequence. The molecular weight of the β-catenin protein is of 92KDa. The overall structure of β-catenin consists of three distinct domains, an N-terminal tail of approximately 130 amino acids containing the α-catenin-binding site, a central arm domain of 550 amino acids that binds to the cytoplasmic region of cadherin and a C-terminal tail of 100 amino acids (Figure 10) (Shapiro and Weis 2009; Akiyama 2000; Nuse and Willert 1998). The N-terminal region contains consensus phosphorylation sites for GSK3β, while the C-terminal region possesses the transactivator function required for activation of target genes. The central region contains 12 imperfect sequence repeats of 42 amino acids known as armadillo repeats, which are sequentially packed together through hydrophobic interfaces into a superhelical structure (Huber et al. 1997) (Figure 10). The individual armadillo repeats consist of three α-helices that are connected by short loops and which are required for the interaction with various proteins, including cadherins, APC and TCF/LEF (Akiyama 2000).

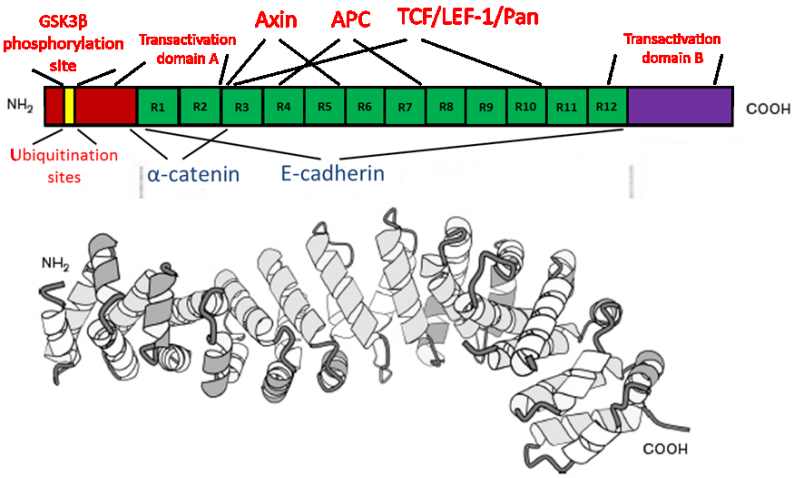


Figure 10: Functional and structural domains of β-catenine. (Adapted from Nusse and Willert 1998)

3.2 The Wnt/β-catenin signaling

The term ‘‘Wnt’’ was introduced 20 years ago fusing the names of two orthologous genes: Wingless (Wg), a Drosophila segment polarity gene, and Int-1, a mouse protooncogene (Giles et al. 2006). Wnt signaling is involved in various aspects of embryogenesis and development, including cell proliferation, differentiation, migration, cell polarity, cell-to-cell communication, and survival (Li et al. 2006; Moon et al. 1997; Miller et al. 1999). As a result, mutations in the Wnt pathway are often linked to human birth defects, cancer and other diseases (Clevers 2006).

The 19 proteins of the Wnt family are small, secreted glycoproteins that are highly conserved in a wide variety of organisms, from “Caenorhabditis elegans” to humans, suggesting a critical role for Wnt signaling in cell fate (Miller et al. 1999, Moon et al. 2002, Schneider et al. 2003). Wnt proteins signal through two types of receptors: seven-pass transmembrane receptors known as Frizzled proteins (Fz), and the single-pass transmembrane co-receptors lipoprotein- receptor related proteins (LRPs) (Huang et al. 2004).

Wnt ligands are classified into two distinct groups based on the subcellular signaling elements. The first group, referred to as the Wnt1 group, predominantly uses the “canonical” pathway for intracellular signaling and includes Wnt1, Wnt2a, Wnt3a, and Wnt8 (Figure 11). The second group, the Wnt5a group, includes Wnt4, Wnt5a, and Wnt11 and uses a very different subcellular signaling network, the “non canonical” pathway (Figure 12).

In the canonical pathway, in the absence of Wnt proteins, cytoplasmic β-catenin protein is constantly degraded by the action of the Axin complex, which is composed of the scaffolding protein Axin, the tumor suppressor *adenomatous polyposis coli* gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3β). CK1 and GSK3β sequentially phosphorylate the amino terminal region of β-catenin (the first in the serine

45 and the second in the threonine 41 and serine 37 and 33), resulting in β-catenin recognition by β-Trcp, an E3 ubiquitin ligase subunit, and subsequent β-catenin ubiquitination and proteasomal degradation (He et al. 2004). This continual elimination of β-catenin prevents β-catenin from reaching the nucleus, and Wnt target genes are thereby repressed by the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins. The Wnt/β-catenin pathway is activated when a Wnt ligand, such as Wnt1, Wnt2, Wnt3, Wnt3a, and Wnt8, binds to a seven-pass transmembrane Frizzled (Fz) receptor and its co-receptor, low-density lipoprotein receptor related protein 6 (LRP6) or its close relative LRP5. The formation of a likely Wnt-Fz-LRP6 complex together with the recruitment of the scaffolding protein Dishevelled (Dsh) results in LRP6 phosphorylation and activation and the recruitment of the Axin complex to the receptors. These events lead to inhibition of Axin-mediated β-catenin phosphorylation and thereby to the stabilization of β-catenin, which accumulates and travels to the nucleus to form complexes with TCF/LEF and activates Wnt target gene expression (Figure 11) (Emami et Corey 2007). These Wnt/β-catenin targets include gene products responsible for proliferation (MYC, MYB, C-JUN, Cyclin D1), antiapoptosis (survivin), invasion (matrix metalloproteinases (MMPs) and angiogenesis (VEGF). Thus, the activation of Wnt targets not only regulates tissue development and regeneration but it can also influence tumor growth and progression in the case of aberrant nuclear accumulation of β-catenin (Breuhahn et al. 2006).

In the ‘‘non-canonical’’ Wnt pathway, Wnts proteins such as Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, and Wnt11 bind to FZ, but transduction of the signal does not require β-catenin (Kuhl et al. 2000). The activation of the FZ receptor and co receptors such as Ror2 and Ryk and transduction proceeds through diverse mechanisms dependent on either the Dishevelled (Dsh) downstream effector or Ca^{2+}. Dsh can be activated by FZ directly or through the induction of heterodimeric G-proteins. Dsh has been shown to activate the small GTPase RhoA and its effector ROK (Rho-associated kinase) in order to regulate actin

cytoskeleton. Another downstream effector of Dsh is JNK which can be activated by RhoA to regulate PCP (planar cell polarity) during eye development. Signal transduction through Ca^{2+} activates nemo-like kinase (NLK) and the nuclear factor of activated T cells (NFAT) with NLK inhibiting canonical Wnt signalling through the phosphorylation of TCF/LEF (Veeman et al. 2003a; Veeman et al. 2003b) (Figure 12).

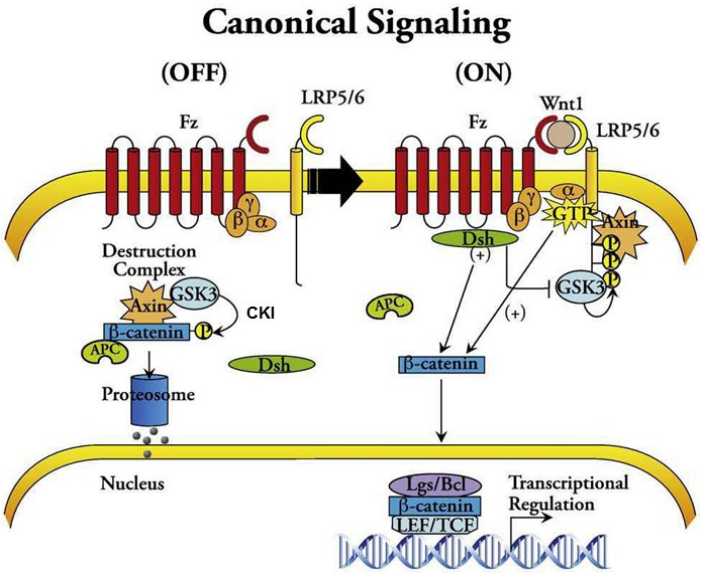


Figure 11: Wnt/β-catenin signaling. (From Flaherty and Dawn 2008)

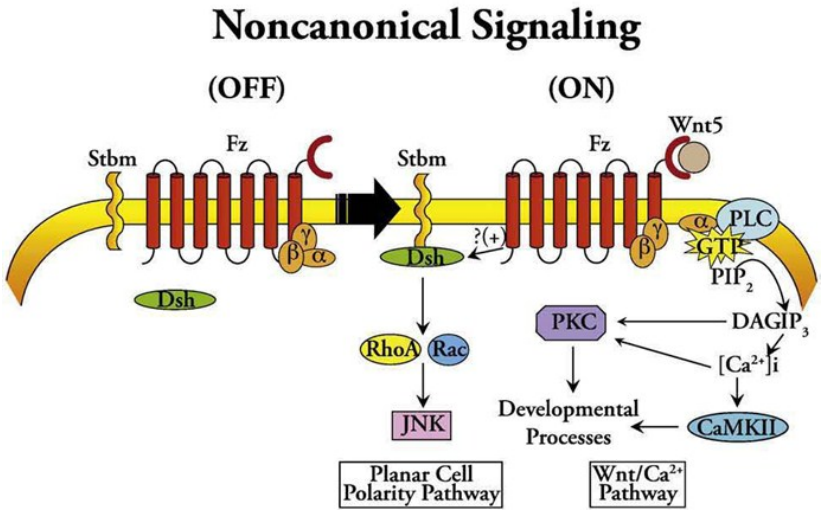


Figure 12: non canonical Wnt signaling. (From Flaherty and Dawn 2009)

Several extracellular secreted factors regulate Wnt signaling. Secreted Frizzled-related proteins (SFRP), Wnt inhibitory factor-1 (WIF-1) and Cerberus sequester Wnts and prevent their association with the FZ receptors (Suzuki et al. 2005) and therefore inhibit the canonical and non-canonical Wnt pathways (Logan amd Nusse 2004). Dickkopf proteins (DKKs), which belong to a multigene family, are another type of inhibitor of Wnt pathways (Zorn et al. 2001). DKKs inhibit Wnt signaling by binding to and antagonizing LRP5/6 receptors and promoting their internalization to lysosomes (Levasseur et al. 2005; Davidson et al. 2002).

3.3 Wnt/β-catenin signaling in HCC

Mutations affecting the Wnt/β-catenin pathway appear to be the most frequent genetic event in human HCC (Laurent-Puig and Zucman-Rossi 2006). Interestingly, recent data showed that the Wnt/β-catenin signaling pathway exerts a critical role in many aspects of liver development and physiology (Ober et al. 2006; Tan et al. 2008; Decaens et al. 2008; Benhamouche et al. 2006). It is tightly controlled by multiple factors, and understanding such regulation opens a unique field for the development of targeted treatments.

Various molecular and genetic factors participate to such aberrant activation of the Wnt/β-catenin pathway. Firstly, mutations are frequently identified in genes encoding for the main actors of the pathway. Gain-of-function mutations of *CTNNB1* (encoding for β-catenin) are encountered in about one third of HCCs (de La Coste et al. 1998) and define *CTNNB1* as the most frequently mutated gene in HCCs. Conversely, loss-of-function mutations of negative regulators of the pathway are also observed, namely mutations of the *AXIN1* and *AXIN2* genes (<5%) and of the *APC* gene (exceptional) (Laurent-Puig and Zucman-Rossi 2006). The activation of the Wnt/β-catenin signalling pathway is mainly attributed to the mutations of the APC gene in colon cancer (Cottrell et al. 1992). However, in human HCC, APC mutations are rare; instead, promoter methylation plays a major role

in APC inactivation (Lee et al. 2003b; Yang et al. 2003). Beside such mutational events, the Wnt/β-catenin pathway can be also activated in HCCs as a consequence of a deregulated dialogue between the tumor cells and their microenvironment. For instance, an autocrine Wnt stimulation loop can be established following epigenetic events that change the expression profiles of the ligands, the extracellular inhibitors and the receptors of the Wnt family (Bengochea et al. 2008). A modified crosstalk with other signaling pathways such as HGF or TGF-β can also account for the aberrant activation of the Wnt/β-catenin pathway in HCCs (Hoshida et al. 2009; Monga et al. 2002).

Mutations in each of the components of the complex may cause abnormal cytosolic stabilization of β-catenin, mutations of the β-catenin gene itself appear to be the most common cause for stabilization in pathological situations. These mutations usually affect residues at position 33 (Ser), 37 (Ser), 41 (Thr) or 45 (Ser) located in exon 3 of the human β-catenin gene that are the targets of priming phosphorylation by CKIα (Ser45) or subsequent phosphorylation by GSK3β (Ser33, Ser37 and Ser41) (De La Coste et al. 1998; Hsu et al. 2000; Satoh et al. 2000; Wong et al. 2001). Moreover, β-catenin mutations and exon 3 deletions have been identified in 48% of sporadic hepatoblastomas, a type of childhood malignant liver tumor (Koch et al. 1999). The above results strongly suggest that the APC/β-catenin pathway is an important player in liver tumorigenesis.

Activation of β-catenin finally leads to the transcriptional activation of a variety of genes (Lee et al. 2007). However, not much is known about the role of specific genes activated with regard to their role in tumor development. On the other hand, glutamine synthetase (GS), one of the enzymes identified to be regulated by nuclear β-catenin, may be a candidate that contributes to enhanced malignancy of HCCs (Cadoret et al. 2002; Loeppen et al. 2002). In fact, Osada *et al*. demonstrated that GS expression may enhance the metastatic potential in HCC, and that GS immunostaining identifies HCC patients with a high risk for disease recurrence after curative hepatectomy (Osada et al. 2000). In the

intact liver, GS is confined to a small population of hepatocytes located around the hepatic terminal venules and is regulated in a highly complex manner (Gebhardt et al. 2007).

Deregulated expression of β-catenin, which may result from APC defects, activating mutations in the β-catenin gene itself, or other alterations in the Wnt pathway, has been implicated as an important step in carcinogenesis. β-catenin immunostaining showed increased membranous and cytoplasmic expression in most HCCs by comparison with non tumoral hepatocytes (Ihara et al. 1996). More importantly, abnormal nuclear localization of β-catenin was revealed in a 43% of HCCs. There is a strong correlation between nuclear β-catenin staining and somatic mutations of the β-catenin gene in HCC, indicating that activation of the Wnt/β-catenin pathway in HCC occurs predominantly through activating mutations in the β-catenin gene itself. It differs from colorectal cancers, in which APC mutations are responsible for β-catenin stabilization in 80% of the cases (Rubinfeld et al. 1996), and from melanoma or ovarian and uterine carcinomas, in which very occasional mutations can be found despite frequent β-catenin overexpression (Palacios et al. 1998; Fukuchi et al. 1998; Rimm et al. 1999).

β-catenin mutations and interstitial deletions have been reported in both primary HCC and hepatoma cell lines (Miyoshi et al. 1998; De La Coste et al. 1998). Once again, the alterations affect putative phosphorylation residues in the regulatory site, but Ser37 was rarely mutated in HCC. Fifty percent of HCCs that develop in transgenic mice expressing c-myc or H-ras in the liver contain β-catenin mutations, suggesting that β-catenin activation can cooperate with ras or myc in HCC progression (De La Coste et al. 1998). Previously it has been shown that β-catenin gene mutation and nuclear accumulation of the protein in HCCs ranged from 13 to 34% and from 11 to 43% respectively (De La Coste et al. 1998; Hsu et al. 2000; Taniguchi et al. 2002; Wong et al. 2001). HCV-associated HCCs tend to have higher frequencies of both β-catenin mutations and nuclear accumulation than HBV-associated ones. In addition, mutation of Axin1 has been found in approximately 5–10% of human HCCs. Axin1 mutations including point mutations and small deletion are more

frequently observed at the N-terminal half of the protein, which might stabilize β-catenin by impeding the formation of the APC/GSK3β/β-catenin complex (Satoh et al. 2000; Taniguchi et al. 2002). Besides Axin and β-catenin mutations, inappropriate activation of the Wnt/β-catenin signalling pathway can be brought about when the upstream mediators are dysregulated. Overexpression of the Wnt ligand and the Frizzled receptor has been demonstrated in human HCC. On the other hand, the Wnt antagonist, sFRP1 has been found to be epigenetically silenced in human cancers including HCC (Suzuki et al. 2002). Likewise, a recent study has shown that Dsh-1 and Dsh-3 are overexpressed in human HCC (Chan et al. 2006). Overexpression of Dsh is associated with β-catenin accumulation and Wnt/β-catenin signalling activation (Uematsu et al. 2003a; Uematsu et al. 2003b). In addition, it has been recently demonstrated that two Dsh inhibitors, HDPR1 and Prickle-1, are also frequently underexpressed in human HCC (Chan et al. 2006; Yau et al. 2005). PIN1 is another Wnt signalling regulator found to be dysregulated in HCC. Overexpression of PIN1 stabilizes β-catenin by inhibiting its interaction with APC (Ryo et al. 2001). Interestingly, overexpression of PIN1 and mutation of β-catenin appear to be mutually exclusive events in Wnt signalling activation in HCC (Pang et al. 2004). Apart from the above-mentioned genes, other members of the Wnt/β-catenin signalling pathway, such as LPR5/6 and their antagonists DKK1/3, are also subjects of investigation (Niehrs 2006). It would not be surprising that more Wnt/β-catenin regulators would be identified and implicated in carcinogenesis (Liao and Lo 2008).

4. Crosstalk between Wnt/β-catenin and EGFR system in cancer.

Cancer development is a complex progress in which many signaling pathways are involved. Cross-communication between different pathways allows the integration of the great diversity of stimuli. Wnt and EGFR pathways been reported to closely interact in

tumorigenesis, but how they crosstalk and co-activate tumor progression remains an unanswered, interesting topic.

Crosstalk between Wnt and EGFR has been identified in some tumors. In breast cancers, Wnt overexpression activates signaling via EGFR (Faivre and Lauge 2007; Musgrove 2004). In HC11 mammary epithelial cells, constitutive expression of Wnt1 and Wnt5a accompanies activation of EGFR and MAPK. Inhibition of EGFR kinase activity and addition of sFRP1 both prevent this effect. TGFα and other EGFR ligands are not induced by Wnt-1 or Wnt-5a, but addition of metalloproteinase inhibitors blocks the stimulation of EGFR and ERK phosphorylation. Thus, Wnt activation of EGFR is apparently mediated by an increase in the availability of EGFR ligands (Civenni et al. 2003). Further studies showed that in breast cancers, Wnt1 transactivates EGFR, implying that constitutive Wnt signaling might impact not only the canonical pathway but also EGFR activity by augmenting ligand availability (Schlange et al. 2007). In liver-specific non-mutated β-catenin-overexpressing transgenic mice, EGFR seems to be a direct target of the activated Wnt signaling pathway, and EGFR activation might contribute to some mitogenic effect of increased β-catenin in the liver (Tan et al. 2005). In NSCLC, there is a positive correlation between activated EGFR mutation and nuclear accumulation of β-catenin (Suzuki et al. 2007). All of these results suggest a close correlation between Wnt and EGFR signal pathways in cancers.

Many studies indicate that Wnt and EGFR signaling crosstalk via receptor tyrosine kinase pathways. EGFR mediated PI3K/AKT activation promotes β-catenin transactivation and tumor cell invasion, suggesting that EGFR activation transactivate β-catenin activity via receptor tyrosine kinase pathways in tumor cells (Lu et al. 2003; Ji et al. 2009; Sharma et al. 2002; Agarwal et al. 2005). In breast cancers, upregulation of Wnt-1 induces EGFR and Erk1/2 MAPK activation (Faivre et al. 2007). In APC deficient mice, Wnt activity causes EGFR/PI3K/AKT activation (Moran et al. 2004).

The crosstalks between Wnt and EGFR are summarized in Figure 13. Both Wnt and EGFR signaling are closely related with tumorigenesis. In recent years a considerable body of evidence shows that Wnt and EGFR crosstalk with each other in cancer development. Addition of Wnt ligands transactivates EGFR signaling, possibly through Frizzled and its downstream partners. EGFR can form a complex with β-catenin and further activate Wnt pathway. In cancers, mutations or dysregulation in the Wnt pathway often induce EGFR activation. This review also points out several possible convergence points between Wnt and EGFR signaling, such as Frizzled, β-catenin and NKD2. Tight regulation of those proteins maintains the homeostasis and prevents from tumorigenesis. Further studies will surely disclose more convergence points between Wnt and EGFR signaling. Mutations in key proteins of Wnt and EGFR pathways have been found in most of the cancers. 80% of colon cancers have APC mutations (Powel et al. 1992) and 50-70% of breast, colon and lung cancers have EGFR and ErbB3 mutations (Normanno et al. 2003). However, what percentage of conincidence of mutations in both EGFR and Wnt pathways in those patients, remain a very important and interesting topic.

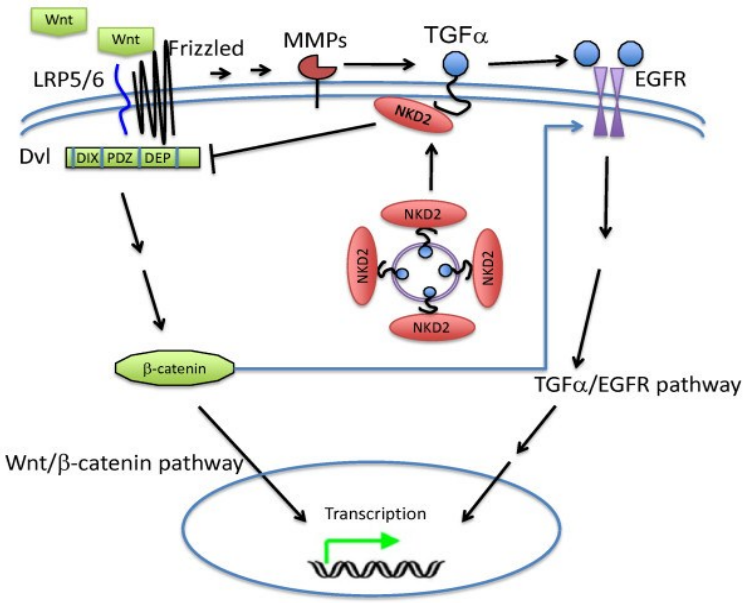


Figure 13: Convergence between Wnt and EGFR pathways. (From Hu and Li 2010)

5. Fibroblast growth factor (FGF)/FGF receptor (FGFR) system

5.1 Generality

The fibroblast growth factor (FGF) family consists of 22 structurally related proteins (FGF1 to FGF14, FGF16 to FGF23) that are involved in various biological processes including embryonic development, wound healing, angiogenesis and metabolic signaling (Ornitz and Itoh 2001; Itoh and Ornitz 2004; Popovici et al. 2005; Beenken and Mohammadi 2009). Many of the FGFs are secreted proteins involved in paracrine cell signaling. Because these FGFs display high affinity for heparin sulfate proteoglycans in the extracellular matrix of tissues, their signaling tends to remain spatially near their site of release.

Most of these signaling molecules bind to and activate members of the FGF receptor (FGFR) family. The FGFR family is composed of four receptor tyrosine kinase designated FGFR1-4 and one receptor which lacks a cytoplasmatic tyrosine kinase receptors domain, designated FGFR5. Within the FGFR family, FGFR4 is the dominant isoform expressed in mature hepatocytes and is expressed at higher levels in the liver compared to other organs (Lin and Desnoyers 2012).

FGF signaling is activated by a ligand-receptor interaction that results in the autophosphorylation of tyrosine residues in the intracellular region of an FGFR. The signal is further relayed through four distinct pathways: the Janus kinase/signal transducer and activator of transcription (Jak/Stat) (Hart et al. 2000; Deo et al. 2002; Udayakumar et al. 2002), phosphoinositide phospholipase Cγ (PLCγ) (Burgess et al 1990; Mohammadi et al 1991), PI3K (Hart et al. 2001) and Erk pathways (Hadari et al. 2001; Dailey et al. 2005) (Figure 14). The formation of a complex between FGFR, fibroblast growth factor receptor substrate 2 (Frs2a), Src homology region 2 domain containing phosphatase 2 (Shp2) and growth factor receptor-bound protein 2 (Grb2) facilitates son of sevenless homology (Sos)-Ras-Erk activation, whereas FGFR-Frs2a-Grb2 complex formation drives PI3K

activation via Grb2-associated binding protein 1 (Gab1). FGF signaling is tightly regulated and modulated at multiple levels, both intracellularly and extracellularly. For example, the bioavailability of secreted FGF ligands is regulated by their binding to extracellular heparan sulfate proteoglycans (HSPGs). HSPGs also act as important co-receptors for FGF ligands by facilitating the assembly of activated ligandreceptor complexes (Ornitz and Itoh 2001). Intracellularly, the FGF signaling cascade is further regulated by negative-feedback mechanisms at multiple levels by dual specificity phosphatases (DUSPs), similar expression to FGF (Sef), Spred and Sprouty proteins (Dailey et al. 2005). Several of these inhibitors are themselves downstream transcriptional targets of the FGF pathway.

FGFs range in molecular weight from 17 to 34KDa in vertebrates; all members share a conserved sequence of 120 amino acids that show 16-65% sequence identity (Ornitz and Itoh 2001). The FGFs gene is located on chromosome 11q13.3 and the size complete gene is 6.10kb, containing 3 exons.

Within the family of FGFs there are seven subfamilies consisting of FGFs that display increased structural and functional similarity. These subfamilies can also be classified into three groups: the intracellular FGF11/12/13/14 subfamily also known as FGF homology factors that do not signal through the FGFRs; the hormone-like (endocrine) subfamily also known as FGF19 subfamily that comprise the human FGF19/21/23 and the mouse fgf15 proteins that have a reduced affinity for heparin sulfate proteoglycan and contain intramolecular disulfide bonds, which may function to increase their stability in plasma and allow them to function as hormones (Harmer et al. 2004), and the canonical FGF subfamily comprising the FGF1/2/5, FGF3/4/6, FGF7/10/22, FGF8/17/18 and FGF9/16/20 subfamilies, by their action mechanisms (Itoh and Ornitz 2004).

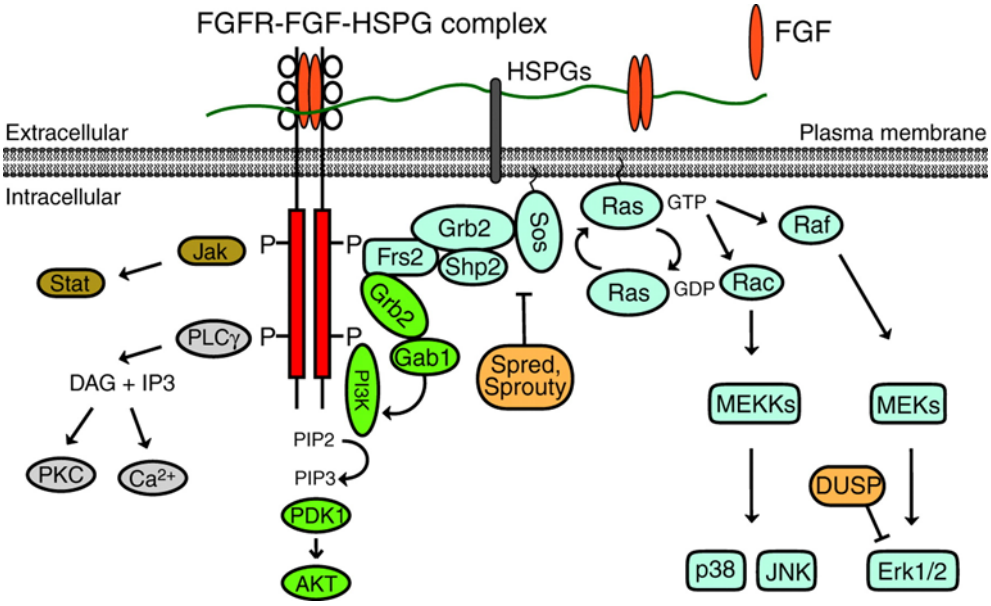


Figure 14: The FGF-FGFR signaling pathway. (Adapted from Lanner and Rossant 2010)

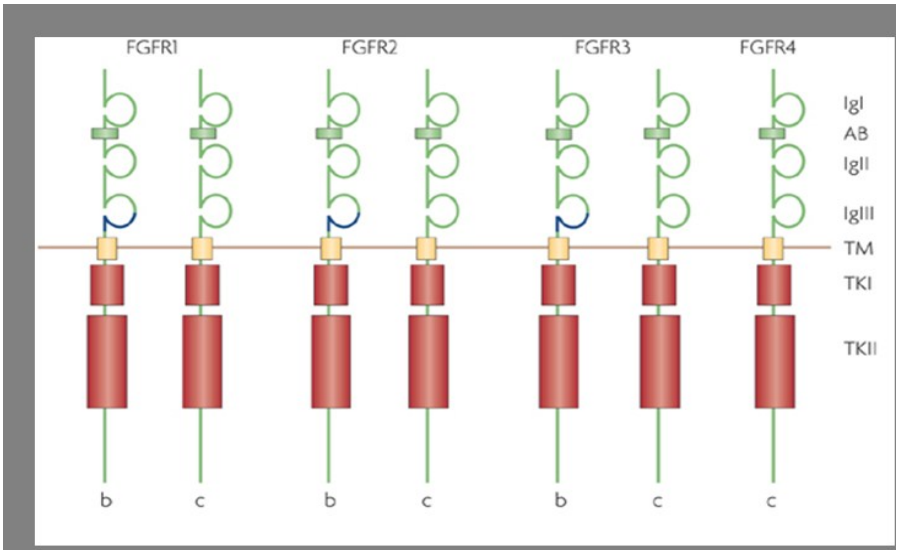


Figure 15: FGFR structure. (From Mason 2008)

The prototypical FGFR is composed by an extracellular region containing three immunoglobulin (Ig)-like domains (D1-D3), a single transmembrane helix and a cytoplasmatic domain with protein tyrosine kinase activity. A unique hallmark of FGRs is the presence of a contiguous stretch of acidic residues in the linker between D1 and D2 which is termed “acid box” (AB) (Mohammmadi et al. 2005). Ligand binding requires both D2 and D3 domains. The D1 domain and the D1-D2 linker are dispensible for ligand binding and suppress FGF and HS binding affinity of the D2-D3 region. Alternative splicing

can generate a range of FGFR1–4 isoforms, some of which are secreted proteins. Most significantly, alternative splicing within the region encoding the C-terminal part of the third extracellular immunoglobulin loop in FGFR1–3 generates IIIb or IIIc isoforms, dramatically affecting ligand–receptor binding specificity (Mason 2007). The D3 alternative splicing event elaborates the number of principal FGFRs from four to seven: FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c, FGFR4 (Figure 15).

5.2 FGF19/FGFR4 signaling

FGF19 was cloned by homology to its mouse ortholog fgf15 (Iwata et al. 2009, Nishimura et al. 1999). The FGF19 gene is located on human chromosome 11q13 and belongs to an evolutionary conserved cluster of genes that includes FGF3, FGF4 and Cyclin D1 (Wu et al. 2010). The full-length cDNA encodes a 216 amino acid protein containing an amino-terminal signal sequence, two highly conserved cysteine residues and a clear relationship to other members of the FGF family. FGF19 mRNA is found in brain, skin, cartilage, retina, gall bladder, small intestine, kidney, placenta and umbilical cord (Nishimura et al. 1999; Xie et al. 1999).

As mentioned before most FGFs function primarily in a paracrine and/or autocrine fashion. However the FGF19 subfamily members can function as endocrine factors or hormones. FGF19 can act as a classic endocrine hormone to regulate bile acid homeostasis as well as glucose and lipid metabolism. In the liver, FGF19 is able to suppress bile acid production by downregulating the key enzyme in bile acid biosynthesis (Pai et al. 2008). In adipose tissue it may increase glucose uptake by regulating a glucose transporter. A deregulating FGF19 signaling may be critical for development and progression of a numbers of cancers, because it can contribute to cell proliferation, survival, invasion, migration and tumor angiogenesis (Turner and Grose 2010).

The effects of FGF19 on downstream signaling MAPK and β-catenin pathways are mediated by its cognate receptor FGFR4. Unlike the other members of the FGF family, FGF19 binds only the FGFR4 that is the most widely distributed member of the FGFR family. Under normal circumstances, FGFR4 is expressed in liver, lung, gall bladder, small intestine, pancreas, colorectal, lymphoid, ovary and breast tissue (Lin et al. 2007). Typically the binding interaction requires stabilization by heparin sulfate glycosaminoglycan cofactors that are present in the extracellular matrix (HS) (Ornitz and Ithoh 2001; Beenken and Mohammadi 2009; Asada et al. 2009). The interaction with HS also limits the diffusion of FGFs from their site of release and creates a low-affinity FGF reservoir that enhances paracrine signaling (Beenken and Mohammadi 2009; Asada et al. 2009). In contrast the FGF19 subfamily members have reduced affinity for HS which increases the radius of diffusion and enables endocrine signaling. FGF19 can bind FGFR4 in the presence of a coreceptor belonging to the Klotho family (Wu et al. 2007) that comprised Klotho (KL) and β-Klotho (KLB). The Klotho proteins are type I transmembrane glycoproteins containing extracellular regions that contain two beta-glycosidase-like repeats with restricted tissue distribution (Kurosu and Koro-o 2009). KLB is the coreceptor of FGFR4, is required for the activity of FGF19 (Kurosu et al. 2007; Lin et al. 2007). KLB is a 130kDa trans-membrane protein with a short (29 amino acids) intracellular domain that has no predicted kinase activity and two extracellular glycosidase domains that lack a characteristic glutamic acid residue essential for enzymatic activity. KLB exhibits a more restricted expression profile in adipose, liver and pancreas tissues (Lin et al. 2007). KLB and FGFR4 are both expressed at high levels in mature hepatocytes (Lin et al. 2007), where KLB stabilizes FGF19-FGFR4 binding to regulate the expression of cholesterol 7a-hydroxylase (CYP7A1) and hepatocyte proliferation (Lin et al. 2007, Wu et al. 2010, Kurosu et al. 2007).

5.3 FGF19/FGFR4 in cancer.

The FGF19/FGFR4 signaling axis promotes hepatocyte proliferation and is involved in several human cancers, in particular HCC. High expression levels of FGF19 were detected in several human tumors including colon adenocarcinoma, lung squamous cell carcinoma and hepatocellular carcinoma. FGF19 activates several signaling events in the liver that might contribute to tumorigenesis. Besides the MAPK pathway, it is known that the Wnt pathway crosstalks with FGF signaling (Pai et al. 2008) and 44% of neoplastic hepatocytes in FGF19 transgenic mice have nuclear staining for β-catenin (Nicholes et al. 2002) (Figure 16). FGF19 increase tyrosine phosphorylation of β-catenin within the E-cadherin- β-catenin-α-catenin complex, and induces loss of E-cadherin binding to β-catenin in colon cancer cell lines, potentially contributing to tumor growth and invasion (Pai et al. 2008).

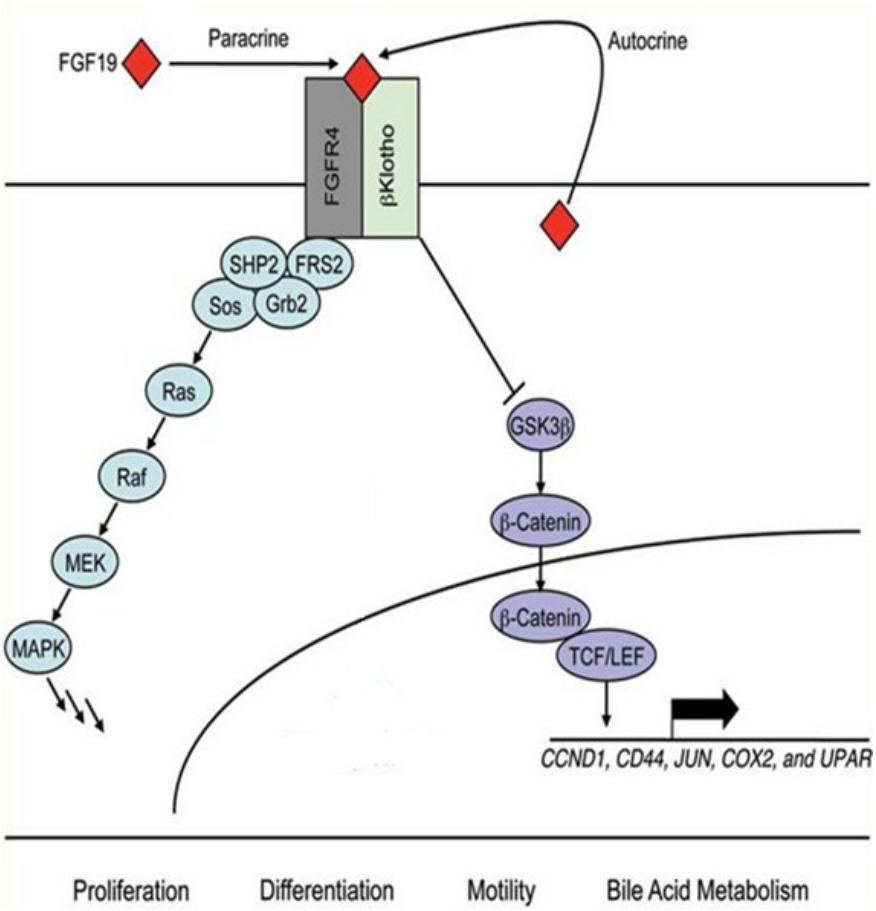


Figure 16: FGF19-FGFR4 Signaling Pathway in cancer. (Adapted from Lin and Desnoyers 2012)

FGFR4, contrary to the other FGFR family members, does not have alternatively spliced variants, but its functions appear to be altered by polymorphisms (Ho et al. 2009). There are many reports in the literature that implicate FGFR4 in tumorigenesis. The Gly388Arg polymorphism is associated with increased risks of several cancers and FGFR4 is frequently overexpressed in patients with HCC. The anti-apoptotic effects of FGFR4 have been associated with resistance to chemotherapy and knockdown of FGFR4 expression by siRNAs in the liver cancer cell line Huh7 was able to suppress α-fetoprotein production (Ho et al. 2009, Roidl et al. 2009). However apparently contradictory contradicting effects have also been reported, including the observation that the genetic deletion of FGFR4 in mice accelerates the progression of DEN-induced HCC (Huang et al. 2009), and the overexpression of FGFR4 and KLB induce apoptosis and inhibit tumor cell proliferation in vitro (Luo et al. 2010), suggesting a potential protective role of FGFR4 in suppressing hepatoma proliferation.

PURPOSE

HCC is the most frequent form of primary liver cancer and deadly disease, with limited therapeutic options. The only therapeutic agent that has shown some efficacy is the multi-target kinase inhibitor sorafenib, suggesting the interference with dysregulated cell signaling pathway could be exploited for HCC treatment. To this end the identification of key signaling pathway in hepatocarcinogenesis, and understanding their extensive crosstalk, are essential to devise efficacious therapies and to avert resistance.

The EGFR signaling system is considered as a "signaling hub" where different growth and survival signal converge. This interaction with other signal pathways may occur at different levels. The EGFR system is commonly activated in HCC, and is currently being evaluated as a therapeutic target in combination therapies. As previously demonstrated the EGFR ligand AR, plays a central role in proliferation, survival and chemoresistance of cancer cells, including HCC cells and is frequently up-regulated in HCC tissue and cells through mechanisms not completely known.

Another one important signaling pathway, most dysregulated in a number of human tumors, including HCC, is the Wnt/β-catenin system. Several mechanisms account for β-catenin pathway activation in HCC cells, which includes mutations in β-catenin gene or in components of the β-catenin degradation complex, as well as dysregulation in the expression of Wnt/Frizzled signaling elements. Crosstalks between Wnt/β-catenin signaling and EGFR system have been reported in different cell types, including liver parenchymal cells.

Knowing the existence of a variety of crosstalk between the most important signaling pathways during hepatocarcinogenesis, the first purpose of this thesis was to evaluate whether the expression of AR could be regulated through the β-catenin signaling pathway in human HCC cells.

In second place, knowing that the FGF19 is a soluble growth factor involved in hepatocarcinogenesis and an important β-catenin pathway activator, we asked if the AR gene expression can be induced by FGF19 through the β-catenin pathway in human HCC cells and if the AR up-regulation is required for FGF19-elicited HCC cell proliferation.

Finally we tried to demonstrate a positive correlation between the AR and FGF19 gene expression in human HCC tissue in order to evaluate a possible clinical and therapeutical relevance of the FGF19/FGFR4 axis in HCC.

MATERIALS & METHODS

1. Cell culture and treatments.

The HCC cell lines HepG2, Hep3B and PLC/PRF5 from ATCC (Rockville, MD) were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2mM L-glutamine, 100units/mL Penicillin, 100mg/mL Streptomycin and 10% Fetal Bovine Serum (FBS) (Castillo et al. 2006, Pardo-Saganta et al. 2009). The human HCC cell line Huh7 (0403) was obtained from the Japanese Collection of Research Bioresources (JCRB, Tokyo, Japan) and maintained according to their instructions.

Cells were treated with the GSK3β; inhibitors LiCl and SB-415286 (Sigma, St. Louis, MO); the EGFR inhibitor PD153035 (Calbiochem, San Diego, CA); the AR goat polyclonal neutralizing antibody (αAR) (#AF262) or the control goat IgG (R&D Systems, Minneapolis, MN).

Medium, Trypsin, FBS and antibiotics were from Gibco/Invitrogen (Paisley, United Kingdom). The materials for the cell culture (plates, scrapper, pipettes and plastic tubes) were from Costar/Corning (Corning, NY, EEUU) and Sarstedt (Newton, NC, EEUU).

2. Plasmid constructs and transfections.

OneShot® Top10 the competent bacteria cells (Invitrogen) were transformed with the indicated plasmids using the chemical transformation protocol suggested by manufacturer. The transformation reaction was spread in broth (LB, Sigma) containing a specific antibiotic (depending on plasmid resistance) and were incubated at 37ºC overnight.

The plasmids were extracted with the kit *Endofree Plasmid Maxi Kit* (Qiagen, Hilden, Germany) and plasmid DNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc, Wilmintong, DE, EEUU).

A 1255bp fragment of human AR gene 5' region, immediately upstream of the 5' end of the mRNA start site, nucleotides -210 to -1464 from the ATG (Plowman et al 1990), was

generated by PCR amplification of HepG2 cells genomic DNA using the primers described in the Table 1.

This AR 5' DNA fragment was cloned upstream from the luciferase reporter gene in the pGL3-Basic vector (Promega, Madison, WI). Site-directed mutagenesis of the Tcf-binding elements (TBEs) was performed on this construct using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the protocol supplied by the manufacturer. Mutations were as follows:

- TBE1: CTTTG**TA** → CTTTG**CC**;
- TBE2: CTTTG**AA** → CTTTG**GC**;
- TBE3: **TA**CAAA**C** → **GC**CAAA**G**.

The primers used for site-directed mutagenesis are reported in the Table 1.

The expression vectors for the dominant stable β-catenin T41A mutant and the dominant negative Tcf4 variant (ΔNTcf4) were kindly given by Dr. Marie-Annick Buendia (Pasteur Institute, Paris, France).

The cells were plated in 60mm dishes until 70% confluence and transiently transfected with lipofectamine reagent (Invitrogen) with the wild type and mutant AR-promoter reporter constructs, the empty pGL3-Basic vector as control and the TOPflash Tcf reporter plasmid (Millipore, Billerica, MA). Cells were also co-transfected with the Renilla luciferase reporter vector (Promega) as an internal control for transfection efficiency. Luciferase activity in cell lysates was determined using the Dual-Luciferase Reporter Assay System (Promega).

Huh7 cells were stably transfected with the expression vector for β-catenin T41A mutant and the empty expression vector (pcDNA3) and subsequently selected in complete medium containing 0.6mg/mL of Geneticin sulfate (G418, Invitrogen). After 2 weeks, individual colonies were harvested and each transfected clone was expanded.

Gene	Name	Primers	Tª hibridation
AR	S	5' CAGCCCACCCGAGTAGCTGGGACTA 3'	60ºC
	AS	5' GTAGGGCGGCGCGCACCTGCCGCTTTATA 3'	
TBE1	S	5' TCTGTTGTAGATGTTAAGTA**GC**CAAAGAGGTTGTCAGAGTTTGAAAC 3'	55ºC
	AS	5' GTTTCAAACTCTGACAACCTCTTTG**GC**TACTTAACATCTACAACAGA 3'	
TBE2	S	5' AAGAATTCATATCCACCTGGCTTTG**GC**CATTATCGGCTGTGAGATGG 3'	55ºC
	AS	5' CCATCTCACAGCCGATAATG**GC**CAAAGCCAGGTGGATATGAATTCTT 3'	
TBE3	S	5' CATCACGCCCAGCTAATTTC**C**TTTG**GC**TTTTTAGTAAAGATGGGGTT 3'	55ºC
	AS	5' AACCCCATCTTTACTAAAAA**GC**CAAA**G**GAAATTAGCTGGGCGTGATG 3'	

Table 1: primers used for amplification and for site directed mutagenesis.

3. RNA isolation and quantitative real-time PCR (qPCR).

Total RNA was extracted after homogenization with the TriReagent (Sigma) based on the Chomczynsky and Sacchi's Method (Chomczynsky and Sacchi, N. 1987). The RNA pellet was resuspended in sterile water treated with diethyl pyrocarbonate (H_2O DEPC, Sigma). RNA concentrations were measured with a Nanodrop ND-1000 Spectrophotometer.

Two µg of total RNA was reverse transcribed into complementary DNA (cDNA), after DNase treatment and denaturation at 95ºC, using the M-MLV reverse transcriptase as instructed by the manufacturer (Invitrogen). Total RNA (2µg) was mixed with a solution containing 50mM Tris-HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$, 10ng/µl random primers, 0.5mM desoxinucleotides triphosphates (dNTPs: dATP, dTTP, dGTP and dCTP), 5mM dithiothreitol (DTT), 1,2U/µl RNase inhibitors (RNase out, Invitrogen) and 6U/µl reverse transcription enzyme M-MLV (Invitrogen) and incubated 60 minutes at 37°C. The cDNA samples were purified by Spin Columns Centrisep (Princeton Separation, Adelphia, NJ, EEUU) and stored at -20°C.

The differences in the expression of the different genes were analyzed by Real Time PCR (qPCR) that was performed by using an iCycler (BioRad, Hercules, CA) and the iQ SYBR Green Supermix (BioRad). The quantification of the expression of the mRNA was calculated using as the Histone gene as a control with the following formula:

$$2^{\Delta Ct} = 2 \text{ (CT Histone – CT gene of interest)}$$

The temperature of hybridization and the reading of the fluorescence were different depending on each gene but the general reaction conditions were:

Initial denaturation:	95ºC, 1min 30sec	
Denaturation of each cycle:	95ºC, 30sec	45 cycles
Hybridization:	XºC, 15sec	
Extension:	72ºC, 25sec	
Lecture:	XºC, 10sec	
Melting curve:	from 70ºC at 99.5ºC in intervals of 0.5ºC (60x)	

We designed all primers to distinguish between genomic and cDNA amplification and all PCR (polymerase chain reaction*)* products have been sequenced to confirm the specificity. The sequences of primers used for qPCR are described in the Table 2.

Gene	Name	Primers	T[a] hibridation/ Lecture
AR	S	CTGTCGTCTTGATACTCGG	60ºC/80ºC
	AS	GCCAGGTATTTGTGGTTCGT	
Tbx3	S	GAAGAAGAGGTGGAGGACGA	62ºC/82ºC
	AS	GACATCCACTGTTCCCCAGT	
Cyclin D1	S	TGGAACACCAGCTCCTGTGC	64ºC/84ºC
	AS	TCCAGGTAGTTCATGGCCAG	
FGF19	S	CTCTCCAGCTGCTTCCTGCGCATC	62ºC/84ºC
	AS	TTGTAGCCATCAGGGCGGATCTCC	
FGFR4	S	GCTGGCTTAAGGATGGACAG	62ºC/84ºC
	AS	CGTTGATGACGATGTGCTTC	
β-Klotho	S	AAGACACCACGGCCATCTAC	62ºC/78ºC
	AS	ATTCAGTGACACCCCAGGAG	
Histone	S	AAAGCCGCTCGCAAGAGTGCG	62ºC/82ºC
	AS	ACTTGCCTCCTGCAAAGCAC	

Table 2: primers sequences and hybridation temperatures.

4. Chromatin immunoprecipitation (ChIP).

$20x10^6$ cells for each condition were plated and grown in complete DMEM medium. After the indicated treatments cells were treated with 1% formaldehyde (Parneac, Barcelona) for 10 minutes at room temperature to induce crosslinking DNA-protein. The crosslinking reaction was stopped by adding 0.125M glycine during 5 minutes. Cells were washed with ice-cold PBS, scrapped in ice-cold PBS containing 1mM PMSF (Sigma) and centrifuged at 5000rpm for 5 minutes. The pellets were re-suspended in lysis buffer solution (3mM $MgCl_2$,10mM NaCl, 10mM Tris-HCl pH 7.4, 0.5% NP40 (Roche), 1mM PMSF, 10µg/mL aprotinin (Roche), 10µg/mL leupeptin (Sigma) and incubated in ice 10 minutes, disrupted in a potter B (10 strokes) and centrifuged at 5000rpm for 5 minutes. Nuclei pellets were re-suspended in lysis buffer solution (50mM Tris-HCl pH 8.1, 10mM EDTA, 1% sodium dodecyl sulfate (SDS) and protease inhibitors) and incubated 10 minutes in ice.

Samples were sonicated in order to obtain DNA fragments of 500-600pb, centrifuged at 14000rpm for 10 minutes and supernatants were collected.

The obtained chromatin was pre-cleared using *Dynabeads protein G* (Invitrogen) previously blocked for 2 hours in orbital agitation with salmon sperm DNA. The samples were separated in three tubes: one was incubated with 5µg of the specific antibody (Table 3), other one with IgG (of the same isotype) and the third without antibody (the last two were used as negative controls), overnight at 4ºC in orbital agitation. Subsequently the magnetic support was added to each tube for 2 hours at 4ºC in constant agitation. The supernatants were collected as total chromatin *input.* The *Dynabeads* were then resuspended and washed three times with a buffer composed of 2mM EDTA, 50mM Tris-HCl pH 8.0 and 0.2% sarkosyl, and another four times with the IP washing buffer (100mM Tris-HCl pH 9.0, 500mM LiCl, 1% NP40 and 1% deoxycholic acid). Samples were eluted with an elution buffer solution (50mM $NaHCO_3$ and 1% SDS) and vortexed during 15

minutes. 0.3M NaCl and RNase (100mg/mL, Qiagen) were added to the supernatants of and samples were heated at 67ºC overnight to reverse the formaldehyde cross-link.

The DNA was then precipitated with ethanol for two hours at -80ºC and centrifuged at 14000 rpm for 15-20 minutes at 4ºC. The pellet were resuspended in Tris-EDTA (TE) buffer pH 7.5 containing 10mM Tris-HCl pH 7.5, 5mM EDTA, 0.25% SDS supplemented with 15µg of Proteinase K (Sigma) and incubated for 2 hours at 45ºC.

Afterwards samples RNA were extracted with phenol/chloroform/isoamylic alcohol (25:24:1, Invitrogen) and chloroform/isoamilyc alcohol (24:1, Invitrogen) and precipitated overnight at -20ºC with NaCl, 5µg of tRNA (Roche), 5µg of glycogen (Roche) and a double volume of absolute ethanol. Afterwards samples were centrifuged at 14000rpm for 20 minutes at 4ºC and the pellets were re-suspended in sterile H_2O.

The *AR* 5' regions -1459 to -1287, -987 to -811 and -600 to -364, which include three putative Tcf/Lef binding sites previously identified by DNA sequence analysis (Katohy and Kathoy 2006), were analyzed by qPCR with the primers described in the Table 4.

Immunoprecipitations with IgG control antibody gave no amplification products upon PCR. Values were normalized to average values of inputs.

antibody	manufacture	Ref.No
anti-β-catenin	Cell signaling	#v9581
anti-Tcf4	Millipore	#05-511
control rabbit IgG	Santa Cruz	Sc-2027

Table 3: antibodies used for ChIP.

Gene	Name	Primers	T^a hibridation/Lecture
TBE1	S	5'-CACCCGAGTAGCTGGGACTA-3'	62ºC/80ºC
	AS	5'-CAGTGGCTCATGCCTGTAATC-3'	
TBE2	S	5'-TGCCAGTATGCCACCAGTAG-3'	62ºC/78ºC
	AS	5'-CACCATCTCACAGCCGATAA-3'	
TBE3	S	5'- TGTTGGAAACATCAGGCAAA-3'	62ºC/80ºC
	AS	5'- CGTAAGGATTCGCTGAGAGG-3'	

Table 4: primers sequences.

5. Western blot analysis.

The cells were scraped and collected by centrifuging at 4000rpm for 5 minutes in PBS in a refrigerated centrifuge. Samples were resuspended in 50-60µl of RIPA lysis buffer (150mM NaCl, 50mM Tris pH 7.5, 0.1% SDS, 1% triton X-100, 0.5% sodium deoxycholate, 1mM sodium orthovanadate, 10mM sodium fluoride, 100mM β-glycerolphosphate and a cocktail of protease inhibitors de Roche Diagnostics), sonicated 30 seconds at medium intensity, with a Bioruptor sonicator (Diagenode, Liège, Bélgium) and centrifugated at 13000rpm for 15 minutes in a refrigerated centrifuge. The supernatants were collected and quantitated by the *Bicinchoninic Acid* (BCA, Sigma) *Protein Assay*.

SDS-PAGE using 10% resolving gels was used to separate total proteins. A total of 15-30µg of total protein extract was boiled for 5 minutes in loading buffer 5X (50mM Tris pH 6.8, 100mM β-mercaptoethanol, 2% SDS, 10% glycerol, 0.01% bromophenol blue) and eletrophoresed in running buffer (25mM Tris, 192mM Glycine, 0.1% SDS) on a Protean II Minigel Apparatus (Bio-Rad, Sydney, Australia). SDS-PAGE gels were run at 100V until the loading dye had reached the end of the stacking gel and then at 100-120V through in the resolving gel. Pre-stained molecular weight protein markers (Precision Plus Protein Standard Dual Color, Bio-Rad) were used to determine the molecular sizes of the resulting bands.

The proteins separated by SDS-PAGE were electrotransferred onto a nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences, Buckinghamshire, UK) using a transfer buffer (25mM Tris, 192mM Glycine and 20% Methanol) for 45 minutes at 4ºC using un costant voltage (120V).

The nitrocellulose membranes were bloqued for 1 hour at room temperature in dry milk 5% for not phophorylated protein or bovin serum albumin (BSA) 5% for phosphorylated protein in TBS-Tween (TBS-T) (25mM Tris pH 7.5, 200mM NaCl, 0.1% Tween 100). Afterwords membranes were incubated overnight with the primary antibody

at the concentration recomended by the manufacter in 5% milk or BSA in TBS-T (Table 5). Following primary antibody incubation and washing in 0.1% TBS-T (3x5min), membranes were incubated 1 hour with the secondary antibody (rabbit or mouse) diluited 1:6000 in 5% milk or BSA. Afterwords membranes were washed three times in TBS-T (5min) and once in TBS 1X (10min). Membranes were developed using the chemiluminescence Western Lightning kit (PerkinElmer. Inc, Waltham, MA, EEUU) according to the instructions of the supplier using Amersham Hyperfilms ECL (Amersham Biosciences).

antibody	manufacture	Ref.No	diluition	blocking solution	antibody 2º
anti-phospho-GSK3β (Ser9)	cell signalling	#9336	1:1000	5% BSA	Rabbit
anti-Active β-catenin	Millipore	#05-665	1:1000	5% Milk	Mouse
anti-Tcf4	Millipore	#05-511	1:1000	5% Milk	Mouse
Polyclonal anti-β-actin antibody	Sigma	#A2066	1:2000	5% Milk	Rabbit

Table 5: antibodies used for the western blot.

6. *RNA interference.*

Cells were plated in a 6 multiwell plates, in 10% FBS DMEM without antibiotics. Subsequently cells were transfected with specific siRNA oligonucleotides (siAR1 and siAR2) and control siRNAs (siGL) at a concentration of 100nm/well. The sequences of the siRNAs are reported on the Table 6.

Transfections were performed using the RNasi Mix (Invitrogen) according to the manufacturer's instructions in 10% FBS OPTIMEM without antibiotics, medium was change after 6 hours for complete DMEM. Tuelve hours post-transfection medium was changed to 0% FBS DMEM and 8 hours after treatments the RNA and proteins were extracted from the cells.

Gene		Name	Primers	Manufacter
siGL		S	5' CGUACGCGGAAUACUUCGA [dT] [dT]	Sigma
		AS	5' UCGAAGUAUUCCGCGUACG [dT] [dT]	
siAR	1	S	5' CCACAAAUACCUGGCUAUA [dT] [dT]	Dharmacon
		AS	5' UAUAGCCAGGUAUUUGUGG [dT] [dT]	
	2	S	5' AAAUCCAUGUAAUGCAGAA [dT] [dT]	
		AS	5' UUCUGCAUUACAUGGAUUU [dT] [dT]	

Table 6: siRNA sequences.

7. AR determination by ELISA.

The concentration of soluble AR was evaluated in the cells conditioned media by an Enzyme Linked Immunosorbent Assay (ELISA). The cell culture was maintained in absence of serum, the conditioned medium was collected and 1mM phenylmethylsulfonyl fluoride (PMSF) was added, medium was pre-cleared by centrifugation at 4500rpm for 5 minutes and then lyophilized.

This technique is based on the antibody sandwich principle. First, 100µl (2µg/mL concentration) of a capture antibody specific to the analyte of interest (monoclonal anti-AR capture antibody #MAB262, R&D System) is bound to a PVC microtiter plate to create the solid phase overnight a room temperature. Unbound antibody is removed by washing the plate with a washing buffer (0.05% Tween 20 in PBS pH 7.4). Therefore the remaining protein-binding sites were blocked in the coated well by adding a blocking reagent (PBS, 1% BSA, 5% sucrose and 0.05% NaN_3) for 2 hours at room temperature, following by wash with the washing buffer.

A standard curve using recombinant human AR (#AR A7080, Sigma) was prepared in double starting to a concentration of 2ng/µl. Samples and controls were prepared in a diluent buffer (20mM Tris pH 7.3, 150mM NaCl, 0.1% BSA and 0.05% Tween20) and incubated 2 hours at room temperature with the solid phase antibody, which captures the

analyte. After 3 washes to remove unbound analyte, a conjugated detection antibody (biotinylated anti-human AR antibody, polyclonal, BAF262, R&D System) was added and incubated for 2 hours at room temperature. This detection antibody binds to a different epitope of the molecule being measured and completes the sandwich. Following 3 washes to remove unbound detection antibody, a detection reagent (Streptavidin-HRP) is added in concentration of 1:1000 and incubated 1 hour at room temperature. The plate is washed 3 times, and 100µl of a substrate solution 3,3',5,5'-tetramethylbenzidine (TMB)/hydrogen peroxide is added and color develops in proportion to the amount of bound analyte. The color development is stopped with 50µl of stopping solution 2N SO_4H_2 and the intensity of the color is measured at 370nm in the microplate reader 680XR (Bio-Rad).The standard curve using recombinant human AR (#AR A7080, Sigma) was used to calculate AR concentrations in the conditioned medium.

8. Immunofluorescence staining.

Cells were cultured on coverslips. After treatments cells were fixed with 4% paraformaldehyde (15min) and subsequently permeabilized with 0.1% Triton X-100. After blocking in Supper blocking Buffer (Biorad, Hercules, CA) (30min) fixed cells were incubated with anti-β-catenin antibodies (Santa Cruz Biotechnology, #sc-7963) (1:100 in 1% Albumin) overnight at 4ºC. Slides were washed with saline and incubated with Alexa Fluor 488-conjugated rabbit anti-mouse secondary antibody (1:500 in 1% Abumin) (Invitrogen, Carlsbad, CA) for 1 ½ h at room temperature, then washed and mounted. Finally, sections were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) and examined under a fluorescence microscope.

9. Data mining in gene expression datasets from human HCC tissues.

Data mining was performed using public high-throughput gene expression datasets from human HCC tissues obtained from Gene Expression Omnibus (GEO) database. AR and FGF19 expression values were obtained from the following sets of data: GSE1898 (90 HCC tissue samples) (Lee et al. 2006) and GSE5975 (238 HCC tissue samples) (Jia et al. 2007).

10. Statistical analysis.

Statistical analysis was performed with the Graph Pad Prism version 5.00 software (Graph Pad Software, San Diego, CA). Experiments were performed at least twice in triplicates. Data are means ± SEM. Data were compared among groups using the Student *t* test. The Spearman's correlation coefficient was used to analyse the correlation between *AR* and *FGF19* gene expression values obtained from the above-mentioned datasets A *P* value of <0.05 was considered significant.

RESULTS

1. *Activation of β-catenin signaling induces AR gene expression in human HCC cells.*

In order to examine the regulation of *AR* gene expression through the activation of the β-catenin signaling pathway we first used two well-known inhibitors of GSK3β, LiCl and SB-415286 (Stambolic et al. 1996; Coghlan et al. 2000). GSK3β is a multifunctional serine/threonine kinase implicated in diverse cellular processes including proliferation, differentiation, motility and survival. Aberrant regulation of GSK3β has been implicated in a range of human pathologies including non-insulin-dependent diabetes mellitus, cardiovascular disease, some neurodegenerative diseases, and bipolar disorder and also participates in neoplastic transformation and tumor development. One of the most well-known substrates of GSK3β is β-catenin, and GSK3β is an important regulator of the Wnt/β-catenin signaling pathway. GSK3β phosphorylates β-catenin leading to its ubiquitination and degradation by the proteasomal pathway. GSK3β inhibition, by phosphorylation, prevents it from phosphorylating β-catenin, thus stabilizing β-catenin in the cytoplasm. As β-catenin accumulates, it translocates into the nucleus where it binds to TCF/LEF complexes and dramatically increases their transcriptional activity (Luo 2009; Dahmani et al. 2011; Aberle et al. 1997).

To demonstrate that, we treated Huh7 cells for 3 and 12 hours with LiCl or SB-415286 at a concentration of 20mM and 10μM respectively and we performed a western blot analysis in order to evaluate the levels of dephosphorylated and active β-catenin which, as shown in Figure 17, increase upon stimulation with both LiCl and SB-415286 as compared to control cells.

These data are further confirmed by immunocytochemical staining, which shows, as reported in Figure 18, the accumulation and nuclear translocation of β-catenin in the Huh7 cells treated for 12 hours with LiCl or SB-415286 compared with negative control cells. Nuclei were stained with DAPI.

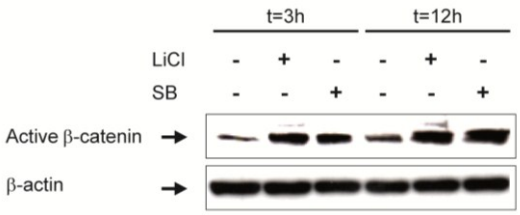


Figure 17: Huh7 cells were treated with LiCl (20mM) or the GSK3β inhibitor SB-415286 (10μM) (SB) for the indicated time points and active β-catenin was detected by immunoblotting with β-catenin specific antibody (dephosphorylated on Ser37 and Thr41).

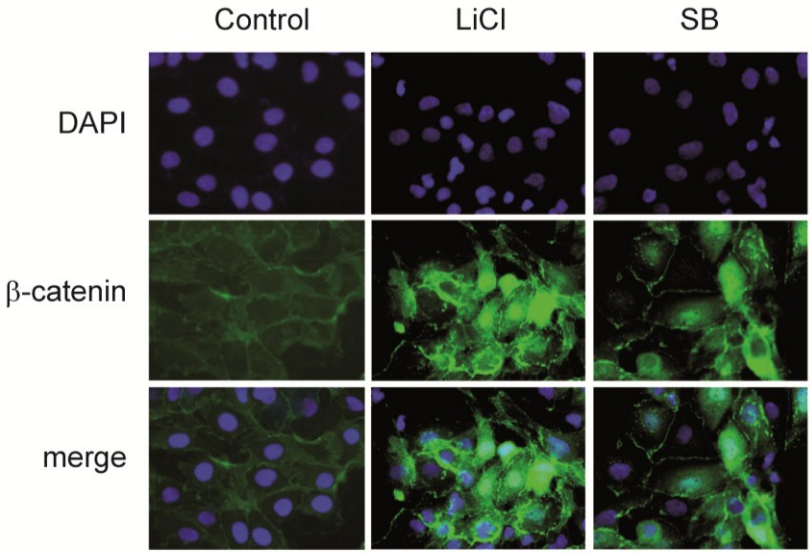


Figure 18: Immunofluorescent demonstration of increased β-catenin protein levels and nuclear distribution in Huh7 cells treated with LiCl (20mM) or SB-415286 (10μM) (SB) for 12h. Representative images are shown.

We also demonstrate that AR mRNA levels increase in a time and dose dependent manner upon LiCl stimulation of Huh7 cells (Figure 19A) as well as in Hep3B cells (Figure 19B) and HepG2 human HCC cells (Figure 19C). The SB-415286 β-catenin agonist also induced AR mRNA expression in all three cell lines (Figure 19A-C). Furthermore we also demonstrate that activation of the β-catenin pathway induces AR protein secretion in human HCC cells. For this we treated cells with LiCl (20mM) or SB-415286 (10μM) for 24 hours in serum-free medium, and we detected, in the conditioned medium of Huh7 cells, increased levels of AR protein consistently with the up-regulation of AR mRNA previously shown (Figure 20).

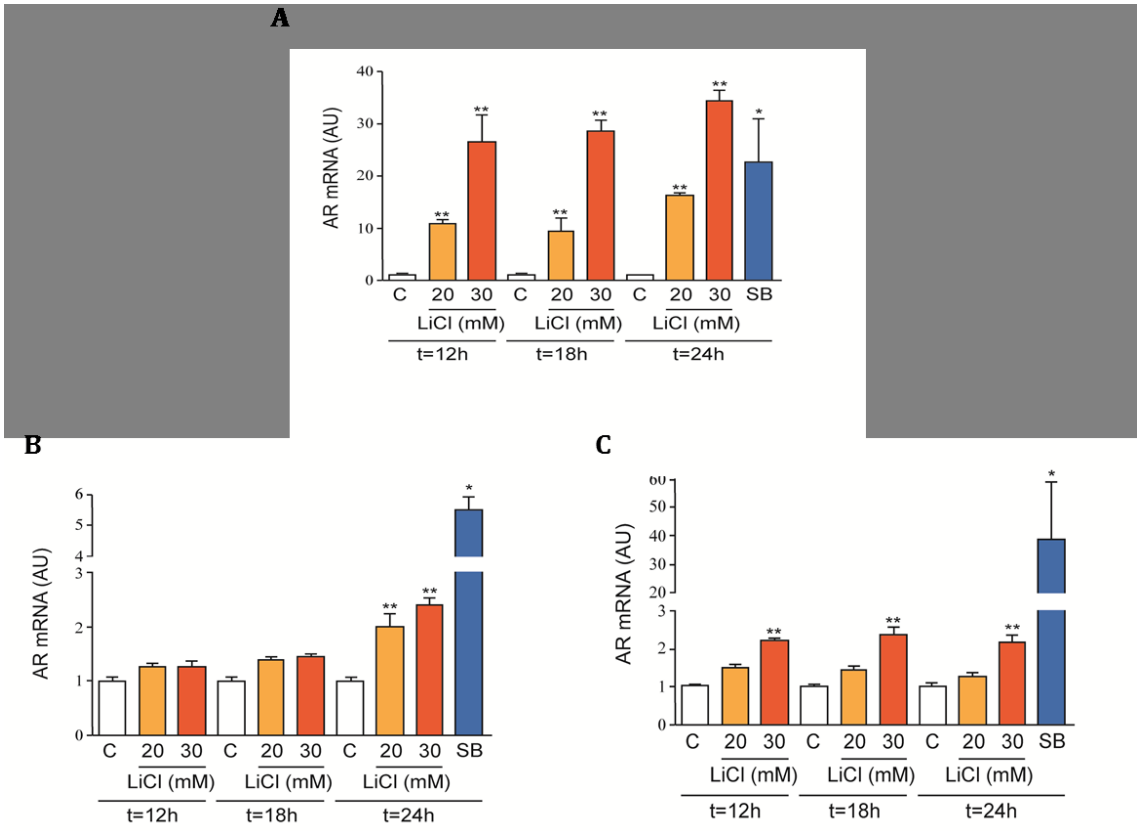


Figure 19: Time- and dose-dependent induction of *AR* gene expression in Huh7 cells upon β-catenin pathway activation by LiCl treatment. The effect of SB-415286 (10μM) (SB) at 24h of treatment is also shown (A) Huh7 cells, (B) Hep3B cells, (C) HepG2 cells. * *P*<0.05 *vs* control, ** *P*<0.01 *vs* control. AU: arbitrary units.

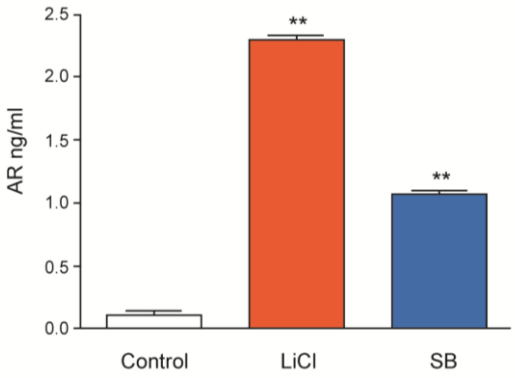


Figure 20: Huh7 cells were treated with LiCl (20mM) or SB-415286 (10μM) (SB) for 24h in serum-free medium, and AR protein contents were determined by ELISA in the conditioned media. ** *P*<0.01 *vs* control.

2. Activation of β-catenin pathway promotes β-catenin and Tcf4 recruitment to AR promoter in human HCC cells.

As previously mentioned activation of β-catenin signalling involves its nuclear translocation and interaction with members of the TCF/LEF family of transcription factors, leading to the formation of regulatory complexes that bind Tcf-binding elements (TBEs) on the promoters of target genes (Dahmani et al. 2011; MacDonald et al. 2009, White et al. 2012). A previous *in silico* study on the potential regulation of the expression of EGF family members by the Wnt/β-catenin pathway had identified three putative consensus TBE sites in the 5' region of the human *AR* gene (Katoh and Katoh 2006). These sequences are shown in Figure 21.

To characterize the mechanisms involved in AR gene regulation by β-catenin pathway we examined the *in vivo* binding of Tcf4 and β-catenin to these TBEs indicated as TBE1, TBE2 and TBE3. As shown in Figure 22A, ChIP analysis indicated that binding of β-catenin to these three TBEs was detected under basal conditions and it was significantly increased upon 24 hours of LiCl 20mM treatment of Huh7 cells as compared to control cells. Chromatin immunoprecipitation with a Tcf4 specific antibody also showed enhanced binding to these TBEs after LiCl stimulation when compared to the controls cells (Figure 22B). Immunoprecipited genomic DNA was PCR amplified and resolved in agarose gels. The induction of Tcf4 and β-catenin binding upon LiCl stimulation to TBEs in AR 5' region was further confirmed by quantitative analysis of immunoprecipitated chromatin (qChIP), as indicated in the Methods, shown in Figure 22A and B. These findings indicated that Tcf4/β-catenin specifically bound to AR 5' region under conditions of active β-catenin pathway signalling.

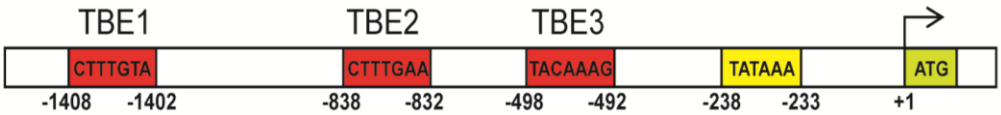


Figure 21: Identification in the human *AR* promoter sequence of three putative Tcf binding sites (TBE): TBE1, TBE2 and TBE3. The positions of these elements, and that of the TATA box, are indicated with numbering in reverse starting from the ATG.

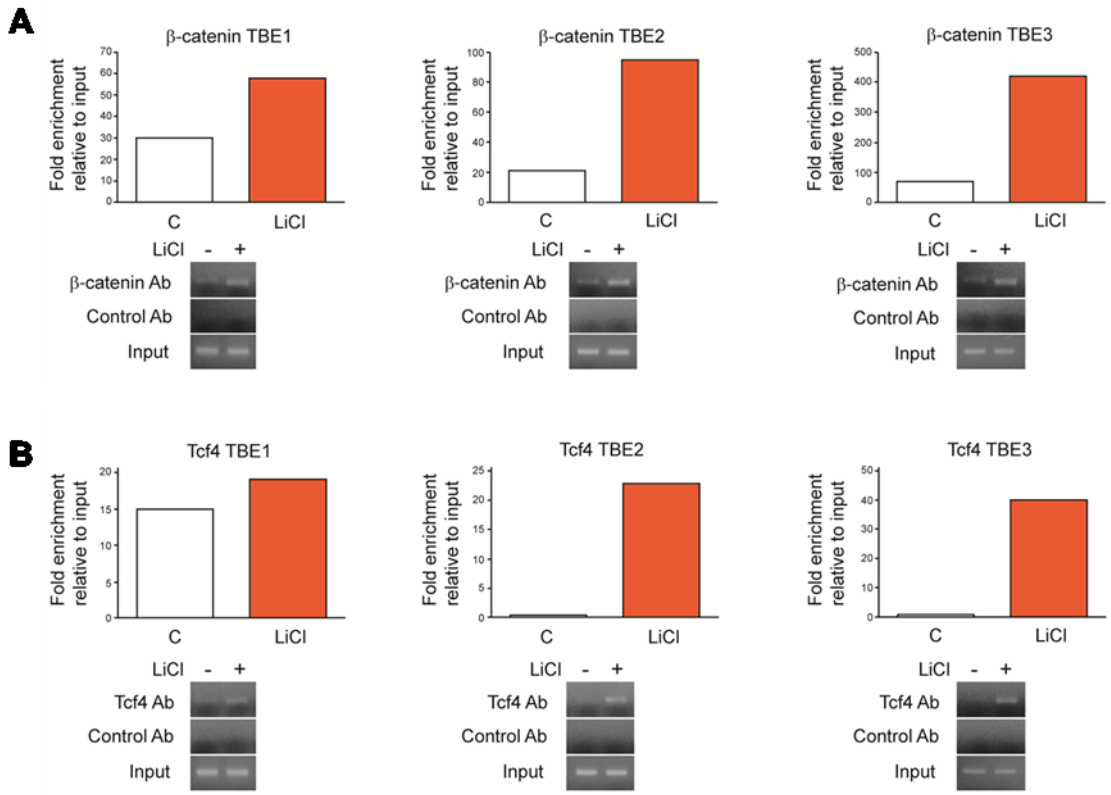


Figure 22: ChIP assay measuring the binding of β-catenin (A) or Tcf4 (B) to the three AR promoter regions encompassing the TBE1, TBE2 and TBE3 sites in control (C) and LiCl (20mM, 24h) treated Huh7 cells. Upper panel DNA obtained after immunoprecipitation of chromatin with β-catenin (A) and Tcf4 (B) antibodies from control and LiCl treated cells was quantified by qPCR as indicated in Materials and Metods section. Lower panel: ChIP assay evaluating the binding of β-catenin (A) and Tcf4 (B) to the three AR promoter regions encompassing the TBE1, TBE2 and TBE3 sites in control (C) and LiCl (20mM, 24h) treated Huh7 cells as indicated. Data are representative of two experiments performed in duplicates.

3. Expression of an active β-catenin mutant promotes AR gene expression in human HCC cells.

To further investigate the involvement of β-catenin in the regulation of AR gene expression we stably transfected Huh7 cells with an active β-catenin mutant called β-catenin T41A or the corresponding empty vector, pcDNA 3.1. This β-catenin T41A mutant protein has increased stability, and has been previously shown to significantly up-regulate the expression of β-catenin target genes (Renard et al 2007; Wei et al. 2000). Consistently we observed increased β-catenin protein levels in β-catenin T41A expressing Huh7 cells compared with control cells stably transfected with the empty expression vector, as well as increased mRNA expression of the Wnt/β-catenin target gene *Tbx3* (Renard et al 2007) (Figure 23A and B). T41A β-catenin expressing Huh7 cells also displayed increased levels of AR mRNA and secreted more AR protein to the culture medium (Figure 24A and B). These observations confirmed the notion that β-catenin pathway activation can trigger AR gene expression in HCC cells. To gain further insight on the mechanisms involved in the regulation of AR gene transcription by β-catenin/Tcf, we performed a site-directed mutation of the TBE sites found in AR 5' regulatory region, in the context of a 1255bp AR promoter luciferase reporter plasmid as described in Materials and Methods section. Huh7 cells stably transfected with the control vector pcDNA3.1 or with the T41 β-catenin active mutant were transiently transfected with the empty luciferase reporter plasmid (pGL3) or with the wild type AR promoter luciferase reporter construct (ARwt pGL3). Transfection of the wild type AR promoter construct in Huh7 cells expressing the active T41A β-catenin mutant resulted in a significant stimulation of AR promoter activity compared to control Huh7 cells (Figure 25).

Huh7 cells expressing the T41A β-catenin active mutant were also transfected with three AR promoter constructs in which the three TBE sites had been individually mutated (ART1mut pGL3, ART2mut pGL3 and ART3mut pGL3). The mutation in either TBE site

markedly reduced T41A β-catenin-induced AR promoter activity (Figure 25). Altogether, these evidences indicate that β-catenin pathway activation triggers *AR* gene expression in HCC cells and that *AR* would be a direct transcriptional target of the β-catenin/Tcf complex.

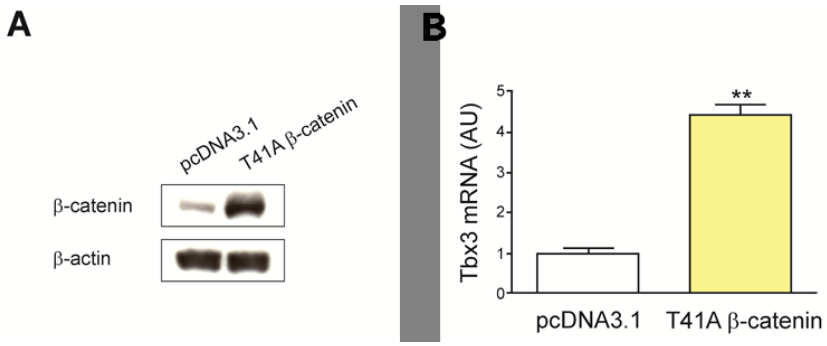


Figure 23: (A) Western blot of β-catenin protein levels in Huh7 cells stably transfected with an empty control vector (pcDNA3.1), or with the same vector harbouring a human stable β-catenin mutant (T41A β-catenin). (B) mRNA expression levels of the β-catenin target gene *Tbx3* in Huh7 cells stably transfected with control vector (pcDNA3.1) or with the T41A β-catenin active mutant. ** *P*<0.01 *vs* control.

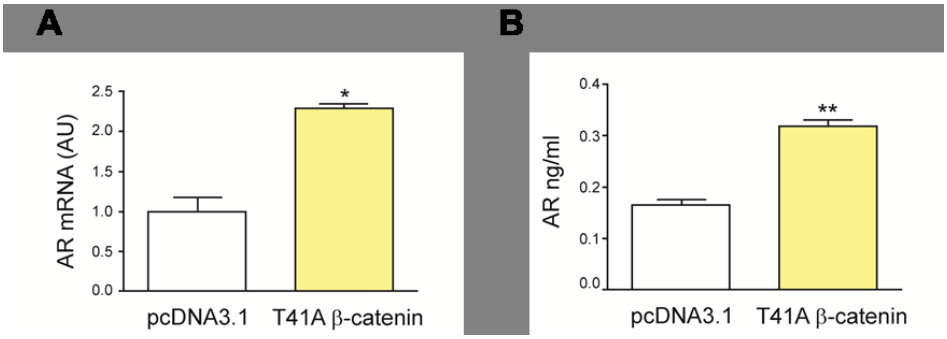


Figure 24: (A) Expression levels of AR mRNA in Huh7 cells stably transfected with control vector (pcDNA3.1) or with the T41A β-catenin active mutant. * *P*<0.05 *vs* control. (B) AR protein contents as determined by ELISA in the conditioned media of Huh7 cells stably transfected with the control vector pcDNA3.1 or with the T41A β-catenin active mutant. Media was collected after 24h culture in serum-free conditions. ** *P*<0.01 *vs* control.

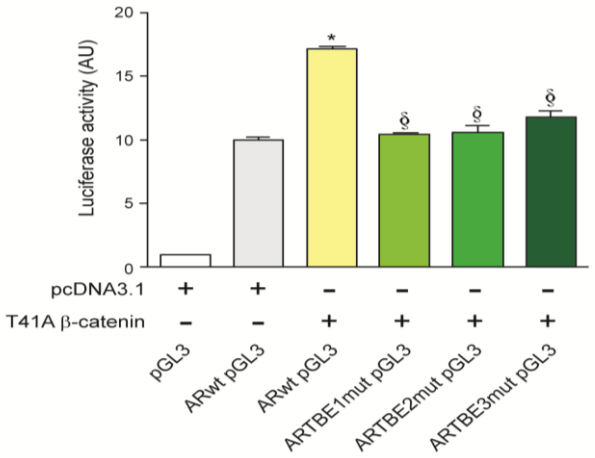


Figure 25: Huh7 cells stably transfected with the control vector pcDNA3.1 or with the T41 β-catenin active mutant, were transiently transfected with the empty luciferase reporter plasmid (pGL3) or with the wild type AR promoter luciferase reporter construct (ARwt pGL3). Huh7 cells expressing the T41A β-catenin active mutant were also transfected with three AR promoter constructs in which the three TBE sites had been individually mutated (ART1mut pGL3, ART2mut pGL3 and ART3mut pGL3). Luciferase activity was measured as described in Methods. * $P<0.05$ *vs* ARwt pGL3-derived luciferase activity in pcDNA3.1 transfected cells. § $P<0.05$ *vs* ARwt pGL3-derived luciferase activity in T41A β-catenin transfected cells. AU: arbitrary units.

4. β-catenin activation of HCC cell proliferation is mediated in part through the induction of AR.

Signaling through the β-catenin pathway has been thoroughly demonstrated to contribute to HCC cell growth (Renard et al 2007; Merle et al. 2004; Zeng et al. 2007). This is supported in part by the growth-promoting effects of constitutively active β-catenin mutants, such as S33Y, in human fetal hepatoblasts and HCC cells (Wege et al. 2011). We observed that Huh7 cells stably expressing the T41A β-catenin active mutant showed enhanced proliferation compared with cells transfected with the control empty vector pcDNA3.1 (Figure 26). Interestingly, the growth of T41A β-catenin expressing Huh7 cells was significantly reduced when cells were treated with the EGFR inhibitor PD153035, or incubated whit a specific AR neutralizing antibodies (αAR) for 72 hours as compared with cells treated with control purified goat IgG (αIgG) or Huh7 cells transfected with the empty vector pcDNA3.1 (Figure 27). In cells treated with PD153035 we found an 87.7% of reduction over the stimulation conferred by T41A β-catenin expression, while with the specific AR neutralizing antibody we detect a reduction of 46.1%. The combination of αAR

and PD153035 did not further increase the already potent effect of the EGFR inhibitor. The involvement of AR on the enhanced proliferation of T41A β-catenin expressing cells was additionally demonstrated upon AR gene expression knockdown with AR specific siRNAs (Figure 28). These findings indicate that at least part of the growth-promoting effects of β-catenin pathway activation may be mediated through the up-regulation of *AR* gene expression.

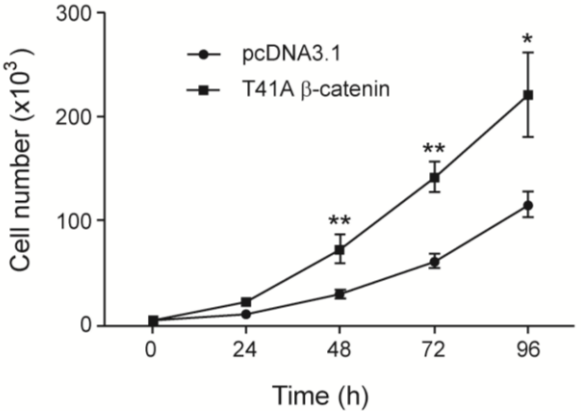


Figure 26: Proliferation of Huh7 cells stably transfected with control vector pcDNA3.1 or the T41A β-catenin active mutant. * *P*<0.05 and ** *P*<0.01 *vs* pcDNA3.1 transfected cells.

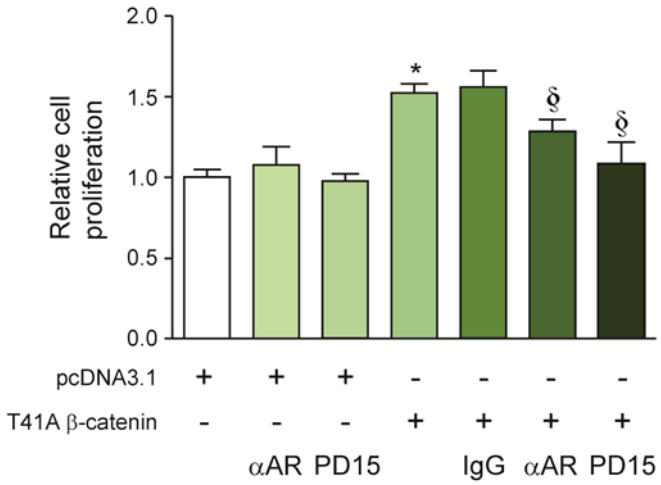


Figure 27: Proliferation induced by T41A β-catenin active mutant is attenuated by AR neutralizing antibodies or the EGFR inhibitor PD153035. T41A β-catenin expressing Huh7 cells were incubated in the absence or presence of AR neutralizing antibodies (αAR, 20μg/mL), control purified goat IgG (IgG, 20μg/mL) or PD153035 (1μM, PD15) for 72h and cell proliferation relative to pcDNA3.1 transfected cells was measured. The effect of these treatments on control pcDNA3.1 transfected cells is also shown. * *P*<0.05 *vs* pcDNA3.1 transfected cells, § *P*<0.05 *vs* untreated T41A β-catenin transfected cells.

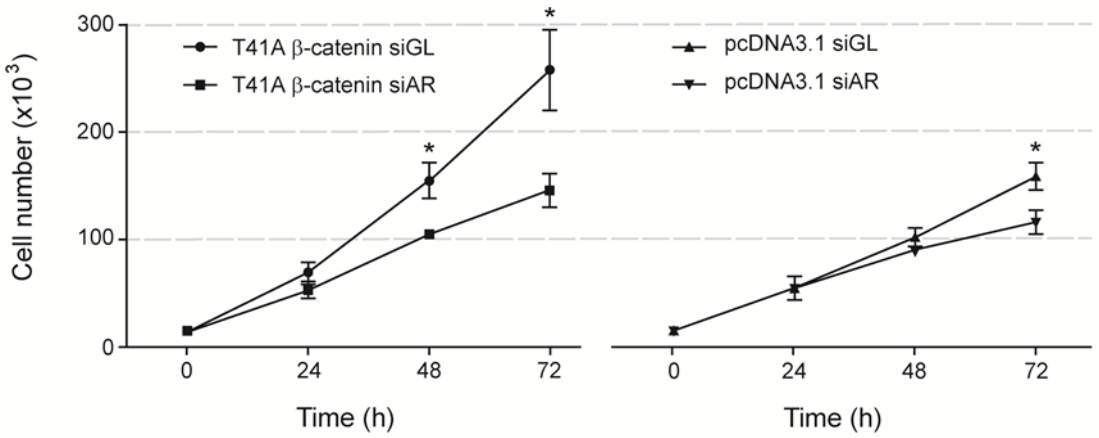


Figure 28: Proliferation of Huh7 cells stably transfected with control vector pcDNA3.1 or the T41A β-catenin active mutant silenciated with siGL or siAR* $P<0.05$ and ** $P<0.01$ *vs* pcDNA3.1 transfected cells.

5. FGF19 activates β-catenin signalling and AR gene expression in human HCC cells.

Aberrant activation of the Wnt/β-catenin pathway in HCC can be accounted for by mutations in the *CTNNB1* gene as well as by altered expression of ligands, receptors and inhibitors of the Wnt pathway (Dahmanin et al. 2011, Bengochea et al. 2008). In addition, β-catenin signalling can be activated through crosstalks with other signalling pathways deregulated in HCC (Dahmanin et al. 2011, Pai et al. 2008; Desbois-Mouthon et al. 2001; Hoshida et al. 2009; Yang et al. 2011). Among these pathways the one driven by FGF19, and its tyrosine kinase receptor FGFR4, is perhaps the best characterized in its functional interaction with the β-catenin system in HCC cells (Sawey et al. 2011). Activation of β-catenin by FGF19 has been already shown in Huh7 cells (Sawey et al. 2011). We decide to examine if this response could be extended to Hep3B cells. Interestly, compared to other HCC cell lines, Hep3B cells expressed higher basal levels of FGF19 (Figure 29A), FGFR4 (Figure 29B), β-Klotho (Figure 29C) and AR (Figure 29D) mRNA. As shown in Figure 30, treatment with FGF19 (50ng/mL), for 24 hours, increased luciferase activity in cells transfected with Tcf-luciferase reporter plasmid as compared to controls. Upon FGF19 treatment cells also displayed increased levels of active dephosphorylated β-catenin and phospho-GSK3β as analyzed by immunoblotting showed in Figure 31, and showed

elevated mRNA levels of the Wnt/β-catenin target gene *Tbx3* (Renard et al. 2007) (Figure 32)when compared to control cells after 5 hours of stimulation with of FGF19.

We next evaluated if FGF19 could affect *AR* gene expression in HCC cells. FGF19 treatment at different concentration for 5 hours resulted in a dose and time-dependent increase in AR mRNA levels (Figure 33A and B) and in an enhanced accumulation of AR protein in the cells conditioned media after a treatment for 24 hours with FGF19 (Figure 33C) in both Hep3B and Huh7 cells.

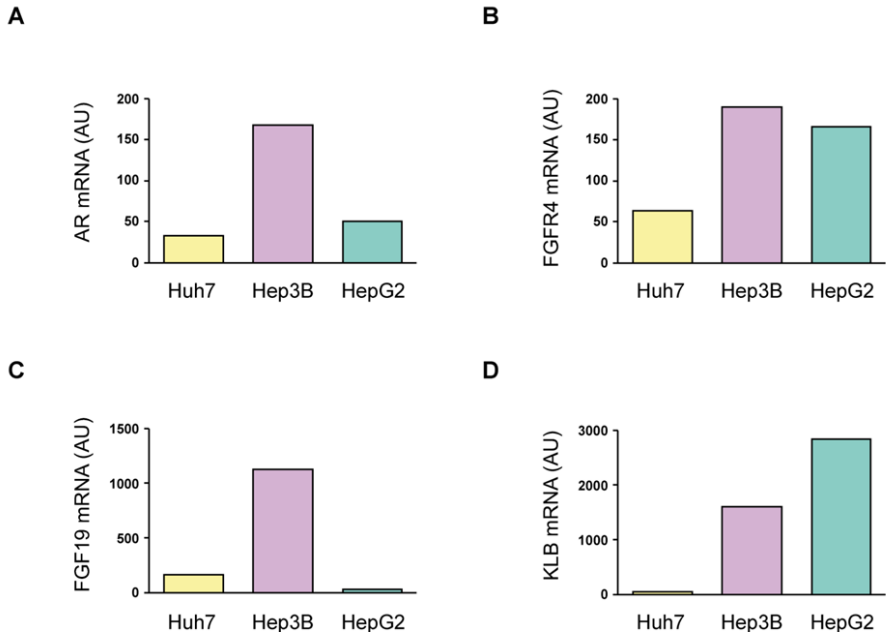


Figure 29: mRNA basal levels of FGF19 (A), FGFR4 (B), β-Klotho (C) and AR (D) mRNA in different HCC cell lines.

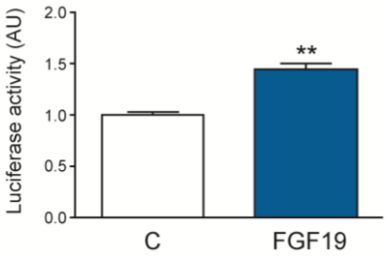


Figure 30: Hep3B cells transfected with a Tcf-regulated reporter vector were treated or not with FGF19 (50ng/mL) for 24h and then was measured luciferase activity. **P*<0.05 *vs* control (C) cells.

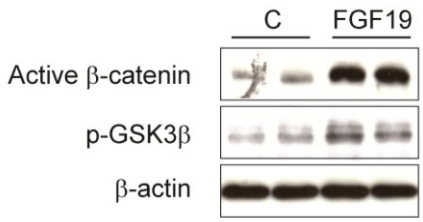


Figure 31: Hep3B cells were treated or not with FGF19 (50ng/mL, 10min) and active β-catenin or phosphorylated GSK3β (p-GSK3β) were detected by western blotting. Representative blots of two experiments performed in duplicates are shown.

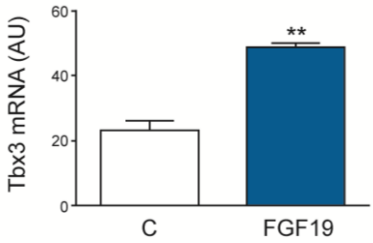


Figure 32: mRNA levels of the β-catenin target gene *Tbx3* in Hep3B cells treated or not with FGF19 (50ng/mL, 5h). **P<0.01 *vs* control.

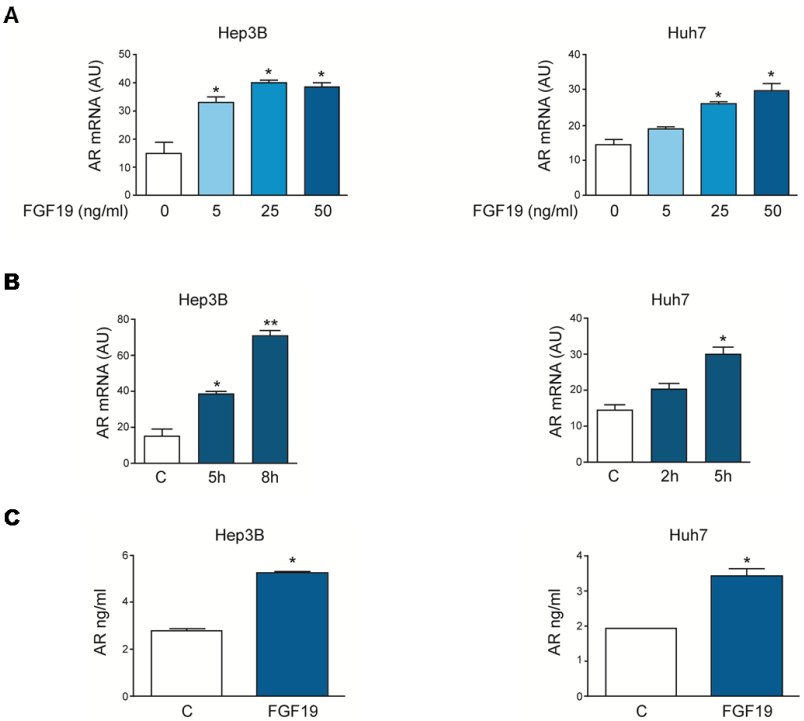
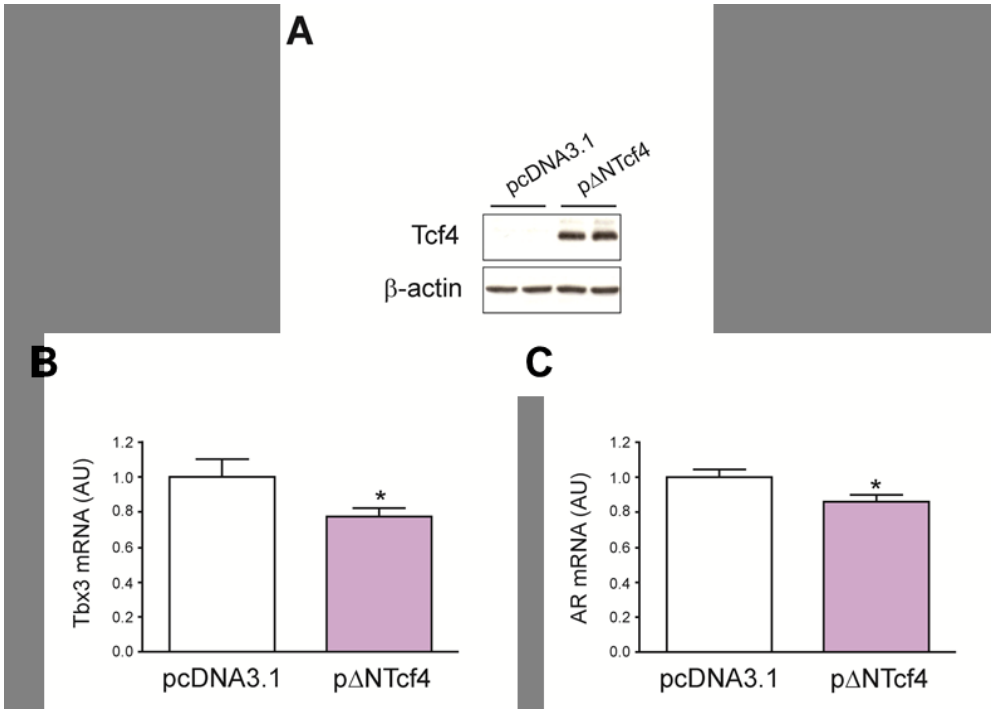


Figure 33: (A) Dose-dependent induction of AR expression (mRNA) in Hep3B and Huh7 cells treated with the indicated concentrations of FGF19 for 5h. (B) Time-dependent induction of AR expression (mRNA) in Hep3B and Huh7 treated with 50ng/mL of FGF19 at the time indicated. (C). AR protein contents were determined by ELISA in the conditioned media of FGF19 treated (50ng/mL, 24h) or control (C) in Hep3B and Huh7 cells. *P<0.05 *vs* control ** P<0.005 *vs* control cells. AU: arbitrary units.

6. Inhibition of β-catenin activity by dominant negative Tcf4 (ΔNTcf4) reduces basal and FGF19-induced AR gene expression in HCC cells.

To evaluate the role played by the β-catenin system in FGF19-mediated AR expression, we made use of a previously reported dominant negative ΔNTcf4 expression vector (Renard et al. 2007). First we transiently transfected HepG2 cells, which express a more stable truncated β-catenin allele (Wege et al. 2011; Yuzugullu et al. 2009), with the control pcDNA3.1 vector or with the ΔNTcf4 expression vector. As shown in Figure 34A, we demonstrated increased Tcf4 protein levels in ΔNTcf4 transfected HepG2 cells analysed by western blotting as compared with control cells. We also demonstrated that expression of ΔNTcf4 resulted in reduced basal expression of the Wnt/β-catenin target gene *Tbx3* and also in decreased AR mRNA levels, confirming the biological activity of this dominant negative Tcf4 mutant and the regulation of *AR* gene expression in response to the manipulation of the β-catenin pathway (Figure 34B and C).

Next we examined the response of ΔNTcf4 expressing HCC cells to FGF19 treatment. Huh7 cells were transiently transfected with the control pcDNA3.1 vector or with the ΔNTcf4 cDNA. The western blot analysis shown in Figure 35A, displays Tcf4 protein levels in control and ΔNTcf4 transfected Huh7 cells 24 hours after transfections. Transfected cells were treated or not for 5 hours with FGF19 (50ng/mL) and we observed that the up-regulation of *Tbx3* expression in Huh7 cells treated with FGF19 was attenuated in cells expressing ΔNTcf4 compared to cells transfected with control plasmid, and a similar response was observed regarding AR expression (Figure 35B and C).



Figure·34: HepG2 cells, that express an active mutant β-catenin allele, were transiently transfected with the control pcDNA3.1 vector or with this vector harbouring ΔNTcf4 cDNA. (A) Tcf4 protein levels in control and ΔNTcf4 transfected HepG2 cells analyzed by western blotting 24h after transfections. In these cells were also analyzed the mRNA levels of the β-catenin target gene *Tbx3* (B) and those of *AR (C)*. *P<0.05 vs cells transfected with pcDNA3.1.

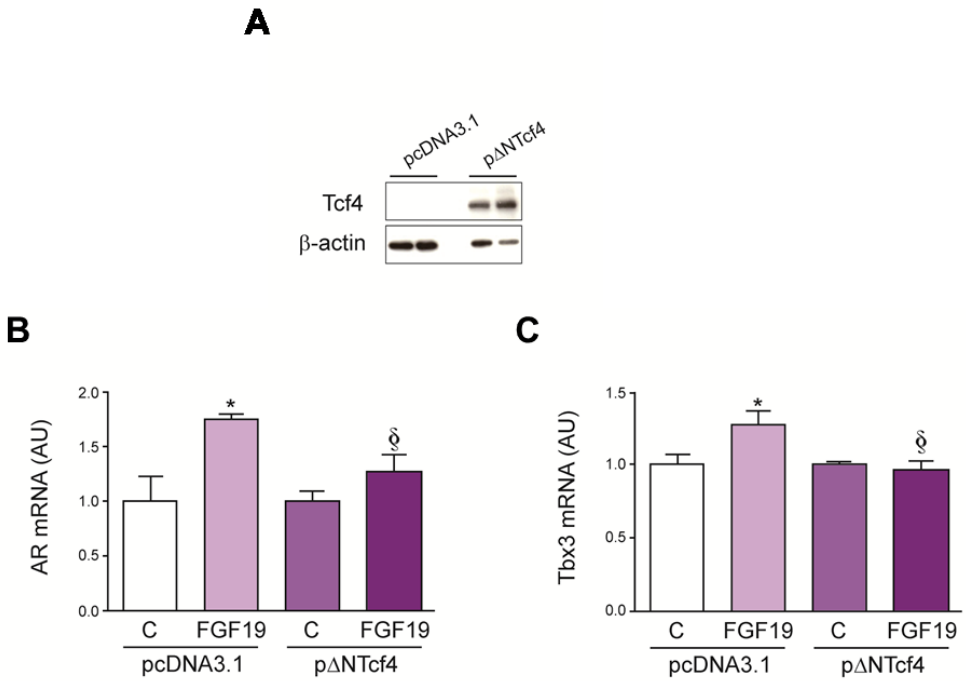


Figure 35: Huh7 cells were transiently transfected with the control pcDNA3.1 vector or with the ΔNTcf4 cDNA. (A) Tcf4 protein levels in control and ΔNTcf4 transfected Huh7 cells analyzed by western blotting 24h after transfections. Transfected cells were then treated or not with FGF19 (50ng/mL, 5h), and the mRNA levels of the the β-catenin target gene *Tbx3* (B) and those of AR (C) were measured. *P<0.05 *vs* control cells (C) transfected with pcDNA3.1. §P<0.05 *vs* cells transfected with pcDNA3.1 and treated with FGF19.

7. *Transactivation of AR promoter by FGF19 treatment depends on the integrity of TBE sites.*

To further evaluate the implication of β-catenin signalling on the FGF19-mediated regulation of AR gene expression, we examined the response of the above-described wild type AR promoter luciferase reporter construct and the three TBE mutants to FGF19 treatment. Hep3B cells were transiently transfected with the empty luciferase reporter plasmid (pGL3), with the wild type AR promoter luciferase reporter construct (ARwt pGL3) and with the three AR promoter constructs in which the three TBE sites had been individually mutated (ART1mut pGL3, ART2mut pGL3 and ART3mut pGL3). Cells were treated with FGF19 for 12 hours and luciferase activity was measured as described in Materials and Methods. As shown in Figure 36, FGF19 stimulation resulted in a significant increase in AR promoter activity. In line with our previous findings in T41A β-catenin active mutant expressing cells, the FGF19-triggered activation of *AR* promoter was markedly reduced when either TBE site was mutated.

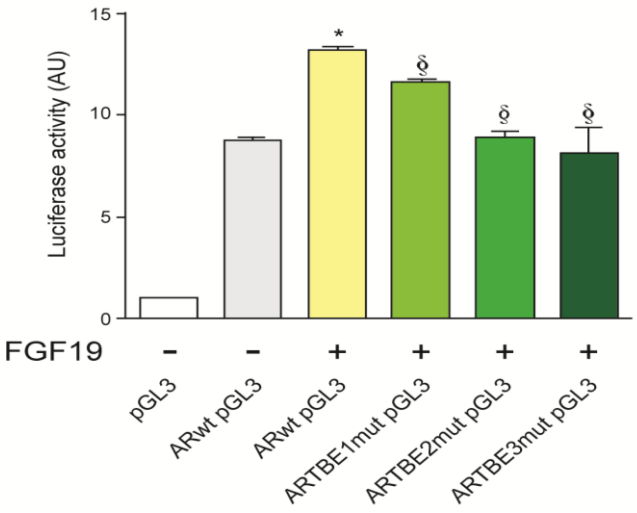


Figure 36: Cells were treated as indicated with FGF19 (25ng/mL, 12h) and luciferase activity was measured as described in Methods. **P*<0.05 *vs* untreated ARwt pGL3 transfected cells. §*P*<0.05 *vs*ARwt pGL3 transfected cells treated with FGF19. AU: arbitrary units.

8. Functional relevance of AR up-regulation in FGF19-induced HCC cell proliferation.

FGF19 has been recently identified as a new growth factor capable of inducing the proliferation of HCC cells and as a driver gene in hepatocarcinogenesis (Sawey et al. 2011; Desnoyers et al. 2008; Miura et al. 2012). Induction of Cyclin D1 expression by FGF19 involves the activation of the β-catenin pathway, and was found to be essential for the promotion of HCC cell growth (Sawey et al. 2011). Considering that AR is a mitogenic factor for HCC cells (Castillo et al. 2006; Desbois-Mouthon et al. 2006), and an FGF19 target gene according to our current findings, we examined the role of FGF19-induced AR in HCC cell proliferation. To do so we knocked down AR expression by transfecting Hep3B cells with AR-specific (siAR) or control siRNAs (siGL) and 24 hours later cells were treated with FGF19 for 5 hours. As shown in Figure 37A, AR mRNA levels elicited by FGF19 treatment were effectively reduced upon transfection with AR-specific siRNAs when compared with those induced in cell transfected with control siGL. Interestingly the stimulation of Cyclin D1 gene expression induced by FGF19, that is two-fold over controls, was blunted when AR mRNA levels were knocked down (Figure 37B) and we also shown in Figure 37C the effect of AR-specific siRNA transfection on AR protein concentration in Hep3B cells conditionated medium. This suggested that AR would be an important mediator of FGF19 effects on the expression of this key cell cycle regulatory gene. To further explore the role of AR in the proliferative effects of FGF19 we measured the growth of Hep3B cells treated with FGF19 in the presence or absence of specific AR neutralizing antibodies (αAR,), control purified goat IgG (αIgG) or PD153035 (PD15) for 48 hours. Under these conditions we could demonstrate a significant reduction, of an 88.7%, in FGF19-induced cell proliferation that was not observed in cells incubated with control IgGs (Figure 38). A role for AR/EGFR signalling in FGF19-mediated cell

proliferation was further supported by the inhibitory effects of the EGFR inhibitor PD153035 on FGF19-mediated cell growth (Figure 38).

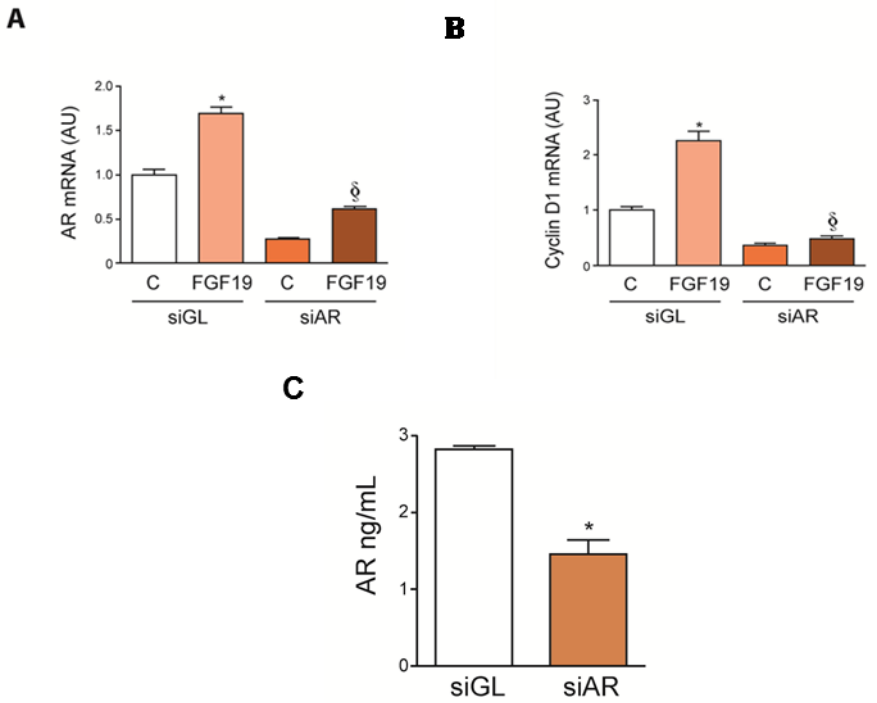


Figure 37: Hep3B cells were transfected with control siRNAs (siGL) or AR specific siRNAs (siAR) and 24h later cells were treated with FGF19 (25ng/mL, 5h). mRNA levels of the the AR (A) and Cyclin D1 (B) were measured. **P*<0.05 *vs* siGL transfected control (C) cells. §*P*<0.05 *vs* siGL transfected and FGF19 treated cells. (C) AR protein concentration in condiotioned medium from siGL and siAR transfected Hep3B cells was determined by ELISA 72 hours after transfections. * *P*<0.05 *vs* siGL transfected control. AU: arbitrary units.

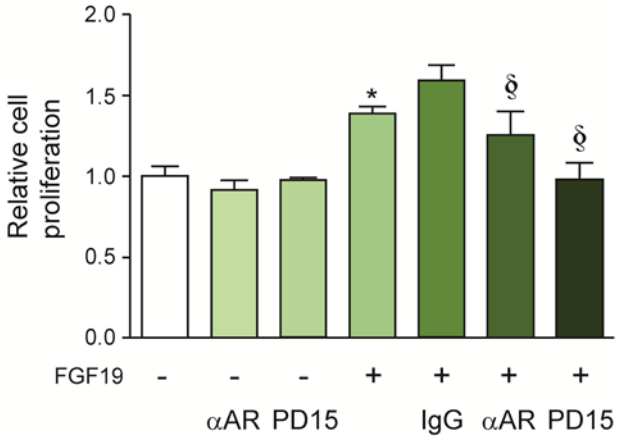


Figure 38: Proliferation induced by FGf19 is attenuated by AR neutralizing antibodies or the EGFR inhibitor PD153035. Hep3B cells were incubated with FGF19 (25ng/mL) as indicated in the absence or presence of AR neutralizing antibodies (αAR, 20μg/mL), control purified goat IgG (IgG, 20μg/mL) or PD153035 (1μM, PD15) for 48h, and cell proliferation relative to untreated cells was measured. The effect of αAR and PD153035 on control (FGF19 untreated) cells is also shown. **P*<0.05 vs untreated cells. §*P*<0.05 *vs* FGF19 treated cells.

9. Correlation between FGF19 and AR gene expression in human HCC tissues.

To further support our present experimental observations on the regulation of AR gene expression by FGF19 in human HCC cell lines we analyzed previously published microarray data from two independent gene expression profiling studies. Data was available from Gene Expression Omnibus (GEO) database for 90 and 239 HCC tumor tissues respectively (Lee et al. 2006; Jia et al. 2007). As shown in Figure 39A and B, our data-mining approach revealed a significant positive correlation between FGF19 and AR mRNA levels in these ample groups of human HCC tissues. In addition, we also examined the expression of AR and FGF19 in the two subclasses of tumors with differential prognosis that were identified in the GSE1989 data set according to their gene expression profile (Lee et al. 2006). Interestingly, we found that the expression of *AR* and *FGF19* gene was higher in the subclass of tumors with strong cell proliferation and anti-apoptosis gene expression signatures, corresponding to patients with poorer prognosis (reduced survival) (Lee et al. 2006) (Figure 39C and D).

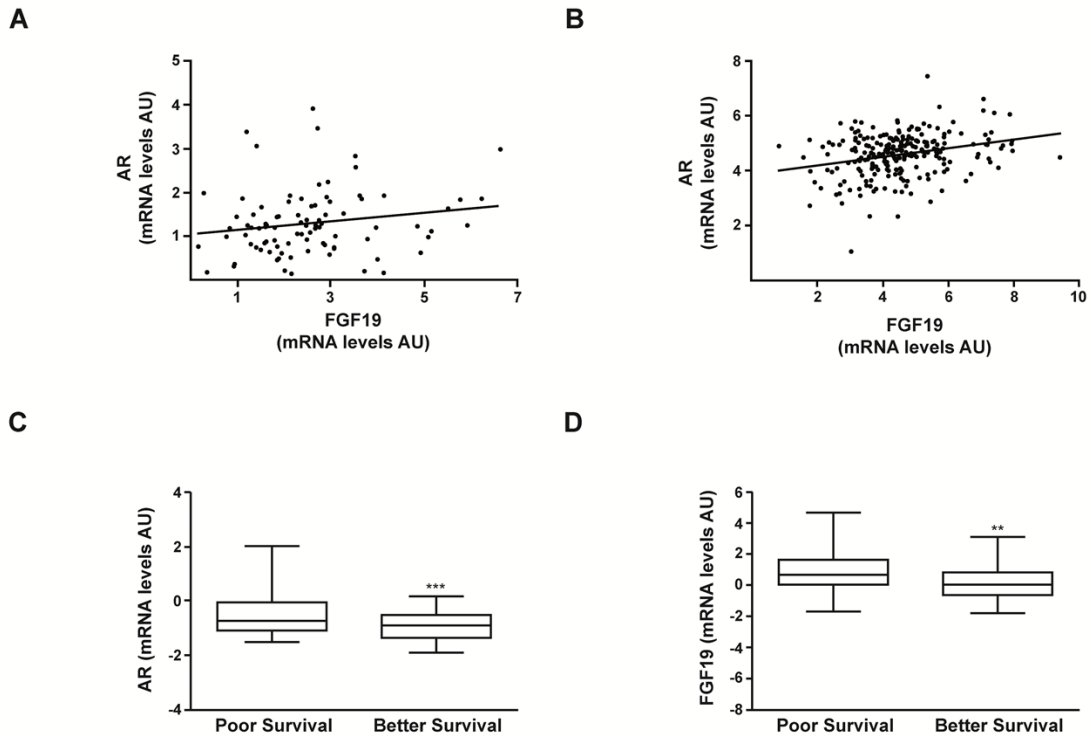


Figure 39: Gene expression data was obtained for *AR* and *FGF19* from two independent microarray studies and correlation of expression levels was analyzed as described in Methods. (A) *AR* and *FGF19* gene expression values were obtained from the GSE1898 dataset (90 HCC tissue samples) and showed a significant positive correlation r = 0.18, *P*<0.05. (B) AR and FGF19 gene expression values were obtained from the GSE5975 dataset (238 HCC tissue samples) and showed a positive correlation r = 0.25, *P*<0.0001. Gene expression data of AR (C) and FGF19 (D) in the subclasses of tumors corresponding to patients with poor and better survival originally identified in the GSE1898 dataset (90 HCC tissue samples) ***P= 0.0005, ** *P*<0.0068.

DISCUSSION

The EGFR ligand AR is increasingly recognized as a potent oncogenic factor over-expressed in a variety of human cancers including HCC (Busser et al. 2011; Berasain et al. 2007a). AR has been demonstrated to participate in most of the characteristic traits of transformed cells including self-sufficiency in generating growth signals, limitless replicative potential, tissue invasion and metastasis, angiogenesis and resistance to apoptosis. The mechanisms responsible for AR upregulation in transformed cells are complex and not fully understood and may vary from one tumor subtype to another. In addition, it is difficult to assess whether AR mediates the acquisition of these various neoplastic phenotypes by itself or in cooperation with other growth factors and/or receptors. However, the intrinsic role of AR in tumorigenesis has been demonstrated in multiple types of cancer by direct and/or indirect mechanisms without compensation by other EGF family members. On the other hand, even if the EGFR is the only receptor for AR, some studies have shown that AR is able to activate other receptors. Various molecules are known to crosstalk with the EGFR, such as cMET (Puri and Salgia 2008), integrins (Ricono et al. 2009), G-protein coupled receptors (Thomas et al. 2006) and the androgen receptor (Recchia et al. 2009). Moreover, direct interactions or heterodimerization between the ErbB members have been well characterized (Yarden and Sliwkoski 2001), as have interactions with other membrane receptors, such as the PDGFR (Saito et al. 2001) and the IGF1R (Morgillo et al. 2006). Thus, the AR-induced oncogenic signaling network is dramatically more complex than previously thought. AR has pleiotropic effects on cancer cells, and it may function as a stimulatory or inhibitory growth factor depending on the phenotype of and environment surrounding a given cell (Busser et al. 2011).

The lack of clinical efficacy of existing systemic chemotherapies and subsequent dismal prognosis in unresectable HCC patients with macrovascular invasion or extrahepatic metastasis have prompted many biologic and clinical researchers to study in detail the molecular mechanisms implicated in hepatocarcinogenesis. As known the molecular

pathogenesis of HCC is complex and heterogeneous, comprising a multistep process that accounts for the serial accumulation of genomic alterations involved in the development and progression of the tumor (Farazi and de Pinho 2006; Fransvea et al. 2009). Further understanding of pathogenetic characterization of HCC at the molecular level, which mainly consists of the specific interaction of both the extracellular growth factors-receptors and the intracellular signal transduction pathways, also helps researchers to identify the potential therapeutic targets in a certain step of signaling cascades for the treatment of this tumor (Song 2009). A multitude of different molecular signaling pathways are affected in liver cancer cells making it difficult to focus molecular treatments. Of major interest are the growth factors and their receptors as well as their signaling pathways. The molecular targeting of these factors has become a promising approach for the effective treatment of HCC. HCC is highly resistant to conventional chemotherapy. However, the impressive advances in the knowledge of tumor biology taken place during the last years and the remarkable success of targeted therapy in other tumors (either alone or in combination with cytostatics) has opened promising avenues for HCC therapy. In HCC multiple molecular alterations ensure the incessant growth of the neoplastic cells. Anti-EGFR agents were initially tested for the treatment of epithelial cancers such as non-small-cell lung cancer (NSCLC), in which the expression and function of the EGFR signaling system is dysregulated (Dannemberg et al. 2005; Ciardiello and Tortora 2008). Two classes of EGFR antagonists are currently available: anti-EGFR monoclonal antibodies and small-molecule EGFR tyrosine kinase inhibitors (Ciardiello and Tortora 2008). Monoclonal antibodies, such as cetuximab, bind to the extracellular domain of the EGFR, blocking ligand binding and receptor activation (Ciardiello and Tortora 2008; Sattler et al. 2008). Tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, compete with ATP to bind to the intracellular catalytic domain of EGFR tyrosine kinase and inhibit downstream signaling from the receptor (Ciardiello and Tortora 2008; Sattler et al. 2008). Phase 2 trials with the EGFR TKI erlotinib have yielded encouraging results in

advanced HCC (Philip et al. 2005). However, the somatic mutations in exons 18 to 21 of EGFR that correspond to the response of non–small cell lung cancers to gefitinib (Lynch et al. 2004) have not been demonstrated in HCC, suggesting that this therapy may not be useful as a single-agent therapy for HCC (Su et al. 2005). Combination therapies may prove more effective because blockade of EGFR by monoclonal antibodies or TKIs can enhance the antitumor activity of radiation and conventional chemotherapeutic agents (Huether et al. 2005a; Huether et al. 2005b; Zhu et al. 2005; Buckley et al 2008). Crosstalks with other signaling systems that are also dysregulated in HCC, such as the IGF2/IGF1R, and the constitutive activation of downstream signaling effectors, have been invoked. It was demonstrated that while IGF-2 exerted its mitogenic effect on HCC cells through AR mediated transactivation of the EGFR, its pro-survival activity was mediated through the PI3K pathway and was completely resistant to EGFR inhibitors (Desbois-Mouthon et al. 2006). Interestingly, combination of EGFR and IGF1R inhibitors overcomes resistance to EGFR blockade and results in enhanced HCC cell killing in preclinical studies (Höpfner et al. 2008; Desbois-Mouthon et al. 2006). Moreover, simultaneous targeting of downstream signaling effectors of the EGFR and IGF1R pathways such as mTOR enhanced therapeutic efficacy in experimental models (Höpfner et al. 2008; Villanueva et al. 2008). These findings highlight the molecular complexity of HCC and provide a rationale to test combination of targeted therapies in HCC patients. These combinations may also include inhibitors of the COX2 system, which as previously stated extensively crosstalks with the EGFR axis (Wu et al. 2006) or newly developed inhibitors of ADAM17, which have shown promising results in the inhibition of colorectal cancer cell growth (Merchant et al. 2008). Additionally, although ErbB2 overexpression is not common in HCC, given the capacity of ErbB receptors to form heterodimers and the potent intracellular signaling generated from heterodimeric complexes (Bublil et al. 2007), a dual EGFR and ErbB2 inhibitor, lapatinib, is currently being tested in experimental HCC and early clinical trials (Llovet and Bruix 2008; Berasain et al. 2009).

Most probably, the combination of agents with different molecular targets as the growth factor receptor inhibitors or cytostatics will improve the clinical response in patients with advanced HCC with tolerable and manageable side effects. Furthermore, the use of targeted therapies such as Sorafenib, that are already approved for therapy in advanced HCC, might also improve HCC recurrence in patients who undergo resection, local ablation and transarterial chemoembolization, which is currently being tested in several ongoing clinical trials (Faloppi et al. 2011). Since the blockade of a specific target can be overcome by other molecular abnormalities, it is likely that HCC could easily develop resistance to compounds that hit a single molecule. Drugs designed to block different growth-promoting pathways (promiscuous drugs such as multiple kinase inhibitors) or combination of different targeted therapies might however attain success in the control of liver cancer. As shown in the case of breast cancer, targeted therapy against molecules conveying growth-promoting signals may render HCC sensitive to specific chemotherapeutic protocols. In the future methods to analyse the molecular signature of each HCC might make possible to select the appropriate combination of targeted therapies that should be used in the particular patient. However toxic side effects of drug combinations will be a risk especially in patients with underlying liver cirrhosis and/or poor liver function (Avila et al. 2006). AR is also involved in resistance to several cancer treatments and therefore constitutes a promising therapeutic target and a validable predictive and prognostic biomarker. Down-regulation of AR expression and availability, as well as the use of AR-neutralizing antibodies, have been demonstrated to attenuate the growth of hepatocellular carcinoma cells (Castillo et al. 2006) and abolish the anti-apoptotic activity of AR in lung adenocarcinoma cells (Hurbin et al. 2002), showing promising pre-clinical results suggesting that these strategies could enhance the effectiveness of other targeted and conventional antitumoral approaches (Busser et al. 2011; Blivet-Van Eggelpoël et al. 2012; Berasain et al. 2007a; Miyamoto et al. 2009). To this end, understanding the mechanisms leading to AR overexpression in cancer cells,

including HCC cells is an important albeit unresolved issue (Busser et al. 2011). Through different complementary approaches we demonstrate in this study that *AR* gene expression can be activated in human HCC cells by β-catenin signalling, a key signalling pathway in hepatocarcinogenesis (Nault and Zucman-Rossi 2011; Avila et al. 2006; Breuhahn et al. 2010; Whittaker et al. 2010; Calvisi et al. 2012; Dahmanin et al. 2011; Bengochea et al. 2008).

Various molecular and genetic factors participate to the aberrant activation of the Wnt/β-catenin pathway in HCC. Firstly, mutations are frequently identified in genes encoding for the main actors of the pathway. Gain-of-function mutations of *CTNNB1* (encoding for β-catenin) are encountered in about one third of HCCs and define *CTNNB1* as the most frequently mutated gene in HCCs. Conversely, loss-of-function mutations of negative regulators of the pathway are also observed, namely mutations of the *AXIN1* and *AXIN2* genes (<5%) and of the *APC* gene (exceptional). Beside such mutational events, the Wnt/β-catenin pathway can be also activated in HCCs as a consequence of a deregulated dialogue between the tumor cells and their microenvironment. For instance, an autocrine Wnt stimulation loop can be established following epigenetic events that change the expression profiles of the ligands, the extracellular inhibitors and the receptors of the Wnt family. A modified crosstalk with other signaling pathways such as HGF or TGF-β can also account for the aberrant activation of the Wnt/β-catenin pathway in HCCs (Dahmanin et al. 2011).

AR gene expression was regulated at transcriptional level by different factors involved in cancer. An evolutionarily conserved cAMP response element (CRE) sequence exists close to the transcriptional start site of the AR gene. It has been demonstrated (Comerford et al. 2003, Taylor et al. 1999, Taylor and Colgabn 1999) a role for the CRE binding protein (CREB) in controlling hypoxia-regulated gene expression. CREB was initially described as a signal-dependent activator of genes such as somatostatin, via PKA-dependent phosphorylation of Ser133 (Andrisani and Dixon 1990; Maxwell and Rotcliffe 2002; Mayr

and Montminy 2001). However, it has been reported (Comerford et al. 2003; Taylor et al. 1999; Taylor et al. 2000; Zhao and Brinton 2004) that CREB may act as a repressor for genes including TNF-α. Thus CREB plays an ambiguous role in the regulation of transcription that appears to be gene specific. In each case, however, CREB binds to an 8bp palindrome (5'-TGACGTCA-3') known as the CRE that regulates transcription. Hypoxic sensitivity of the amphiregulin promoter is retained in a short fragment directly upstream from the TATA box that contains a sole CRE motif. Interestingly, this motif was recently demonstrated to mediate amphiregulin expression in response to prostaglandin E2 (Shao et al. 2003). It is known that hypoxia alters CREB expression and subsequently transcriptional activity in intestinal epithelial cells, an event dependent on decreased activity of protein phosphatase-1γ (Taylor et al. 1999; Taylor and Colgan 1999). Thus hypoxia activates intestinal epithelial amphiregulin expression in transformed cells in a manner dependent on functional activity of a CRE motif situated 37bp upstream of the transcriptional start site. The hypoxia elicited amphiregulin expression may contribute to continued cell proliferation in focal areas of hypoxia found in a range of epithelial tumors (O'Reilly et al. 2005). BRCA1 (Breast Cancer 1) has been reported to regulate transcription on a variety of promoters, however on its own binds DNA only in a sequence non-specific manner (Paull et al. 2001, Simons et al. 2006). BRCA1 can regulate transcription by interacting with other transcription factors which can bind to specific sites on DNA and in this way BRCA1 alters the function of these transcription factors (Mullan et al. 2006; Benezra et al. 2003). Two independent BRCA1-response elements has been identified on the AR promoter located at −202/−182 and +19/+122 positions and exist a clear connection between the tumor suppressor BRCA1 loss of function and the AR upregulation, important for the development and progression of breast cancer (Lamber et al 2010). Here we provide evidence showing that AR is a transcriptional target of the β-catenin/Tcf4 complex, including the demonstration of direct binding of β-catenin and Tcf4 to three putative TBE sites in the AR promoter region upon β-catenin pathway activation.

Endogenous AR expression and secretion to the extracellular medium was induced in HCC cells in response to the ectopic expression of the dominant stable β-catenin mutant T41A (Renald et al. 2007). Conversely, AR mRNA levels, together with those of the well-characterized β-catenin target gene *Tbx3* (Renald et al. 2007), were reduced by the expression of a dominant ΔNTcf4 in HepG2 cells, which express a stable β-catenin allele of 75KDa (Renald et al. 2007; Zeng et al. 2007; Wege et al. 2011). Furthermore, AR promoter-reporter activity triggered by the expression of this active β-catenin variant was reduced upon mutation of the TBE sites within this AR 5' region. Together, these observations strongly suggest that AR is a target of β-catenin signalling in human HCC cells. Nevertheless, β-catenin signalling activates a wide variety of target genes that putatively could be involved in HCC tumorigenesis and malignant behaviour (Dahamanin et al. 2011; Renald et al. 2007; White et al. 2012; Nejak-Bowen and Monga 2011). In this context, establishing the functional significance of β-catenin-stimulated AR gene expression was therefore important. Expression of active β-catenin variants such as the S33Y mutant in Huh7 cells has been shown to increase their growth (White et al. 2012). Here we demonstrate that the T41A β-catenin mutant also enhanced Huh7 cell proliferation. Interestingly, proliferation of T41A β-catenin expressing Huh7 cells was significantly reduced when EGFR activity was inhibited with PD153035, and in the presence of specific AR-neutralizing antibodies. These findings demonstrate that increased AR expression in response to β-catenin pathway activation indeed plays a role in the growth elicited by enhanced β-catenin signalling in HCC cells.

Functional crosstalk between Wnt-triggered signalling and the EGFR system have been previously identified in other cancer cells of epithelial origin such as breast cancer cells, where Wnt overexpression activates signaling via EGFR. In HC11 mammary epithelial cells, constitutive expression of Wnt1 and Wnt5a accompanies activation of EGFR and MAPK (Hu and Li 2010). In these cells Wnt signalling increased the availability of EGFR ligands, leading to EGFR transactivation, cyclin D1 expression and cell proliferation

(Civenni et al. 2003; Schlange et al. 2007). However, these effects occurred through mechanisms independent from the canonical Wnt signalling pathway, and were linked to Src-mediated activation of the metalloproteases that mediate the release of EGFR ligands from the cell surface (Schlange et al. 2007). Many studies indicate that Wnt and EGFR signaling crosstalk via receptor tyrosine kinase pathways. EGFR mediated PI3K/AKT activation promotes β-catenin transactivation and tumor cell invasion, suggesting that EGFR activation transactivate β-catenin activity via receptor tyrosine kinase pathways in tumor cells (Lu et al. 2003; Ji et al. 2009; Sharma et al. 2002; Agarwal et al. 2005). In breast cancers, upregulation of Wnt-1 induces EGFR and Erk1/2 MAPK activation. In APC deficient mice, Wnt activity causes EGFR/PI3K/AKT activation (Hu and Li 2010). Moreover, a recent study demonstrated that the high endogenous expression of AR in breast cancer cells potently and constitutively down-regulates the expression of Dikkopf1, a secreted inhibitor of the Wnt/β-catenin pathway (Baillo et al. 2011). These findings further illustrate the multifarious interactions between the Wnt/β-catenin and EGFR systems in cancer cells. Our current observations provide a novel point of convergence between EGFR and β-catenin pathways in human HCC cells, in this case through the regulation of the expression of the EGFR ligand AR by β-catenin signalling.

β-catenin acts in conjunction with the Tcf family of transcription factors to activate the expression of specific oncogenes and thus promote cell proliferation (Moon et al. 2004, Coleman 2003). In 33–67% of HCC patients, nuclear and/or cellular accumulation of β-catenin have been closely associated with clinico-pathological characteristics such as a poorly differentiated morphology, high proliferative activity, vascular invasion and dismal prognosis (Coleman 2003; Endo et al. 2000). In addition, mutation of β-catenin (leading to its nuclear and/or cytoplasmic accumulation) has been reported in 10–20% of all HCC, and in more than 40% of HCCs associated with hepatitis C virus infection (Hsu et al. 2000; Inagawa et al. 2002; Huang et al. 1999), but with lower frequency in other types of human cancers (Cieply et al. 2009; Sparks et al. 1998). Such oncogenic mutation of β-catenin

renders it capable of bypassing APC-targeted degradation and accumulating in the nucleus to form a complex with Tcf4, thereby aberrantly activating downstream transcriptional events (Huang et al. 1999, Rubinfeld et al. 1997). Thus, interfering with the Wnt/β-catenin signaling pathway may provide a potential opportunity for the development of novel and highly selective drugs for the treatment of HCC. Targeting of the aberrant activation of Wnt/β-catenin pathway has previously been proposed as a promising strategy in cancer therapy (Morin et al. 1997, You et al. 2004). A recent study reported the use of a high throughput screening assay to identify natural compounds that can disrupt the oncogenic Tcf4/β-catenin complex in vitro (Avila et al. 2006; Wei et al. 2010). Several small molecule antagonists such as PKF118-310, PKF115-584, CPG049090 selectively interfered with the binding of β-catenin and Tcf4, and also inhibited the proliferation of colon and prostate and HCC cancer cell lines *in vitro* and *in vitro*. (Wei et al. 2010).

As previously mentioned several mechanisms account for β-catenin pathway activation in HCC cells (Nault and Zucman-Rossi 2011; Dahmanin et al. 2011; Bengochea et al. 2008; White et al. 2012; Yuzugullu et al. 2009; Nejak-Bowen and Monga 2011). In addition, signalling pathways commonly activated in HCC cells such as those triggered by FGF19, TGFβ or the HGF receptor MET, have been reported to stimulate β-catenin signalling (Sawey et al. 2011; Hoshida et al. 2009; Monga et al. 2002). Activation of β-catenin through the FGF19/FGFR4 system has been thoroughly characterized in human HCC and colon cancer cells (Sawey et al. 2011; Pai et al. 2008). This, together with previous evidences showing the ability of the closely-related FGFR1 receptor to up-regulate AR expression in epithelial cells (Bade et al. 2011), led us to examine whether AR could be a target of FGF19/FGFR4 signalling. Here we first demonstrate that FGF19 is a novel inducer of AR gene expression in human HCC cells. Moreover, the inhibitory effect of dominant negative ΔNTcf4 on FGF19 induced AR expression, as well as the attenuated response to FGF19 of AR promoter-reporter constructs with mutated TBE sites; strongly suggest that β-catenin signalling is involved in this response. FGF19 has been recently identified as a driver gene

in human hepatocarcinogenesis (Sawey et al. 2011). FGF19 gene expression is elevated in liver tumors correlating with a poor prognosis, and FGF19 neutralizing antibodies inhibit experimental HCC growth (Desnoyers et al. 2008; Miura et al. 2012). In HCC cells FGF19 signals through the FGFR4/β-klotho complex, which is also frequently up-regulated in human HCC tissues and is emerging as a good candidate anti-tumor target (Bade et al. 2011; Gauglhofer et al. 2011; Poh et al. 2012; French et al. 2012). Although the intracellular pro-tumorigenic mechanisms triggered by FGF19/FGFR4/β-klotho signaling are not completely known induction of cyclin D1 expression is considered a key event, and this effect also involved the activation of β-catenin signaling (Sawey et al. 2011). Interestingly, we found that knockdown of AR expression blunted the induction of cyclin D1 mediated by FGF19 in HCC cells. In line with this finding we also observed that upon EGFR inhibition, or in the presence of AR neutralizing antibodies, the proliferative effects of FGF19 on HCC cells were significatively attenuated.

As known, FGFR4 is required for FGF19-mediated liver tumorigenesis in vivo and show that treatment with an FGFR4 neutralizing antibody (LD1) inhibited FGFR4-mediated signaling, proliferation, and colony formation in cell-based assays and tumor growth in preclinical models of HCC in vivo. Are also known that FGFR4 expression is elevated in several types of cancer, including liver cancer, as compared to normal tissues. These findings provide evidence for a modulatory role of FGFR4 in HCC development and progression and suggest that FGFR4 may be an important and novel therapeutic target in treating this disease (French et al. 2012).

Taken together our results identify AR as a new downstream target of FGF19 in HCC cells, and reveal a functional cross-talk in which the AR/EGFR system mediates, and likely amplifies, the tumor-promoting effects of FGF19. Our finding that *AR* and *FGF19* gene expression levels showed a positive correlation in human HCC tissues supports the clinical relevance for our experimental observations. Moreover, the increased expression of these two genes in a subclass of tumors corresponding to patients with poorer prognosis (Lee et

al. 2006) suggests their potential contribution to the progression of the disease. From a translational point of view, our observations also suggest that the efficacy of FGF19/FGFR4 targeted therapies that are currently being devised for HCC treatment (Desnoyers et al. 2008; Bade et al. 2011; French et al. 2012) could be enhanced by simultaneously interfering with the AR/EGFR system.

CONCLUSIONS

1. AR is a transcriptional target of β-catenin in HCC cells.

2. β-catenin-mediated AR expression participate in the pro-mitogenic effects of mutant β-catenin variant (T41A) in HCC cells.

3. FGF19 is a novel agonist of AR expression in HCC cells through the activation of β-catenin signaling.

4. FGF19-induced AR mediates in part the proliferative effects of FGF19 in HCC cells.

5. AR and FGF19 gene expression levels showed a positive correlation and asociated with a poor prognosis in human HCC tissues supports the clinical relevance for our experimental observations.

6. From a translational point of view, our observations also suggest that the efficacy of FGF19/FGFR4 targeted therapies that are currently being devised for HCC treatment could be enhanced by simultaneously interfering with the AR/EGFR system.

REFERENCES

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. Betacatenin is a target for the ubiquitin-proteasome pathway. 1997, *EMBO J,* **16**:3797-3804.

Adams TE, McKern NM, Ward CW. Signaling by the type 1 insuline-like growth factor receptor: interplay with the epidermal growth factor receptor. 2004, *Growth Factors,* **22**:89-95.

Agarwal A, Das K, Lerner N, Sathe S, Cicek M, Casey G, Sizemore N: The AKT/I kappa B kinase pathway promotes angiogenic/metastatic gene expression in colorectal cancer by activating nuclear factor-kappa B and beta-catenin. 2005, *Oncogene*, **24**:1021-1031.

Akiyama T. Wnt/β-catenin signaling. 2000, *Cytokine & Growth Factor Reviews*, **11**:273-282.

Andrisani OM and Dixon JE. Somatostatin gene regulation. 1990, *Annu Rev Physiol* **52**:793–806.

Angers S, Moon RT. Proximal events in Wnt signal transduction. 2009, *Nat. Rev. Mol. Cell Biol,* **10**(7):468–77.

Anthony P. Pathology of the Liver (eds MacSween R, Burt A, Portmann B, Ishak K, Scheuer P. &Anthony P.) 2002, 711–775 (Churchill Livingstone, London,New York, Sydney, Toronto).

Anzola M. Hepatocellular carcinoma: role of hepatitis B and hepatitis C viruses proteins in hepatocarcinogenesis. 2004, *Journal of Viral Hepatitis*, **11**:383–393.

Asada M, Shinomiya M, Suzuky M et al. Glycosaminoglycan affinity for the complete fibroblast growth factor family. 2009, *BBA*, **1790**:40-48.

Avila MA, Berasain C, Sangro B, Prieto J. New therapies for hepatocellular carcinoma. 2006, *Oncogene*, **25**:3866-3884.

Bade LK, Goldberg JE, DeHut HA, Hall MK, Schwertfeger KL. Mammary tumorigenesis induced by fibroblast growth factor receptor 1 requires activation of the epidermal grwoth factor receptor. 2011, *J Cell Sci*, **124**:3106-3117.

Baillo A, Giroux C, Ethier SP. Knock-down of amphiregulin inhibits cellular invasion in inflammatory breast cancer. 2011, *J Cell Physiol*, **226**:2691-2701.

Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. 2009, *Nature Reviews Drug Discovery*, **8**:235-253.

Benezra M, Chevallier N, Morrison DJ, MacLachlan TK, El-Deiry WS, Licht JD. BRCA1 augments transcription by the NF-kappaB transcription factor by binding to the Rel domain of the p65/RelA subunit. 2003, *J Biol Chem,***278**:26333–41.

Bengochea A, de Souza MM, Lefrançois L, Le Roux E, Galy O, et al. Common dysregulation of Wnt/Frizzled receptor elements in human hepatocellular carcinoma. 2008, *Brit J Cancer*, **99**:143-150.

Benhamouche S, Decaens T, Godard C, Chambrey R, Rickman DS, Moinard C, et al. Apc tumor suppressor gene is the ‘‘zonation-keeper’’ of mouse liver. 2006, *Dev Cell*, **10**:759-70.

Berasain C, Castillo J, Perugorria MJ, Prieto J, Avila MA. Amphiregulin: a new growth factor in hepatocarcinogenesis. 2007a, *Cancer Lett*, **254**:30-41.

Berasain C, Castillo J, Prieto J and Avila MA. New molecular targets for hepatocellular carcinoma: the ErbB1 Signaling system. 2007b, *Liver Int*, **27**(2):174-85.

Berasain C, Garcia-Trevijano ER, Castillo J, Erroba E, Lee DC, Prieto J, et al. Amphiregulin: an early trigger of live regeneration in mice. 2005a, *Gastroenterology* **128**(2):424-32.

Berasain C, Garcia-Trevijano ER, Castillo J, Erroba E, Lee DC, Santamaria M, et al. Novel role for amphiregulin in protection from liver injury. 2005b, *J Biol Chem*, **280**: 19012–19020.

Berasain C, Perugorria MJ, Latasa MU, Castillo J, Goñi S, Santamari´a M,* Prieto J and Avila MA. The Epidermal Growth Factor Receptor: A link between inflammation and liver cancer. 2009, *Exp Biol Med (Maywood)*, **234**(7):713-725.

Blivet-Van Eggelpoël MJ, Chettouh H, Fartoux L, Aoudjehane L, Barbu V, Rey C, Priam S, Housset C, Rosmorduc O, Desbois-Mouthon C. Epidermal growth factor receptor

and HER-3 restrict cell response to sorafenib in hepatocellular carcinoma cells. 2012 *Journal of Hepatology*, **57**: 108-115.

Blobel CP. ADAMS. Key components in EGFR signaling and development. 2005, *Nat Rev Mol Cell Biol*, **6:**32–43.

Bosch FX, Ribes J & Borràs J. Epidemiology of primary liver cancer. 1999, *Semin Liver Dis*, **19**:271–285.

Bréchot C. Molecular mechanisms of hepatitis B and C related to liver carcinogenesis. 1998, *Hepatogastroenterology*, **45**(Suppl 3):1189–1196.

Breuhahn K, Longerich T and Schirmacher P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. 2006, *Oncogene*, **25**:3787–3800.

Breuhahn K, Schirmacher P. Signaling networks in human hepatocarcinogenesis. Novel aspects and therapeutic options. 2010, *Prog Mol Biol Transl Sci,* **97**:251-277.

Brown CL, Meise KS, Plowman GD, Coffey RJ, Dempsey PJ. Cell surface ectodomain cleavage of human amphiregulin precursor is sensitive to a metalloportease inhibitor. 1998, J *Biol Chem*, **273**:17258–17268.

Bublil EM, Yarden Y. The EGF receptor family: spearheading a merger of signaling and therapeutics. 2007, *Curr Opin Cell Biol,* **19**:124–134.

Buckley AF, Burgart LJ, Sahai V and Kakar S. Epidermal Growth Factor Receptor Expression and Gene Copy Number in Conventional Hepatocellular Carcinoma. 2008, *Am J Clin Pathol*, **129**:245-251.

Burgess WH, Dionne CA, Kaplow J, Mudd R, Friesel R, Zilberstein A, Schlessinger J & Jaye M. Characterization and cDNA cloning of phospholipase C-gamma, a major substrate for heparin-binding growth factor 1 (acidic fibroblast growth factor)-activated tyrosine kinase. 1990, *Molecular and Cellular Biology*, **10**:4770–4777.

Busser B, Sancey L, Brambilla E, Coll JL, Hurbin A. The multiple roles of amphiregulin in human cancer. 2011, *Bioch Biophys Acta*, **1816**(2):119–131.

Cadoret A, Ovejero C, Terris B, Souil E, Levy L, Lamers WH, Kitajewski J, Kahn A, Perret C: New targets of beta-catenin signaling in the liver are involved in the glutamine metabolism. 2002, *Oncogene*, **21**:8293-8301.

Calle EE, Rodriguez C, MD, Kimberly Walker-Thurmond BA, and Michael J. Thun. Overweight, Obesity, and Mortality from Cancer in a Prospectively Studied Cohort of U.S. Adults. 2003, *N engl j med*, **348**(17):1625–1638.

Calvisi D, Frau M, Tomasi ML, Feo F, Pascale RM. Deregulation of signalling pathways in prognostic subtypes of hepatocellular carcinoma: Novel insights from interspecies comparison. 2012, *Biochim Biophys Acta*, **1826**:215-237.

Campbell JS, Hughes SD, Gilbertson DG, Palmer TE, Holdren MS, et al. Platelet-derived growth factor C induces liver fibrosis, steatosis, and hepatocellular carcinoma. 2005, *Proc Natl Acad Sci* USA, **102**:3389–3394.

Carraway KL, Weber JL, Unger MJ, Ledesma J, Yu N, Gassmann M, Lai C. Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. 1997, *Nature*, **387**(6632): 512-16.

Castillo J, Erroba E, Perugorria MJ, Santamaria M, Lee DC, et al. () Amphiregulin contributes to the transformed phenotype of human hepatocellular carcinoma cells. 2006, *Cancer Res,* **66**:6129-6138.

Castillo J, Erroba E, Perugorría MJ, Santamaría M, Lee DC, Prieto J, Avila MA and Berasain C. Amphiregulin Contributes to the Transformed Phenotype of Human Hepatocellular Carcinoma Cells.2006, *Cancer Res*; **66**:6129-6138.

Chan DW, Chan CY, Yam JW, Ching YP, Ng IO. Prickle-1 negatively regulates Wnt/beta-catenin pathway by promoting Dishevelled ubiquitination/degradation in liver cancer. 2006, *Gastroenterology*, **131**:1218–27.

Chang H; Riese DJ; Gilbert W; Stern DF; McMahan UJ. Ligands for ErbB-family receptors encoded by a neuregulin-like gene. 1997, *Nature*, **387**(6632): 509-12.

Chomczynsky P and Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenolchloroform extraction. 1987, *Anal Biochem*, **162**:156-159

Ciardiello F and Tortora G. EGFR antagonists in cancer treatment. 2008, *N Engl J Med* **358**:1160–1174.

Cieply B, Zeng G, Proverbs-Singh T, Geller DA, Monga SP. Unique phenotype of hepatocellular cancers with exon-3 mutations in beta-catenin gene. 2009, *Hepatology*; **49**:821–831.

Citri A and Yarden Y. EGF–ERBB signalling: towards the systems level. 2006, *Mol Cell Biol*, **7**(7):505–16.

Civenni G, Holbro T, Hynes NE. Wnt1 and Wnt5a induce cyclin D1 expression through ErbB1 transactivation in HC11 mammary epithelial cells. 2003, *EMBO Reports*, **4**:166-171.

Clevers H. Wnt/beta-catenin signaling in development and disease. 2006, Cell, **127**:469–480.

Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, et al. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene expression. 2000, *Chem Biol*, **7**:793-803.

Coleman WB. Mechanisms of human hepatocarcinogenesis. 2003, *Curr Mol Med*, **3**:573–588.

Comerford KM, Leonard MO, Karhausen J, Carey R, Colgan SP, and Taylor CT. Small ubiquitin-related modifier-1 modification mediates resolution of CREB-dependent responses to hypoxia. 2003, *Proc Natl Acad Sci USA,* **100**:986–991.

Comporti M, Arezzini B, Signorini C, Sgherri C, Monaco B, Gardi C. (2)-isoprostanes stimulate collagen synthesis in activated hepatic stellate cells: alink with liver fibrosis? 2005, *Lab. Invest*, **85**(11):1381–1391.

Cooper O, Vlotides G, Fukuoka H, Greene MI and Melmed S. Expression and function of ErbB receptors and ligands in the pituitary. 2011, *Endocr Relat Cancer*, **18**:R197–R211

Cottrell S, Bicknell D, Kaklamanis L, Bodmer WF. Molecular analysis of APC mutations in familial adenomatous polyposis and sporadic colon carcinomas. 1992, *Lancet*, **340**:626–30.

Dahmanin R, Just PA, Perret C. The Wnt/-catenin pathway as a therapeutic target in human hepatocellular carcinoma. 2011, *Clin Res Hepatol Gastroenterol*, **35**:709-713.

Dailey L, Ambrosetti D, Mansukhani A and Basilico C. Mechanisms underlying differential responses to FGF signaling. 2005, *Cytokine Growth Factor Rev.,* **16**:233-247.

Dannenberg AJ, Lippman SM, Mann JR, Subbaramaiah K, DuBois RN. Cyclooxygenase-2 and epidermal growth factor receptor: pharmacologic targets for chemoprevention. 2005, *J Clin Oncol,* **23**:254–266.

Davidson G, Mao B, Del Barco BI, Niehrs C. Kremen proteins interact with Dickkopf1 to regulate anteroposterior CNS patterning. 2002, *Development*, **129**:5587–5596.

De La Coste A, Romagnolo B, Billuart P, et al. Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. 1998, *Proc Natl Acad Sci USA*, **95**:8847–51.

Decaens T, Godard C, de Reynies A, Rickman DS, Tronche F, Couty JP, et al. Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate. 2008, *Hepatology*, **47**:247—58.

Deo DD, Axelrad TW, Robert EG, Marcheselli V, Bazan NG & Hunt JD. Phosphorylation of STAT-3 in response to basic fibroblast growth factor occurs through a mechanism involving platelet-activating factor, JAK-2, and Src in human umbilical vein endothelial cells. Evidence for a dual kinase mechanism. 2002, J Biol Chem, **277**:21237–21245.

Desbois-Mouthon C, Baron A, Blivet-Van Eggelpoel MJ, Barbu V, Fartoux L, Venot C, Bladt F et al. Insuline-like growth factor-1 receptor inhibition induces a resistance mechanism via the epidermal growth factr receptor/HER3/AKT signaling pathway: rational basis for cotargeting insulin-like growth factor-1 receptor and epidermal growth factor receptor in hepatocellular carcinoma. 2009, *Clin Cancer Res*, **15**:5445-56.

Desbois-Mouthon C, Cacheux W, Blivet-Van Eggelpoe¨l M-J, Barbu V, Fartoux L, Poupon R, Housset C, Rosmorduc O. Impact of IGF1R/EGFR crosstalks on hepatoma cell sensitivity to gefitinib. 2006, *Int J Cancer*, **119**:2557–2566.

Desbois-Mouthon C, Cadoret A, Blivet-Van Eggelpoël MJ, Bertrand F, Cherqui G, et al. Insulin and IGF-1 stimulate the β-catenin pathway through two signaling cascades involving GSK-3b inhibition and Ras activation. 2001, *Oncogene,* **20**:252-259.

Desnoyers LR, Pai R, Ferrando RE, Hötzel K, Le T, et al. Targeting FGF19 inhibits tumor growth in colon cancer xenograft and FGF19 transgenic hepatocellular carcinoma models. 2008, *Oncogene*, **27**:85-97.

Dunn WA, Hubbard AL. Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: ligand and receptor dynamics. 1984, *J Cell Biol*, **98**:2148–2159.

El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. 2007, *Gastroenterology*, **132**:2557-76.

El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. 2004, *Gastroenterology*, **126**(2):460-8.

Emami KH and Corey E. When prostate cancer meets bone: Control by wnts. 2007, *Cancer Letters*, **253:**170–179.

Endo K, Ueda T, Ueyama J, Ohta T, Terada T. Immunoreactive E-cadherin, alpha-catenin, beta-catenin, and gammacatenin proteins in hepatocellular carcinoma: Relationships with tumor grade, clinicopathologic parameters, and patients' survival. 2000, *Hum Pathol*, **31**:558–565.

Faivre EJ, Lange CA: Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. 2007, *Mol Cell Biol*, **27**:466-480.

Faloppi L, Scartozzi M, Maccaroni E, Di Pietro Paolo M, Berardi R, et al. Evolving strategies for the treatment of hepatocellular carcinoma: from clinical-guided to molecularly-tailored therapeutic options. 2011, *Cancer Treat Rev May*; **37**(3): 169–77.

Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. 2006, Nat Rev Cancer; **6**:674-687.

Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. 2004, *Gastroenterology*, **127**(5 Suppl 1):S35-50.

Ferlay J, Bray F, Pisani P and Parkin DM. GLOBOCAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Version 1.0. 2001, *IARC CancerBase*, No.5. Lyon, IARCPress.

Fisher OM, Hart S, Gschwind A, Ullrich A. EGFR signal transactivation in cancer cells. 2003, *Biochem Soc Trans*, **31**:1203-8.

Flaherty MP and Dawn B. Noncanonical wnt11 signaling and cardiomyogenic differentiation. 2008, *Trends Cardiovasc Med*, **18**(7):260–268.

Foley J, Nickerson NK., Nam S, Kah Allen T, Gilmore JL, Nephew KP, Riese DJ. EGFR signaling in breast cancer: Bad to the bone. 2010, *Semin Cell Dev Biol*, **21**(9):951–960.

Foy E, Li K, Sumpter R Jr, Loo YM, Johnson CL, Wang C, Fish PM, Yoneyama M, Fujita T, Lemon SM, Gale M Jr. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. 2005, *Proc. Natl Acad. Sci. USA*, **102**(8):2986–2991.

Fransvea E, Paradiso A, Antonaci S, Giannelli G. HCC heterogeneity: molecular pathogenesis and clinical implications. 2009, *Cell Oncol*; **31**:227-233.

French DM, Lin BC, Wang M, Adams C, Shek T, Hötzel K, et al. Targeting FGFR4 inhibits hepatocellular carcinoma in preclinical mouse models. 2012, *PLoS One*, **7**(5):e36713.

Fukuchi T, Sakamoto M, Tsuda H, Maruyama K, Nozawa S, Hirohashi S: Beta-catenin mutation in carcinoma of the uterine endometrium. 1998, *Cancer Res*, **58**:3526–3528.

Gale M Jr. & Foy EM. Evasion of intracellular hostdefence by hepatitis C virus. 2005, *Nature*, **436**:939–945.

Gauglhofer C, Sagmeister S, Schrottmaier W, Fischer C, Rodgarkia-Dara C, Mohr T, et al. Up-regulation of the fibroblast growth factor 8 subfamily in human hepatocellular carcinoma for cell survival and neoangiogenesis. 2011, *Hepatology*, **53**:854-864.

Gebhardt R, Baldysiak-Figiel A, Krugel V, Ueberham E, Gaunitz F: Hepatocellular expression of glutamine synthetase: an indicator of morphogen actions as master regulators of zonation in adult liver. 2007, *Prog Histochem Cytochem*, **41**:201-266.

Giles RH, van Es JH, Clevers H. Caught up in a Wnt storm: Wnt signaling in cancer. 2003, *Bioch Biophys Acta*, **1653:**1–24.

Graham TA, Weaver C, Mao F, Kimmelman D and Yu W. Crystal structure of a beta-catenin/Tcf complex. 2000, *Cell,* **103:**885-896.

Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. 1997, *EMBO J*, **16**:1647–55.

Hadari YR, Gotoh N, Kouhara H, Lax I & Schlessinger J Critical role for the docking-protein FRS2 alpha in FGF receptor-mediated signal transduction pathways. 2001, *PNAS*, **98**:8578–8583.

Han C, Michalopoulos GK, Wu T. Prostaglandin E2 receptor EP1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. 2006, *J Cell Physiol,* **207**: 558-60.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. 2011, *Cell*, **144:**646–674.

Harari D, Tzahar E, Romano J, Shelly M, Pierce JH, Andrews GC and Yarden Y. Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. 1999, *Oncogene,* **18**:2681-2689.

Harmer NJ, Pellegrini L, Chirgadze D, Fernandez-Recio J, and Blundell TL. The cristal structure of fibroblast growth factor (FGF19) 19 reveals novel features of the FGF family. 2004, *Biochemistry*, **43**:629–640.

Harris RC, Chung E, Coffey RJ. EGF receptors ligands. 2003, *Exp Cell Res*. **283**:2–13.

Hart KC, Robertson SC and Donoghue DJ. Identification of tyrosine residues in constitutively activated fibroblast growth factor receptor 3 involved in mitogenesis, Stat activation, and phosphatidylinositol 3-kinase activation. 2001, *Mol Biol Cell*, **12**:931–942.

Hart KC, Robertson SC, Kanemitsu MY, Meyer AN, Tynan JA & Donoghue DJ Transformation and Stat activation by derivatives of FGFR1, FGFR3, and FGFR4. 2000, *Oncogene*, **19**:3309–3320.

He X, Semenov M, Tamai K, Zeng X. LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. 2004, *Development* (Cambridge, England); **131**:1663–1677.

Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. A heparin-binding growth factor secreted by macrophagelike cells that is related to EGF. 1991, *Science*, **251**:936–939.

Higashiyama S, Lau K, Besner GE, Abraham JA, Klagsbrun M. Structure of heparin-binding EGF-like growth factor. 1992, *J Biol Chem*, **267**:6205–6212.

Hino O, Kajino K, Umeda T & Arakawa Y. Understanding the hypercarcinogenic state in chronic hepatitis: a clue to the prevention of human hepatocellular carcinoma. 2002, *J Gastroenterol*, **37**:883–887.

Ho HK, Pok S, Streit S et al. Fibroblast growth factor receptor 4 regulates proliferation, anti-apoptosis and alpha-fetoprotein secretion during hepatocellular carcinoma progression and represents a potential target for therapeutic intervention. 2009, *J Hepatol*, **50**:118-127.

Hoek JB and Pastorino JG. Ethanol, oxidative stress, and cytokine-induced liver cell injury. 2002, *Alcohol*, **27**:63–68.

Holbro T, Civenni G, Hynes NE. The ErbB receptors and their role in cancer progression. 2003, *Exp Cell Res*, **284**:99–110.

Höpfner M, Schuppan D, Scheru¨ bl H. Growth factor receptors and related signalling pathways as targets for novel treatment strategies of hepatocellular cancer. 2008, *World J Gastroenterol,* **14**:1–14.

Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. 2009, *Cancer Res*, **69**:7385-92.

Hsu HC, Jeng YM, Mao TL, Chu JS, Lai PL, Peng SY. Beta-catenin mutations are associated with a subset of low-stage hepatocellular carcinoma negative for hepatitis B virus and with favorable prognosis. 2000, *Am J Pathol*, **157**:763–770.

Hu T, Li C. Convergence between Wnt-β-catenin and EGFR signalling in cancer. 2010, *Mol Cancer*, **9**:236.

Huang H, Fujii H, Sankila A, et al. Beta-catenin mutations are frequent in human hepatocellular carcinomas associated with hepatitis C virus infection. 1999, *Am J Pathol*, **155**:1795–1801.

Huang HC and Klein PS. The Frizzled family: receptors for multiple signal transduction pathways. 2004, *Genome Biol*, **5**:234.

Huang X, Yang C. Jin C et al. Resident hepatocyte fibroblast growth factor receptor 4 limits hepatocarcinogenesis. 2009, *Mol Carcinog*, **48**:553-562.

Huber AH, Nelson WJ and Weis WI. Threedimensional structure of the armadillo repeat region of betacatenin. 1997, *Cell,* **90**:871-882.

Huether A, Hoepfner M, Baradari V, et al. EGFR blockade by cetuximab alone or as a combination therapy for growth control of hepatocellular carcinoma. 2005a, *Biochem Pharmacol,* **70**:1568-1578.

Huether A, Hoepfner M, Sutter AP, et al. Erlotinib induces cell cycle arrest and apoptosis in hepatocellular cancer cells and enhances chemosensitivity towards cytostatics. 2005b, *J Hepatol,* **43**:661-669.

Hurbin A, Dubrez L, Coll JL, Favrot MC. Inhibition of apoptosis by amphiregulin via an insulin-like growth factor-1 receptor-dependent pathway in non-small cell lung cancer cell lines, 2002, *J Biol Chem*, **277**:49127–49133.

Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. 2005, *Nat Rev Cancer*, **5**:341–54.

Ihara A, Koizumi H, Hashizume R, Uchikoshi T: Expression of epithelial cadherin and a- and β-catenins in nontumoral livers and hepatocellular carcinoma. 1996, *Hepatology*, **23**(6):1441–1447.

Inagawa S, Itabashi M, Adachi S, Kawamoto T, Hori M, Shimazaki J, Yoshimi F, Fukao K. Expression and prognostic roles of beta-catenin in hepatocellular carcinoma: Correlation with tumor progression and postoperative survival. 2002, *Clin Cancer Res*, **8**(2):450–456.

Isokane M, Hieda M, Hirakawa S, Shudou M, Nakashiro K, Hashimoto K, et al. Plasma-membrane-anchored growth factor pro-amphiregulin binds A-type lamin and regulates global transcription. 2008, *J Cell Sci*; **121**:3608–3618.

Ito Y, Takeda T, Sakon M, Tsujimoto M, Higashiyama S, Noda K & cols. Expression and clinical significance of erb-B receptor family in hepatocellular carcinoma. 2001, *Br J Cancer*, **84**:1377-83.

Itoh N, Ornitz DM. Evolution of the Fgf and Fgfr families. 2004, *Trends in Genetics*, **20**(11):563-569.

Iwata T, Hevner RF. Fibroblast growth factor signaling in development of the cerebral cortex. 2009, *Dev Growth Differ*, **51**(3):299-323.

Ji H, Wang J, Nika H, Hawke D, Keezer S, Ge Q, Fang B, Fang X, Fang D, Litchfield DW, Aldape K, Lu Z. EGF-induced ERK activation promotes CK2-mediated disassociation of alpha-Catenin from beta-Catenin and transactivation of beta-Catenin. 2009, *Mol Cell*, **36**(4):547-559.

Jia HL, Ye QH, Qin LX, Budhu A, Forgues M, Chen Y, Liu YK, Sun HC, Wang L, Lu HZ, Shen F, Tang ZY, Wang XW. Gene expression profiling reveals potential biomarkers of human hepatocellular carcinoma. 2007, *Clin Cancer Res*, **13**(4):1133-1139.

Kamegaya, Y. Hiasa Y, Zukerberg L, Fowler N, Blackard JT, Lin W, Choe WH, Schmidt EV, Chung RT. Hepatitis C virus acts as a tumoraccelerator by blocking apoptosis in a mouse model of hepatocarcinogenesis. 2005, *Hepatology*, **41**(3):660–667.

Kataoka Hiromi EGFR ligands and their signaling scissors, ADAMs, as new molecular targets for anticancer treatments. 2009, *J Derm Science*, **56**(3):148–153.

Katoh Y, Katoh M. Canonical WNT signalling pathway and human AREG. 2006, *Int J Mol Med*, **17**:1163-1166.

Kitada T, Seki S, Kawakita, N, Kuroki T & Monna T. Telomere shortening in chronic liver diseases. 1995, *Biochem Biophys Res Commun*, **211**:33–39.

Kobayashi T, Kubota K, Takayama T & Makuuchi M. Telomerase activity as a predictive marker for recurrence of hepatocellular carcinoma after hepatectomy. 2001, *A. J Surg*, **181**:284–288.

Koch A, Denkhaus D, Albrecht S, Leuschner I, von Schweinitz D and Pietsch T. Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the beta-catenin gene. 1999, *Cancer Res.,* **59:**269–273.

Kowdley KV. Iron, hemochromatosis, and hepatocellular carcinoma. 2004, *Gastroenterology*, **127**(5 Suppl 1):S79-86.

Krause DS & van Etten RA. Tyrosine kinases as targets for cancer therapy. 2005, *N Engl J Med*, **353**:172–187.

Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT. The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape, 2000, *Trends Genet*, **16**:279–283.

Kurosu H and Koro-o M. The klotho gene family as a regulator of endocrine fibroblast growth factors. 2009, *Mol Cell Endo*, **299**:72-78.

Kurosu H, Choi M, Ogawa Y, Dickson AS, Goetz R, Eliseenkova AV, Mohammadi M, Rosenblatt KP, Kliewer SA, Kuro-o M. Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. 2007, *J Biol Chem*, **282**(37):26687-95.

Lamber EP, Horwitz AA, and Parvin JD. BRCA1 represses Amphiregulin gene expression. 2010, *Cancer Res*; **70**(3): 996-1005.

Lanner F and Rossant J The role of FGF/Erk signaling in pluripotent cells. 2010, *Development*, **137**:3351-3360.

Laurent-Puig P, Zucman-Rossi J. Genetics of hepatocellular tumors. 2006, *Oncogene*, **25**:3778-86.

Lee CL, Hsieh KS, Ko YC. Trends in the incidence of hepatocellular carcinoma in boys and girls in Taiwan after large-scale hepatitis B vaccination. 2003a, *Cancer Epidemiol Biomarkers Prev,* **12**(1):57-9.

Lee DC, Hinkle CL, Jackson LF, Li S, Sunnarborg SW. EGF family ligands. In: Thomson AW, Lotze MT (Eds.), 2003c, *The Cytokine Handbook. Academic Press,* London, pg. 959–987.

Lee HS, Park MH, Yang SJ, Park KC, Kim NS, Kim YS, Kim DI, Yoo HS, Choi EJ, Yeom YI: Novel candidate targets of Wnt/beta-catenin signaling in hepatoma cells. 2007, *Life Sci*, **80**:690-698.

Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. 2006, *Nat Med*, **12**:410-416.

Lee S, Lee HJ, Kim JH, Lee HS, Jang JJ, Kang GH. Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. 2003b, *Am J Pathol*, **163**:1371–8.

Levasseur R, Lacombe D, de Vernejoul MC. LRP5 mutations in osteoporosis–pseudoglioma syndrome and high-bone-mass disorders. 2005, *Joint Bone Spine*, **72**:207–214.

Li F, Chong ZZ, Maiese K. Winding through the WNT pathway during cellular development and demise. 2006, *Histol Histopathol*, **21**:103–124.

Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, Ray SC, Gale M Jr, Lemon SM. Immune evasion by hepatitis C virus NS3/ 4A protease-mediated cleavage of the Toll-likereceptor 3 adaptor protein TRIF. 2005, *Proc Natl Acad Sci USA*, **102**(8)**:**2992–2997.

Liao YC and Lo SH. Deleted in liver cancer-1 (DLC-1): a tumor suppressor not just for liver. 2008, *Int J Biochem Cell Biol*, **40**(5): 843–847.

Lin BC and Desnoyers LR. FGF19 and cancer. 2012, *Endocrine FGFs and Klothos*, Edited by Makoto Kuro-o.

Lin BC, Wang M, Blackmore C et al. Liver-specific activities of betaklotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activities of FGF19 and FGF21. 2007, *. Biol Chem*, **282**:26687-26695.

Llovet JM and Bruix J. Molecular targeted therapies in hepatocellular carcinoma. 2008, *Hepatology*, **48**:1312–1327.

Loeppen S, Schneider D, Gaunitz F, Gebhardt R, Kurek R, Buchmann A, Schwarz M. Overexpression of glutamine synthetase is associated with beta-catenin-mutations in mouse liver tumors during promotion of hepatocarcinogenesis by phenobarbital. 2002, *Cancer Res*, **62**:5685-5688.

Logan CY and Nusse R. The Wnt signaling pathway in development and disease. 2004, *Annu Rev Cell Dev Biol*, **20**:781–810.

Lu Z, Ghosh S, Wang Z, Hunter T: Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. 2003, *Cancer Cell*, **4**:499-515.

Luetteke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, Chang A, Lee DC. Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. 1999, *Development*, **126**(12):2739–2750.

Luo J. Glycogen synthase kinase 3b (GSK3b) in tumorigenesis and cancer chemotherapy. 2009, *Cancer Letters,* **273**:194–200.

Luo Y, Yang C, Lu W, Xie R, Jin C, Huang P, Wang F, McKeehan WL. Metabolic regulator betaKlotho interacts with fibroblast growth factor receptor 4 (FGFR4) to induce apoptosis and inhibit tumor cell proliferation. 2010, *J Biol Chem*, **285**(39):20069-30078.

Lynch TJ, Bell DW, Sordella R, Daphne W, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, M.D, Louis DN, Christiani DC, Settleman J and Haber DA. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. 2004, *N Engl J Med,* **350**:2129-2139.

Macdonald A, Crowder K, Street A, McCormick C, Saksela K, Harris M. The hepatitis C virus nonstructural NS5A protein inhibits activating protein-1 function by perturbing ras-ERK pathway signaling. 2003, *J. Biol. Chem*, **278**:17775–17784.

MacDonald BT, Tamai K, He X. Wnt/beta-catenin signalling: components, mechanisms, and diseases. 2009, *Dev Cell,* **17**:9-26.

Majumder M, Ghosh AS, Steele R, Zhou XY, Phillips NJ, Ray R, Ray R. Hepatitis C Virus NS5A Protein Impairs impairs TNF-mediated hepatic apoptosis, but not by an anti-FAS antibody, in transgenic mice. 2002, *Virology*, **294**:94–105.

Majumder S, Ghoshal K, Datta J, Bai S, Dong X, Quan N, Plass C, Jacob ST. Role of de novo DNA methyl-transferases and methyl CpG-binding proteins in gene silencing in a rat hepatoma. 2002, *J Biol Chem*, **277**:16048–16058.

Mandayam S, Jamal MM, Morgan TR. Epidemiology of alcoholic liver disease. 2004, *Sem Liver Dis*; **24**(3):217-32.

Mason I. Initiation to end point: the multiple roles of fibroblast growth factors in neural development. 2007, *Nature Reviews Neuroscience*, **8**:583-596.

Massagué J and Pandiella A. Membrane-anchored growth factors. 1993, *Annu Rev Biochem*, **62**:515-41.

Maxwell PH and Ratcliffe PJ. Oxygen sensors and angiogenesis. 2002, *Semin Cell Dev Biol,* **13**:29–37.

Mayr B and Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. 2001, *Nat Rev Mol Cell Biol,* **2**:599–609.

McClain CJ, Hill DB, Song Z, Deaciuc I & Barve S. Monocyte activation in alcoholic liver disease. 2002, *Alcohol*, **27**,53–61.

McCrea PD, Turck CW, Gumbiner B. A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin. 1991, *Science*, **254**(5036):1359-61.

Meng W and Takeichi M. Adherens junction: molecular architecture and regulation. 2009, *Cold Spring Harb Perspect Biol*; **1**(6):a002899.

Merchant NB, Voskresensky I, Rogers CM, LaFleur B, Dempsey PJ, Graves-Deal R, Revetta F, Foutch AC, Rothemberg ML, Washington MK, Coffey RJ. TACE/ADAM-17: a component of the epidermal growth factor receptor axis and a promising therapeutic target in colorectal cancer. 2008, *Clin Cancer Res*, **14**:1182–1191.

Merle P, Kim M, Herrmann M, et al. Oncogenic role of the frizzled-7/beta-catenin pathway in hepatocellular carcinoma. 2005, *J Hepatol*, **43**: 854–62.

Miller JR, Hocking AM, Brown JD, Moon RT, Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. 1999, *Oncogene*, **18**:7860–7872.

Miura S, Mitsuhashi N, Shimizu H, Kimura F, Yoshidome H, et al. Fibroblast growth factor 19 expression correlates with tumor progression and poorer prognosis of hepatocellular carcinoma. 2012, *BMC Cancer*, **12**:56.

Miyamoto S, Fukami T, Yagi H, Kuroki M, Yotsumoto F. Potential for molecularly targeted therapy against epidermal growth factor receptor ligands. 2009, *Anticancer Res*, **29**:823-830.

Miyamoto S, Yagi H, Yotsumoto F, Kawarabayashi T, Mekada E. Heparinbinding epidermal growth factor-like growth factor as a novel targeting molecule for cancer therapy. 2006, *Cancer Sci*, **97**:341–7.

Miyoshi Y, Iwao K, Nagasawa Y, Aihara T, Sasaki Y, Imaoka S, Murata M, Shimano T and Nakamura Y. Activation of beta-catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. 1998, *Cancer Res*, **58**:2524±2527.

Modrell B, McDonald VL, Shoyab M, The interaction of amphiregulin with nuclei and putative nuclear localization sequence binding proteins. 1992, *Growth Factors*, **7**:305–314.

Mohammadi M, Honegger AM, Rotin D, Fischer R, Bellot F, Li W, Dionne CA, Jaye M, Rubinstein M & Schlessinger J A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. 1991, *Mol Cell Biol*, **11**:5068–5078.

Monga SP, Mars WM, Pediaditakis P, Bell A, Mule K, Bowen WC, et al. Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. 2002, *Cancer Res*, **62**:2064-2071.

Moon RT, Bowerman B, Boutros M, Perrimon N, The promise and perils of Wnt signaling through beta-catenin. 2002, *Science*, **296**:1644–1646.

Moon RT, Brown JD, Torres M. WNTs modulate cell fate and behavior during vertebrate development. 1997, *Trends Genet*, **13**:157–162.

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. 2004, *Nat Rev Genet*, **5**:691–701.

Moran AE, Hunt DH, Javid SH, Redston M, Carothers AM, Bertagnolli MM: Apc deficiency is associated with increased Egfr activity in the intestinal enterocytes and adenomas of C57BL/6J-Min/+ mice. 2004, *J Biol Chem*, **279**:43261-43272.

Morgillo F, Woo JK, Kim ES, Hong WK, Lee HY. Heterodimerization of insulin-like growth factor receptor/epidermal growth factor receptor and induction of survivin expression counteract the antitumor action of erlotinib, 2006, *Cancer Res*, **66:**10100–10111.

Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. 1997, *Science*, **275**:1787–1790.

Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Miyazawa T, Ishibashi K, Horie T, Imai K, Todoroki T, Kimura S, Koike K. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus associated hepatocarcinogenesis. 2001, *Cancer Res*, **61**(11):4365–4370.

Mullan PB, Quinn JE, Harkin DP. The role of BRCA1 in transcriptional regulation and cell cycle control. 2006, *Oncogene*, **25**:5854–63.

Murakami Y, Saigo K, Takashima H, Minami M, Okanoue T, Bréchot C, Paterlini-Bréchot P. Large scaled analysis of hepatitis Bvirus (HBV) DNA integration in HBV related hepatocellular carcinomas. 2005, *Gut*, **54**(8):1162–1168.

Murillo MM, del Castillo G, Sanchez A et al Involvement of EGF receptor and c-Src in the survival signals induced by TGF-beta1 in hepatocytes. 2005, *Oncogene* **24**(28):4580–4587.

Musgrove EA: Wnt signalling via the epidermal growth factor receptor: a role in breast cancer? 2004, *Breast Cancer Res*, **6**:65-68.

Nakopoulou L, Stefanaki K, Boletis J, Papadakis J, Kostakis A, Vosnides G, Zeis PM. Immunohistochemical study of epidermal growth factor receptor (EGFR) in various types of renal injury. 1994, *Nephrol Dial Transplant*, **9**(7):764-9.

Nault JC, Zucman-Rossi J. Genetics of hepatobiliary carcinogenesis. 2011, *Semin Liver Dis*, **31**:173-187.

Nejak-Bowen KN, Monga SPS. Beta-catenin signalling, liver regeneration and hepatocellular cancer: sorting the good from the bad. 2011, *Sem Canc Biol*, **21**:44-58.

Nicholes K, Guillet S, Tomlinson E et al. A mouse model of hepatocellular carcinoma: ectopic expression of fibroblast growth factor 19 in skeletal muscle of transgenic mice. 2002, *Am J Pathol*, **160**:2295-2307.

Niehrs C. Function and biological roles of the Dickkopf family of Wnt modulators. 2006, *Oncogene*, **25**:7469–81.

Nishimura T, Utsunomiya Y, Hoshikawa M, Ohuchi H, Itoh N. Structure and expression of a novel human FGF, FGF-19, expressed in the fetal brain.1999, *Bioch Biophys Acta*, **1444**:148-151.

Nollet F, Berx G, Molemeans F and Van Roy F. (1996). Genomic organization of the human 8-catenin gen (CTNNBl). 1996, *Genomics*, **32**,413-424.

Normanno N and Gullick WJ. Epidermal growth factor receptor tyrosine kinase inhibitors and bone metastases: different mechanisms of action for a novel therapeutic application? 2006, *Endocr Relat Cancer*, **13**:3–6.

Normanno N, Bianco C, De Luca A, Maiello MR, Salomon DS: Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. 2003, *Endocr Relat Cancer*, **10**:1-21.

Nusse R and Willert K. Beta-catenin: a key mediator of Wnt signaling. 1998, Curr Opin Gen Dev, **8**(1):95-102.

O'Reilly SM, Leonard MO, Kieran N, Comerford KM, Cummins C, Pouliot M, Lee SB and Taylor CT. Hypoxia induces epithelial amphiregulin gene expression in a CREB-dependent manner. 2005, *Am J Physiol Cell Physiol,* **290**: C592–C600.

Ober EA, Verkade H, Field HA, Stainier DY. Mesodermal Wnt2b signalling positively regulates liver specification. 2006, *Nature*, **442**:688-91.

Ohtsu H, Dempsey PJ, Eguchi S. ADAMs as mediators of EFR receptor transactivation by G protein-coupled receptors. 2006, *Am J Physiol Cell Physiol*, **291**:C1–-C10.

Okuda K. Hepatocellular carcinoma. 2000, *J Hepatol*, **32**:225–237.

Olayioye MA, Neve RM, Lane HA and Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer 2000, *The EMBO Journal*, **19**:3159 -3167.

Ono M, Kuwano M. Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. 2006, *Clin Cancer Res*, **12**:7242–51.

Ornitz DM, Itoh N. Fibroblast growth factors. 2001, *Genomic Biology*, **2**(3):reviews 3005.1-3005.12.

Osada T, Nagashima I, Tsuno NH, Kitayama J, Nagawa H. Prognostic significance of glutamine synthetase expression in unifocal advanced hepatocellular carcinoma. 2000, *J Hepatol*, **33**:247-253.

Osna NA, Clemens DL and Donohue TM Jr. Ethanol metabolism alters interferon-γsignaling in recombinant HepG2 cells. 2005, *Hepatology*, **42**:1109– 1117.

Pachiadakis I, Pollara G, Chain BM, Naoumov NV. Is hepatitis C virus infection of dendritic cells a mechanism facilitating viral persistence? 2005, *Lancet Infect Dis*, **5**(5):296-304.

Pai R, Dunlap D, Qing J et al. Inhibitiomn of fibroblast growth factor 19 reduces tumor growth by modulating beta-catenin signaling. 2008, *Cancer Res*, **68**:5086-5095.

Palacios J, Gamallo C: Mutations in the β-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. 1998, *Cancer Res*, **58**:1344–1347.

Pang R, Yuen J, Yuen MF, et al. PIN1 overexpression and beta-catenin gene mutations are distinct oncogenic events in human hepatocellular carcinoma. 2004, *Oncogene*, **23**: 4182–6.

Pardo-Saganta A, Latasa MU, Castillo J, Alvarez-Asiain L, Perugorria MJ, et al. The EGFR ligand amphiregulin is a negative regulator of hepatic acute phase gene expression. 2009, *J Hepatol,* **51**:1010-1020.

Park KJ, Choi SH, Choi DN et al. Hepatitis C virus NS5A protein modulates c-Jun N-terminal kinase through interaction with tumor necrosis factor receptor-associated factor 2. 2003, *J Biol Chem*, **278**:30711–8.

Parkin DM, Bray FI. & Devesa SS. Cancer burden in the year 2000.The global picture. 2001, *Eur. J. Cancer*, **37**: S4–S66.

Pastore S, Mascia F, Mariani V, Girolomoni G. The epidermal growth factor receptor system in skin repair and inflammation. 2008, *J Invest Dermatol*, **128**:1365–1374.

Paull TT, Cortez D, Bowers B, Elledge SJ, Gellert M. Direct DNA binding by Brca1. 2001, *Proc Natl Acad Sci USA*, **98**:6086–6091.

Philip PA, Mahoney MR, Allmer C, et al. Phase II study of erlotinib (OSI-774) in patients with advanced hepatocellular cancer. 2005, *J Clin Oncol,* **23**:6657-6663.

Piao YF, He M, Shi Y & Tang TY. Relationship between microvessel density and telomerase activity in hepatocellular carcinoma. 2004, *World J Gastroenterol*, **10**:2147–2149.

Plowman GD, Green JM, McDonald VL. Neubauer M.G., Disteche C.M., Todaro G.J., Shoyab M. The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. 1990, *Mol Cell Biol*, **10**:1969–1981.

Poh W, Wong W, Ong H, Aung MO, Lim SG, Chua BT, Lim SG**,** Chua BT and Ho HK. Klotho-beta overexpression as a novel target for suppressing proliferation and fibroblast growth factor receptor-4 signaling in hepatocellular carcinoma. 2012, *Mol Cancer*, **11**:14.

Popovici C, Roubin R, Coulier Fetal. An evolutionary history of the FGF superfamily. 2005, *BioEssays*, **27**:849-857.

Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW. APC mutations occur early during colorectal tumorigenesis. 1992, *Nature*, **359**:235-237.

Puri N and Salgia R. Synergism of EGFR and c-Met pathways, crosstalk and inhibition, in non-small cell lung cancer. 2008, *J Carcinog*, 7-9.

Recchia AG, Musti AM, Lanzino M, Panno ML, Turano E, Zumpano R, Belfiore A, Ando S, Maggiolini M. A crosstalk between the androgen receptor and the epidermal growth factor receptor leads to p38MAPK-dependent activation of mTOR and cyclinD1 expression in prostate and lung cancer cells. 2009, *Int. J. Biochem. Cell Biol*, **41**:603–614.

Renard CA, Labalette C, Armengol C, Cougot D, Wei Y, et al. Tbx3 is a downstream target of the Wnt/β-catenin pathway and a critical mediator of β-catenin survival functions in liver cancer. 2007, *Cancer Res*, **67**:901-910.

Ricono JM, Huang M, Barnes LA, Lau SK, Weis SM, Schlaepfer DD, Hanks SK, Cheresh DA. Specific crosstalk between epidermal growth factor receptor and integrin alphavbeta5 promotes carcinoma cell invasion and metastasis. 2009, *Cancer Res*, **69**:1383–1391.

Riese DJ and Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. 1998, *BioEssays,* **20**:41-48.

Rimm DL, Caca K, Hu G, Harrison FB, Fearon ER: Frequent nuclear/ cytoplasmic localization of β-catenin without exon 3 mutations in malignant melanoma. 1999, *Am J Pathol*, **154**:325–329.

Roidl A, Berger HJ, Kumar S et al. Resistance to chemiotherapy is associated with fibroblast growth factor receptor 4 up-regulation. 2009, *Clin Cancer Res*, **15**:2058-2066.

Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P: Binding of GSK3b to the APC-β-catenin complex and regulation of complex assembly. 1996, *Science*, **272**:1023–1026.

Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P. Stabilization of beta-catenin by genetic defects in melanoma cell lines. 1997, *Science*, **275**:1790–1792.

Ryo A, Nakamura M, Wulf G, Liou YC, Lu KP. Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. 2001, *Nat Cell Biol*, **3**:793–801.

Saito Y, Haendeler J, Hojo Y, Yamamoto K, Berk BC. Receptor heterodimerization: essential mechanism for platelet-derived growth factor-induced epidermal growth factor receptor transactivation. 2001, *Mol Cell Biol*, **21**:6387–6394.

Sanderson MP, Dempsey PJ, Dunbar AJ. Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors. 2006, *Growth Factors*, **24**:121–136.

Satoh S, Daigo Y, Furukawa Y, et al. AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. 2000, *Nat Genet*, **24**:245–50.

Sattler M, Abidoye O, Salgia R. EGFR-targeted therapeutics: focus on SCCHN and NSCLC. 2008, *The Scientific World Journal*, **8**:909–919.

Sawey ET, Chanrion M, Cai C, Wu G, Zhang J, et al. Identification of a therapeutic strategy targeting amplified *FGF19* in liver cancer by oncogenomic screening. 2011, *Cancer Cell*, **19**:347-358.

Schiffer E, Housset C, Cacheux W, et al. Gefitinib, an EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis. 2005, *Hepatology*, **41**:307–14.

Schlange T, Matsuda Y, Lienhard S, Huber A, Hynes NE: Autocrine WNT signaling contributes to breast cancer cell proliferation via the canonical WNT pathway and EGFR transactivation. 2007, *Breast Cancer Res*, **9**:R63.

Schneider SQ, Bowerman B. Cell polarity and the cytoskeleton in the Caenorhabditis elegans zygote. 2003, *Annu Rev Genet*, **37**:221–249.

Shao J, Lee SB, Guo H, Evers BM, and Sheng H. Prostaglandin E2 stimulates the growth of colon cancer cells via induction of amphiregulin. 2003, *Cancer Res,* **63**:5218–5223.

Shapiro L and Weis WI. Structure and biochemistry of cadherins and catenins. 2009, *Cold Spring Harbor Perspect Biol,* **1**:a003053.

Sharma M, Chuang WW, Sun Z: Phosphatidylinositol 3-kinase/AKT stimulates androgen pathway through GSK3beta inhibition and nuclear beta-catenin accumulation. 2002, *J Biol Chem*, **277**:30935-30941.

Shepard HM, Brdlik CM, Schreiber H. Signal integration: a framework for understanding the efficacy of therapeutics targeting the human EGFR family. 2008, *J. Clin Invest*, **118**:3574-81.

Shoyab M, McDonald VL, Bradley JG, Todaro GJ. Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7. 1988, *Proc Natl Acad Sci USA*, **85**:6528–6532.

Simons AM, Horwitz AA, Starita LM, et al. BRCA1 DNA-binding activity is stimulated by BARD1. 2006, *Cancer Res*, **66**:2012–8.

Song IH. Molecular targeting for treatment of advanced hepatocellular carcinoma 2009, *The Kor J Hepatol*; **15**:299-308.

Spangenberg HC, Thimme R and Blum HE. Targeted therapy for hepatocellular carcinoma. 2009, *Nat Rev Gastroent & Hepat*, **6**:423-432.

Sparks AB, Morin PJ, Vogelstein BKinzler KW. Mutational analysis of the APC/betacatenin/Tcf pathway in colorectal cancer. 1998, *Cancer Res*, **58**:1130–1134.

Stambolic V, Ruel L, Woodgett JR. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. 1996, *Curr Biol*, **6**:1664-1668.

Stein RA and Staros JV. Evolutionary analysis of the ErbB receptor and ligand families. 2000, *J Mol Evol*, **50**:397–412.

Sternlicht MD, Sunnarborg SW, Kouros-Mehr H, Yu Y, Lee DC, Werb Z. Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding of epithelial amphiregulin. 2005, *Development*, **132**:3923–3933.

Stoll SW, Johnson JL, Li Y, Rittié L, and Elder JT. Amphiregulin carboxy-terminal domain is required for autocrine keratinocyte growth. 2010, *J Invest Dermatol*, **130**(8):2031–2040.

Su MC, Lien HC, Jeng YM. Absence of epidermal growth factor receptor exon 18-21 mutation in hepatocellular carcinoma. 2005, *Cancer Lett,* **224**:117-121.

Suzuki H, Gabrielson E, ChenW, et al. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. 2002, *Nat Genet*, **31**:141-9.

Suzuki H, Toyota M, Nojima M, Mori M, Imai K. SFRP, a family of new colorectal tumor suppressor candidate genes. 2005, *Nippon Rinsho*, **63**:707–719.

Suzuki M, Shigematsu H, Nakajima T, Kubo R, Motohashi S, Sekine Y, Shibuya K, Iizasa T, Hiroshima K, Nakatani Y, et al: Synchronous alterations of Wnt and epidermal growth factor receptor signaling pathways through aberrant methylation and mutation in non small cell lung cancer. 2007, *Clin Cancer Res*, **13**:6087-6092.

Takano S, Yokosuka O, Imazeki F, Tagawa M & Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. 1995, *Hepatology*, **21**:650–655.

Tan X, Apte U, Micsenyi A, Kotsagrelos E, Luo JH, Ranganathan S, Monga DK, Bell A, Michalopoulos GK, Monga SP: Epidermal growth factor receptor: a novel target of the Wnt/beta-catenin pathway in liver. 2005, *Gastroenterology*, **129**:285-302.

Tan X, Yuan Y, Zeng G, Apte U, Thompson MD, Cieply B, et al. Beta-catenin deletion in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development. 2008, *Hepatology*, **47**:1667-79.

Taniguchi K, Roberts LR, Aderca IN, et al. Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. 2002, *Oncogene*, **21**:4863–71.

Taylor CT and Colgan SP. Therapeutic targets for hypoxia-elicited pathways. 1999, *Pharm Res,* **16**:1498–1505.

Taylor CT, Fueki N, Agah A, Hershberg RM, and Colgan SP. Critical role of cAMP response element binding protein expression in hypoxiaelicited induction of epithelial tumor necrosis factor-α. 1999, *J Biol Chem,* **274**:19447–19454.

Taylor CT, Furuta GT, Synnestvedt K, and Colgan SP. Phosphorylation-dependent targeting of cAMP response element binding protein to the ubiquitin/proteasome pathway in hypoxia. 2000, *Proc Natl Acad Sci USA,* **97**:12091–12096.

Thomas SM, Bhola NE, Zhang Q, Contrucci SC, Wentzel AL, Freilino ML, Gooding WE, Siegfried JM, Chan DC, Grandis JR. Cross-talk between G proteincoupled receptor and epidermal growth factor receptor signaling pathways contributes to growth and invasion of head and neck squamous cell carcinoma. 2006, Cancer Res. 66, 11831–11839.

Turner N and Grose R. Fibroblast growth factor signalling: from development to cancer. 2010, *Nat Rev Cancer*, **10**(2):116-29.

Tzhar E, Yarden Y. The ErbB-2/HER2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. 1998, *Biochim. Biophys. Acta*, **1377**:M25–M37.

Udayakumar TS, Stratton MS, Nagle RB & Bowden GT Fibroblast growth factor-1 induced promatrilysin expression through the activation of extracellular-regulated kinases and STAT3. 2002, *Neoplasia*, **4**:60–67.

Uematsu K, He B, You L, Xu Z, Mccormick F, Jablons DM. Activation of the Wnt pathway in non small cell lung cancer: evidence of dishevelled overexpression. 2003a, *Oncogene*, **22**:7218–21.

Uematsu K, Kanazawa S, You L, et al. Wnt pathway activation in mesothelioma: evidence of dishevelled overexpression and transcriptional activity of beta-catenin. 2003b, *Cancer Res*, **63**:4547–51.

Veeman MT, Axelrod JD, Moon RT. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling, 2003a, *Dev Cell*, **5**:367–377.

Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. Zebrafish prickle, a modulator of noncanonical Wnt/ Fz signaling, regulates gastrulation movements, 2003b, *Curr Biol*, **13**:680–685.

Villanueva A, Chiang DY, Newell P, Peix J, Thung S, Alsinet C, Tovar V, Roayaie S, Minguez B, Sole M, Battiston C, Van Laarhoven S, Fiel MI, Di Feo A, Hoshida Y, Yea S, Toffanin S, Ramos A, Martignetti JA, Mazzaferro V, Bruix J, Waxman S, Schwartz M,

Meyerson M, Friedman SL, Llovet JM. Pivotal role of mTOR signalling in hepatocellular carcinoma. 2008, *Gastroenterology,* **135**:1972–1983.

Wege H, Heim D, Lütgehetmann M, Dierlamm J, Lohse AW, et al. Forced activation of β-catenin signaling supports the transformation of *hTERT*-immortalized human fetal hepatocytes. 2011, *Mol Cancer Res*, **9**:1222-1231.

Wei W, Chua MS, Grepper S, So S. Small molecule antagonosts of Tcf4/β-catenin complex inhibits the growth of HCC cells in vitro and in vivo. 2010, *Int J. Cancer*, **126**:2426-2436.

White BD, Chien AJ, Dawson DW. Dysregulation of Wnt/β-catenin signalling in gastrointestinal cancers. 2012, *Gastroenterology*, **142**:219-232.

Whittaker S, Marais R, Zhu AX. The role of signaling pathways in the development and treatment of hepatocellular carcinoma. 2010, *Oncogene*, **29**:4989-5005.

Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: biology driving targeted therapeutics. 2008, *Cell Mol Life Sci*, **65**:1566–1584.

Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, Schwartz B, Simantov R, Kelley S. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. 2006, *Nat Rev Drug Discov*, **5**(10):835–844.

Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese DJ 2nd. Functional selectivity of EGF family peptide growth factors: implications for cancers. 2009, *Pharmacol Ther*, **122**(1):1-8.

Wong CM, Fan ST, Ng IO. beta-Catenin mutation and overexpression in hepatocellular carcinoma: clinicopathologic and prognostic significance. 2001, *Cancer*, **92**:136–45.

Wu T. Cyclooxygenase-2 in hepatocellular carcinoma. 2006, *Cancer Treat Reviews***,** **32**:28–44.

Wu X, Ge H, Gupte J Weiszmann J, Shimamoto G, Stevens J, Hawkins N,Lemon B, Shen W, Xu J,Veniant MM, Li YS, Lindberg R, Chen JL, Tian H and Li Y. Co-receptor requirements for fibroblast growth factor-19 signaling. 2007, *J Biol Chem*, **282**:29069-29072.

Wu X, Ge H, Lemon B, Vonderfecht S, Weiszmann J, Hecht R, Gupte J, Hager T, Wang Z, Lindberg R, et al: FGF19-induced hepatocyte proliferation is mediated through FGFR4 activation. 2010, *J Biol Chem*, **285**(8):5165-5170.

Xian W, Schwertfeger KL, Vargo-Gogola T and Rosen JM. Pleiotropic effects of FGFR1 on cell proliferation, survival, and migration in a 3D mammary epithelial cell model. 2005, *J. Cell Biol*, **171**:663-673.

Xie MH, Holcomb I, Deuel B, Dowd P, Huang A, Vagts A, Foster J, Liang J, Brush J, Gu Q, Hillan K, Goddard A, Gurney AL. FGF-19, a novel fibroblast growth factor with unique specificity for FGFR4. 1999, *Cytokine*, **11**(10): 729-735.

Yang B, Guo M, Herman JG, Clark DP. Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. 2003, *Am J Pathol*, **163**:1101–7.

Yang W, Xia Y, Ji H, Zheng Y, Liang J, et al. Nuclear PKM2 regulates β-catenin transactivation upon EGFR activation. 2011, *Nature*, **480**:118.122.

Yarden Y and Sliwkowski MX. Untangling the ErbB signalling network. 2001, *Nat Rev Mol Cell Biol*, **2**:127–137.

Yarden Y. The EGFR family and its ligands in human cancer. Signalling mechanisms and therapeutic opportunities. 2001, *Eur. J. Cancer*, **37**(Suppl. 4):S3–8.

Yau TO, Chan CY, Chan KL, et al. HDPR1, a novel inhibitor of the WNT/beta-catenin signaling, is frequently downregulated in hepatocellular carcinoma: involvement of methylationmediated gene silencing. 2005, *Oncogene*, **24**:1607–14.

You L, He B, Xu Z, Uematsu K, Mazieres J, Fujii N, Mikami I, Reguart N, McIntosh JK, Kashani-Sabet M, McCormick F, Jablons DM. An anti-Wnt-2 monoclonal antibody induces apoptosis in malignant melanoma cells and inhibits tumor growth. 2004, *Cancer Res*, **64**:5385–5389.

Yu MC, Yuan JM. Environmental Factors and Risk for Hepatocellular Carcinoma. 2004, *Gastroenterology*, **127**:S72–S78.

Yuzugullu H, Benhaj K, Ozturk N, Senturk S, Celik E, et al. Canonical Wnt signalling is antagonized by noncanonical Wnt5a in hepatocellular carcinoma. 2009, *Mol Cancer*, **8**:90.

Zeng G, Apte U, Cieply B, Singh S, Monga SP. siRNA-mediated beta-catenin knockdown in human hepatoma cells results in decreased growth and survival. 2007, *Neoplasia*, **9**:951-959.

Zhao L and Brinton RD. Suppression of proinflammatory cytokines interleukin-1 and tumor necrosis factor-α in astrocytes by a V1 vasopressin receptor agonist: a cAMP response element-binding protein-dependent mechanism. 2004, *J Neurosci,* **24**:2226–2235.

Zhu BD, Yuan SJ, Zhao QC, Li X, Li Y, Lu QY. Antitumor effect of gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, combined with a cytotoxic agent on murine hepatocellular carcinoma. 2005, *World J Gastroenterol,* **11**:1382-1386.

Zorn AM. Wnt signalling: antagonistic Dickkopfs. 2001, *Curr Biol*, **11**:R592–R595.