WNT/β-CATENIN SIGNALING PATHWAY ACTIVATION IN EPITHELIAL CANCERS

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BY

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ABSTRACT

WNT/β-CATENIN SIGNALING PATHWAY ACTIVATION IN EPITHELIAL CANCERS

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Supervisor: Dr. Can AKCALI and Pr. Dr. Mehmet OZTURK
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Wnt signaling is involved in a large set of cellular and developmental processes, and when mis-regulated can lead to both degenerative diseases and many types of cancer. The involvement of Wnt signaling was already well demonstrated in several types of human cancers such as colorectal cancer. However, in some others such as hepatocellular carcinoma (HCC) and breast cancer, the role of Wnt signaling is not fully understood.

To study the role of Wnt pathway in liver cancer, we first classified human hepatoma cell lines into well-differentiated and poorly differentiated groups using hepatocyte-specific biomarkers. Wnt/β-catenin signaling activity was measured using TCF/LEF-dependent reporter assay. Canonical Wnt/β-catenin signaling was constitutively active in 80% of well differentiated and 14% of poorly differentiated cell lines, respectively. Furthermore, ectopic expression mutant of S33Y β-catenin resulted in strong canonical Wnt/β-catenin activity in well differentiated, but not in poorly differentiated HCC cells. Comprehensive analysis of major Wnt signaling components by a rapid RT-PCR assay showed redundant expression of many Wnt ligands, Frizzled receptors, co-receptors and TCF/LEF factors in HCC. In contrast, canonical signaling-inhibitory Wnt5A and Wnt5B ligands were selectively expressed in poorly differentiated HCC cell lines. Our observations indicate that canonical Wnt/β-catenin signaling is active in well differentiated, but repressed in poorly differentiated HCC cells. Thus, canonical Wnt/β-catenin signaling plays a dual role in HCC.

To study the role of Wnt pathway in breast cancer, we performed a comprehensive expression analysis, by RT-PCR, of Wnt signaling molecules, including 19 Wnt ligands, ten Frizzled receptors, two LRP co-receptors and four Lef/TCF transcription factors in immortalized normal human mammary epithelial cells (HMECs), six breast cancer cell lines (BCCL) and 14 primary breast tumors (PBT). BCCL expressed/over-expressed all Frizzleds except *FZD10,* LRP5/6 and Lef/TCFs. They also overexpressed *WNT4, WNT7B, WNT8B, WNT9A* and *WNT10B*, but the expression of *WNT1, WNT2B, WNT3, WNT5A, WNT5B* and *WNT16* was lost or decreased in most BCCL. Wnt expression correlated with nuclear β-catenin accumulation and *cyclin D1* induction in BCCL, compared to HMECs, indicating a reactivation of the canonical Wnt signaling in malignant cells. Furthermore, the expression of *FZD1, WNT-4, WNT7B*, *WNT8B, WNT9A and WNT10B*, all implicated in canonical Wnt signaling, was upregulated in PBT, whereas the non-canonical *WNT5A* expression was down-regulated.

Our study gave strong evidences for the differential involvement of Wnt pathway in liver and breast cancers. In liver cancer, Wnt pathway activity seems to be linked to the differentiation status of HCC cell lines. Furthermore, our data showed that the canonical Wnt pathway was active in well-differentiated HCC cell lines and repressed in poorly differentiated ones. In contrast, the study of Wnt pathway in breast cancer cell lines showed similarities rather than differences. Indeed, our study revealed a significant correlation between Wnt ligands mRNA expression profile and the induction of Cyclin D and nuclear β-catenin protein accumulation in all breast cancer cell lines studied. We concluded that, although involved in both types of cancers, Wnt signaling is acting differently in liver and breast cancers. More interestingly, in the same type of cancer such as HCC, Wnt signaling displayed differential activity depending on the cell differentiation status.

ÖZET

EPİTEL KANSERLERİNDE Wnt/β-CATENİN YOLAĞININ AKTİVASYONU

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Karaciğer Kanserinde Wnt yolaklarını çalışırken, öncelikle insan hepatoma hücre hatlarını hepatositlere özel biyojik işaretler (marker) kullanarak özelleşmiş ve az özelleşmiş olarak sınıflara ayırdık. Wnt/β-katenin sinyal aktivitesi TCF/LEF raportör testi yapılarak ölçüldü. Standart Wnt/β-katenin sinyalleri, tamamen özelleşmiş hücre hatlarının 80%'inde, ve az özelleşmiş hücre hatlarının %14'ünde sürekli aktifti. Bunun yanısıra, mutant S33Y β-katenin'in ektopik sentezi, tamamen özelleşmiş HCC hücre hatlarında güçlü standart Wnt/β-katenin aktivitesine yol açarken, az özelleşmiş HCC hücre hatlarında bu etki görülmedi. Başlıca Wnt sinyal elemanları üzerine RT-PCR ile yapılan kapsamlı araştırmalar, birçok Wnt ligandları, Frizzled reseptörleri, ko-reseptörler ve TCF/LEF faktörlerinin gereğinden fazla ve birbirinin yerine geçebilecek şekilde sentezlendiğini göstermiştir. Buna karşılık, standart Wnt sinyalini inhibe eden Wnt5A ve Wnt5B ligandları, seçici olarak az özelleşmiş HCC hücre hatlarında sentezlenmektedir. Bizim gözlemlerimize göre standart Wnt/β-katenin sinyali, tamamen özelleşmiş HCC hücrelerinde aktif, fakat az özelleşmiş HCC hücrelerinde baskılanmış durumdadır. Bu nedenle, standart Wnt/β-katenin sinyali HCC hücrelerinde çift rol oynamaktadır.

Meme Kanserinde Wnt yolaklarını çalışırken, ölümsüzleştirilmiş normal insan meme epitel hücreleri (HMECs), 6 meme kanser hücre hattı (BCCL) ve 14 primer meme tümörü kullanarak, Wnt sinyal moleküllerinden, 19 Wnt ligandı, 10 frizzled reseptörü, 2 LRP ko-reseptörü ve 4 Lef/TCF transkripsiyon faktörünün kapsamlı

ekspresyon analizleri yapıldı. BCCL, *FZD10* hariç bütün frizzledları, LRP5/6 ve Lef/TCF'leri sentezliyor/aşırı sentezliyordu. Ayrıca *WNT4, WNT7B, WNT8B, WNT9A ve WNT10B* de aşırı sentezleniyor, fakat *WNT1, WNT2B, WNT3, WNT5A, WNT5B* ve *WNT16* sentezi birçok BCCL de kayboluyor ya da düşüyordu. Wnt ekspresyonun, Normal İnsan Meme Hücre Hattı ile kıyaslandığında, Meme Kanser Hücre hatlarındaki çekirdek β-katenin birikmesi ve cyclin D1 indüksiyonu ile ilintili olması standart Wnt sinyali kanserli hücrelerde yeniden aktive edildiğini göstermekteydi. Bunun yanı sıra, *FZD1, WNT-4, WNT7B*, *WNT8B, WNT9A* ve *WNT10B* gibi standart Wnt sinyali ile ilişkili genlerin ekspresyonu Primer Meme Tümörlerinde arttığı halde, standart olmayan *WNT5A* ekspresyonu baskılanmaktaydı. Bu sonuçlar otokrin standart Wnt sinyalinin insan meme karsinogenezi ile ilişkili olduğunu göstermekteydi.

Bizim çalışmamız, Wnt yolakının karaciğer ve meme kanserleri ile ilgili olan farklı ilşkisi üzerine güçlü kanıtlar sunmaktadır. Karaciğer kanserinde, Wnt yolakının aktivitesi HCC hücre hatlarının özelleşmesi ile ilgili gözükmektedir. Bunun yanı sıra, sonuçlarımız Wnt yolakının tamamen özelleşmiş HCC hücre hatlarında aktif, az özelleşmiş olanlarda baskılanmış durumda olduğunu göstermiştir. Buna karşılık, Meme Kanser Hücre hatlarıyla yapılan Wnt çalışmaları farklılıktan ziyade benzerlikler göstermiştir. Çalışmamız, Meme Kanser Hücre hatlarında, Wnt ligandlarının mRNA ekspresyon profili ile cyclin D indüksiyonu ve çekirdek β-katenin proteininin birikmesi arasındaki kayda değer ilişkiyi ortaya çıkartmıştır. Sonuç olarak, Wnt sinyali iki tip kanserle de ilişkili olmasına karşın, karaciğer ve meme kanserinde farklı roller oynamaktadır. Daha da ilgi çekici olanı, Wnt sinyali, HCC gibi aynı tip kanserde bile, hücrenin özelleşme durumuna göre farklı aktiviteler gösterebilmektedir.

TO

*MY **WIFE***

*MY **YASSINE***

*MY **PARENTS***

*MY BROTHER **NEJI***

*MY PROFESSORS **RAJA** AND **ALI***

*MY FRIENDS **ABU JAAFER, MELLITA** AND **BECHIR***

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ABBREVIATIONS

APC	Adenomatous Polyposis Coli
APS	Ammonium Persulfate
BSA	Bovine Serum albumin
$CaPO_4$	Calcium Phophate
CE	Convergent Extention
CO_2	carbone dioxyde
DEMEM	Dulbecco's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
FCS	Fetal Calf Serum
FZD	Frizzled
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HNF	Hepatocyte Nuclear Factor
HRP	Horse Radish Peroxidase

KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
LB	Luria-Betani medium
LEF	Lynphocyte Enhancing Factor
LRP	LDL receptor Related Protein
MMTV	Mouse Mammary Gland Virus
NaCl	Sodium Chloride
Na_2HPO_4	Sodium Monohydrogen Phosphate
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCP	Plana Cell Polarity
PCR	Polymerase Chain Reaction
PVDF	Polyvinyl Difluoride
Rb	Retinoblastoma
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulfate
TBS	Tris Buffered Saline

TBS-T	Tris Buffered Saline with Tween 20
TCF	T Cell Factor
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
TGF	Transforming Groth Factor
UV	Ultraviolet
Wnt	Wint
WT	Wild Type

1. INTRODUCTION

1.1. WNT SIGNALING NETWORK

Wnt signaling was initially identified in early embryogenesis of *Drosophila* by discovering the segment polarity gene *Wingless* (*Wg*). Then, viral carcinogenesis experiments conducted in mice led to the discovery of the common integration site of the mouse mammary tumour virus (MMTV) within the promoter of the gene named *Int-1* (for 'Integration'). This viral integration resulted in increased production of Int-1 protein and caused mammary tumour development giving a causative role for this protein in mouse mammary development. Sequence analysis revealed that *Int-1* was orthologous to the *Drosophila Wg* gene. Terms were combined to produce the name 'Wnt' for the mammalian *Int-1* gene and its paralogs (Ilyas, 2005).

Wnt signaling is involved in a large set of cellular and developmental processes in the animal kingdom, including embryonic patterning, tissue separation, cell proliferation, differentiation, migration, and apoptosis. There are three different Wnt signaling pathways: the canonical Wnt/β-catenin pathway, and the non-canonical Wnt pathways: Wnt/Ca++ pathway and Wnt/Planar Cell Polarity pathway (Lustig *et al*., 2003; van Es *et al*. 2003; Veeman *et al*. 2003). The canonical Wnt signaling is involved in cell fate choices; stem cell renewal and differentiation, whereas the non-canonical Wnt signaling generally deals with cellular movement, morphological changes and tissue organization (Giles *et al*., 2003; Lustig *et al*., 2003; van Es *et al*. 2003).

Wnt signaling is required also for adult tissue maintenance, and its mis-regulation promotes both degenerative diseases and cancer. Indeed, *LRP5* mutations that make it insensitive to Dkk-mediated Wnt pathway inhibition have been correlated with decreased bone mass (Nusse, 2005). On the other hand, mutations affecting APC, β-catenin or Axin-1 proteins that promote constitutive activation of the Wnt signaling pathway lead to cancers. More recently, numerous reports showed

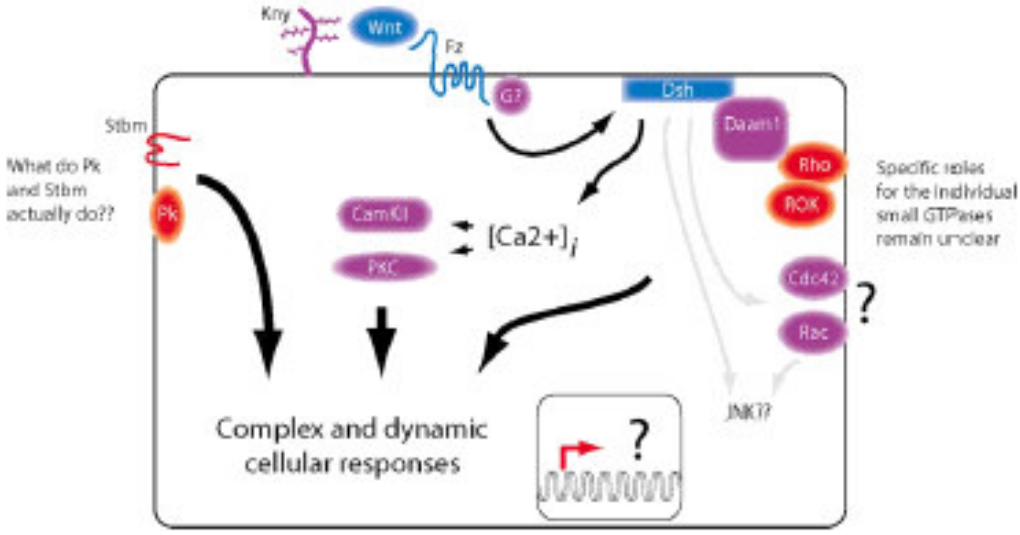
the overexpression of different Wnt ligands in different human cancer types suggesting a possible autocrine mechanism for constitutive Wnt pathway reactivation in human cancer. This hypothesis was recently well demonstrated by Bafico *et al*., 2004 in breast and ovarian cancers.

1.1.1 Non-canonical Wnt signaling pathways

Non-canonical Wnt signaling refers to the β-catenin-independent Wnt pathways that can signal through calcium flux, JNK, Rho family of GTPases or heterotrimeric G proteins. Non-canonical Wnt signaling pathways have been shown to control numerous cellular processes such as cell behavior and cell fate determination, cellular movements, cardiogenesis, myogenesis and tissue separation. The best-characterized non-canonical Wnt pathways are the Wnt/calcium pathway and the *Drosophila* planar cell polarity (PCP) pathway.

1.1.1.1 Wnt/Ca^{++} signaling pathway

Wnt ligands, in vertebrates, can activate two pathways and can be classified accordingly. Canonical Wnt ligands such as Wnt-1, Wnt-3a and Wnt-8b can transform mammalian cells and induce axis duplication in amphibian embryos. Whereas, non-canonicals Wnt-4, Wnt-5a and Wnt-11 do not have transforming activity or induce axis duplication, they rather cause defects in cell movement during gastrulation when injected to Xenopus embryos. Non-canonical Wnt ligands are shown to activate a signal that will induce intracellular calcium release and a subsequent activation of calcium-sensitive kinases: protein kinase C (PKC) and calcium/calmodulin-dependent kinase (CamKII) (Veeman *et al*., 2003). This pathway has been called Wnt/calcium signaling pathway to distinguish it from the Wnt/β–catenin signaling pathway, and thought to act through heterotrimeric G proteins with a controversial participation of Dishevelled (Figure 1.1).



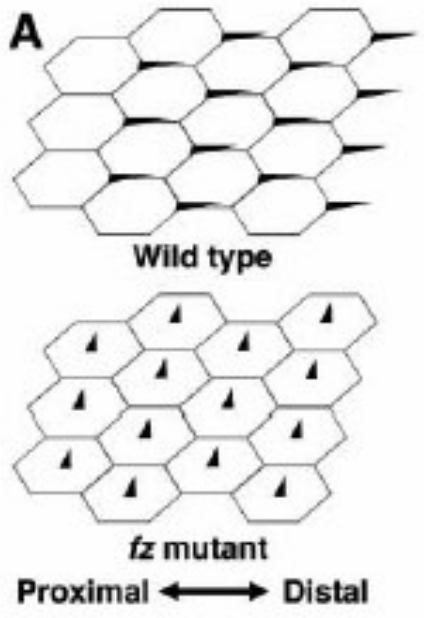
(Veeman *et al*., 2003)

Figure 1.1: Wnt/Ca^{++} signaling pathway

Recent studies showed that Wnt-5a and Wnt-11 activate the Wnt/calcium pathway and control cell polarization during vertebrate gastrulation indicating an overlap with the *Drosophila* planar cell polarity (PCP) pathway. On the other hand, the early observation that overexpression of Wnt-5a blocked the secondary axis induction by Wnt-8 in Xenopus embryos revealed the antagonistic relation between the Wnt/calcium and Wnt/β–catenin pathways. This finding was further supported and a couple of mechanisms have been proposed to explain the antagonistic effect of Wnt-5a on the canonical pathway. Indeed, Topol *et al*. showed that Wnt-5a inhibited the canonical Wnt pathway by degrading β-catenin via *Siah2* transcriptional up-regulation (Topol *et al*., 2003). This antagonistic relation between Wnt pathways raised the question if Wnt/calcium pathway would have any kind of tumor suppressor activity since the opposite canonical Wnt pathway is hyperactivated in cancer. Two reports gave clear evidence that the non-canonical Wnt5-a displayed a tumor suppressor activity in thyroid carcinoma cells (Kremenevskaja *et al*., 2005) and melanoma cells (Liang *et al* 2003).

1.1.1.2 Planar cell polarity pathway

In *Drosophila*, the planar cell polarity (PCP) pathway initiated by Frizzled and mediated by Dishevelled through Rho protein controls the correct cell polarization during the development of the fly embryo in many tissues such as the wing, the eye and the dorsal epidermis. In the wing, the PCP pathway is required for the correct orientation of the hairs that are produced by each cell. Normally, each cell produces a single hair on its apical surface at the distal vertex of the cell. The hair then grows towards the distal edge. Frizzled mutation, for example, will lead to the loss of both the right localization and orientation of the hair (Strutt, 2003) (Figure 1.2).

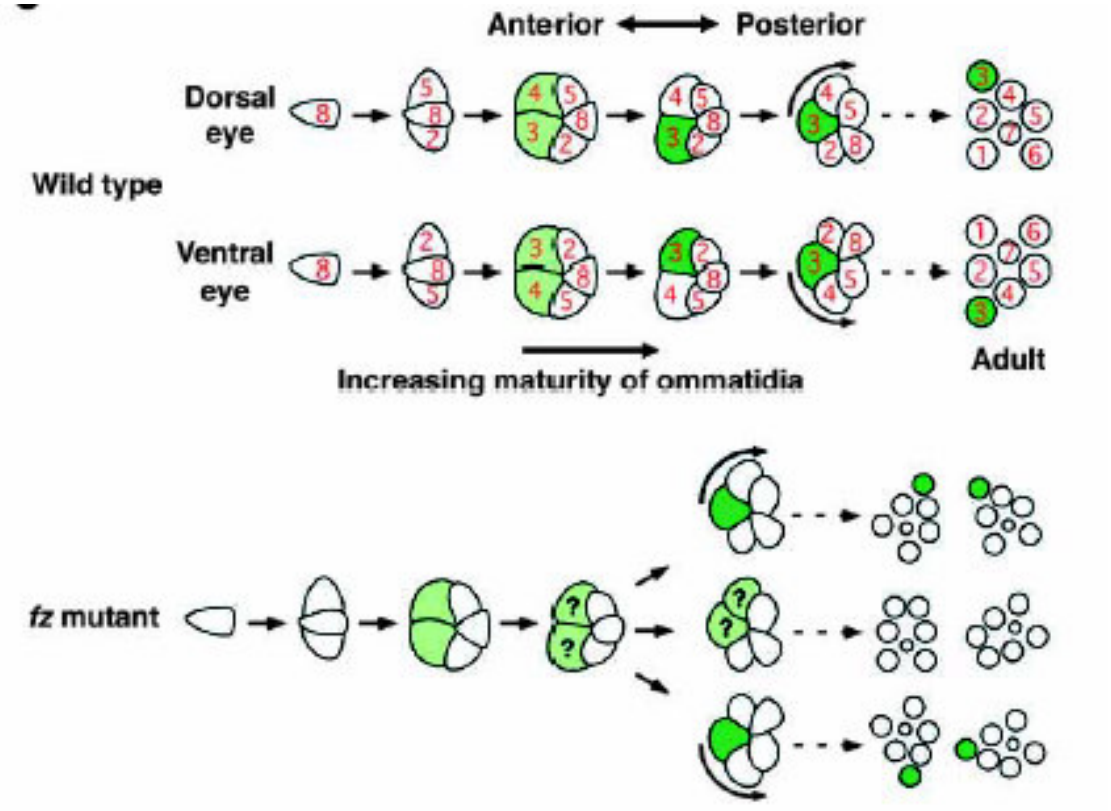


(Strutt, 2003)

Figure 1.2: The PCP pathway is required for the correct orientation of the hairs produced by each cell in the *Drosophila* wing

In the eye, the PCP pathway determines the polarity of the ommatidial units made of 12 supporting cells. During development, each ommatidium undergoes two

distinct events that determine its polarity in the adult eye. Firstly, it adopts a correct chirality according to its position above or below the dorsoventral midline of the eye. Secondly it rotates exactly 90 degree in the appropriate direction (Figure 1.3) (Strutt, 2003).

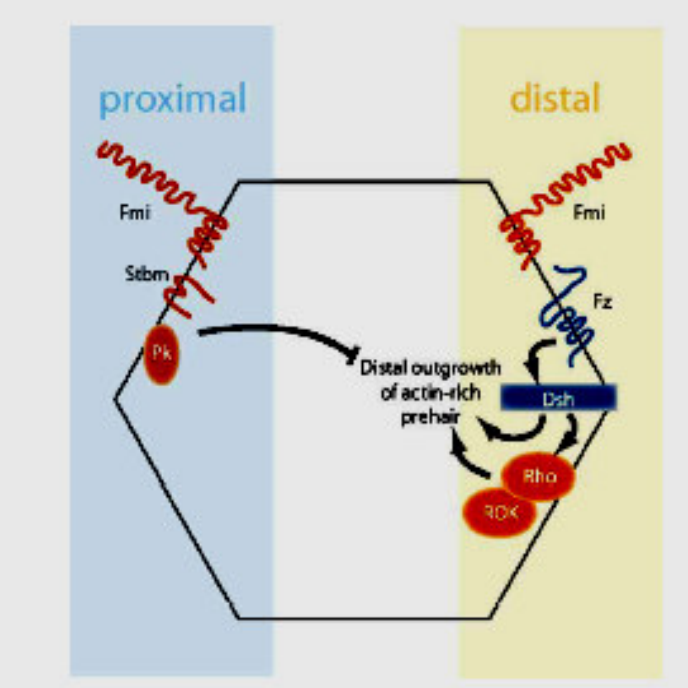


(Strutt, 2003)

Figure 1.3: The PCP pathway determines the polarity of the ommatidial units in the *Drosophila* eye

Some of the PCP pathway proteins including Flamingo, localize to both the proximal and the distal sides of the cell. Some others, however, including Frizzled, Dishevelled, and Rho become localized specifically to the distal side, whereas Pickle and Strabismus become localized to the proximal side (Figure 1.4). The molecular mechanism underlying this spatial distribution of the *Drosophila* PCP proteins

remains unclear. Nevertheless, many studies showed that lack of any one of the PCP pathway proteins resulted in similar polarity defects in the wing and eye cells demonstrating the requirement of these molecules in the control of the correct development of the planar cell polarity (Veeman *et al*. 2003).

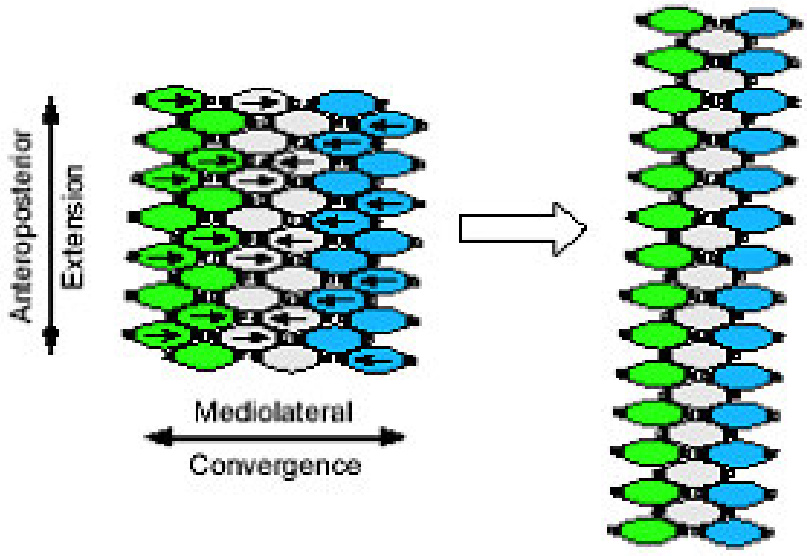


(Veeman *et al*. 2003)

Figure 1.4: The spatial distribution of the *Drosophila* PCP proteins

The entire *Drosophila* PCP pathway genes are conserved in vertebrates where they control developmental patterning events, similar to those seen in *Drosophila*, such as convergent extension during gastrulation and the coordinated orientation of the sensory hair cells in the inner ear. The gastrulation of vertebrate embryos is ensured by a complex cell movements and rearrangements that are mediated by multiple processes. Convergent extension (CE) is one of these processes that can be described by the narrowing and the lengthening of a group of cells. This process is important

in the lengthening of the anteroposterior axis of the embryos, and contributes to neurolation and organogenesis. The vertebrate homologues of the *Drosophila* PCP pathway proteins can also affect the CE of neural tissues in amphibian embryos and some of them such as Stbm, Pk and Diego can act in vertebrates via the JNK pathway that could be considered as the vertebrate analogue of the *Drosophila* PCP pathway (Strutt, 2003).



(Strutt, 2003)

Figure 1.5: CE pathway controls the lengthening of the neural tissues in amphibian embryos

1.1.2 Canonical Wnt signaling pathway

Wnt/β-catenin signaling pathway, named also canonical Wnt pathway, refers to the molecular cascade initiated, in normal cells, by a Wnt ligand and culminating in the stabilization and increase of the β-catenin protein level. It was firstly identified by the discovery of the common origin of the *Drosophila* segment polarity gene *Wingless* and the murine proto-oncogene *Int-1*. Canonical Wnt pathway activation

starts, normally, by the binding of a Wnt ligand to its cognate receptor complex made of a Frizzled receptor and LRP co-receptor and succeeded by the phosphorylation of the integrator molecule Disheveled. By associating with Axin, Disheveled blocks the formation of scaffolding complex responsible for presenting β-catenin to be phosphorylated by GSK-3 β. Unphosphorylated β-catenin escapes ubiquitination and proteasomal degradation, accumulates in the cytoplasm and, in certain circumstances, may migrate to the nucleus. There β-catenin cooperates with LEF/TCF family of transcription factors to activate the transcription of target genes (Polakis 2000; Giles *et al*., 2003).

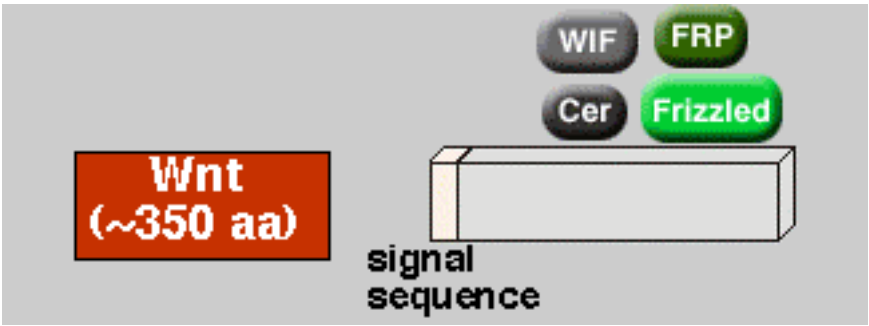
Canonical Wnt signaling regulates, in general, cell proliferation, differentiation and cell fate determination during animal development. In adult organism, it regulates tissue homeostasis, stem cells maintenance and if perturbed leads to cancer. Tremendous amount of work in different animal systems showed the implication of the canonical Wnt pathway in different aspects of embryonic development. Indeed, mutation studies of different genes in the canonical Wnt pathway revealed its importance in the gastrulation process. Indeed, mutation in a single *Wnt, Wnt3*, disrupts gastrulation by blocking primitive streaks formation and consequently, mesoderm and definitive endoderm formation. Similar patterning defects are seen as well in the double mutant Lrp5/6 mice, while *β-catenin* mutant display additional defects in orienting the distal visceral endoderm to the anterior side. In the absence of β-catenin, the endodermal cells change their fate and form cardiac mesoderm instead. Mouse embryos carrying the *Apc* mutation have truncated forebrain and abnormal dorsal localization of the cardiac mesoderm.

Canonical Wnt signaling has also been linked to tumor development since the discovery of *Int-1* integration site in viral carcinogenesis experiments in mice. To date, besides colorectal cancer and hepatocellular carcinoma which harbor the highest rate of Wnt pathway gene mutations, canonical Wnt pathway abnormal reactivation has been linked to many other cancer types including those which do not harbor any activating mutation such as breast cancer.

1.1.3 Major components of the canonical Wnt signaling pathway

1.1.3.1 Wnt

In human, 19 Wnt genes were identified and the finished genomes of mammals and invertebrates revealed 19 Wnt genes in mouse, seven in *Drosophila* and five in *C. elegans*. Sequence analysis in different organisms revealed an extensive conservation of Wnt genes (Nusse 2005). Wnt proteins are secreted lipid-modified signaling molecules that regulate different cellular processes in animal development and tissue homeostasis in adult organisms (Nusse 2005). They are defined by characteristic primary amino acid sequences rather then functional properties. They contain a signal sequence followed by a highly conserved cysteine distribution (figure 1.6)



(Nusse)

Figure 1.6: Wnt protein structure and interacting proteins

It has been recently demonstrated that Wnt proteins are palmitoylated on a conserved cysteine. This modification was shown by mutation studies to be essential for function and explained the fact that Wnt proteins are more hydrophobic then

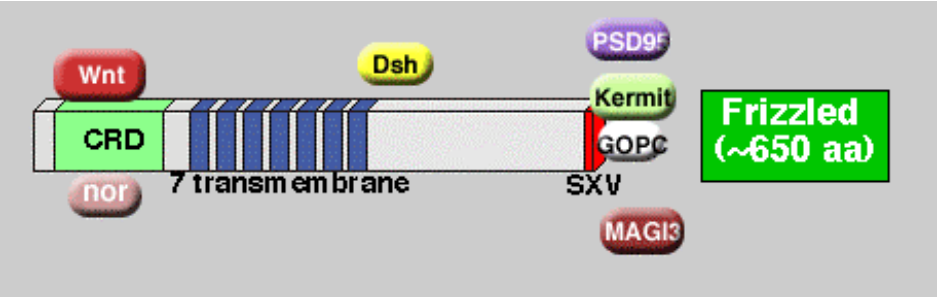
predicted by the primary amino acid sequence (Willert *et al*., 2003). Although this palmitoylation is essential for a normal Wnt signaling, its precise function is not known. Wnt mutant gene constructs at the palmtyolation site can produce a weak signal when overexpressed (Willert *et al*., 2003). This observation could be explained by the fact that the palmityl group may help to target the Wnt protein to the membranes but its absence could be overcome by a higher protein concentration (Logan *et al*., 2004). The spread antibody staining of Wingless in *Drosophila* has demonstrated that Wnt proteins function as long-range morphogenetic molecules in a concentration-dependent manner to act on distant neighboring cells. Although, heparan sulfate proteoglycans have been shown to have a role in stabilizing Wnt protein or aiding them to move between cells, the transport mechanism of Wnt proteins remains to be fully characterized at the molecular level.

Wnt proteins bind to their primary receptors, Frizzleds, which are seven-transmembrane receptors with a long N-terminal cysteine-rich head called Cysteine-Rich Domain (CRD). In addition to the Frizzled CRD, Wnt proteins interact with the single-pass transmembrane protein of the LRP family to form the necessary trimeric receptor complex to initiate Wnt signaling. The Wnt-Frizzled-LRP complex formation can be inhibited by Dikkopf, a potent Wnt signaling inhibitor, which binds to LRP with a higher affinity then Wnt.

1.1.3.2. Frizzled

Frizzleds, the primary receptors of the Wnt proteins, are seven-transmembrane receptors with a long N-terminal extension called a cysteine-rich domain (CRD). The human genome counts ten different *Frizzleds* genes: *Frizzled-1* to *-10*. All frizzled proteins contain a conserved extracellular CRD followed by seven transmembrane segments. Contrarily, their C-terminal cytoplasmic regions differ significantly in length and sequence. As transmembrane receptors, Frizzled proteins engage in multiple interactions with different partners in the extracellular and intracellular milieu (Figure 1.7). Firstly, the Frizzled CRD interacts with the Wnt ligand in the extracellular milieu. Secondly, the frizzled C-terminal intracellular tail

interacts with one of the signal integrating molecules such as Disheveled, GTPases or heterotrimeric G proteins to activate the appropriate downstream signaling cascade depending on the cellular context.



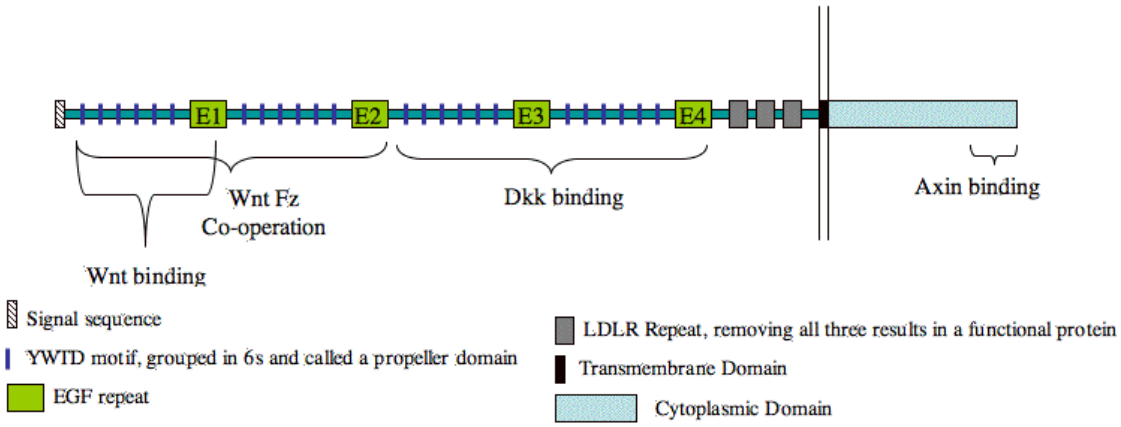
(Nusse)

Figure 1.7: Frizzled protein structure and interacting proteins

Similarly to Wnt ligands, Frizzled receptors have been shown to activate distinct Wnt signaling pathways and can be loosely classified accordingly. A subset of Frizzled receptors such as *Xenopus* Frizzled-3, -4, and –7, have been shown to activate Wnt/β–catenin pathway (Umbhauer *et al*. 2000). Others such as human Frizzled-6 and rat Frizzled-2 have been shown to inhibit it. Furthermore, a conserved motif (Lys-Thr-X-X-X-Trp) located two amino acids after the seventh transmembrane domain was shown to be engaged in the Frizzled/Dishevelled interaction and required for Wnt/β-catenin activation via mediating Disheveled relocalization and phosphorylation (Umbhauer *et al*. 2000).

1.1.3.3 LRP/Arrow co-receptors

In addition to Frizzled receptor, Wnt ligand also requires the presence of LRP5/6 co-receptor in vertebrate or Arrow in *Drosophila*. LRP (LDL receptor related protein) is a single pass transmembrane protein with a cytoplasmic tail containing several proline-rich motifs [Pro-Pro-Pro-(Ser/Trp)-Pro] (Figure 1.8).



(Nusse)

Figure 1.8: LRP protein structure and interacting proteins

The uncontroversial requirement of LRP in Wnt/β–catenin signaling was demonstrated by the discovery of Dikkopf protein as an LRP interacting protein that inhibits specifically the Wnt/β–catenin signaling. Wnt ligand bind to LRP and frizzled to form a trimeric receptor complex. Following Wnt binding, the proline motifs become phosphorylated allowing the sequestering of Axin to the cytoplasmic tail of LRP near the cytoplasmic membrane (Logan *et al*., 2004).

1.1.3.4 Extracellular inhibitors

In order to ensure a tight spacio-temporal regulation of the Wnt signaling, cells developed several regulatory mechanisms, which can act at different levels (DasGupta *et al*., 2005). In the extracellular milieu, a first group composed of secreted Frizzled-related proteins (sFRPs), Wnt-inhibitory factor-1 (WIF-1), Cerberus and Coco can sequester Wnt ligand and prevent its interaction with the receptors. In human, sFRP family consists of five members. They contain a cysteine-rich domain (CRD), which shares 30-50 % sequence homology with the CRD of Frizzled receptors (Ilyas, 2005). WIF-1contains a unique conserved WIF domain and five EGF–like repeats. Cerberus and Coco are related proteins

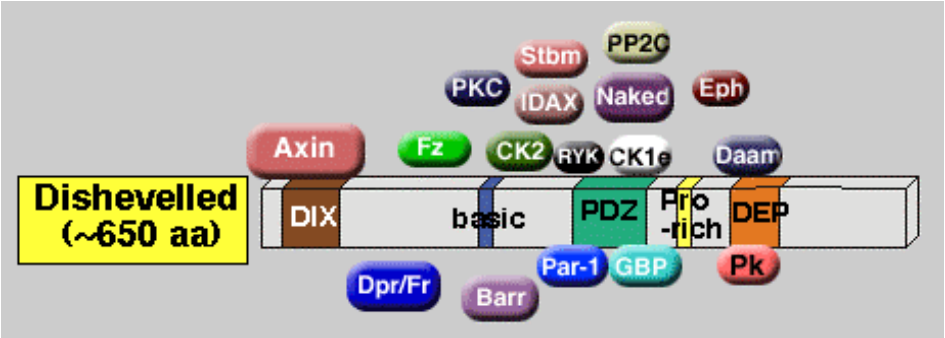
interacting with a variety of growth factors including Wnt ligands and bone morphogenetic protein (BMP) to inhibit the signaling of the respective pathways in Xenopus. However, the inhibitory effect of Cerberus and Coco mammalian orthologs on Wnt signaling has not yet been proved.

The second group of extracellular Wnt signaling inhibitors is the Dickkopf (Dkk) family of secreted proteins. *Dkks* have not been identified in invertebrates but in human three members of the *Dkk* family (*Dkk-1, -2* and *–4*) were found. They inhibit Wnt signaling by inactivating LRP5 and LRP6, which are essential for the activation of the canonical Wnt signaling pathway. The inhibitory effect of Dkk-2 and Dkk-4 proteins requires the participation of Kremen2 to form a tertiary complex with LRP co-receptor leading to the internalization of LRP and makes it unavailable for Wnt binding (Logan *et al*., 2004).

1.1.3.5 Dishevelled

Dishevelled is a constitutively expressed cytoplasmic protein, which is an essential component for the Wnt/β-catenin, the *Drosophila* PCP and the vertebrate CE pathways. Whereas, its ability to activate the Wnt/calcium signaling has been shown to be modest (Veeman *et al*., 2003). In mammals there are three homologs of the *Drosophila Dishevelled: Dishevelled-1, -2* and *–3*. Dishevelled protein is composed of three conserved domains, an N-terminal DIX domain, a central PDZ domain and a C-terminal DEP domain (Boutros *et al*., 1999) (Figure 1.9).

These domains are differentially required for the integration of different signaling functions of this mysterious molecule. While a residual Wnt/β-catenin signaling activity can be seen in the absence of any of the three conserved Dishevelled domains, DEP domain is absolutely required in *Drosophila* PCP and vertebrate CE pathways (Veeman *et al*., 2003). Although the mechanism by which Dishevelled transduces the Wnt signal remains debatable, some evidence elements in this regard have been assembled.



(Nusse)

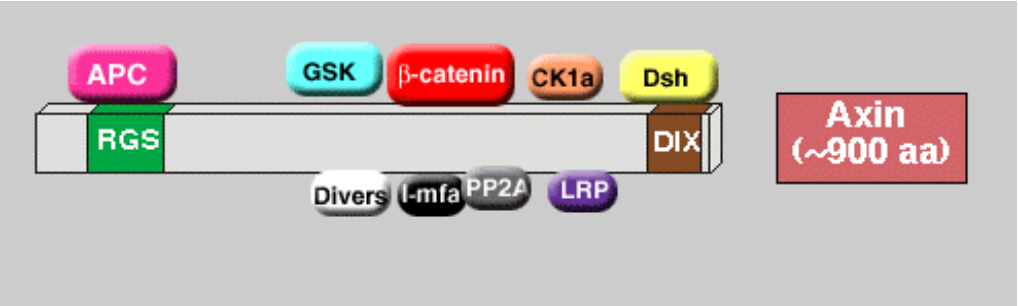
Figure 1.9: Dishevelled protein structure and interacting proteins

Firstly, upon Wnt stimulus, Dishevelled can interact with the C-terminal cytoplasmic tail of Frizzled and gets phosphorylated most likely by the Wnt – regulated protein kinase Par-1 (Sun *et al*., 2001). Secondly LRP proteins can interact with the cytosolic protein Axin (Mao *et al*., 2001; Tolwinski *et al*., 2003), therefore, it is possible to think that following Wnt activation, LRP could be phosphorylated on the PPP(S/T)P motif allowing the attachment of Axin to the cytoplasmic tail of the activated LRP (Logan *et al*., 2004). Thirdly, both Dishevelled and Axin contain the so-called DIX domain. Dishevelled and Xenopus Axin can heterodimerize through their DIX domains (Itoh *et al*., 2000), therefore, it was proposed that Wnt binding to Frizzled and LRP could promote a direct interaction between Axin and Dishevelled through their DIX domains to dissociate the protein complex that regulate β-catenin degradation (Logan *et al*., 2004).

1.1.3.6 Axin

In human, there are two *AXIN* genes: *AXIN-1* and *AXIN-2*, which encode for 900 amino acid proteins. Axin is an inhibitor of the Wnt/*β*–catenin signaling pathway acting as a scaffolding protein that binds, in addition to Dishevelled and LRP, to all the components of the β-catenin phosphorylation complex: β-catenin,

APC, GSK-3β, CK 1α and PP2A (Figure 1.10).



(Nusse)

Figure 1.10: Axin protein structure and interacting proteins

It has been shown that Axin mRNA injection into frog embryos inhibited dorsal axis formation (Zeng *et al*., 1997). Furthermore, truncating mutations of AXIN-1 leads to the nuclear accumulation of β-catenin in hepatocellular carcinomas and the adenoviral transfer of wild-type *AXIN-1* into these cell or HepG2 cells decreased the nuclear accumulation of β-catenin and lowered TCF/LEF-1 mediated transcriptional activity (Satoh *et al*., 2000). Therefore, Axin is considered a critical component of the Wnt/*β*–catenin signaling pathway and it has been attributed the qualification of tumor suppressor. Indeed, Axin-1 loss of function has been found in Hepatocellular carcinomas [8 to10%] (Satoh *et al*., 2000; Taniguchi *et al*., 2002), in hepatoblastomas [7%] (Taniguchi *et al*., 2002), in medulloblastoma [4 to 12%] (Dahmen *et al*., 2001; Yokota *et al*., 2002) and in 4% of endometroid type of ovarian carcinomas (Wu *et al*., 2001). Axin-2 mutations are rare in human cancer and have been found only in 3% of Hepatocellular carcinomas (Taniguchi *et al*., 2002) and 2% of endometroid type of ovarian carcinomas (Wu *et al*., 2001) so far.

1.1.3.7 APC

The adenomatous polyposis coli, APC, is a very large protein of 312 kDa that has many functions in cell migration and adhesion, cell cycle regulation and

chromosome stability (Peifer *et al*., 2000). But its critical function as a negative regulator of the Wnt/β-catenin signaling pathway by controlling the cellular β-catenin level remains the most important in tumorigenesis. APC binds to many cellular proteins other then Axin and *β*–catenin and its amino acid primary sequence shows the presence of three structural motifs, which are responsible for its *β*–catenin-regulating function: the first is made of three 15-amino acid repeats involved in β-catenin and plakoglobine binding, the second is seven 20-amino acid repeats involved in downregulating these proteins and the third is SAMP repeats facilitating Axin and Conductin binding (Figure 1.11).

(Nusse)

Figure 1.11: APC protein structure and interacting proteins

Besides its role in presenting β-catenin to the phosphorylation complex, APC appears to shuttle between the cytoplasm and the nucleus whereby it captures nuclear β-catenin and escort it to the cytoplasmic destruction machinery.

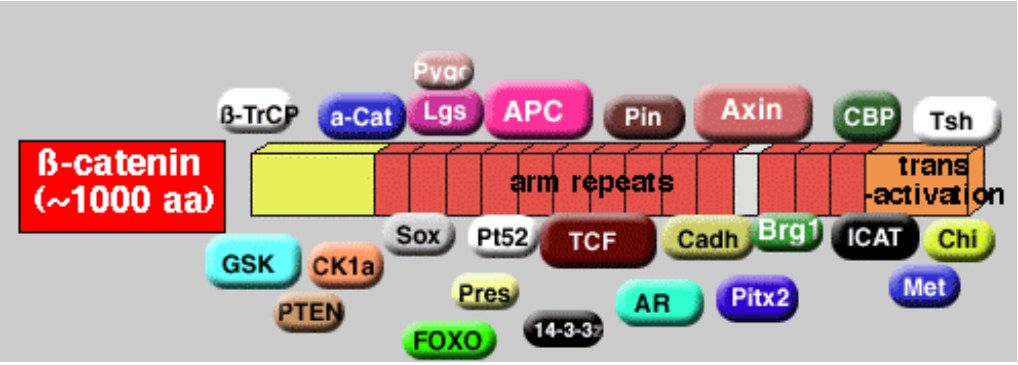
The *APC* gene was initially discovered in the hereditary cancer syndrome named familial adenomatous polyposis [FAP]. Patients with FAP have inherited monoallelic-inactivating mutation in *APC* gene, and most of them display mutations in the remaining wild-type allele in the polyps. *APC* mutations are common in many other human cancers but their frequency in the sporadic colorectal type that goes up to 85% is spectacular. The vast majority of these mutations, which are insertions,

deletions or non-sens mutations, lead to frame-shifts or premature stop codons in the APC transcript and non-functional APC protein (Giles *et al*., 2003).

1.1.3.8 β-catenin

β-catenin protein was initially discovered as a component of the adherens junctions. It promotes cell adhesion by linking the cadherins to the actin filament through the adaptation molecule β-catenin. This adhesion function is based on a membrane associated and stable form of β-catenin. Variable level of free β-catenin was found in the cytoplasm and occasionally in the nuclei depending on the cell type indicating the presence of a second function of β-catenin related with gene transcriptional regulation. Contrarily to the stable membrane bound form, the cytoplasmic β-catenin is unstable and continuously degraded by the proteasomal machinery in most of the normal cells of an adult organism.

The dual function of the β-catenin has structural basis as revealed by primary structure of protein, which is made of three main domains (Figure 1.12). The N-terminal domain of about 130 amino acids that is responsible for the tight regulation of the β-catenin protein level. This domain contain key serine/threonine residues at the positions 29, 33, 37, 41 and 45 which are subject to phosphorylation to earmark the protein for ubiquitination by the E3-ubiquitin ligase to be degraded by the proteasomal machinery. The central domain made of 12 imperfect armadillo repeats that are engaged in the interaction of β-catenin with a wide list of interacting proteins, to name few: APC, Axin, TCF and E-cadherin. The C-terminal domain, spanning the last 100 amino acids, confers to the protein its transcriptional activation property.



(Nusse)

Figure 1.12: β-catenin protein structure and interacting proteins

β -catenin is considered as a transcription factor although no DNA–*β*inding domain or activity was identified, it uses the DNA binding propriety of the LEF/TCF family of transcription factors to transactivate its target genes expression.

In the normal physiological conditions, free β-catenin protein level in the cytoplasm is tightly regulated and kept very low. In the absence of any Wnt stimulus or activating mutation, β–catenin is continuously labeled by phosphorylation on specific residues and degraded by the ubiquitin-mediated proteolysis. Axin-1 and APC are thought to hold and present the β-catenin to be phosphorylated by casein kinase Iα (CK I α) and glycogene synthase kinase–3β (GSK-3β). Once the phosphorylation complex is formed, it is further stabilized by the phosphorylation of Axin and APC by GSK-3β and the protein phosphatase 2A (PP2A) when it is in he trimeric form that contains the B56 subunit (Ilyas, 2005). CK I α phosphorylates β catenin on a single site: serine 45 that will prime the subsequent ordered phosphorylation by GSK-3β on threonine-41, serine-37 and serine-33. Phosphorylated *β*–catenin is then recognized by the *β*–transduction repeat containing protein (*β*–TrCP) of the E3 ubiquitin ligase complex and led to the proteasomal degradation system (Ilyas, 2005). It is noteworthy to mention that all four residues,

in the destruction box of β-catenin, must be phosphorylated in order to be recognized by the *β*–TrCP. Although GSK-3β -mediated degradation of β-catenin is the main mechanism that controls its cytoplasmic low level, another GSK-3β -independent mechanism has been shown to promote β-catenin degradation. *Siah-1*, which is induced by p53, can associate with β-catenin and promote its proteasomal degradation via E2 conjugating enzyme and the ubiquitin E3 ligase.

The β-catenin gene (*CTNNb1*) is frequently mutated in many types of cancer (Giles *et al*., 2003). Most of these mutations occur in the destruction box, encoded by the exon3 of the *CTNNb1* gene, on the GSK-3β phosphorylation target residues. Some deletions covering this region are seen as well in hepatoblastomas. The outcome of these mutations is a stabilized β-catenin which, may translocate to the nucleus, cooperates with the LEF/TCF family of transcription factors to exercise transcriptional activation of target genes depending on the cell type and context. The highest frequency of *CTNNb1* mutations was seen in hepatocellular carcinomas, which harbor between 13 and 41% (Giles *et al*. 2003; Prange *et al*. 2003), in gastric carcinomas 20% (Giles *et al*., 2003) and colorectal cancer 10% (Giles *et al*., 2003).

1.2 WNT SIGNALING IN CANCER:

Aberrant activation of the Wnt/β-catenin signaling pathway is one of the most frequent abnormalities in human cancer. In colorectal cancers, canonical Wnt signaling is aberrantly activated by mutations affecting either APC tumor suppressor gene (85%) or β-catenin-encoding *CTNNb1* oncogene (10%)(Giles *et al*., 2003). In liver cancer, frequent accumulation of β-catenin protein correlated with *CTNNb1* and *AXIN-1* mutations (Giles *et al*., 2003), and *p53* mutations (Cagatay *et al*., 2002).

Theoretically, reactivation of the canonical Wnt pathway can occur either by abnormal expression of Wnt ligand and receptor or by activating mutations affecting one of the downstream components in the pathway. Numerous studies in different

types of human cancers showed that mutations in *CTNNb1, AXIN* and *APC* genes have implicated this pathway in the genesis of cancer (Giles *et al*., 2003). Particularly, colorectal cancer is an important example since 85% of sporadic colorectal cancers have mutations in APC (Laurent-Puig *et al*., 1998).

1.2.1 WNT SIGNALING AND LIVER CANCER:

1.2.1.1 Liver cancer

Hepatocellular carcinoma [HCC] or hepatoma, which arises from hepatocytes, is the major type of primary liver cancer. It is the fifth most frequent neoplasm worldwide (>500,000 deaths/year), and its incidence is steadily increasing in the West (Bruix *et al*., 2004). In addition to HCC, there two other rare types of liver cancer which are: cholangiocellular carcinoma or bile duct cancer arising from bile duct cells, and hepatoblastoma or childhood liver cancer which is common in young children under the age of three years. HCC, classified histologically in the epithelial group, is linked etiologically to many factors (Ozturk, 1999):

- Chronic infection with hepatitis B virus [HBV] and hepatitis C virus [HCV] is involved in about 80% of HCC cases worldwide.
- Dietary intake of chemical carcinogens such as aflatoxin B1
- Alcoholism
- Environmental factors: chemicals, cigarette smoking...
- Genetic factors: Hereditary tyrosinemia, a1-antitrypsin deficiency and idiopathic hemochromatosis.

At the molecular level, HCCs are linked to the alteration of four growth regulatory pathways (Ozturk, 1999):

- P53 pathway: The tumor suppressor gene *p53* is inactivated in HCC by mutations [28% of HCC cases] or allelic deletions [24 –65%] (Ozturk , 1999).

- Wnt/β–catenin pathway: β–catenin gene, *CTNNb1*, is mutated in approximately 22% of HCC, and *Axin-1* gene is mutated in about 7% of these tumors (Ozturk, 1999).

- RB1 pathway: three genes in the RB1 pathway namely *RB1, p16INK4A* and *cyclin D* undergo structural changes in HCCs. Firstly, LOH at the *RB1* gene locus is quite frequent in HCC and *RB1* gene is mutated in 15% of these tumors (Ozturk, 1999). Secondly, the *p16INK4A* gene displays both germ line and somatic mutations in HCC and about 50% of these tumors have *de novo* methylation on *p16INK4A* gene. Thirdly, *Cyclin D* gene is amplified in 10 to 20% of HCCs. All together, these mutations on the RB1 pathway will lead to a loss of growth control in more than 30% of HCCs (Ozturk, 1999).

- TGF–β pathway: it is involved in growth inhibition and apoptosis. In overall, *TGF–*β is altered in about 25% of HCCs. Three genes involved in the TGF–β pathway are mutated in HCCs. The mannose-6-pohosphate/insuline-like growth factor-2 receptor (M6P/IGF2R) that is involved in the activation of *TGF–*β is mutated in 18 to 33% of HCCs (De Souza *et al*., 1995). *SMAD2* and *SMAD4* genes, which are intracellular mediators of the TGF–β pathway, are mutated in less then 10% of HCCs (Kawate *et al*., 1999; Yakicier *et al*., 1999).

According to most accepted hypothesis, HCC arises as a well differentiated tumor and proliferates with a stepwise process of dedifferentiation (Kojiro, 2005). High-grade dysplastic nodules are considered to be true preneoplastic lesions giving rise to well differentiated HCC. This early form then progresses into moderately and poorly differentiated tumors, followed by undifferentiated tumors (Kojiro, 2005). Early well-differentiated tumors are highly proliferative and become less

differentiated when they reach 1-1.5 cm. At this stage, angiogenesis, tissue invasion and metastasis become evident. Later on, HCC cells become undifferentiated and are able to invade vessels and form extra-hepatic metastases (Bruix *et al*., 2004). This dedifferentiation process is associated with a progressive accumulation of genomic changes including chromosomal gains and losses, as well as *p53* mutations (Thorgeirsson *et al*., 2002). A rare exception to this picture is the status of the *CTNNb1* (β-catenin) gene, which encodes a key component of the canonical Wnt/β-catenin signaling pathway.

1.2.1.2 Wnt/*β*–catenin signaling aberration in liver cancer

Canonical Wnt pathway is one of the altered growth regulatory pathways in HCC (Ozturk, 1999). In HCC, *β-catenin* mutations have been found in 22% of cases in average, and an additional 7% display *Axin1* mutations (Buendia, 2002). Thus, almost a third of HCCs display mutations affecting the canonical Wnt/β-catenin signaling. The hallmark of Wnt/β-catenin activation is the accumulation of β-catenin protein in the cytoplasm and its translocation under certain cellular circumstances to the nucleus. On the other hand, Wnt/*β*–catenin activation could be due to activating mutations or the aberrant expression of a ligand/receptors. Several studies have shown a significant correlation between Wnt/*β*–catenin pathway mutational activation and *β*–catenin nuclear accumulation in HCC. These studies revealed a common finding that the frequency of nuclear *β*–catenin is higher than the mutation rate of the *β–catenin* gene [39% versu 24% and 66% versus 34%]. Additional mutation in *Axin-1* gene, which account for 7% in HCC would not explain this discrepancy. Therefore, additional mechanisms capable of inducing *β*–catenin translocation to the nucleus must be considered. Theoretically, aberrant overexpression of canonical Wnt pathway activators such as canonical Wnt ligands and Frizzleds or down-regulation of other repressors such as non-canonical *WNT5A* would be a logical hypothesis.

Several years ago, two independent studies showed that *β-catenin* mutations are associated with distinct subsets of HCCs (Hsu *et al*., 2000; Laurent-Puig *et al*., 2001). According to Hsu *et al*., *β-catenin* mutations are associated with a subset of well differentiated and low-stage HCCs with a favorable prognosis (Hsu *et al*., 2000). On the other hand, Laurent-Puig *et al*. determined that HCCs form two distinct groups according to the chromosome stability status. One group demonstrates chromosome stability, *β-catenin* mutation and chromosome 8p losses. The other group demonstrates chromosome instability and frequent *Axin1* and *p53* mutations (Laurent-Puig *et al*., 2001). These observations have now been confirmed and extended by many other studies based on mutation analysis or nuclear β-catenin staining. High frequencies of *β-catenin* mutation and nuclear β-catenin staining were detected in early stage well differentiated HCCs, but both aberrations were declining in late stage less differentiated HCCs (Wong *et al*., 2001; Mao *et al*., 2001; Inagawa *et al*., 2002; Fujito *et al*., 2004). Although a few studies did not agree with some of the findings (Suzuki *et al*., 2002; Tien *et al*., 2005), these observations are consistent with the hypothesis that β-catenin aberrations in HCC occur during the initial step of neoplastic transformation at the time when the well-differentiated HCC lesions emerge from dysplastic nodules. Indeed, dysplastic nodules display no *β-catenin* mutation (Prange *et al*., 2003; Park *et al*., 2005). As *APC* mutations (leading to aberrant activation of β-catenin) are the earliest changes detected in colorectal cancers (Gregorieff *et al*., 2005), a similar finding in HCC does not come as a surprise. However, the progressive decline of β-catenin aberrations in less differentiated and more aggressive HCCs is unexpected. As stated earlier, constitutive activation of the canonical Wnt/β-catenin signaling as a result of aberrant β-catenin accumulation is considered to play a key role in colorectal cancers.

1.2.2 WNT SIGNALING IN BREAST DEVELOPMENT AND CANCER:

1.2.2.1 Breast development

The human adult breast is made of four main structures: lobules, ducts, fat and connective tissue. In addition, the breast has a nipple, which is a protruding point

surrounded by a dark tissue: the areola. Lobules are the milk-producing units that form the lobes during lactation. Ducts are the branching tubes connecting the lobules and lobes. They converge all to the larger collecting ducts towards the nipple. Ducts carry milk from the lobules towards the nipple during lactation. Fat and connective tissues surround the lobes and the branching ducts and constitute a supporting stroma. Like all organs, the breast is connected to the main circulation system by blood and lymphatic vessels (**www.mammary.nih.gov**).

In human, the breast starts to form at embryonic week 7 to 8 and continues to develop throughout the female life. The breast tissue originates from the ectoderm following mesenchymal-epithelial interactions that will direct the mesenchymal differentiation and epithelial proliferation leading to the first nodule structure formation. The nodule forms next the breast bud that will start to branch and form the secondary buds. The secondary buds will form canals and branches, which by elongation and invasion of the mesenchyme will form the ductal structure (Howard *et al*., 2000).

At birth, the ductal system opens to the surface through a cavity on the skin to form the nipple. Up on this stage, both female and male breast tissues show the same development. At puberty, and following hormone stimulation in female, the breast will develop further. Hormone stimulates ducts growth, invasion into the fat pads and formation of lobular structures (Howard *et al*., 2000).

During pregnancy and lactation, the human breast will display an increase in the number of lobules and loss of fat. Lobulo-acinar structures, which have milk-secreting alveolar cells, form due to the high proliferation followed by terminal differentiation. At weaning, the removal of suckling stimulus leads to involution, which means the elimination of secretory epithelial cells by apoptosis and phagocytosis. At each pregnancy, terminal duct lobular units expand in size and then involutes. At menopause, a greater ductal and lobular involution occurs and the removed tissues are replaced by fat (Howard *et al*., 2000).

1.2.2.2 Wnt/β–catenin signaling aberration in breast cancer

Since the discovery of *Wnt-1* as a mouse mammary tumor virus-induced oncogene in mouse breast tumors, Wnt signaling has become a center of interest in human breast carcinogenesis. Surprisingly however, human breast cancers do not display genetic alterations in known mutational target genes of the Wnt signaling, including *APC, CTNNB1* and *AXIN1* (Brown 2001; Ueda *et al*., 2001; Giles *et al*. 2003; Brennan *et al*., 2004; Howe *et al*., 2004). Nevertheless, there are observations, which indicate the reactivation of Wnt/β-catenin signaling in human breast cancer. Firstly, several reports showed that about 60% of breast cancers display accumulation of β-catenin, which is a sign for Wnt/β–catenin pathway activation (Brown, 2001). Secondly, *Cyclin D1*, a well-known transcriptional target of the canonical Wnt signaling is overexpressed in primary breast tumors (Lin *et al*., 2000). Thirdly, an increasing number of studies showing the overexpression of many Wnt ligands in breast cancer enforce the hypothesis that Wnt/β–catenin signaling pathway could be activated through the abnormal overexpression of Wnt proteins in the presence of the required receptors.

Although there is no systematic study of all 19 Wnt genes in breast cancer so far, there has been reports showing the overexpression of *WNT2, WNT2B, WNT4, WNT5A, WNT7B* and *WNT10B* in a certain proportion of breast tumors (Brown, 2001). These observations were not sufficient to claim that Wnt/β-catenin is activated by mis-regulation of Wnt pathway components in breast cancer until Bafico *et al*. presented the first demonstration for Wnt/β–catenin signaling reactivation by an autocrine mechanism (Bafico *et al*., 2004). This report showed that specific extracellular Wnt/β–catenin pathway inhibitors FRP1 and DKK1 caused a dramatic decrease in the transcriptional active form of β-catenin in breast cancer cells.

2. AIMS

Although still debated, HCC originate from hepatocytes as a well-differentiated tumor and proliferates with a stepwise process of dedifferentiation (Kojiro *et al*., 2005). Early well-differentiated tumors are highly proliferative and become less differentiated when they reach 1-1.5 cm. At this stage, HCC cells start to acquire angiogenesis, tissue invasion and metastasis properties. Later on, they become undifferentiated and are able to invade vessels and form extra-hepatic metastases (Bruix *et al*., 2004). This dedifferentiation process is associated with a progressive accumulation of genomic changes including chromosomal gains and losses, as well as *p53* mutations (Thorgeirsson *et al*., 2002). Laurent-Puig *et al.* showed that HCCs could be classified into two distinct groups according to the chromosome stability status. One group demonstrates chromosome stability, *β-catenin* mutation and chromosome 8p losses. The other group demonstrates chromosome instability and frequent *Axin1* and *p53* mutations (Laurent-Puig *et al.,* 2001).

Almost a third of HCCs display constitutive activation of Wnt/β-catenin signaling caused by the mutations in *CTNNb1* or *Axin1* genes (Reya *et al*., 2005). Indeed, in HCC, *β-catenin* mutations have been found in 22% of cases in average, and an additional 7% display *Axin1* mutations (Buendia, 2002). Interestingly, the status of the *CTNNB1* (β-catenin) gene, which encodes a key component of the canonical Wnt/β-catenin signaling pathway, makes the exception for the general picture. Unlike the other genomic changes accumulating during HCC evolution such as chromosomal instability, p53 and Axin1 mutations, high frequencies of *CTNNb1* mutation and nuclear β-catenin protein staining were detected in early stage well differentiated HCCs, but both aberrations were declining in late stage less differentiated HCCs (Wong *et al.*, 2001; Mao *et al.*, 2001; Inagawa *et al.*, 2002; Fujito *et al.*, 2004).

In attempt to understand the particular behavior of β-catenin during HCC evolution we hypothesize that the canonical Wnt signaling pathway has a differentiation-dependent regulation in HCC.

In breast cancer, however, the implications of Wnt signaling are quite puzzling. On one hand, there is ample evidence for a direct role of aberrant Wnt signaling in mouse breast carcinogenesis. On the other hand, human breast cancers do not display mutations on *APC, CTNNB1* and *AXIN1* genes that are known to be frequent mutational targets in other human cancers (Brown 2001; Ueda *et al*., 2001; Giles *et al*., 2003; Brennan *et al*., 2004; Howe *et al*., 2004). Nevertheless, there are observations, which indicate the reactivation of Wnt/β-catenin signaling in human breast cancer. Firstly, several reports showed that about 60% of breast cancers display accumulation of β-catenin, which is a sign for Wnt/β-catenin pathway activation (Brown, 2001). Secondly, *Cyclin D1*, a well-known transcriptional target of the canonical Wnt signaling is overexpressed in primary breast tumors (Lin *et al*., 2000). Thirdly, an increasing number of studies show the overexpression of many Wnt ligands in breast cancer cell lines and primary breast tumors.

Therefore, we also hypothesize that Wnt/β-catenin signaling pathway could be activated through the abnormal overexpression of Wnt proteins in the presence of the required receptors in breast cancer cell lines and primary breast tumors.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacterial strain:

The bacterial strain used in this work was: *E. coli*, DH5α, JM 109.

3.1.2 Cell lines:

Ten HCC (Huh7, Hep40, Hep3B, FOCUS, Mahlavu, SNU182, SNU 387, SNU 398, SNU 449 and SNU 475), one hepatoblastoma (HepG2), six Breast cancer cell lines: MCF 7, T47-D, BT-474, BT-20, MDA-MB–453, MDA-MB–468 all from ATCC and the telomerase-immortalized human mammalian epithelial cell line hTERT-HME1 (HMEC; Clontech) were used in this study.

3.1.3 Tumor samples:

Non-tumor-matched primary breast tumor samples were obtained from 14 women who underwent surgery for infiltrating ductal carcinoma. Freshly frozen samples were used for RNA isolation.

3.1.4 Enzymes:

Taq DNA Polymerase was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

3.1.5 Antibodies and chemiluminescence:

Mouse anti-human β–catenin monoclonal antibody (clone 6F9) was purchased from Abcam Ltd (Cambridge, UK). HRP-conjugated rabbit anti-mouse Ig was from DAKO Corporation (CA, USA). Detection of immobilized proteins on membranes was done by using ECL Plus Western Blotting Detection System from Amersham Biosciences UK Limited (Little Chalfont Buckinghamshire, England) or Lumi-Light Western Blotting Substrate from Roche Diagnostics GmbH (Mannheim, Germany).

3.1.6 Nucleic acids:

DNA molecular weight standard: GeneRulerTM 100 bp DNA Ladder (catalogue number: SM 0241) was purchased from MBI Fermentas GmbH (Germany). pEGFP-N2 expression vector was purchased from CLONTECH Laboratories, Inc. (CA, USA). pGL3-OT and pGL3-OF constructs were a gift from Bert Vogelstein (John Hopkins Oncology Center, Baltimore, MD, USA).

3.1.7 Oligonucleotides:

The primers used for PCR were synthesized by IONTEK (Istanbul, Turkey).

Table 3.1: Wnt primers list

Genes	**Primer sequence (Forward * Reverse)**
WNT1	CAGTGGAAGGTGCAGTTGCAGC *CAGTGGAAGGTGCAGTTGCAGC
WNT2	CGGGAATCTGCCTTTGTTTA * TCCTTTCCTTTCCTTTCCTT
WNT2B	AAGATGGTGCCAACTTCACC * ATTTCTGCATTCCTTGCACC
WNT3	TCGGCGTGTTAGTGTCCAGG * CTTGTGCCAAAGGAACCCGTC
WNT3A	GCACCACCGTCCACGACAGC * CCTCGCTACAGCCACCCCAC
WNT4	CCTTCGTGTACGCCATCTCT * TCAGAGCATCCTGACCACTG
WNT5A	TGGCTTTGGCCATATTTTTC * CCGATGTACTGCATGTGGTC
WNT5B	ACGCATCTGTCTTTGGGAGA * CCCTGCTCCTCTGATCCTTT
WNT6	GGTTATGGACCCTACCAGCA * GGTTATGGACCCTACCAGCA
WNT7A	CCCACCTTCCTGAAGATCAA * ACAGCACATGAGGTCACAGC
WNT7B	ATGCACAGAAACTTTCGCAA * TGCATCCGGTCCTCTAGAAC
WNT8A	AACCTGTTTATGCTCTGGGC * GAAGAGCATTTTCAGGGCAG
WNT8B	TTCCAAGCAGTTTGTCGATG* GAGATGGAGCGAAAGGTGTC
WNT9A	TGGAGGCCGTGAGCATGAGT* CTTAAGGTTGTCTCCGCAGC
WNT9B	TGCACCTGTGATGACTCTCC * CTGATACGCCATGGCACTTA
WNT10A	TCTTCCTACTGCTGCTGGCT * TAGGGGATCTTGTTGCGAGT
WNT10B	CATCCAGGCACGAATGCCAATC * AGGCTCCAGAATTGCGGTTGTG
WNT11	GTGTGCTATGGCATCAAGTG * AGCTCAATGGAGGAGCAGTT
WNT16	CCAAGGAAACTGGATGTGGT * TCATGCAGTTCCATCTCTCG

Table 3.2: *Frizzled, LRP, LEF/TCF* primers list

Genes	**Primer sequence (Forward * Reverse)**
FZD1	TCAACTACCACTTCCTGGGG * CAGCACAGCACTGACCAAAT
FZD2	CTTCTGGGCCACACGAACCAG * GGCACCCGGCTGCAGTCCCGG
FZD3	GTATGGAATATGGACGTGTCACAC* TATGAGATCCTTGTGTCACTGTGG
FZD4	GCCAATGTGCACAGAGAAGA * GGTTTTGTGAGGTAAGGGCA
FZD5	TTCTGGATAGGCCTGTGGTC * AGGTAGCAGGCTGACAGGAA
FZD6	CAAATTCATGTGGTTCCACCT * TGCCTTGGACACCAAAATCCA
FZD7	CCAACGGCCTGATGTACTTT * GAGAACGGTAAAGAGCGTCG
FZD8	AAGACAGGCCAGATCGCTAA * GACACGAAGAGGTAGCAGGC
FZD9	TGCCCCTCTCTGGCTACCTG * GGGCACCGTGTAGAGGATGG
FZD10	CCTCCAAGACTCTGCAGTCC * CTCCAAGACTCTGCAGTCC
LRP5	AAACAGCAGTGCGACTCCTTC * TGGCACACAAAATAGACACCA
LRP6	TCAAGCACCAAAGGCACTTAC * CTTGGCTGTTGCCACTGA
LEF1	TCAGGTACAGGTCCAAGAATGA * AAGAGGGGTTGGCAGTGATT
TCF1	TGGCTTCTACTCCCTGACCT * TCTCTGCCTTCCACTCTGCT
TCF3	TCGAGAAGAACAGGCCAAGT * GGGGCAGGCTGAACACATTA
TCF4	TGTACCCAATCACGACAGGA * GCCAGCTCGTAGTATTTCGC

3.1.8 Electrophoresis and photography:

Horizontal electrophoresis apparatus was from Stratagene (Heidelberg, Germany). Densitometric Fluorescence-Chemiluminescence image analyzer and the Molecular Enalyst software used in agarose gel profile visualizing was from BioRad Laboratories (CA, USA).

3.1.9 Tissue culture reagents:

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were obtained from BIOCHROM AG Seromed (Berlin, Germany). Penicillin/ Streptomycin mixture was obtained from Biological Industries (Haemal, Israel).

3.2 SOLUTIONS AND MEDIA:

3.2.1 General solutions:

1X Phosphate–βuffered saline (PBS):	Per liter: 8 g NaCl, 0.2 g KCl, 1.44g Na2HPO4 and 0.24 g KH2PO4 (pH 7.4)
1X Tris–βuffer saline (TBS):	10 mM Tris- Cl (pH 8) and 150 mM NaCl.
1X Tris-acetic acid-EDTA (TAE):	0.04 M Tris-acetate and 1 mM EDTA (pH 8)
5X formaldehyde gel-running buffer:	0.1 M MOPS, 40 mM sodium acetate (pH 7) and 5 mM EDTA (pH 8). Sterilized by filtration through 0.2–μm-pore size filter.

1X Tris-glycine electrophoresis buffer:	25 mM Tris base, 250 mM glycine (pH 8.3) and 0.1% SDS.
Transfer buffer:	39 mM glycine, 48 mM Tris-Cl, 0.037% SDS and 20% methanol (pH 8.3).
30% acrylamide mix (29:1):	Per 100 ml: 29 g acrylamide and 1 g bis-acrylamide in double-distilled water. Sterilized by filtration through 0.45–μm-pore size filter.
10% ammonium persulfate (APS):	0.1 g/ml in double-distilled water. Prepared freshly.
Ethidium bromide:	10 mg/ml in double-distilled water.
6X gel-loading buffer:	0.25% Bromophenol blue, 0.25% Xylene Cyanol FF and 30% glycerol in double-distilled water.
Formaldehyde gel-loading buffer:	50% glycerol, 1mM EDTA (pH 8), 0.25 bromophenol blue and 0.25% xylene cyanol FF. Prepared with DEPC-treated water.
1X SDS gel loading buffer:	50 mM Tris- Cl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue and freshly added 1% 2-β-mercaptoethanol.
Solution I:	50 mM glucose, 25 mM Tris-Cl (pH 8) and 10 mM EDTA (pH 8). Sterilized in autoclave and stored at 4^{o}C.

Solution II:	0.2 N NaOH and 1% SDS. Prepared freshly.
Solution III:	per 100 ml: 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml double-distilled water.
Bradford stock solution:	4.75 ml of 95% ethanol, 10 ml of 85% phosphoric acid and 17.5 mg Coomassie brillant blue. Stored in dark at 4°C.
Bradford working solution:	21.25 ml H2O, 0.75 ml of 95% ethanol, 1.5 ml of 85% phosphoric acid and 1.5 ml of Bradford stock solution. Filtered through Whatman No.1 paper and prepared freshly.
2X BES–βuffered saline:	50 mM BES, 280 mM NaCl and 1.5 mM Na2HPO4 (pH 6.96). Sterilized by filtration through 0.45–μm-pore size filter.
Ampicillin:	100 mg/ml in double-distilled water (stock solution). Sterilized by filtration through 0.45-μm-pore size filter and stored at –20°C. Working solution was 100 μg/ml.
Kanamycin:	30 mg/ml in double-distilled water (stock solution). Sterilized by filtration through 0.45-μm-pore size filter and stored at –20°C. Working solution was 30 μg/ml.

Geneticin (G418 Sulfate):	500 mg/ml in double-distilled water (stock solution). Sterilized by filtration through 0.45-µm-pore size filter and stored at –20°C.

3.2.2 Media:

3.2.2.1 Luria–Bertan medium (LB):

Per liter: 10 g bacto-tryptone, 5 g bacto-yeast extract and 10 g NaCl. For LB agar plates, add 15 g/l bacto-agar. Sterilized by autoclave.

3.2.2.2 Growing medium:

DMEM supplemented with 10% FCS, 0.1mM non-essential amino acids and 100 mg/ml of penicillin/streptomycin.

3.2.2.3 Freezing medium:

70% DMEM, 20% FCS and 10% DMSO.

3.3 METHODS:

3.3.1 Cell culture techniques:

3.3.1.1 Cell thawing:

A vial of frozen cell line was taken from the liquid nitrogen tank, and thawed in a 37°C water bath. Then, the cells were resuspended in 10 ml fresh medium and centrifuged at 1500 rpm for five minutes. The supernatant was discarded and the cell pellet was resuspended in an adequate volume of fresh medium to be plated into a culture flask. Cells were incubated in a 37°C incubator with an atmosphere of 5% CO_2 in air. The medium was refreshed the following day.

3.3.1.2 Cell culture:

All HCC and breast cancer cell lines were grown in the growing medium described previously. The telomerase-immortalized human mammary epithelial cell line hTERT-HME1 (HMEC) was grown in DMEM/Ham's F12 (V/V) medium containing 3.5 mg/ml insulin, 0.1 ng/ml EGF, 0.5 mg/ml hydrocortisone, 10% FCS, 0.1mM non essential amino acids and 50 mg/ml of penicillin/streptomycin. HMEC and all cancer cell lines were incubated in a 37^{o}C incubator with an atmosphere of 5% CO_2 in air. The medium was refreshed every two to three days. Cells were split before reaching confluence; the growth medium was aspirated and the cells were washed once with PBS, trypsin was added to the plates and incubated for five minutes at 37^{o}C to remove the monolayer cell from the plate surface. An adequate volume of fresh growing medium was added to the plate and the suspension was pipeted gently to disperse cells. Cells were transferred to fresh plates using adequate dilutions depending on the requirements.

3.3.1.3 Cell freezing:

Exponentially growing cells were harvested by trypsin treatment as described previously and resuspended in growing medium. Cells were counted and centrifuged at 1500 rpm for five minute. The pellet was resuspended in freezing medium at a concentration of 4 x 106 cells/ml. Aliquots of one ml of this suspension were placed into a screw cap cryotubes and left at –70^{o}C overnight. The next day tubes were transferred to the liquid nitrogen storage tank.

3.3.1.4 Transient transfection of mammalian cells using $CaPO_4$ technique:

Cells were plated into 10 cm culture plates the day before transfection in a way to obtain 50 to 60% confluence on the transfection day. Before starting DNA precipitation, the cells were washed once with PBS and 9 ml of fresh growing medium were added to the plate. The following mixture was prepared:

20 µg of foreign DNA

Up to 450 µl H_2O

50 µl $CaCl_2$ (2.5 M) drop wise.

The mixture was left at room temperature for 30 minutes, then 500 µl of 2X BES were added drop wise to the mixture and left at room temperature for 40 minutes. The $CaPO_4$-DNA mixture was then added drop wise to the cells, the plate was stirred gently and incubated in a 37°C incubator with an atmosphere of 5% CO_2 in air. After 18 hours, cells were washed with PBS, fed with fresh growing medium and incubated in the same conditions.

3.3.1.5 Transient transfection of mammalian cells using Lipofectamine:

Cells were plated into 6-well plates the day before transfection in a way to obtain 80 to 90% confluence on the transfection day. Before starting DNA precipitation, the cells were washed once with PBS and 2.25 ml of fresh growing medium without antibiotics were added to the plate. The following mixtures were prepared:

Mixture 1: 2 µg of foreign DNA in up to 125 µl DMEM

Mixture 2: 5 µl of Lipofectamine and 120 µl DMEM

Two mixtures were incubated at room temperature for five minutes bfore mixing them. The resulting mixture was left at room temperature for 30 minutes, then added drop wise to the cells. The plate was stirred gently and incubated in a 37°C incubator with an atmosphere of 5% CO_2 in air. After six hours, cells were washed with PBS, fed with fresh growing medium and incubated in the same conditions.

3.3.2 Western blotting

Equal amounts of proteins were analyzed by 10% SDS-PAGE, then transferred onto a PVDV membrane and probed with the appropriate antibodies. ECL kit (Amersham

Life Science, Inc., Piscataway, NJ) was used for detection of antigen-antibody complexes. Equal protein loading was verified by Western blot assay with Calnexin or GSK-3β antibodies.

3.3.3 Luciferase Assay

The pGL3-OT and pGL3-OF reporter plasmids (gift from Bert Vogelstein) were used to test β-catenin-Lef/TCF-dependent transcriptional activity as described previously (Erdal *et al*., 2005) with minor modifications. Cells were transfected using Lipofectamin 2000 reagent (Invitrogen), following instructions provided by the supplier. Mutant β-catenin-induced TCF/LEF-dependent transcriptional activity was tested after co-transfection of cells with pCI-Neo-mutant β-catenin (S33Y) expression plasmid (1.75 μg/well) together with the reporter plasmids. pCI-Neo (1.75 μg/well) was used as a negative control. At 48 hours following transfection, the luciferase assay was performed by using the Luciferase Reporter Gene Assay, constant light signal kit (Roche Diagnostics GmbH., Mannheim, Germany). Luciferase activity was read with The Reporter® Microplate Luminometer (Turner BioSystems Inc., Sunnyvale, CA) and data was normalized according to transfection efficiency as estimated by counting the GFP expressing cell obtained with each transfection. All experiments were performed in triplicate and TCF activity was reported as the ratio of normalized luciferase activities obtained with pGL-OT and pGL-OF plasmids, respectively.

3.3.4 RNA extraction

3.3.4.1 From cultured cells:

Total RNAs were isolated from cultured cells using the NucleoSpin RNA II Kit (MN Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol.

3.3.4.2 From tissues:

Tissue samples were homogenized using a Potter homogenizer in Tridity G (Applichem, Germany), phenol/chloroform-extracted and purified with the NucleoSpin RNA II kit.

3.3.5 CDNA synthesis:

The cDNAs were synthesized from total RNA as a template (2 µg RNA for cell lines, 1 µg RNA for tumor and non-tumor samples), using oligo-dT primers and RevertAid First Strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania). A negative control without reverse transcriptase (1 µl ddH_2O instead) was also prepared for each sample.

3.3.6 RT-PCR

The list of primers used for these analyses is provided in Table 1. The PCR reactions were usually carried out with 1µl cDNA, at the corresponding annealing temperatures and $MgCl_2$ provided in Table 3.3 for 35 cycles except *WNT2* and *WNT7A*, which were amplified for 30 cycles. Negative controls without reverse transcriptase were included for each set of primers. PCR products were analyzed on a 2% (w/v) agarose gel prepared with Tris/Acetic acid/EDTA (TAE) buffer and stained with 5µg/ml ethidium bromide and visualized under UV transillumination.

Table 3.3: PCR conditions

Wnts				FZD, LRP and LEF/TCF			
	Tm	$MgCl_2$	Size		Tm	$MgCl_2$	Size
WNT1	57	2	469	*FZD1*	52	2	136
WNT2	57	1.5	227	*FZD2*	55	1.5	350
WNT2B	55	1.5	281	*FZD3*	55	2.5	581
WNT3	62	1.5	396	*FZD4*	52	1.5	211
WNT3A	57	1.5	210	*FZD5*	52	2	128
WNT4	52	1	142	*FZD6*	52	1.5	229
WNT5A	56	2.5	199	*FZD7*	50	2	104
WNT5B	50	1	283	*FZD8*	55	2	215
WNT6	58	1.5	250	*FZD9*	50	1.5	164
WNT7A	60	1.5	183	*FZD10*	50	1.5	160
WNT7B	55	1.5	590	*LRP5*	55	2	170
WNT8A	57	2	205	*LRP6*	52	2	242
WNT8B	57	1.5	313	*LEF1*	55	1.5	245
WNT9A	58	1	272	*TCF1*	57	1.5	206
WNT9B	53	1	223	*TCF3*	55	1.5	218
WNT10A	53	1	274	*TCF4*	60	1.5	420
WNT10B	60	2	217				
WNT11	53	1.5	227				
WNT16	54	4	200				

PCR conditions: Initial denaturation 5min, then 30 seconds denaturation , 30 seconds annealing, 30 seconds extension. Final extension was 10 minutes.

3.3.6 Multiplex RT-PCR

Multiplex RT-PCR reactions were carried out using 8 pmol/µl of cyclin D1 or c-Myc primers along with 2 pmol/µl of GAPDH primers for a total cycle number of 30 and 35 for Cyclin D1 and c-Myc, respectively. PCR products were analyzed on a 2 % (w/v) agarose gel prepared with Tris/Acetic acid/EDTA (TAE) buffer and stained with 5µg̃/ml ethidium bromide and visualized under UV transillumination.

3.3.7 Nuclear extract preparation

For nuclear extraction, cells were harvested with a scraper in cold PBS, washed twice in the same buffer and resuspended in 5 volumes of fresh cold solution A (10 mM HEPES; pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT and 0.5 mM PMSF). Resuspended cells were left on ice for 10 min. then centrifuged at 2000 rpm for 10 min at 4^o C. The pellet was resuspended in 2 volumes of solution A, and transferred to a Dounce homogenizer. Twenty strokes were applied with pestle B. Then, 0.1 volume of restoring solution (1ml 10X solution A and 9 ml of 75% sucrose) was added and 4 more strokes were applied. The nuclear pellet was recovered by centrifugation at 10000 rpm for 30 seconds at 4 ^{0}C. The supernatant (cytosolic fraction) was removed and the nuclear pellet was resuspended in 1 volume of nuclear resuspension buffer (9ml solution A and 1ml restoring solution) and centrifuged at 10000 rpm for 30 seconds. The supernatant was removed and the nuclear pellet volume (NPV) was measured. The nuclear pellet was resupended in 5 NPV of solution B (10mM HEPES pH 7.9, 400mM NaCl, 1.5mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, 0.5mM PMSF and 5% glycerol) and homogenized by applying 5 strokes with pestle B. The homogenate was transferred to a 15 ml snug cap white test tube, shacked on ice for 30 min., then centrifuged at 13000 rpm for 30 minutes at 4º C to remove nuclear membranes. The supernatant containing the nuclear extract was used for western blotting.

4. RESULTS

4.1 DUAL ANTAGONISTIC ROLE OF Wnt SIGNALING IN HCC

4.1.1 Disparate canonical Wnt/β-catenin signaling in hepatocellular carcinoma cell lines according to their differentiation status

4.1.1.1 HCC cell lines grouping

In order to investigate Wnt signaling in different types of HCC cell lines, we first grouped them according to their differentiation state using hepatocyte nuclear factor (HNF) family members *HNF-4α* and *HNF-1α* as molecular markers. These two genes that are involved in liver development and hepatocyte specification have previously been identified as specific markers for HCC cells with well-differentiated function and morphology (Ishiyama *et al*., 2003). By RT-PCR, we showed that out of 11 cell lines reported here, four expressed both *HNF-1α* and *HNF-4α* transcripts, strongly. All other cell lines were negative for both markers, except SNU449 cells, which displayed weak *HNF-4α* expression and SNU182 cells, which displayed weak *HNF-1α* (Figure 4.1). This allowed us to classify our cell lines into two groups: the first group of well differentiated cell lines expressing *HNF-1α* and *HNF-4α* including HepG2, Huh7, Hep3B and Hep40 and the second group of poorly differentiated cell lines not expressing *HNF-1α* and *HNF-4α* composed of SNU182, SNU387, SNU398, SNU449, SNU475, FOCUS and Mahlavu. Well-differentiated cell lines, but not poorly differentiated cells also expressed albumin and α-foetoprotein, as additional markers for hepatocyte-like differentiation (Erdal *et al*., unpublished data).

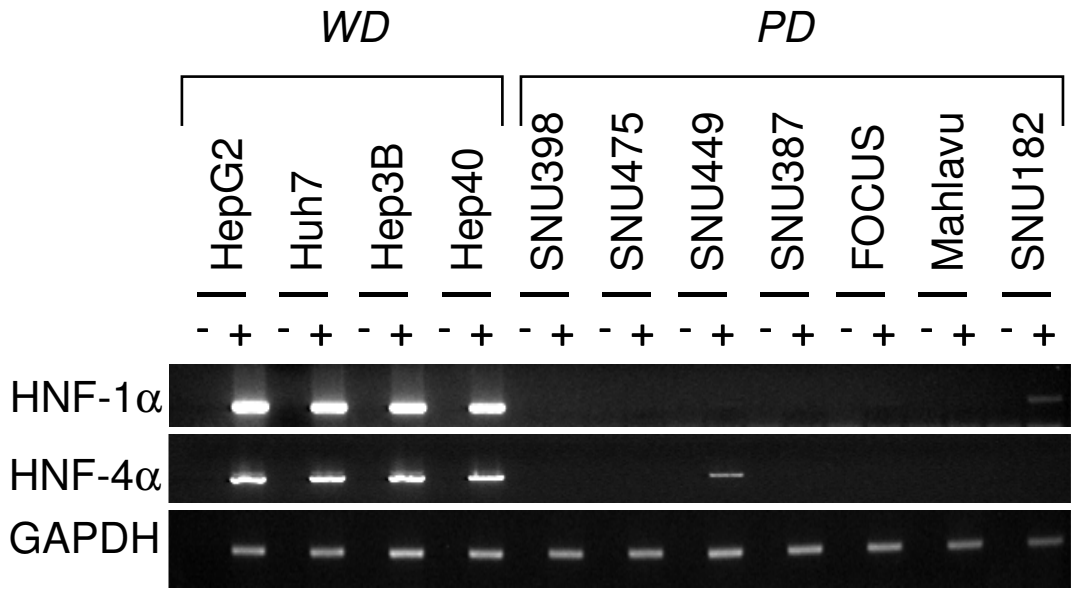


Figure 4.1: Expression profile of *HNF-1α* and *HNF-4 α* transcripts in HCC cell lines.

RT-PCR analysis of HCC differentiation markers *HNF-1 α* and *HNF-4α* allows to classify hepatoma cell lines into well differentiated (expressing *HNF-1 α* and *HNF-4α* transcripts) and poorly differentiated (not expressing *HNF-1 α* and *HNF-4α* transcripts) groups.

4.1.1.2 Canonical Wnt/β-catenin signaling activity in hepatocellular carcinoma cell lines

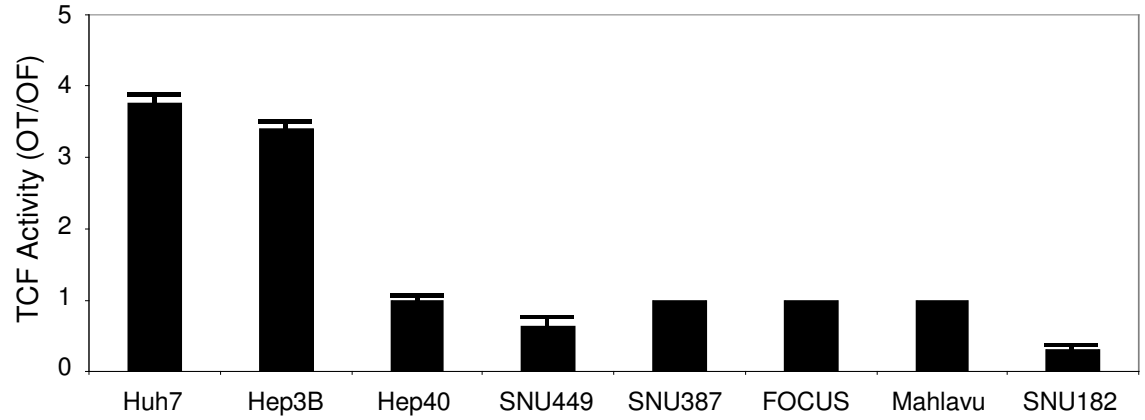
Canonical Wnt/β-catenin signaling activates TCF/LEF-dependent transcription, which can be monitored by reporters containing TCF-responsive elements (Morin *et al*., 1997). We surveyed canonical Wnt/β-catenin signaling activity in well differentiated and poorly differentiated cell line groups, using TCF/LEF reporter pGL3-OT plasmid, as described previously (Erdal *et al.*, 2005). First, we compared TCF/LEF activity in three cell lines with known mutations in Wnt/β-catenin signaling pathway. The well-differentiated, Hepatoblastoma-derived, cell line HepG2 displays β-catenin mutation (N-terminal deletion of 116 amino acids

at the destruction box). Poorly differentiated SNU398 cell line displays β-catenin missense mutation S37Y and SNU475 cell line harbor Axin1 mutation (Satoh *et al.*, 2000; Cagatay *et al*., 2002) (Table 4.1). Normalized TCF/LEF activity was the highest in HepG2 cells. SNU398 cells displayed 50% less activity compared to HepG2. More interestingly, despite a homozygous deletion leading to a loss of Axin1 expression (data not shown; Satoh *et al.*, 2000; Cagatay *et al*., 2002), there was no detectable TCF/LEF activity (PLG3-OT/pGL3-OF ratio equals 1.07) in SNU475 cells (Figure 4.2). In contrast, another *Axin-1*-mutant, but well differentiated HCC cell line, namely PLC/PRF/5 or Alexander, displayed high TCF/LEF activity (Satoh *et al.*, 2000; Erdal *et al*. unpublished data).

Next, we compared TCF/LEF activity of eight other cell lines that display wild type β-catenin and Axin1 expression (Satoh *et al.*, 2000; Cagatay *et al*., 2002; Erdal *et al*., 2005). Hep40 cells harbor a missense Axin1 mutation/polymorphism (R454H), but its functional significance is unknown (Erdal *et al*., 2005). In contrast to Cha *et al.* (Cha *et al.*, 2004), we detected weak but significant TCF/LEF activity (more than 3 folds) in well differentiated Huh7 and Hep3B cell lines. On the other hand, all five poorly differentiated cell lines as well as the well-differentiated Hep40 cells displayed no detectable activity (Figure 4.2).

Taken together, we collected TCF/LEF activity data from 12 hepatoma cell lines. Independent of β-catenin or Axin1 status, TCF/LEF activity was detected in four out of five (80%) well differentiated cell lines, whereas only one out of seven (14%) poorly differentiated cell lines had constitutive TCF/LEF activity. These findings clearly indicate that canonical Wnt/β-catenin signaling in HCC is closely dependent on the differentiation status. Well-differentiated HCC cells usually express constitutive signaling, whereas poorly differentiated HCC cells do not.

A



B

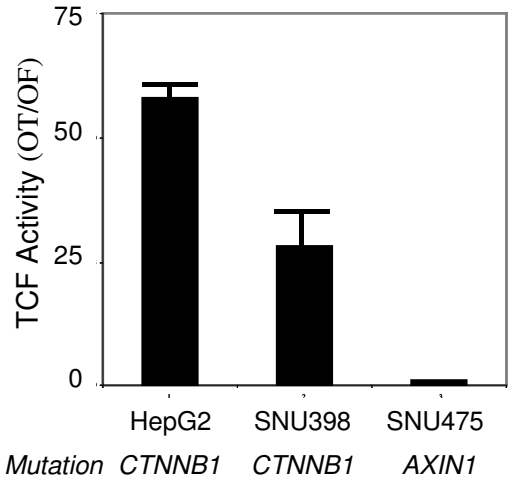


Figure 4.2: Frequent constitutive activation of the canonical Wnt/β-catenin signaling in well differentiated, but not in poorly differentiated hepatoma cell lines.

A: Comparative analysis of the canonical Wnt/β-catenin signaling in HCC cell lines with wild-type *β-catenin* or *Axin-1* genes. Huh7 and Hep3B cell lines (both well differentiated) display weak but significantly increased TCF reporter activity. Other cell lines (all poorly differentiated, except Hep40) display no detectable TCF reporter activity. B: Comparative analysis of the canonical Wnt/β-catenin signaling in hepatoma cell lines with known mutations of *β-catenin* or *Axin-1* genes. TCF reporter assay shows that well differentiated HepG2 cells display high signaling activity. In contrast, it was attenuated in the poorly differentiated SNU398, and undetectable in poorly differentiated SNU475 cell line. Lipofectamine was used for transfection. Assays in triplicate, error bars; standard deviation. TCF activity was normalized as the ratio of signals detected with pGL3-OT and pGL3-OF plasmids, respectively. Assays were done in triplicate.

Table 4.1: List of Wnt pathway mutations in HCC cell lines

	CTNNb-1	*Axin-1*	*APC*
HepG2	Δ 25-140/WT	WT	WT
Huh7	WT	WT	WT
Hep3B	WT	WT	WT
Hep40	WT	WT	WT
SNU398	S37C/WT	WT	WT
SNU475	WT	Δ exon 1-2	WT
SNU449	WT	WT	WT
SNU387	WT	WT	WT
FOCUS	WT	WT	WT
MAHLAVU	WT	WT	WT
SNU182	WT	WT	WT

4.1.2 Canonical Wnt/β-catenin signaling is repressed in poorly differentiated hepatocellular carcinoma cells

The presence of canonical Wnt/β-catenin signaling in well-differentiated hepatoma cells was apparently due to β-catenin or Axin-1 mutation, as well as other unknown factors. On the other hand, lack of canonical Wnt/β-catenin activity in poorly differentiated cells could be due to either a lack of significant signaling activity, or alternatively to an active repression. To test these possibilities, we compared TCF/LEF activity in Huh7, SNU449 and SNU182 cell lines following transient expression of a mutant (S33Y)-β-catenin. Transfection with S33Y-β-catenin resulted in an increase in total β-catenin protein levels in all three cell lines. Changes in β-catenin levels in Huh7 and SNU449 cells were comparable, though SNU182 behaved slightly differently (Figure 4.3a). Well differentiated Huh7 cells responded to S33Y-β-catenin expression by a strong activation of TCF/LEF reporter activity (pGL3-OT/pGL3-OF ratio >130). Under the same experimental conditions, the response of SNU449 cells was minimal (pGL3-OT/pGL3-OF ratio equals 5). More importantly, SNU182 cells were totally unresponsive (pGL3-OT/pGL3-OF ratio <1) (Figure 4.3.b).

These drastic differences between well differentiated Huh7 and two different poorly differentiated cell lines (SNU449 and SNU182) could not be explained by experimental differences, as the measured activities have been corrected for transfection efficiencies (see material and methods section). Therefore, our findings strongly support the hypothesis that canonical Wnt/β-catenin signaling is actively repressed in poorly differentiated HCC cells. This repression appears to occur downstream to β-catenin accumulation, at least in SNU449 and SNU182 cells tested here. These unexpected findings led us to conclude that the regulation of Wnt signaling in well differentiated and poorly differentiated HCC cell lines is complex. This complexity could arise from the cellular context, including epigenetic differences in the expression of multiple Wnt signaling components.

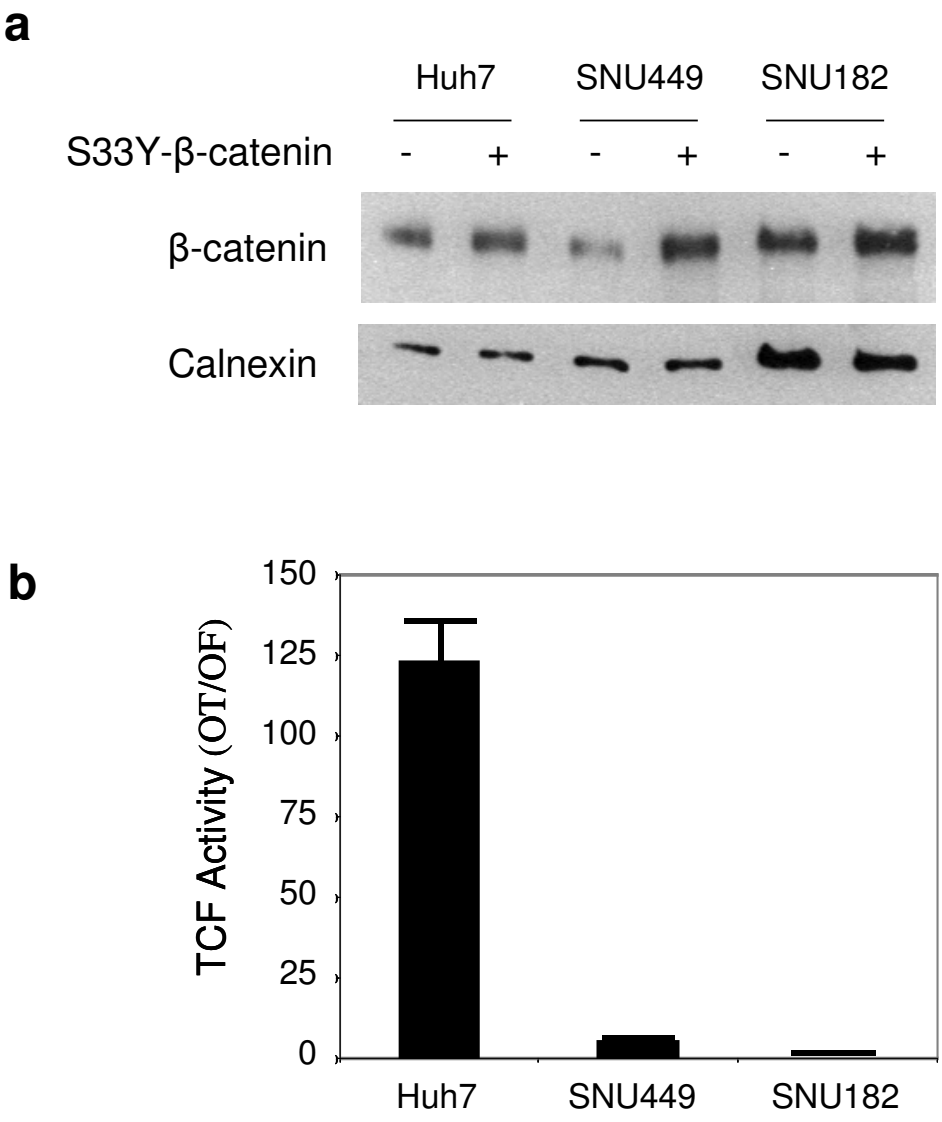


Figure 4.3: Ectopic expression of mutant β-catenin (S33Y) results in high canonical Wnt/β-catenin activity in well differentiated, but not in poorly differentiated hepatocellular carcinoma cell lines.

Well differentiated Huh7, and poorly differentiated SNU449 and SNU182 cell lines were co-transfected with either pCI-neo-mutant β-catenin (S33Y) plasmid (S33Y-β-catenin +) or empty pCI-neo plasmid (S33Y-β-catenin -) using Lipofectamine. (**a**) The cellular β-catenin levels at post-transfection 48h were tested by immunoblotting using anti-β-catenin monoclonal antibody. Calnexin was used as a loading control. (**b**) pCI-neo-mutant β-catenin (S33Y)-transfected cells were subjected to TCF reporter assay (lower panel). TCF activity was normalized as the ratio of signals detected with pGL3-OT (OT) and pGL3-OF (OF) plasmids, respectively. Assays were made in triplicate. Co-transfections included pGL-OT or pGL-OF, in addition to pCI-neo plasmids.

4.1.3 Expression of non-canonical Wnt5A and Wnt5B ligands is restricted to poorly differentiated hepatocellular carcinoma cell lines

Theoretically, reactivation of the canonical Wnt pathway can be caused either by abnormal expression of Wnt ligand and receptor or by activating mutations affecting one of the downstream components in the pathway. Numerous studies in different types of human cancers showed that mutations in *CTNNb1, Axin* and *APC* genes have implicated this pathway in the genesis of cancer (Giles, 2003).

Particularly, colorectal cancer is an important example since 85% of sporadic colorectal cancers have mutations in *APC* (Laurent-Puig *et al*., 1998). In addition, mutations in the beta-catenin gene, *CTNNb1,* account for 18% in human hepatocellular carcinomas (HCC)(Giles, 2003). Recently, two reports gave clear evidence for the presence of an autocrine mechanism behind the constitutive Wnt pathway reactivation in human breast and ovarian cancer cell lines (Bafico *et al*., 2004) and in acute myeloid leukaemia (Simon *et al*., 2005). Nevertheless, currently, there is no clear evidence for any autocrine regulatory role of the Wnt pathway in HCC. Therefore and given the previous data showing the disparate Wnt activity in well differentiated and poorly differentiated HCC cell lines, we hypothesize that the differential expression of the canonical Wnt pathway major components may play a role in its differential regulation according to the differentiation status of HCC cell lines. To validate our hypothesis we performed a large-scale expression analysis using the RT-PCR technique of all major Wnt signaling components in the 11 cell lines studied here.

4.1.3.1 LEF/TCF, LRP and Frizzled receptor expression profile in HCC cells

The hallmark of the canonical Wnt pathway reactivation is β-catenin stabilization and its translocation to the nucleus. It is also known that β-catenin does not have any transcriptional promoting activity; rather it interacts with LEF/TCF family of transcription factors to promote the transcription of target genes. Thus, TCF/LEF transcription factors are considered as major components of the Wnt/β–catenin signaling pathway and their expression is required to have an active pathway.

Therefore, we investigated the expression profile of LEF/TCF family of transcription factors in the HCC cell lines. Our data showed that *TCF-1* and *TCF-4* were highly expressed in all HCC cell lines while *TCF-3* was highly expressed in seven out of 11 HCC cell lines (Figure 4.4). LEF1 transcript expression was weak and restricted to the five cell lines: Huh7, SNU398, Mahlavu, FOCUS and SNU182 cells. In general, our data showed that all HCC cell lines express strongly at least two members of the TCF/LEF transcription factor family (Figure 4.4). We concluded that the expression profile of TCF/LEF transcription factors is in agreement with previous TCF activity data since the presence of at least one member of the TCF/LEF transcription factor family is absolutely required for an active Wnt/β-catenin pathway.

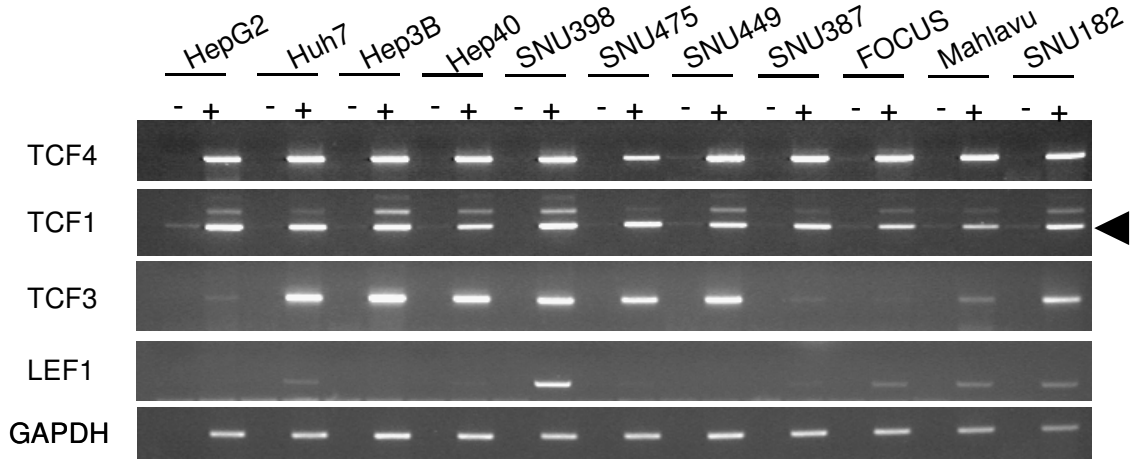


Figure 4.4: Comparative analysis of *LEF/ TCF* transcription factors in hepatoma cell lines by RT-PCR.

With the exception of *LEF1* these components of Wnt signaling are expressed redundantly and broadly in the cell lines tested.

Wnt signaling is, mostly, initiated by the binding of Wnt ligand to its Frizzled receptor. Frizzled receptor is a key molecule in the Wnt/β–catenin pathway by transmitting the Wnt signal from the extracellular milieu to the intracellular

transducing molecule Dishevelled. Thus, Frizzled receptor expression can also be a determinant factor in order to have an active Wnt/β–catenin pathway. Therefore, we investigated, by RT-PCR technique, the expression profile of all ten members of the Frizzled receptor family in 11 HCC cell lines. Our results showed that HCC cell lines expressed redundantly a large set of Frizzled transcripts. Every cell line expressed at least seven out of ten *Frizzleds*. With few exceptions, we can say that our HCC cell lines expressed uniformly *Frizzled-1* to *-7*. Some cell lines weekly expressed *Frizzled-8* and *Frizzled-9*, while *Frizzled-10* was not expressed in any HCC cell line (Figure 4.5). The interaction between Wnt ligand and Frizzled receptor is mediated by the co-receptor LRP-5 or LRP-6 (Wehrli *et al*., 2000). Therefore, in addition to Frizzled receptors we studied the expression profile of LRP-5 and LRP-6 co-receptors in HCC cell lines. Our results revealed that both co-receptors were expressed in all cell lines at almost a similar level (Figure 4.5). These data are in commitment with the presence of TCF activity in Huh7 and Hep3B cells which do not harbor any activating mutation on the Wnt/β–catenin pathway.

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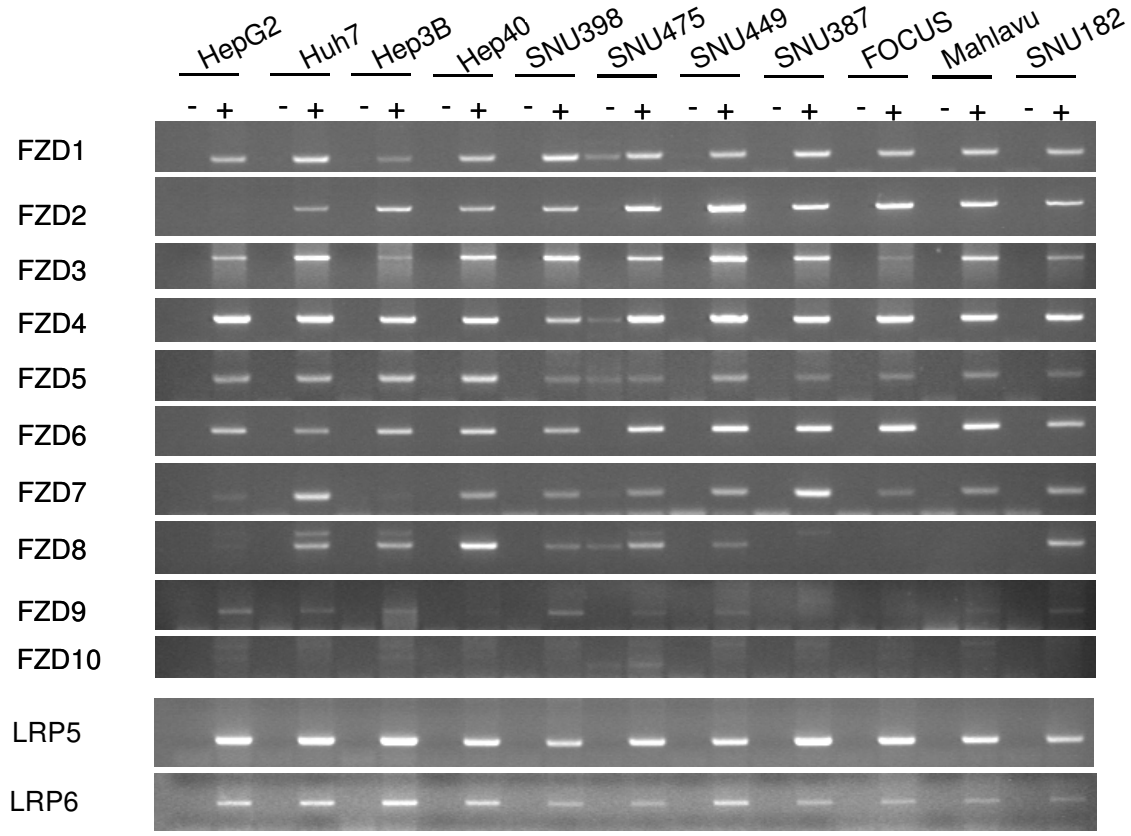

Figure 4.5: Comparative analysis of Frizzled receptors and LRP co-receptors in hepatoma cell lines by RT-PCR.

With the exception of Frizzled-10, all Frizzled and LRP transcripts are expressed redundantly and broadly in the cell lines tested

4.1.3.2 Wnt expression profile in HCC cell

In human, there are 19 known Wnt ligands encoded by 19 different genes. Wnt ligands are secreted lipid modified glycoproteins which initiate the Wnt signaling pathways by interacting with their cognate Frizzled receptors. We studied the expression profile of the complete list of human Wnt ligands by RT-PCR in the 11 HCC cell lines and our results revealed the following:

Firstly, a redundant expression of Wnt ligands by all HCC cells lines. Indeed, except Huh7, which expressed only *WNT2B, WNT3* and *WNT8B*, all HCC cell lines expressed many Wnt ligands. Notably, SNU182 cells expressed the highest number: 17 out of 19 Wnts (Figure 4.6).

Secondly, out of 19 known Wnt genes tested, seven (*WNT2B, WNT3*, *WNT5A, WNT5B, WNT7B, WNT9A* and *WNT10B)* were consistently expressed in the majority of HCC cell lines (Figure 4.6). We noticed that Wnt3, Wnt2B, Wnt7B, Wnt9A and Wnt10B ligands were expressed quite similarly in well-differentiated and poorly differentiated cell lines. In addition, we noticed that nine Wnt genes (*WNT1, WNT2, Wnt3A, WNT6, WNT8A, WNT9B, WNT10A, WNT11* and *WNT16*) were scarcely or not expressed at all by any HCC cell line (Figure 4.7).

Thirdly, the expression of *WNT4, WNT5A, WNT5B and WNT8B* ligands was closely associated with differentiation status of the HCC cell lines. While the expression of the three ligands *WNT4, WNT5A* and *WNT5B* was restricted to poorly differentiated cell lines and one well differentiated cell line, namely Hep40 (Figure 4.6), which is also the only well differentiated cell line lacking constitutive Wnt/β-catenin signaling (Figure 4.2), *WNT8B* ligand was exclusively expressed by well differentiated cell lines with the exception of SNU449.

As shown in Figure 4.6, Wnt ligands that appear to be expressed in both well differentiated and poorly differentiated cell lines (*WNT2B, WNT3, WNT9A* and *WNT10B)* are implicated in canonical Wnt/β-catenin signaling (Shimizu *et al*., 1997;

Person *et al*., 2005; Bennett *et al*., 2002; Wang *et al*., 2005; Grandage *et al*., 2005). We were able to identify four ligands, namely *WNT4, WNT5A, WNT5B* and *WNT8B* that are differentially expressed between well differentiated and poorly differentiated HCC cells. Interestingly, both Wnt5A and Wnt5B are non-canonical signaling ligands that inhibit canonical Wnt/β-catenin signaling (Topol *et al*., 2003; Ishitani *et al*., 2003; Kanazawa *et al*., 2005), have a restricted expression to the group of poorly differentiated cell lines lacking (SNU182, SNU387, SNU475, SNU449, FOCUS and MAHLAVU) or showing reduced (SNU398) TCF activities in addition to the well differentiated HCC cell lines plus Hep40 which lacks also any TCF activity. Wnt4, which is classified as non-canonical Wnt (Shimizu *et al.*, 1997; Maurus *et al*., 2005) although few reports showed its ability to induce canonical Wnt signaling in some particular contexts (Lyons *et al*., 2004), showed almost a similar expression pattern to WNT5A and Wnt5B. On the other hand, Wnt8B, which is considered as a canonical Wnt (Katoh, 2005) had a restricted expression to the well-differentiated HCC cell lines (HepG2, Huh7, Hep3B and Hep40). Although the role of these ligands in liver cells is not known, their expression may play a role in the differential regulation of canonical Wnt/β-catenin signaling according to the differentiation status of HCC cell lines.

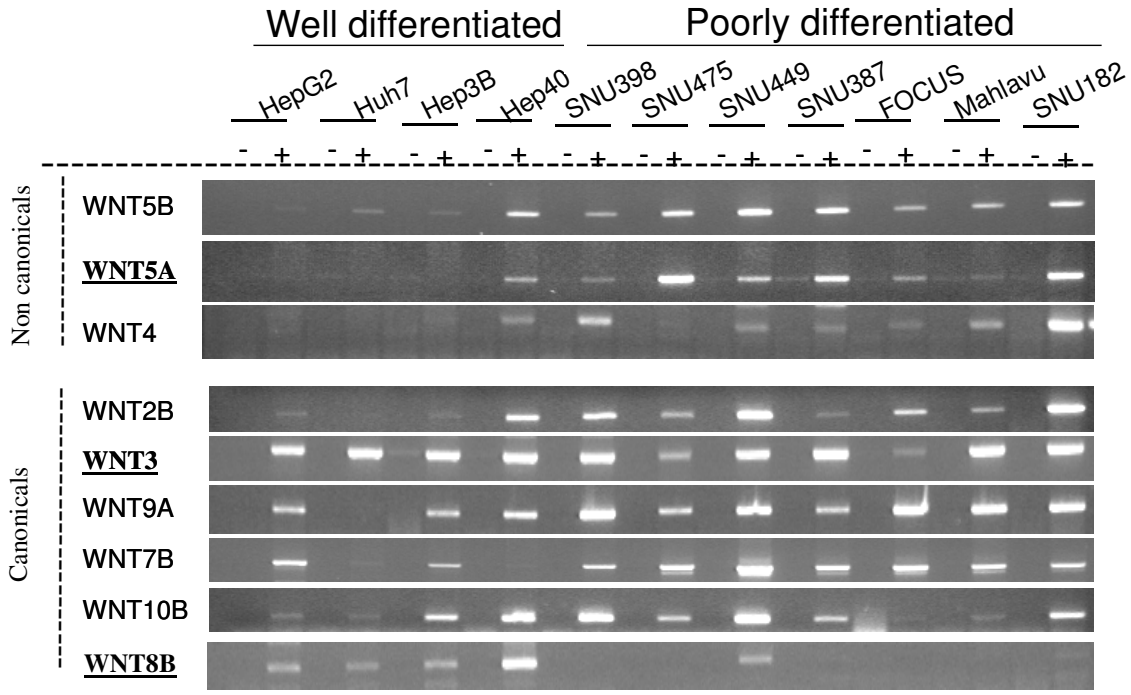

Figure 4.6: Analysis of Wnt ligand expression in HCC cell lines by RT-PCR.

Ligands involved in canonical Wnt/β-catenin and non-canonical signaling pathways were indicated. The expression of non-canonical Wnt4, Wnt5A and Wnt5B ligands is associated with poorly differentiated state. Wnt8B expression was expressed in the well differentiated group and the poorly differentiated SNU449 cells.

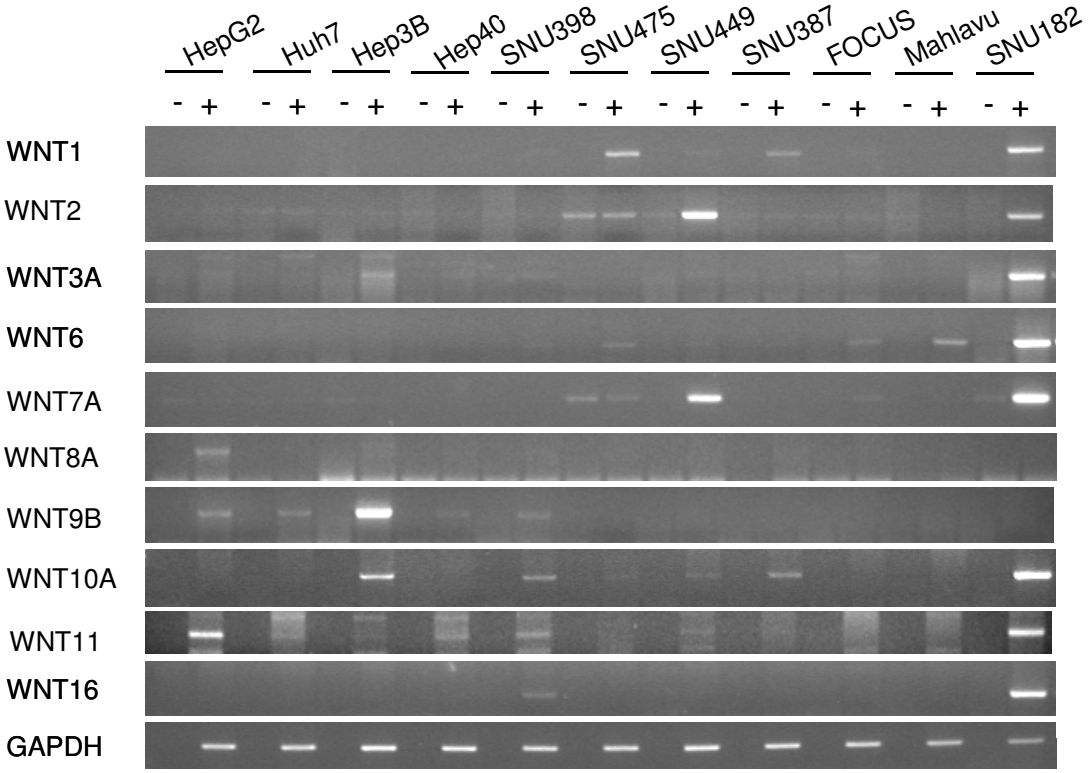

Figure 4.7: Analysis of Wnt ligand expression in HCC cell lines by RT-PCR.

Ligands involved in Wnt/β-catenin signaling pathway which are scarcely or not expressed in HCC cell lines.

4.2 β- CATENIN AND CYCLIN D UPREGULATION IS LINKED TO MULTIPLE WNT LIGANDS EXPRESSION IN BREAST CANCER CELLS

In order to know whether and how the Wnt pathway genes play a role in human breast cancers, we performed a comprehensive analysis of major components of the Wnt signaling pathways in normal and malignant breast cell lines. Telomerase-immortalized human mammary epithelial cell line hTERT-HME (HMEC) was used as normal control. We also used a panel of six breast cancer cell lines that are commonly used for studies related to human breast cancer biology and genetics. Primary breast tumors and a pool of non-tumor breast tissues were also used for confirmatory studies.

4.2.1 Redundant expression of Frizzled receptors, LRP co-receptors and Lef/TCF transcription factors in human breast cancer cells

In mammalians, Wnt signaling is mediated by ten different Frizzled (*FZD*) receptors. In addition, *LRP5* and *LRP6* co-receptors are also involved in the canonical Wnt/β-catenin signaling pathway. We analyzed the expression of these receptors in normal and breast cancer cell lines by RT-PCR. We found that firstly, HMEC and all breast cancer cell lines showed a redundant expression of Frizzled receptor transcripts. Moreover, our analysis revealed that HMEC and the breast cancer cell lines expressed generally more then five out of ten *Frizzled* genes. Secondly, we found that all frizzled receptors, except *FZD-9* and *FZD-10* were expressed in both HMEC and most breast cancer cell lines. Different Frizzled receptors have been implicated in canonical and noncanoncial Wnt signaling. As shown in Figure 3.6, both canonical (*FZD-1, FZD-7, FZD-8*) (Gazit *et al*., 1999; Umbhauer *et al*., 2000) and noncanonical (*FZD-2, FZD-3, FZD-4, FZD-6*) (Gazit *et al*., 1999; Umbhauer *et al*., 2000) Frizzled receptors were expressed in both normal and malignant breast epithelial cells. The *FZD-5* and *FZD-9* were not expressed in HMEC, and only a few malignant cell lines displayed their robust expression, whereas *FZD-10* was negative for all cell lines (Figure 4.6).

The LRP5 and LRP6 co-receptors are required at the cell membrane to mediate the interaction between the Wnt ligand and its Frizzled receptor. Therefore were also tested the expression of LRP5/6 co-receptors in HMEC and our breast cancer cell lines. Our data showed that both co-receptor transcripts were expressed at a comparable level in HMEC and all breast cancer cell lines tested (Figure 3.8).

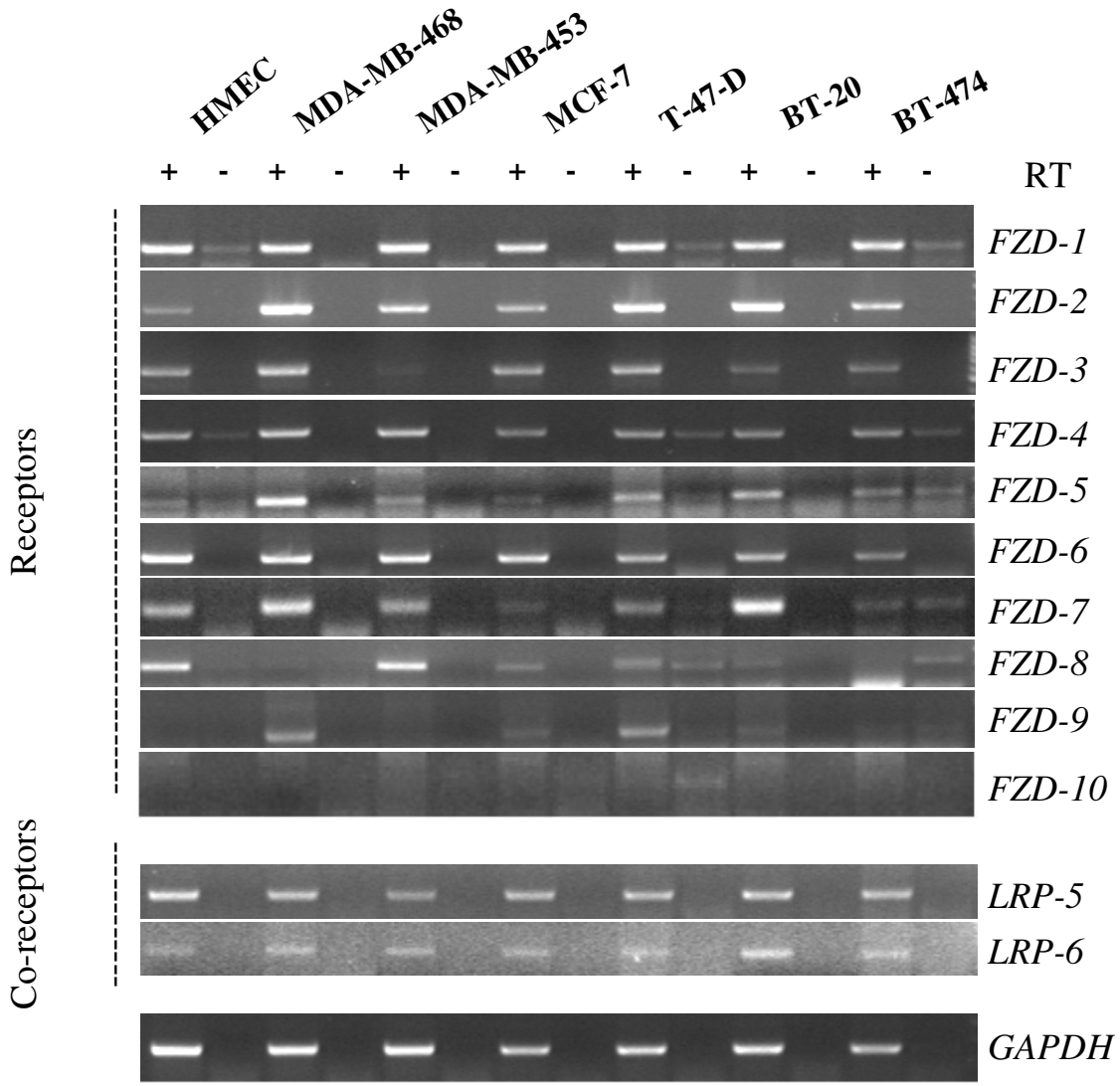

Figure 4.8: Expression profile analysis of Frizzled receptors and co-receptors in HMEC and breast cancer cell lines.

Total RNA from each cell line was reverse transcribed and amplified with primers specific for each indicated gene. For all cell lines, an RT negative control noticed (-) without reverse transcriptase was used in parallel with the normal cDNA noticed as (+). GAPDH is used as an internal control for equal cDNA amount. Upper panel: Redundant expression of FZD receptors in HMEC and breast cancer cell lines. Lower panel: HMEC and breast cancer cell lines express uniformly LRP-5 and LRP-6. C: Canonicals, NC: Noncanonicals, RT: Reverse Transcriptase.

Transcriptional regulation by canonical Wnt signaling is mediated by β-catenin, which forms active complexes with the family of four *LEF/TCF* transcription factors (Kikuchi *et al*., 2000; Nusse, 2005). Our results showed the expression of *LEF-1, TCF-1, TCF-3* and *TCF-4* in HMEC cells. Redundant expression of *TCF-1, TCF-3* and *TCF-4* was also observed in most of the cancer cell lines tested. The *LEF1* gene, on the other hand, displayed increased expression in three of the six cell lines (**Figure 3.6**).

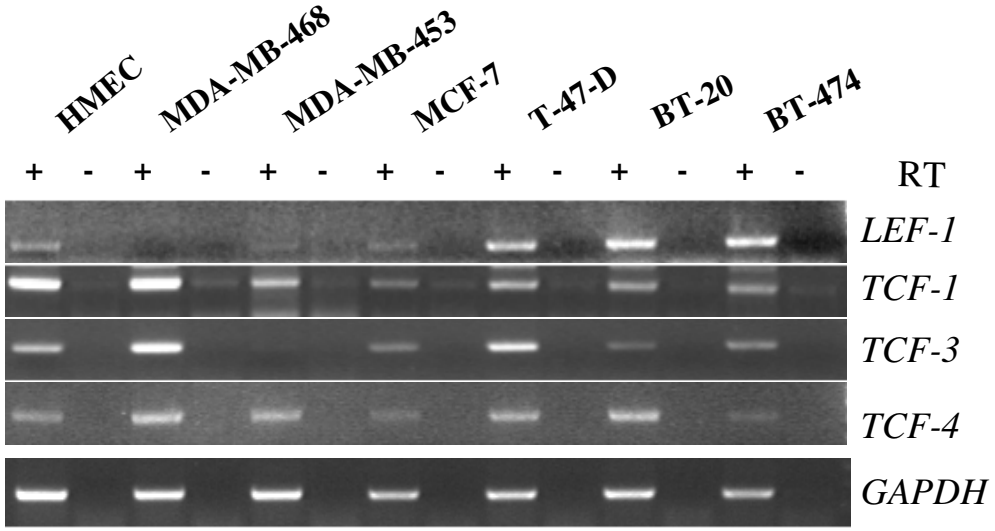

Figure 4.9: Expression profile analysis of Lef/TCF transcription factors in HMEC and breast cancer cell lines.

Total RNA from each cell line was reverse transcribed and amplified with primers specific for each indicated gene. For all cell lines, an RT negative control noticed (-) without reverse transcriptase was used in parallel with the normal cDNA noticed as (+). GAPDH is used as an internal control for equal cDNA amount. HMEC and six breast cancer cell lines redundantly express LEF-1, TCF-1, TCF-3 and TCF-4 transcripts. C: Canonicals, NC: Non canonicals, RT: Reverse TreanscriptaseRT-

4.2.2 Redundant expression of canonical Wnt ligands in breast cancer cells

Experimental data from different animal models indicate that Wnt pathway generates different signals mediated by the so-called canonical and non-canonical signaling systems. Although the exact mechanisms that differentiate between these signaling pathways are not well defined, Wnt ligands appear to play the major role in defining the cellular choices between canonical and non-canonical pathways, probably by interacting with selective subsets of Frizzled receptors (Wong *et al*., 1994; Shimizu *et al*., 1997; Gazit *et al*., 1999; Umbhauer *et al*., 2000). Therefore, we next compared the expression pattern of all Wnt ligands in HMEC with that of breast cancer cell lines (Figure 4.7).

The most striking feature of Wnt ligand expression was its redundancy in both HMEC and breast cancer cell lines. Normal HMEC expressed *WNT-7B, WNT-5B, WNT-1, and WNT -5A, WNT-2B, WNT-3* and *WNT-16* strongly, as well as *WNT4, WNT-9A* and *WNT-10B*, but only weakly. In contrast, these cells did not express *WNT-8B, WNT-3A, WNT-6, WNT-7A, WNT-9B, WNT-8A, WNT-2* and *WNT-11*. Breast cancer cell lines expressed or over-expressed *WNT-7B, WNT9A, WNT-4, WNT-10B, WNT-8B, WNT-3A, WNT-6, WNT-7A* and *WNT-10A*. In contrast, the expression of *WNT-16, WNT-5A, WNT-1, WNT-3, WNT-5B* and *WNT-2B* was either lost or decreased (Figure 4.7). Amongst the Wnt ligands that are expressed or over-expressed in breast cancer cell lines, six of them (*WNT-6, WNT9A, WNT-10B, WNT-8B, WNT-3A* and *WNT-10A*)(Benhaj *et al*., 2006) activate canonical signaling, and two of them *(WNT-4* and *WNT-7B)* activate both canonical and non-canonical Wnt signaling (Benhaj *et al*., 2006). Five of these ligands are also known to have a transforming activity when tested with mouse mammalian epithelial cells, and one, *WNT-10B*, is an oncogene in the mouse (Lane *et al*., 1997).

The second group of ligands that are lost or decreased in breast cancer cells included three canonical (*WNT-1, WNT-2B* and *WNT-3*) and two non-canonical Wnt ligands (*WNT-5A* and *WNT-5B*). One of these down-regulated ligands, namely WNT-5A is also known as an inhibitor of canonical Wnt/β-catenin signaling (Topol

et al., 2003; Ishitani *et al*., 2003). Taken together, these observations indicate that Wnt ligand expression in human breast carcinoma cell lines is dominated by canonical Wnt ligands. This conclusion strongly suggests that the types of Wnt ligand expression alterations in human breast cancers may provide a cell-autonomous source for autocrine activation of the canonical Wnt/β-catenin signaling.

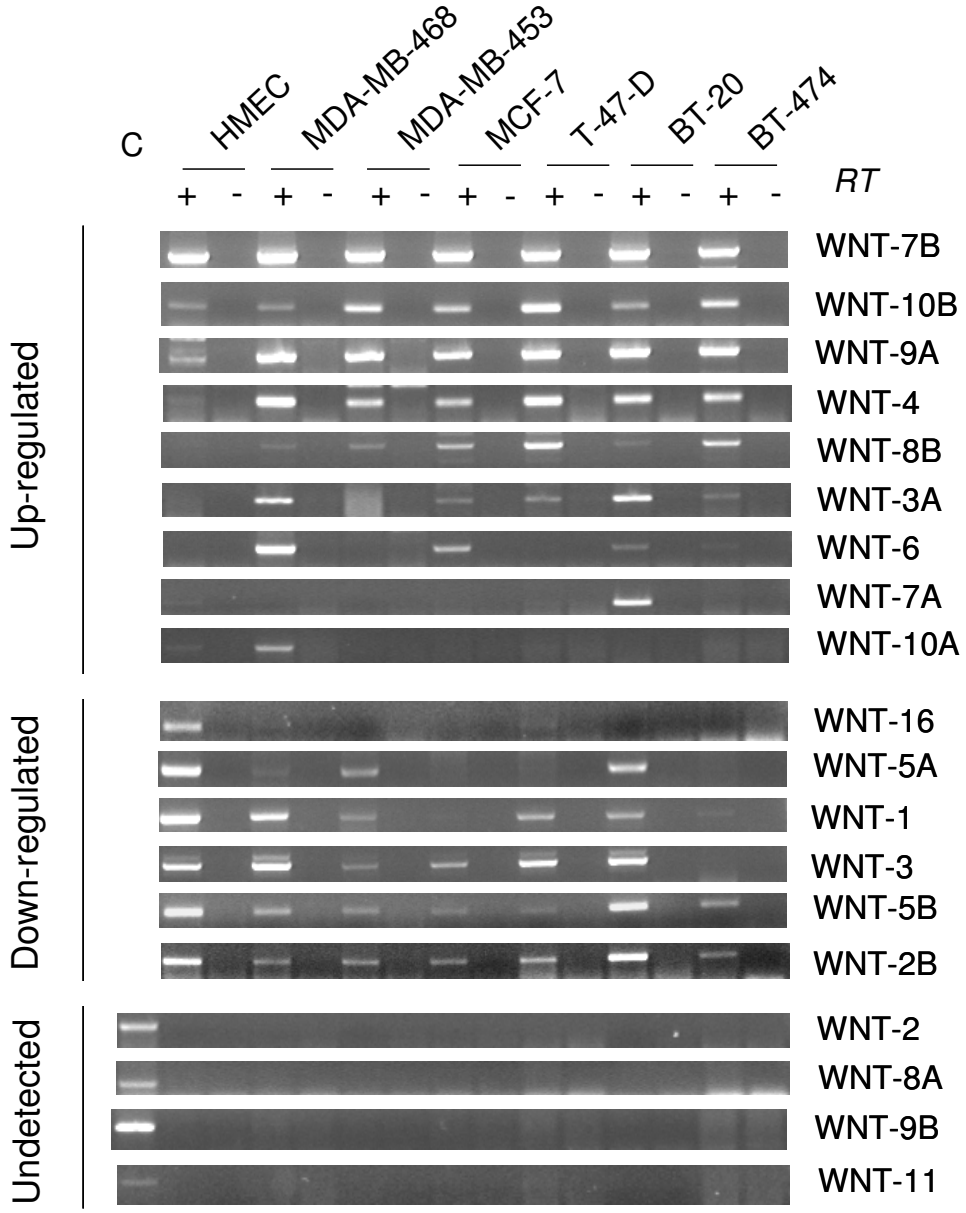

Figure 4.10: Expression profile analysis Wnt ligands in immortalized mammary epithelial (HMEC) and breast cancer cell lines.

RT-PCR analysis of WNT transcripts in HMEC and six breast cancer cell lines. Total RNA from each cell line was reverse transcribed and amplified with specific primers for each indicated *WNT* gene. For all cell lines, an RT negative control noticed (-) without reverse transcriptase was used in parallel with the normal cDNA noticed as (+). Upper panel: Up-regulated *WNTs: WNT7B, -9A, -4,10B, -4, 8B, 3A, 6, 7A* and *–10A*. Middle panel: Down-regulated *WNTs: WNT-16, -5A, -1, -3, -5B* and *–2B*. Lower panel: Non-expressed *WNTs:WNT -2, -8A, -9B and -11*. C: positive control, RT: Reverse transcriptase.

4.2.3 Up-regulation of canonical Wnt ligands in breast cancer cells correlates with nuclear β-catenin accumulation and cyclin D1 up-regulation

Canonical Wnt/β-catenin signaling leads to the cytoplasmic and/or nuclear accumulation of β-catenin (Lin *et al*., 2000; kikuchi, 2000; Giles, 2003). In order to support our preliminary observation that Wnt ligand expression in breast cancer cell lines is more balanced towards the reactivation of the canonical Wnt signaling pathway, we compared β-catenin protein expression levels in HMEC and the breast cancer cell lines by western blotting using a monoclonal anti-β-catenin antibody. Our result showed that except MDA-MB453 in which β-catenin was undetectable, all breast cancer cell lines expressed a higher level of total β-catenin protein than HMEC (Figure 4.11).

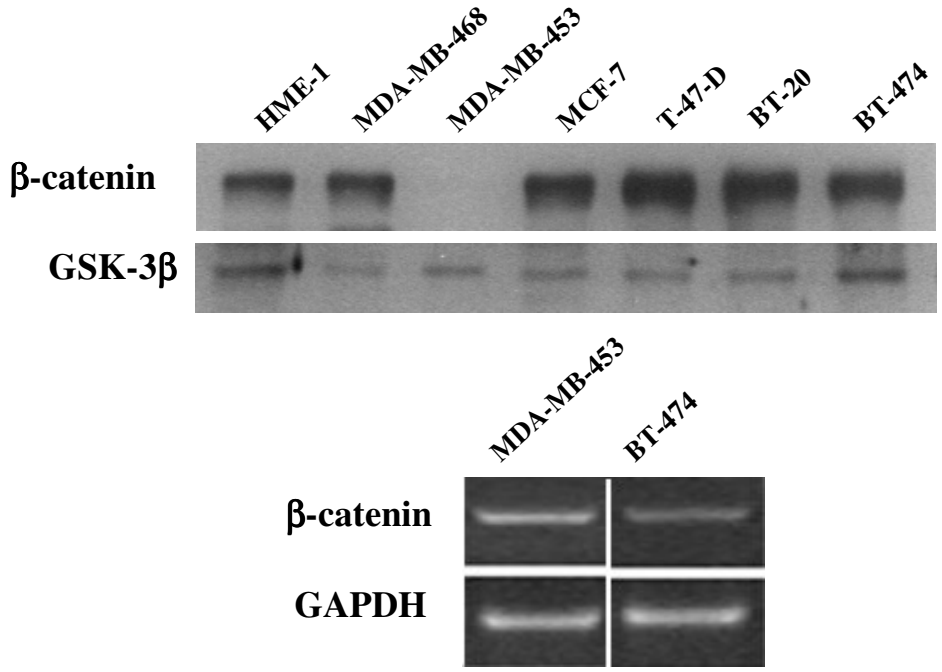

Figure 4.11: total β-catenin protein accumulation in breast cancer cell lines compared to HMEC.

Except MDA-MB-453, all breast cancer cell lines displayed an increased β-catenin protein level in total cell lysates compared to HMEC cells.

Moreover, nuclear accumulation of β-catenin has been reported to be a good indication for canonical Wnt signaling activity (Nusse, 2005; Kikuchi, 2000). Therefore, we next characterized the status of nuclear β-catenin protein in breast cancer cell lines and compared to that of HMEC. As shown in figure 4.12, no nuclear β-catenin was detected in hTERT-HME cells while all breast cancer cell lines displayed nuclear accumulation of β-catenin, as an indication of active canonical Wnt signaling.

Canonical Wnt signlaing, when activated, leads to the up-regulation of *Cyclin D1* (Tetsu *et al*., 1999) and *c-Myc* (He *et al*., 1998) transcripts in colorectal cancers. Therefore, we also analyzed transcript levels of these two target genes, by RT-PCR. Our results showed that the expression of *Cyclin D1* was weak in HMEC, but up-regulated in all breast cancer cell lines tested (Figure 4.12). We observed a close correlation between the up-regulation of *Cyclin D1* and nuclear accumulation status of β-catenin, although *Cyclin D1* induction was not proportionally related to nuclear β-catenin levels. In contrast to *Cyclin D1, c-Myc* transcript levels were high in HMEC and they did not correlate with nuclear β-catenin accumulation.

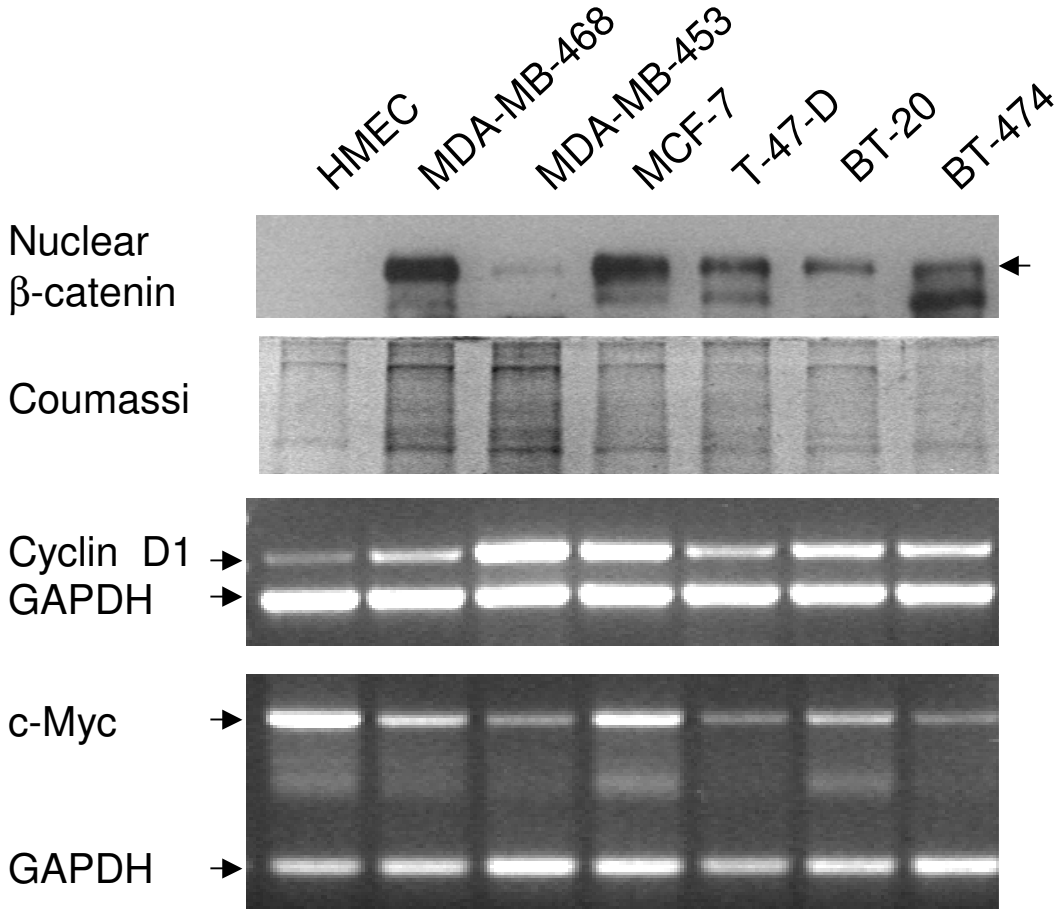

Figure 4.12: Nuclear accumulation of β-catenin protein and induction of cyclin D1 transcripts in breast cancer cell lines.

Upper panel: Nuclear protein fraction from HMEC and breast cancer cell lines were subjected to 10% PAGE and probed with a monoclonal anti-β-catenin antibody (Abcam). Loading control was made by Coomassi blue staining of total nuclear proteins. Middle and bottom panel shows multiplex RT-PCR analysis of *cyclinD1* and *c-Myc* together with *GAPDH* as an internal control

4.2.4 Wnt and Frizzled expression in primary breast tumors

Since our data showed the activation of canonical Wnt signaling in breast cancer cell lines, we decided to test the status of selected Wnt signaling components in fourteen primary breast tumors. We found a strong expression of canonical *FZD-1* receptor in all tumors tested, whereas the expression of non-canonical *FZD-2* and *FZD-6* receptors was down-regulated compared to immortalized HMEC control (Figure 4.13). Three Wnt ligands (*WNT-7B, WNT-10B and WNT-9A*) were strongly positive in most tumors tested. Two additional Wnt ligands (*WNT-4* and *WNT-8B*) were also expressed in most tumors, but moderately. In contrast, no tumor expressed *WNT-5A* and *WNT-16* at detectable levels. Weak expression of *WNT-3A* and *WNT-6* ligands was detectable only in some tumor samples (Figure 4.13). It was noteworthy that all five Wnt ligands that are expressed in most tumors (*WNT-7B, WNT-10B, WNT-9A, WNT-4 and WNT-8B*) are able to signal through canonical Wnt/β-catenin pathway, whereas the down-regulated *WNT-5A* ligand is involved in non-canonical Wnt signaling pathway.

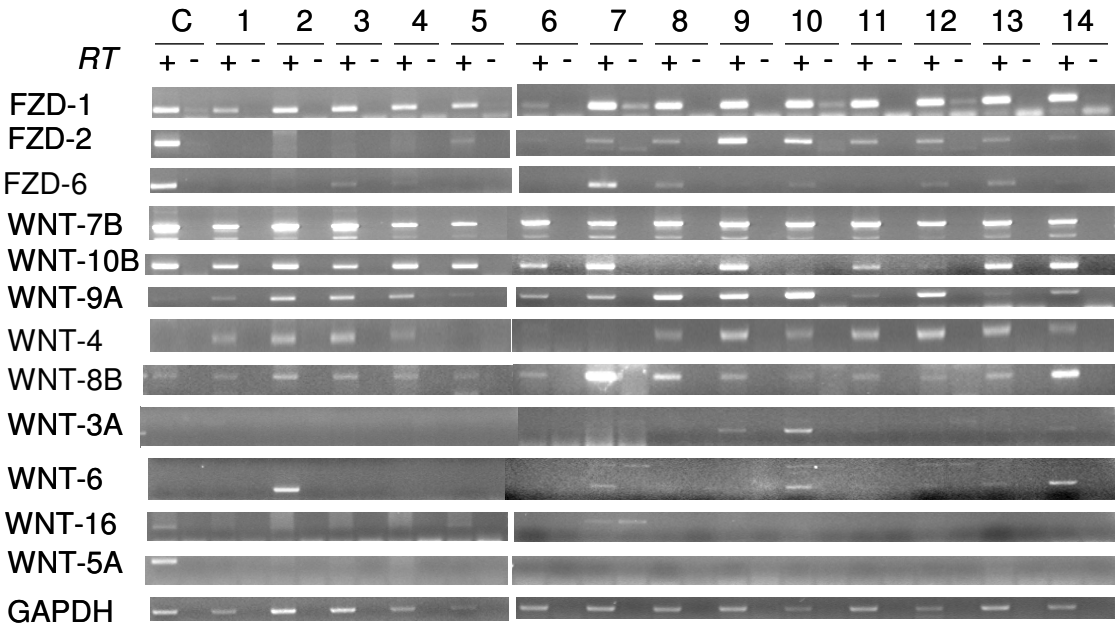

Figure 4.13: Expression of selected Frizzled receptors and Wnt ligands in primary breast tumors.

RT-PCR data shows that FZD-1 is expressed in all tumors, whereas the expression of FZD-2 and FZD-6 was down-regulated in most tumors. RT-PCR data also show the up-regulation of WNT-4 and WNT-9A with down-regulation of WNT-5A in most tumors. C: HMEC, RT: Reverse transcriptase. (+) and (-) indicate samples prepared for cDNA synthesis with and without reverse transcriptase, respectively. GAPDH is used as an internal control.

5. DISCUSSION

5.1 WNT SIGNALING IN HCC

Our observations provide evidence for dual involvement of canonical Wnt/β-catenin signaling in HCC. This signaling is activated in most well differentiated HCC cell lines, but it is inactive if not repressed in most poorly differentiated HCC cell lines. We detected constitutive canonical Wnt/β-catenin signaling in 80% of well differentiated HCC cell lines, including two cell lines (Huh7 and Hep3B) with wild-type *β-catenin* and *Axin-1* genes. This strongly supports the hypothesis that canonical Wnt/β-catenin signaling is active in most well differentiated HCC cells. This can occur either by β-catenin/Axin1 mutations or by an autocrine mechanism, as shown recently in breast and ovarian cancer cell lines (Bafico *et al.*, 2005). Constitutive canonical Wnt/β-catenin signaling has been linked to both stem cell and cancer cell self-renewal in other cancer types. It was proposed that some adult cancers derive from stem/progenitor cells and that canonical Wnt/β-catenin signaling in stem and progenitor cells can be subverted in cancer cells to allow malignant proliferation (Reya *et al*., 2005). Thus, our observations raise the interesting possibility that well differentiated HCCs represent a group of liver tumors originating from liver stem cells. In line with this hypothesis, our preliminary studies indicate that well differentiated cell lines studied here display liver stem cell-like features, such as self-renewal, and generation of differentiated progeny (Erdal-Yildiz *et al*., 2003).

In sharp contrast with well differentiated HCC cell lines, most poorly differentiated HCC cell lines display no detectable canonical Wnt/β-catenin activity. More interestingly, we failed to detect TCF/LEF reporter activity even in SNU475 cells which display homozygous Axin1 deletion (Figure 4.2). Furthermore, ectopic expression of mutant β-catenin, highly active in well differentiated Huh7 cells, failed to generate significant canonical Wnt/β-catenin

signaling, as tested by TCF/LEF reporter activity in two different poorly differentiated cell lines (Figure 3.2). Thus, canonical Wnt/β-catenin signaling is not only inactive, but also repressed in the great majority of poorly differentiated HCC cells.

Studies with transient expression of S33Y-β-catenin (Figure 4.2) provide evidence that the signal generated by this mutant protein is blocked downstream probably at the level of its interaction with TCF/LEF factors and DNA. As all poorly differentiated cell lines express both TCF1 and TCF4, a deficit at this level is unlikely. However, in some contexts Wnt5A and Wnt5B inhibit the canonical pathway, probably at the level of β-catenin function (Topol *et al*., 2003; Ishitani *et al*., 2003; Kanazawa *et al*., 2005). Interestingly, both Wnt5A and Wnt5B are expressed in all poorly differentiated HCC cell lines, but not in well differentiated cell lines with the exception of Hep40 which also lacks canonical Wnt/β-catenin activity (Figure 4.5). Although, it is not known at this time how the canonical Wnt/β-catenin signaling is repressed in poorly differentiated HCC cell lines, it is clear that this type of HCC cells do not need canonical Wnt/β-catenin signaling for survival and self-renewal. Pathological analyzes, and "nodule-in-nodule" HCC lesions strongly support the hypothesis that the progression of HCC is a stepwise process of dedifferentiation. In other words, less differentiated HCCs are apparently clonal progeny of well differentiated HCC cells (Kojiro, 2005). However, primary tumor data showing a gradual decrease in β-catenin mutation and nuclear accumulation frequencies in less differentiated HCCs (Hsu *et al*., 2001; Wong *et al*., 2001; Mao *et al*., 2001; Inagawa *et al*., 2002; Fujito *et al*., 2004) is in apparent contradiction with this prediction.

Our *in vitro* data provide additional evidence that canonical Wnt/β-catenin signaling is not active in most poorly differentiated cells. Taken together, these observations raise the possibility that poorly differentiated HCCs form a distinct types of tumor not directly related to well differentiated HCCs with β-catenin mutation and constitutively active canonical Wnt/β-catenin signaling. One

possibility is that well differentiated HCCs with activated canonical Wnt/β-catenin signaling rarely progress by a dedifferentiation process. Otherwise, the hypothesized dedifferentiation process would have to take into account a mechanism for selective elimination of mutant β-catenin, or active repression of the canonical Wnt/β-catenin signaling in the progeny of well differentiated HCC cells during tumor progression. Repression of canonical Wnt/β-catenin signaling, as shown here experimentally with SNU449 and SNU182 cell lines (Figure 4.2) seems possible. Alternatively, elimination of mutant β-catenin gene, although highly unlikely, is possible theoretically, since β-catenin mutations in HCC are heterozygous, leaving one allele in the wild-type form (de La Coste *et al*., 1998).

5.2 WNT SIGNALING IN BREAST CANCER

Our studies provide several new findings on the role of the Wnt pathway in human breast cancer. Firstly, a large set of ligands, receptors and transcription factors of the Wnt signaling pathway is expressed in human mammary epithelial cells. This strongly suggests that both canonical and non-canonical Wnt signaling pathways are operating in the adult mammary epithelial tissue. Secondly, malignant transformation of breast epithelial cells in humans is accompanied with subtle changes in the expression profiles of Wnt signaling components. The most striking change is the up-regulation of the canonical Wnt ligands together with a decrease in non-canonical *WNT5A* in cancer cells. A previously published report indicated the expression of *WNT3*, *WNT4* and *WNT7B,* but not *WNT3A* and *WNT7A* in human breast cancer cell lines (Huguet *et al*., 1994). Our study not only confirmed these observations but also provided evidence that *WNT9A*, *WNT10B* and *WNT8B* were also up-regulated in all breast cancer cell lines studied. Previous reports indicating *WNT8B* as a target of estrogen signaling in MCF-7 cells (Saitoh *et al*., 2002), and *WNT10B* as a known breast oncogene (Lee *et al*., 1995) and its role in the development of mammary adenocarcinomas in the mouse (Lane *et al*., 1997) increase the importance of our results. Other Wnt ligands, *WNT6*, *WNT7A* and *WNT10A* were also over-expressed,

but only in a few breast cancer cell lines tested. It is noteworthy that all Wnt ligands that are expressed or over-expressed in breast cancer cell lines are able to trigger canonical Wnt signaling.

Several other Wnt ligands, including *WNT1, WNT2B, WNT3, WNT5A, WNT5B* and *WNT16* were expressed in HMEC, but down-regulated in breast cancer cell lines. Three of these ligands (*WNT1, WNT2B* and *WNT3*) are known to signal for the canonical pathway, whereas, at least two, namely *WNT5A* and *WNT5B* signal for non-canonical pathways (Topol *et al*., 2003; Ishitani *et al*., 2003; Kanazawa *et al*., 2005). It therefore appears that, overall, Wnt ligand expression profile in breast cancer cell lines displays a shift towards implication of more canonical Wnt ligands.

Canonical Wnt/β-catenin signaling plays a major role in cytoplasmic and/or nuclear accumulation of β-catenin (Lin *et al*., 2000; Kikuchi, 2000; Giles, 2003). Accumulation of β-catenin protein with induction of cyclin D1 expression is known to be a marker of poor prognosis in breast cancer (Lin *et al*., 2000). In line with these observations, we provide evidence for nuclear accumulation of β-catenin in breast cancer cell lines. In contrast, HMEC did not display any nuclear accumulation. It is known that breast cancer cells do not display mutations in known genes of the canonical Wnt-β-catenin signaling (Brown, 2001; Udea *et al*., 2001; Giles *et al*., 2003; Brenann *et al*., 2004; Howe *et al*., 2004). Therefore, nuclear β-catenin expression in breast cancer cells probably results from autocrine activation of canonical signaling through the help of Wnt ligands that we have shown to be over-expressed in malignant cells.

As shown in Figure 4.9, there are differential nuclear β-catenin levels amongst the breast cancer cell lines that we tested. This difference may be an indication that the autocrine Wnt signaling in these cells occurs at variable intensities due to heterogeneous expression of canonical and non-canonical Wnt ligands. An autocrine Wnt signaling mechanism has recently been proposed for breast cancer (Bafico *et al* 2004). Therefore, our results support this contention. We also noticed that, nuclear β-catenin accumulation shows a moderate correlation with cyclin D1

transcript levels in HMEC and breast cancer cell lines, whereas, c-myc, another known target of the canonical Wnt-β-catenin signaling, appears to act independently (Figure 4.9).

In conclusion, we propose that redundant expression of several Wnt ligands is involved in the activation of canonical Wnt signaling, nuclear accumulation of β-catenin and induction of cyclin D1 overexpression in human breast cancer cell lines. This autocrine mechanism may provide an explanation for the lack of mutations of the genes located in the downstream of Wnt-mediated Frizzled activation in human mammary cancers, despite the demonstrated role of Wnt genes in mouse breast cancers.

Finally, our study provided strong evidences for the differential involvement of Wnt pathway in liver and breast cancers. In liver cancer, we showed that Wnt pathway activity was linked to the differentiation status of HCC cell lines. Indeed, our data in HCC showed that the canonical Wnt pathway was active in well-differentiated HCC cell lines and repressed in poorly differentiated ones. However, the study of Wnt pathway in breast cancer cell lines showed similarities rather than differences. Then, we studied the expression profile of the major components of Wnt/β–catenin signaling in both HCC and breast cancer cell lines. Interestingly, our data showed a redundent expression of Wnt signaling components in both cancer models. In HCC, we found a correlation between the expression profile of both non-canonical Wnt5A and Wnt5B and the TCF activity data. In the breast cancer cell lines, our study revealed a significant correlation between Wnt ligands mRNA expression profile and the induction of *Cyclin D* and nuclear β-catenin protein. We concluded that, although involved in both types of cancers, Wnt signaling is acting differently in liver and breast cancers. More interestingly, within the same type of cancer such as HCC, Wnt signaling displayed differential activity depending on the cell differentiation status.

6. FUTURE PERSPECTIVES

1. Study the effect of Wnt5a and Wnt5b overexpression in well differentiated HCC cell lines such as Huh7 and Hep-3B and check if there will be a negative effect on TCF activity.

2. Shut down the expression of Wnt5a and Wnt5b by siRNA in poorly differentiated HCC cell lines and check if there will be relief of TCF activity repression.

3. Investigate further the repression mechanism on TCF activity in poorly differentiated HCC cell lines: β–catenin localization, TCF protein expression

4. Study other components of the Wnt pathway that may cause the repression of the canonical Wnt signaling in poorly differentiated HCC cell lines such as extracellular inhibitors secreted Frizzled related protein (*sFRP*), *Wnt-inhibitory factor-1 (WIF-1)* and *Dikkopf (Dkk).*

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