CHARACTERIZATION OF GENE EXPRESSION ALTERATIONS IN β-CATENIN ACTIVATED Huh7 HEPATOCARCINOMA CELL LINES BY CONSTRUCTION AND VERIFICATION OF A SAGE LIBRARY

by

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To my beloved family,

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ABSTRACT

CHARACTERIZATION OF GENE EXPRESSION ALTERATIONS IN β-CATENIN ACTIVATED Huh7 HEPATOCARCINOMA CELL LINES BY CONSTRUCTION AND VERIFICATION OF A SAGE LIBRARY

β-catenin, a cytoplasmic component of the Wnt signaling pathway, is observed to be aberrantly activated in several types of tumors, including hepatocellular carcinoma. The aim of this study is to identify novel genes that are regulated by active β-catenin/TCF signaling in hepatocellular carcinoma cell line Huh7 and to investigate their relationship with cell growth control.

SAGE libraries from Huh7-derived cells with high and low β-catenin/TCF activities were constructed and 431 differentially expressed genes were identified; 329 of them showed decreased expression profile in high activity cells, whereas the remaining 102 tags showed increase. Transient overexpression of β-catenin resulted in down regulation of mRNA expression levels of two of these genes, Gene 3 and Gene 6. Gene 6 was also analysed for the possible role in cell growth control and a significant effect on Huh7 proliferation was observed.

Our findings suggest two novel candidate targets of Wnt/β-catenin pathway in hepatocellular carcinoma cell line Huh7 whose direct interaction with β-catenin/Tcf complex should be further analysed. One of these genes, Gene 6 might play a role in cell growth control.

ÖZET

β-KATENIN İLE AKTİVE EDİLMİŞ Huh7 HEPATOKARSİNOM HÜCRE HATTINDA GEN ANLATIM FARKLILIKLARININ SAGE KÜTÜPHANESİ OLUŞTURULUP DOĞRULANARAK KARAKTERİZE EDİLMESİ

Wnt sinyal yolağının sitoplazmada yer alan öğesi β-katenin'in anormal aktivasyonu hepatokarsinoma dahil olmak üzere çeşitli tumor tiplerinde gözlenmiştir. Bu çalışmanın amacı hepatokarsinom hücre hattı Huh7'da β-katenin/TCF sinyaliyle kontrol edilen yeni genlerin tespit edilmesi ve hücre büyümesindeki olası rollerinin incelenmesidir.

Yüksek ve düşük β-katenin/TCF aktivitesi gösteren hücrelerden SAGE kütüphaneleri oluturulmuş ve 431 tane ekpresyon farkı gösteren SAGE etiketi belirlenmiştir. Bunlardan 329'unda azalma, 102'sinde artış gözlenmiştir. Bu genlerden ikisinin, Gen 3 ve Gen 6, haberci RNA seviyeleri β-katenin gen anlatımının hücrelerde geçici olarak arttırılması sonucu azalma göstermiştir. Gen 6'nın hücre büyümesi üzerindeki olası etkisi de incelenmiştir ve Huh7 hücrelerinde önemli bir etki gözlenmiştir.

Bulgularımız Huh7 hepatokarsinom hücre hattında, iki yeni genin Wnt/β-catenin yolağıyla olası ilişkilerine işaret etmektedir. Her iki genin β-katenin/Tcf kompleksiyle direk ilişkileri araştırılmalıdır. Buna ek olarak, Gen 6'nın hücre büyümesi kontrolünde rol alma ihtimali vardır.

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LIST OF SYMBOLS / ABBREVIATIONS

A	Adenine
C	Cytosine
G	Guanine
S	Serine
T	Thymine
Y	Tyrosine

AP	Activator protein
APC	Adenomatous polyposis coli gene product
APS	Ammonium peroxidisulfate
BCL	B-cell lymphoma
BRG	Brahma-related gene
b-TrCP	ß-transduction repeat containing protein
c AMP	Cyclic adenosine mono phosphate
c DNA	Complementary deoxyribonucleic acid
CaCl2	Calcium chloride
CAMK	Calmodulin-dependent kinase
CBP	cAMP response element binding protein
CDC	Cell division cycle
CHIP	Chromatin immunoprecipitation
CK1γ	Casein kinase 1 γ
CKIε	Casein kinase Iε
CO2	Carbondioxide
CRD	Cysteine rich domain
CsCl	Cesium chloride
CtBP	C-terminal binding protein
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxiribonuclease
DPH	DEPC treated water
DTT	Dithiothreitol
DVL	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
EMBO	European Molecular Biology Organization
EMSA	Electro mobility shift assay
ERLB	EtBr RNA loading buffer
EtBr	Ethidium bromide
EtOH	Ethanol
FAP	Familial adenomatous polyposis coli
FBS	Fetal bovine serum
FCS	Fetal calf serum
FRAT	Frequently rearranged in advanced T-cell lymphomas
FRP	Frizzled-related protein
GFP	Green flourescent protein
GITC	Guanidium isothiocyanate
GLT-1	Glutamate transporter 1
Gro/TLE	Groucho/transducin-like Enhancer-of-split
GS	Glutamine synthetase
GSK3ß	Glycogen synthase kinase 3 beta
HCC	Human hepatocellular carcinoma
HMG	High mobility group
Huh	Human hepatoma
IL	Interleukin
ITF-2	Intestinal trefoil factor 2
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
LB	Luria-Bertani
LDL	Low density lipoprotein
LEF	Lymphoid enhancer factor

LiCl	Lithium chloride
LRP	Low density lipoprotein receptor-related protein
m RNA	Messenger ribonucleic acid
MAPK	Mitogen activated protein kinase
MED	Mediator
MgCl2	Magnesium chloride
MgSO4	Magnesium sulphate
MOPS	Morpholino propane sulfonic acid
MSI+	Microsatellite-instability-positive
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NaOAc	Sodium Acetate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NF κB	Nuclear factor κB
NFAT	Nuclear factor of activated T-cells
NLK	NEMO-like kinase
NLS	Nuclear localization sequence
OAT	Ornithine aminotransferase
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PKC	Protein kinase C
PP2A	Protein phosphotase 2A
QRT-PCR	Quantitative reverse transcriptase mediated PCR
RGS	Regulator of G-protein signaling
RNA	Ribonucleic acid
Rnase	Ribonuclease
RPM	Revolutions per minute

RT-PCR	Reverse transcriptase-polymerase chain reaction
SAGE	Serial Analysis of Gene Expression
SDS	Sodium dodecyl sulphate
SOB	Super optimal broth
SOC	Super optimal catabolite repressed broth??
TAE	Tris-acetic acid EDTA
TAK	TGF-beta activated kinase
TBE	Tris base EDTA
TBP	TATA-binding protein
TCF	T cell factor
TEMED	N,N,N,N-tetramethylethylenediamine
TES	Tris-EDTA sodium chloride
WIF	Wnt interacting factor
WISP-1	WNT1 inducible signaling pathway protein 1
WNT	Wingless-type mouse mammary tumor virus integration site family member

1. INTRODUCTION

1.1. Wnt Signaling Pathways

Wnt proteins constitute a family of secreted cysteine-rich glycoproteins that show distinct expression patterns in embryo and adult organisms. Functions of the Wnt protein family have been widely investigated via genetic studies in *Drosophila melanogaster*, mouse, zebrafish and *Caenorhabditis elegans*, and biochemical and cell biology studies in *Xenopus laevis*, sea urchin, chicken embryos and mammalian cultured cells (Wodarz and Nusse, 1998). Wnt proteins regulate cell growth, motility, and differentiation during embryonic development. They are also responsible for regulating stem cell number, and differentiation of adult stem cell systems. They act in a paracrine fashion through members of the Frizzled receptor family, seven transmembrane spanning proteins, activating more than one pathway (Veemen *et al*., 2003) (Figure1.1).

1.1.1. Wnt/β-catenin Pathway

This pathway, also called 'the canonical Wnt pathway' is the best understood Wnt signaling pathway. As the name implies, the Wnt/β-catenin pathway acts through the protein β-catenin. Activation of the Wnt pathway leads to the stabilization of β-catenin which in turn translocates to the nucleus where it interacts with TCF/LEF family of transcription factors in order to activate target gene expression.

1.1.2. Wnt/Calcium Pathway

The Wnt/Calcium pathway interferes with the canonical Wnt/β-catenin pathway but the underlying molecular mechanism is poorly understood. Wnt5a is the main stimulator of this pathway and the Wnt5a knock-out studies in mice gives the evidence about the antogonism between the two pathways. Wnt5a knock-out mice had increased levels of Wnt/β-catenin signaling due to loss of the inhibitor Wnt5a. Wnt/Calcium signaling acts through the calmodulin-dependent kinase II (CAMKII), cell division cycle 42 (CDC42) and the transcription factor nuclear factor of activated T-cells (NFAT).

1.1.3. Wnt/Planar Cell Polarity Pathway (PCP)

As the name implies, this pathway, also called 'the non-canonical Wnt pathway' controls cell polarity. It was first characterised in *Drosophila melanogaster*. Mutations in the Dishevelled protein interfering with the PCP leads to normal differentiation of wing hair cells but they point in random directions.

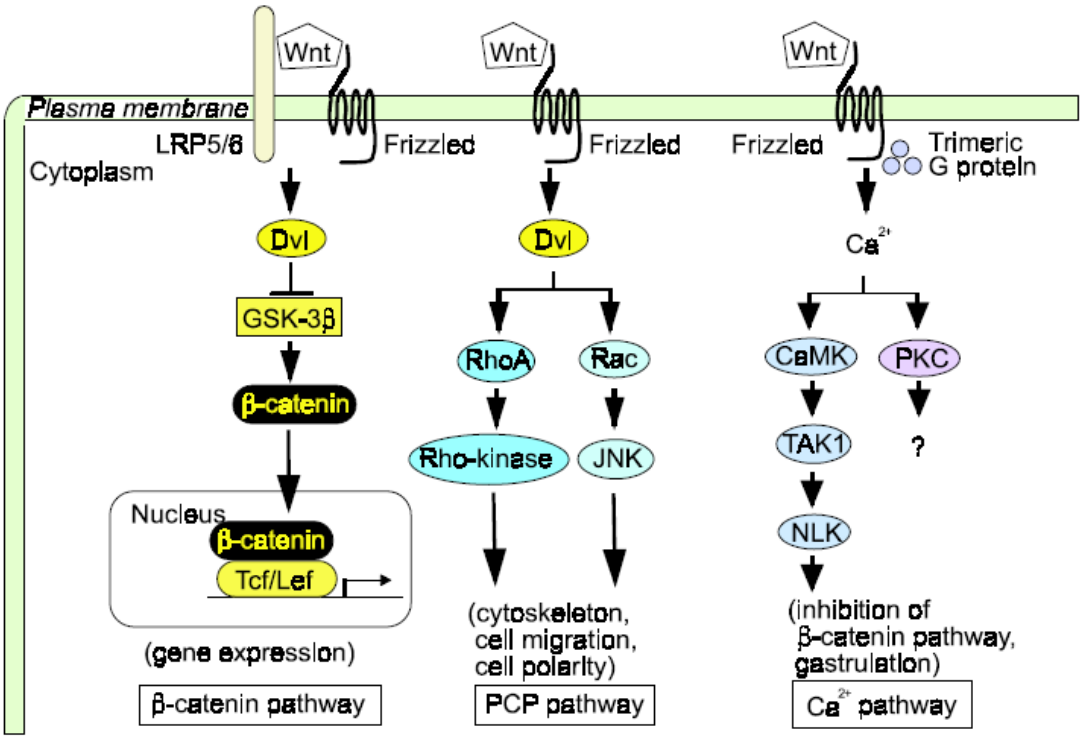


Figure 1.1. Wnt pathways**.** Binding of Wnt to its receptors stimulates 3 different pathways; canonical Wnt pathway, planar cell polarity pathway (PCP) and Wnt/Calcium pathway. Canonical pathway acts through stabilization and accumulation of β-catenin. PCP regulates cytoskeleton, cell migration and cell polarity through activation of Dvl, small G proteins (Rho or Rac), Rho-kinase or JNK. Ca^{+2} pathway activates CaMK, PKC, TAK and NLK. It antagonizes β-catenin pathway and regulates gastrulation.(Kikuchi *et al*., 2006)

1.2. Canonical Wnt/β-catenin Signaling Pathway

The canonical Wnt pathway is initiated by binding of a Wnt ligand to the frizzled receptor and progresses through sequential events leading to the stabilization and translocation of β-catenin, a cytoplasmic component, into the nucleus. ß-catenin plays a major role in the transduction of the canonical Wnt/β-catenin signal. It is localised in the membrane bound to E-cadherin or is free in the cytoplasm. In the absence of Wnt signals, free ß-catenin is constitutively degraded in proteasomes, whereas in their presence, ß-catenin is stabilized and travels to the nucleus where it can associate with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and act as transcriptional activators of Wnt target genes. The canonical Wnt pathway is summarised in Figure 1.2.

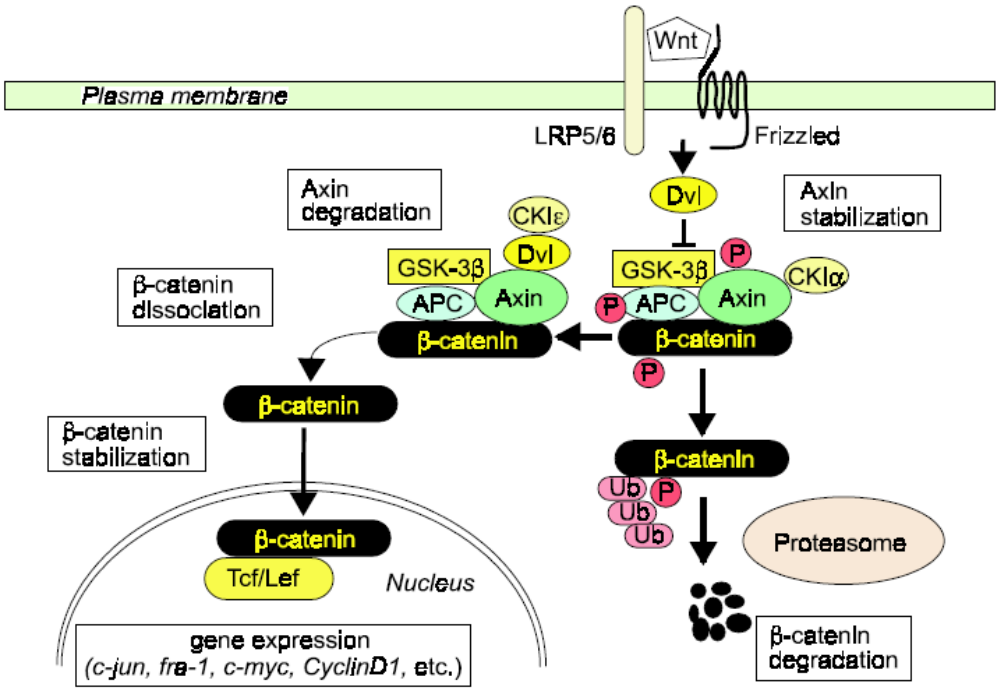


Figure 1.2. Wnt/β-catenin pathway. In the absence of Wnt signal, cytoplasmic β-catenin is degraded upon phosphorylation in the multiprotein complex containing Axin, GSK3β, CKIα and APC. When Wnt binds to its receptors LRP5/6 and Frizzled, its degradation is inhibited and it is stabilized. Stabilized β-catenin accumulates in the cytosol and translocates to the nucleus. β-catenin binds TCF/LEF in the nucleus and activates transcription of target genes. (Kikuchi *et al*., 2006)

1.2.1. Major Components of Canonical Wnt Pathway

Wnts are highly conserved 19 Wnt genes described in human genome. They direct the synthesis of secreted signaling molecules that regulate cell differentiation during development and tissue homeostasis, stem cell number in adult organism. Wnt proteins are cysteine rich and the cysteine distrubition is highly conserved among family. The mechanism that Wnts move between cells remains unknown.

Frizzleds are the main receptors of Wnt proteins. There are 10 Frizzled genes described in the human genome. All are seven-transmembrane receptors with a highly conserved amino-terminal cysteine rich domain (CRD). Binding of Wnts occurs at the amino-terminal CRD however the activation mechanism is not fully understood. Wnts require another receptor, LRP. LRPs are long single pass transmembrane molecules. Wnts bind to LRP5/6 and Frizzled forming a trimeric complex.

Several extracellular proteins interact with Wnts and prevent their binding to Frizzled. Among these are Cerberus, (Wnt interacting factor) WIF-1, and secreted frizzled-related proteins (sFRPs). Another group of extracellular inhibitors act through blocking LRP co-receptor. Dickkopf family proteins inhibit Wnt pathway through internalization of LRP thereby making it unavailable for Wnts (Mao and Niehrs, 2003).

There are three different mammalian Dishevelled proteins; Dishevelled-1, -2 and -3. Dishevelled is functional in planar cell polarity pathway as well as canonical Wnt pathway. Dishevelled proteins have three conserved domains; amino-terminal domain, central domain and carboxy-terminal domain. Amino-terminal domain is shown to be essential for canonical Wnt pathway (Penton *et al.*, 2002). Activated Dishevelled protein functions in stabilization of β-catenin, thus transduction of Wnt signal as discussed in the following sections.

Axin is the core scaffold protein of the β-catenin destruction complex. It has separate binding sites for proteins APC, GSK3β, PP2A and β-catenin. Axin inhibits Wnt signaling by facilitating the GSK3β-dependent phosphorylation of β-catenin. The amino-terminal regulator of G-protein signaling (RGS) domain binds APC. GSK3β and β-catenin bind

separate domains located in the centre of the protein. The carboxy-terminal domain, common with Dishevelled, is responsible for homodimerization as well as binding Dishevelled.

Adenomatous Polyposis Coli Gene Product (APC) is an important component of the β-catenin destruction complex. APC interacts with axin and β-catenin within the destruction complex and both interactions are essential for degradation of β-catenin. APC contains heptad repeats at its amino-terminus and seven armadillo repeats. The central part of the protein has three '15 amino acid repeats' and seven '20 amino acid repeats'. Three SAMP repeats are interspersed within the 20 amino acid repeat region. The amino-terminal heptad repeats are responsible for homo-oligomerization. Central repeat regions; both 15 and 20 amino acid, can bind β-catenin. Studies of colorectal cancer cells showed that the 20 amino acid region was sufficient for down-regulation of β-catenin (Munemitsu *et al.*, 1995).

Another component of the β-catenin destruction complex is the serine/threonine kinase Glycogen Synthase Kinase 3 beta (GSK3ß). In the absence of the Wnt signalling β-catenin phosphorylated by Casein Kinase I at the serine 45 residue is subject to phosphorylation by GSK3ß. GSK3ß dependent phosphorylation of β-catenin occurs at the threonine 41, serine 37 and serine 33. Phosphorylation of β-catenin by GSK3ß is essential for down regulation of Wnt signalling. Inactivation of GSK3ß by lithium chloride (LiCl) or the point mutations on β-catenin phosphorylation site leads to increased Wnt signalling (Aberle *et al.*, 1997; Orford, 1997).

β-catenin is the key mediator of canonical Wnt pathway and is transcribed from the *CTNNB1* gene in humans. Transduction of Wnt signal to the nucleus occurs via stabilization and nuclear transport of β-catenin. Primary structure of β-catenin contains an amino-terminal domain formed of 130 amino acids, a region composed of arm repeats (12 imperfect repeats of 42 amino acids). The so-called arm repeats are common with APC. The amino-terminal region of β-catenin containing the phosphorylation sites is mainly functional in stabilization of β-catenin, whereas the carboxy-terminal region (also called carboxy-terminal activating arm) functions in transcriptional activation by binding several transcriptional co-activators. The amino terminal region was also shown to bind co-

activators and referred as the amino-terminal activating arm. Central region of the protein has binding sites for APC, TCF/LEF and E-cadherin. It has no DNA binding domain and serves as a transcriptional activator through binding TCF/LEF family transcriptional factors (Willert and Nusse, 1998). β-catenin has dual function in cells. In addition to its transcriptional activity, it also functions in cadherin-based cell adhesion system. It binds the transmembrane protein E-cadherin and regulates actin filament assembly via α-catenin (Gates and Peifer, 2005). The two functions of β-catenin are independent and separable as shown in mutational studies (Orsulic *et al*., 1996; Hoffmans *et al.*, 2004).

TCF/LEF family proteins are transcription factors that bind DNA through their high mobility group (HMG) domains. In the absence of β-catenin, these proteins bind Groucho/transducin-like enhancer-of-split (Gro/TLE). Gro/TLE family repressors are long-range transcriptional repressors and have been shown to interact with histone deacetylases. Another repressor that binds TCF is the carboxy-terminal binding protein (CtBP). CtBPs are general short-range transcriptional co-repressors that also interact with histone deacetylases. A conserved 55 amino acid amino-terminal domain of TCFs binds β-catenin. β -catenin displaces Gro/TLE through binding to a second carboxy-terminal binding site that overlaps with Gro/TLE binding site. Some nuclear components that bind β-catenin and act as co-activators are (Brahma-related gene1) Brg-1, Mediator subunit 12 (Med12) and cAMP response element binding protein (CBP). Brg-1 is a component of mammalian SWI/SNF chromatin remodelling complex and binds armadillo repeats 7-12 of β-catenin. CBP has intrinsic and associated histone acetyltransferase activity and alters chromatin structure. Med12 is a component of the Mediator complex (MED) which is first described in yeast. MED links transcriptional regulators to RNA polymerase II and general transcription factors (Kim *et al*., 1994; Flanagan *et al*., 1991). Mammalian counterparts of yeast MED components were identified (Stadeli *et al*., 2006).

1.2.2. The On/Off States of Canonical Wnt Pathway

1.2.2.1. Off State. In the absence of a Wnt signal, free β-catenin levels are kept low avoiding the accumulation and translocation to the nucleus. There is a multiprotein destruction complex that is responsible for targeting free cytosolic β-catenin to the proteasome. The transcriptional activity of TCF/β-catenin complex can also be blocked by posttranslational modifications and the TCF target genes can be suppressed by transcriptional co-repressors that bind TCFs.

Cytosolic β-catenin, but not the β-catenin bound to cadherins is constitutively subject to degredation by the proteasome. The multiprotein complex that targets β-catenin to proteasome is assembled around the proteins axin or conductin/axil. Those scaffold proteins have seperate binding sites for adenomatous polyposis coli gene product (APC), β-catenin and glycogen synthase kinase 3beta (GSK3ß), so it can bind all three proteins simultaneously. Axin also provides binding sites for casein kinase Iα (CKIα) and protein phosphotase 2A (PP2A). The complex is stabilized by phosphorylation of Axin and APC by GSK3ß. β-catenin is phosphorylated by CKIα at serine 45 which leads to phosphorylation by GSK3ß on the serine/threonine residues 41, 37 and 33. GSK3ß dependent phosphorylation of β-catenin results in ubiquitination by ß-transduction repeat containing protein (β-TrCP) and finally degradation in proteasome.

In the absence of β-catenin in the nucleus, transcriptional co-repressors such as Groucho/transducin-like Enhancer-of-split (Gro/TLE) and C-terminal binding protein (CtBP) bind to DNA bound TCFs. It was shown that co-expression of Gro/TLE family members with TCF results in suppression of TCF target gene expression, even in the presence of β-catenin (Levanon *et al.*, 1998).

Genetic and biochemical studies in *C. elegans*, Xenopus and mammalian cells reveals that TCF/β-catenin complexes can be phosphorylated by a mitogen activated protein kinase (MAPK)-related pathway. The MAPK-dependent phosphorylation of TCF/β-catenin complexes prevents their binding to the DNA that results in non-activated TCF target genes (Seidensticker and Behrens, 2000).

1.2.2.2. On State. Wnt signaling is initiated by the binding of Wnt signals to Frizzled and lipoprotein receptor-related protein 5/6 (LRP5/6). The basis of specificity and the mechanism by which the signal is transduced remains unknown. It is known that the cytoplasmic component β-catenin is stabilized upon Wnt ligand binding and the signal is carried to the nucleus by stabilized β-catenin. There are two main models of β-catenin stabilization in the on state of Wnt signaling pathway (Kikuchi *et al*., 2006) (Figure 1.3.).

According to the first model, β-catenin is stabilized through interaction of Dishevelled with Frat. Dishevelled was shown to bind casein kinase Iε (CKIε) and Axin. CKIε phosphorylates Dishevelled in the presence of a Wnt signal. Phosphorylated Dishevelled binds frequently rearranged in advanced T-cell lymphomas (Frat) protein with a high affinity and Frat binds and inhibits GSK3β. Based on these facts, the model suggests that in the presence of Wnt signal, Dishevelled, phosphorylated by CKIε binds to Frat which inhibits GSK3β function. As a result β-catenin bound to Axin can not be phosphorylated by GSK3β.

According to the second model, β-catenin is stabilized through interaction of Axin with LRP5/6. Axin is recruited to the membrane and interacts with LRP5/6 upon Wnt ligand binding. This interaction is facilitated through phosphorylation of LRP6 by casein kinase 1 γ (CK1γ) and GSK3β. Axin is stabilized on the membrane through additional interactions. Axin and Dishevelled were shown to bind each other and Dishevelled bound to Axin binds to Frizzled receptor. As a result Axin is trapped in the membrane through interactions between Axin and LRP5/6 and between Dishevelled and Frizzled. The destruction complex can not be formed in the absence of the central scaffold protein Axin, β-catenin can not be phosphorylated resulting in stabilization.

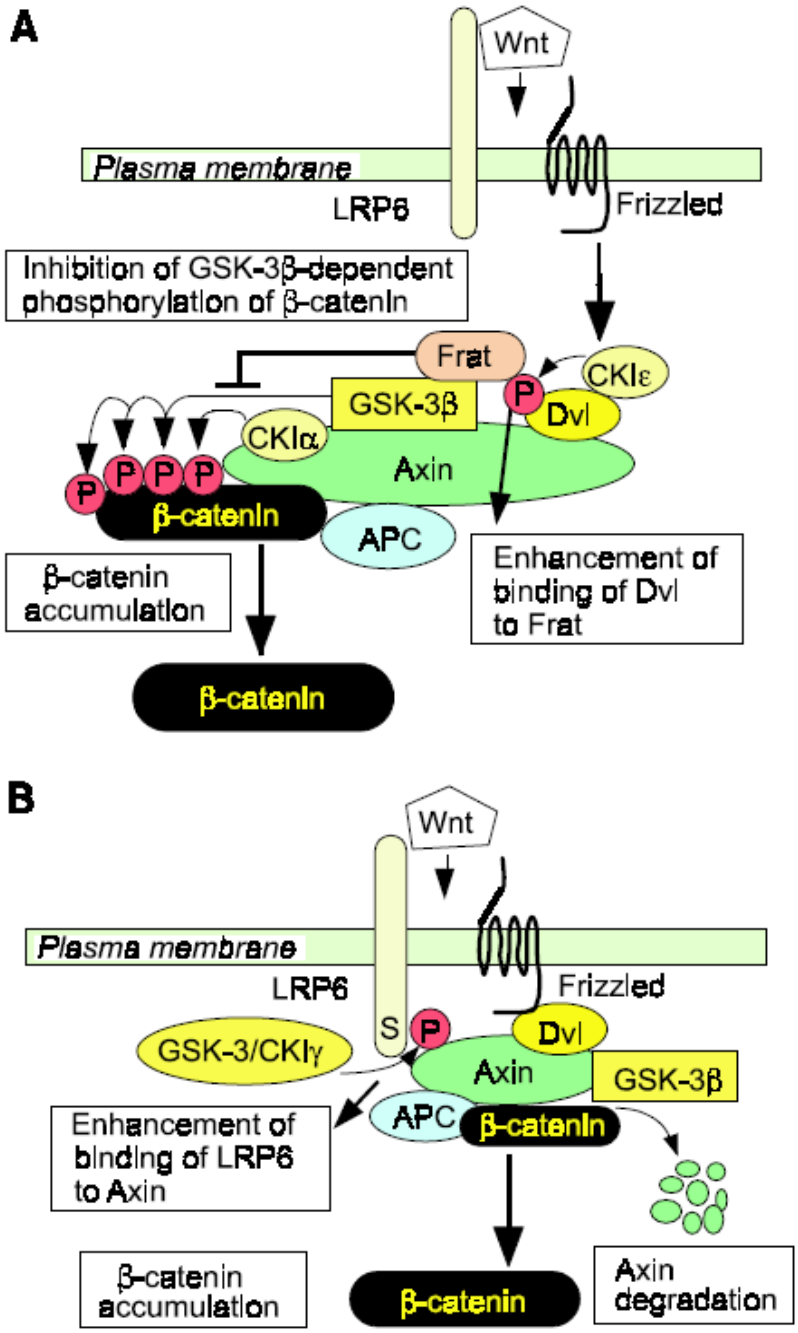


Figure 1.3. Wnt-dependent stabilization of β-catenin. A) Involvement of Dvl/Frat complex. Wnt triggers CKIε-dependent phosphorylation of Dvl which leads to binding of Dvl to Frat. Frat binds GSK3β in the β-catenin destruction complex, inhibiting GSK3β-dependent phosphorylation of β-catenin. B) Involvement of the Axin and LRP6 complex. Wnt triggers the phosphorylation of LRP6 by CKIγ and GSK3β which leads to binding of Axin to LRP6. Axin is recruited to the plasma membrane resulting in the reduced phosphorylation of β-catenin. (Kikuchi *et al*., 2006)

Once β-catenin is stabilized through mechanisms described above, cytosolic β-catenin level increases and β-catenin becomes free to translocate to nucleus. Since β-catenin lacks the nuclear localization sequence (NLS), it is speculated that β-catenin is translocated to the nucleus by the help of other proteins. There are models proposed for explaining transport mechanism of β-catenin to the nucleus (Seidensticker and Behrens, 2000). One model suggests that β-catenin is imported to the nucleus along with newly synthesized or shuttling TCF proteins. β-catenin may also enter nucleus independent of TCFs. Supporting this possibility, it was shown that mutant forms of β-catenin that are unable to bind TCFs can also translocate to the nucleus. Translocation of β-catenin was shown to be independent of importins. β-catenin may be interacting directly with nuclear pore components. Based on recent studies it is thought that binding partners of β-catenin (TCF4, BCL9, APC and Axin) do not affect the import/export rate of it but simply tend to keep β-catenin where they are localised (Stadeli *et al*., 2006). The mechanism underlying nuclear translocation of β-catenin remains unknown.

β -catenin now localised in the nucleus binds the amino terminal domain of TCF/LEF transcription factors. Through this binding β-catenin activates TCF and together they act as transcriptional activators. Co-activator function of β-catenin depends on two domains; the amino terminal activating arm (NTAA) and the carboxy terminal activating arm (CTAA). Both domains are essential for transcriptional activation function of β-catenin/TCF complex and recruit different co-activators. NTAA recruits Pontin, CTAA recruits TATA-binding protein (TBP), Brahma-related gene1 (Brg-1), Mediator subunit 12 (MED12), cAMP response element binding protein/p300 (CBP/p300) (Stadeli *et al*., 2006). The β-catenin/TCF complex binds to a concensus sequence on DNA through the DNA binding high mobility group (HMG) domain. The concensus sequence recognised by TCF/LEF is (A/T) (A/T) CAA (A/T) GG.

TCF/LEF activated by β-catenin controls expression of several target genes. Canonical Wnt target genes include c-Myc, cyclinD1, components of AP-1 complex, extracellular matrix protease matrilysin. Examples to target genes involved in developmental aspects of canonical Wnt signaling are siamois, ultrabithorax, nodal related 3, and twin (Seidensticker and Behrens, 2000).

1.2.3. Canonical Wnt Signaling in Cancer

Nuclear accumulation of ß-catenin due to aberrant activation of Wnt signaling is observed in gastrointestinal tumor (Kolligs *et al*., 2002), colorectal tumor (Brabletz *et al.*, 2002), hepatocellular tumor (Kim *et al*., 2000) and breast tumor (Howe and Brown, 2004). Mutations in components of the pathway were identified in different types of cancer (Polakis, 2007). APC mutations were identified as the main cause of familial adenomatous polyposis coli (FAP), a heritable predisposition to colorectal cancer. Mutations of APC are frequent in sporadic colorectal cancer cases. They are also identified in sporadic lung, ovarian and breast cancers but the mutation rates are very low (Furuuchi *et al*., 2000; Ohgaki *et al*., 2004). Mutations of the APC gene are loss of function mutations and generally lead to truncated proteins that are unable to downregulate β-catenin. Mutations in β-catenin itself are also identified in some cancer types; sporadic colorectal cancers (Korinek *et al*., 1997; Morin *et al*., 1997), melanoma (Rubinfeld *et al*., 1997) and endometroid ovarian cancer (Bell, 2005). Mutations of β-catenin are gain of function mutations and inhibits its degradation by the β-catenin destruction complex. Another component of the pathway, Axin is also mutated in a variety of human cancers (Salahshor and Woodgett, 2005). Mutations of Axin are loss of function mutations and leads to inactive Axin proteins. TCF4, one of the TCF family transcription factors is mutated in nearly half of the microsatellite-instability-positive (MSI+) colorectal cancers (Duval *et al*., 1999; Fukushima et al., 2001; Shimizu *et al*., 2002). Those mutations are recently proposed to eliminate binding of TCF4 to the transcriptional repressor CtBP (Cuilliere-Dartigues *et al*., 2006). Rare inactivating mutations of sFRP1 were also identified in colorectal cancers (Caldwell *et al*., 2004).

As a consequence of the described mutations, Wnt/β-catenin target genes are activated leading to tumorigenesis. Some target genes are functional in cell proliferation; c-Myc and gastrin (He *et al*., 1998; Watson *et al*., 2001), some inhibit apoptosis; survivin (Zhang *et al*., 2001), some are responsible for tumor progression; Laminin γ2 (Hlubek *et al.*, 2001).

1.3. A Transcriptome Analysis Method, Serial Analysis of Gene Expression

Transcriptome is defined as the set of all transcripts in one or a population of biological cells for a given set of environmental circumstances. Unlike genome, transcriptome is a dynamic concept, changing based on different conditions of the cell. Learning about the genes differentially expressed in two different states of a group of cells or tissue; such as normal and disease, gives very important data for characterization of the situation. There are several methods that are used for comparing gene expression differences. Complementary deoxyribonucleic acid (cDNA) subtraction or differential display can be used for this purpose but they are not able to give direct information about abundance. Some other techniques, such as the ribonucleic acid (RNA) blotting, ribonuclease (RNase) protection and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis can evaluate only a limited number of genes at a time. Currently, there are two expression profiling methods that are able to quantitate a high number of transcripts at a time; deoxyribonucleic acid microarrays (Schena *et al*., 1995) and serial analysis of gene expression (SAGE) (Velcelescu *et al.*, 1995).

SAGE is a gene expression profiling technique based on high-throughput sequencing, which allows qualitative and quantitative transcriptome analysis. It is based on the following three principles. It extracts a short tag (about 10 base pair) from the 3' end of each messenger ribonucleic acid (mRNA) within a certain sample. These tags are used to identify the gene by aligning the gene to the Unigene database. SAGE does not require prior knowledge of sequence information; it allows identification of novel genes. By forming concatamers from the short tags (a typical concatamer contains approximately 40 tags) it allows rapid and direct quantification of mRNAs.

SAGE is widely used as a gene expression profiling technique. The power of SAGE technique as a qualitative and quantitative tool makes it a popular transcriptome analysis method along with DNA microarrays. SAGE is used in cancer studies (Bosma. *et al*., 2002; Untergasser *et al*., 2002), human disease studies (Graca *et al*., 2002; Myerowitz, 2002), pathway analysis studies (Torocsik *et al*., 2002; Potapova *et al*., 2002) and plant and model organism studies (Fujii and Amrein, 2002; Steen *et al.*, 2002).

1.3.1. Experimental Outline and Principles of SAGE

Experimental procedure of SAGE is schematized in Figure 1.4. Double stranded cDNA is synthesized from input RNA with biotinylated oligo (dT) primers. Then the cDNA is cleaved with a restriction endonuclease (anchoring enzyme). The choise of the anchoring enzyme is of great importance. It should be a frequent cutter to be able to cut all transcripts at least once. Restriction endonucleases with four base pair recognition sites are used as the anchoring enzyme because they cleave every 256 base pair on average whereas most transcripts are larger. Cleaved cDNAs, the most three prime (3') portion are then isolated by streptavidin beads. Those cDNAs are unique sequences, each representing the transcript it is coming from. The pool of cDNAs is divided in half and ligated to two different adaptors both containing the same type IIS restriction site but different primer sequences. Type IIS restriction enzymes (tagging enzyme) cleave at a defined distance away from the recognition site (Szybalski, 1985). When the adaptor linked cDNAs are cleaved with the tagging enzyme, small cDNA sequences along with the adaptor sequences are released. Those small cDNA sequences are called the 'SAGE tags'. Two sets of adaptor linked tags are then pooled and ligated to create the 'ditags' surrounded by the adaptor sequences. The adaptor sequences allow the polymerase chain reaction (PCR) amplification of the ditags. Resulting PCR amplification products are ditag sequences flanked by four base pair anchoring enzyme recognition sites. The ditags are than ligated to form the concatamers in order to reduce the time and effort spent for the sequencing step. The anchoring enzyme sites flanking the ditag sequences can be used to define and extract the tag sequences from the sequence data by suitable software. Resulting data is the tag sequence and the number of times the tag is identified. Tags may or may not match a described gene which leads to quantification of gene expression or identification of novel genes.

Combinations of different anchoring enzymes with different tagging enzymes are used to create tags of varying length. Long-SAGE that produces longer tags, 17 base pair in order to increase specificity is described as an alternative approach (Saha *et al.*, 2002).

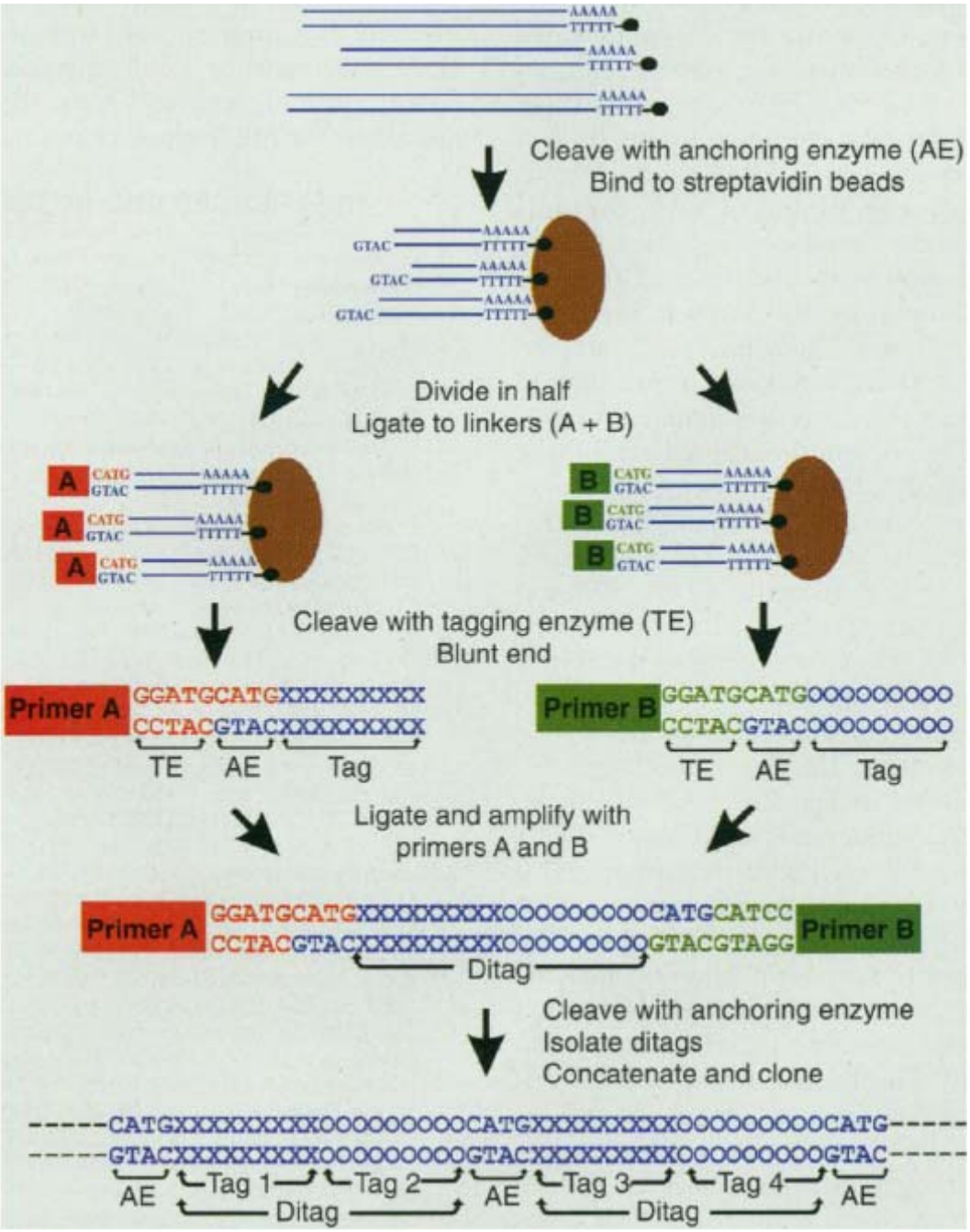


Figure 1.4. Schematic of SAGE. The anchoring enzyme is Nla III and the tagging enzyme is Fok I in this example. Sequences colored red and green represent primer-derived sequences. Blue sequences represent transcript-derived sequences. X and O represent nucleotides of different tags. (Velculescu *et al*., 1995)

1.4. Cell Lines with High and Low β-Catenin/TCF Activity

This study was started based on observations in Bilkent University on isogenic clones from hepatocellular carcinoma-derived Huh7 cells with high β-catenin/TCF activities which showed correlation to more aggressive tumor formation. These cells had been chosen because they do not show any mutations in the three important components of Wnt/ β-catenin pathway; *CTNNB1, Axin-1* and *APC*. The β-catenin /TCF activity is known to be low in this cell line as shown with a TCF4-dependent luciferase reporter gene assay (Lee *et al*., 2006). Huh7 cells had been transfected with the pCI-Neo-mutant *β-catenin* (S33Y) expression plasmid, or the empty pCI-Neo vector and selected clones expanded. S33Y represents the point mutation serine to tyrosine at point 33; in the phosphoylation site of β-catenin. β-catenin/TCF activity was shown to be higher in pCI-S33Y transfected cells when compared to control cells with luciferase gene reporter assay (Figure 1.5). Tumor formation studies showed that pCI-S33Y transfected cells form larger tumors when injected to CD-1 nude mice (Figure 1.6).

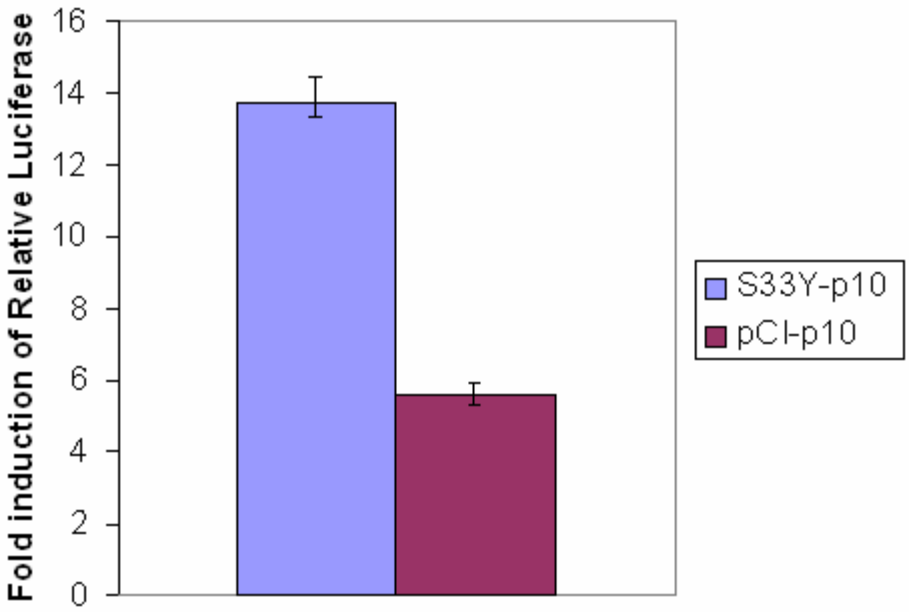


Figure 1.5. TCF4-dependent luciferase activity. Luciferase activity in Huh7 cells transfected with S33Y-β-catenin plasmid (S33Y-p10) as compared to pCI-transfected cells (pCI-p10) both tested at passage 10. S33Y stable has increased at least 2.5 TCF4 activity compared to pCI stable at passage 10. All experiments were carried out in triplicate.

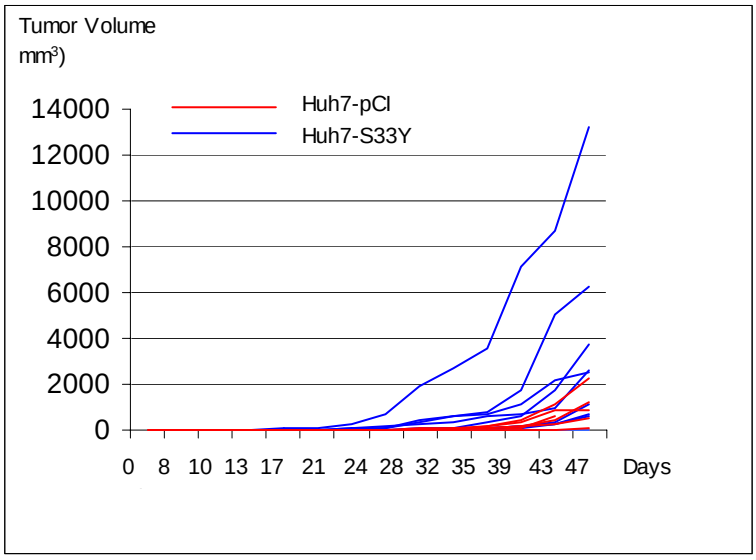


Figure 1.6. Tumor formation profiles of pCI-S33Y and pCI-Neo stables in nude mice. 5x103 cells were injected subcutaneously into nude mice at either right or left sides. Tumor growth was calculated by measuring tumor size in three dimensions. Tumor formation by Huh7-S33Y started earlier and the resulting tumors were larger.

2. PURPOSE

The aim of this study was to identify genes that are differentially expressed in cells with high β-catenin/TCF activity and to investigate their eventual relationship with the canonical Wnt pathway and possible roles in cell growth control.

In order to identify differentially expressed genes in cells with high β-catenin/TCF activity, SAGE libraries from cells with high and low β-catenin/TCF activity will be constructed. For further investigation of their relationship with the canonical Wnt pathway expression levels will be analysed upon overexpression of β-catenin. For investigation of the possible role in cell growth control proliferation assays will be performed upon overexpression of candidate target gene.

3. MATERIALS

3.1. Chemicals

All laboratory chemicals were analytical grade from Sigma (St. Louis, MO, USA) and Merck (Schucdarf, Germany), unless stated otherwise in the text. Tissue culture media and solutions were purchased from Invitrogen (San Diego, CA USA), Apllichem (Darmstadt, Germany), and Biochrom AG (Berlin, Germany). In vitro transfection reagent, Exgen 500 was purchased from Fermentas (Burlington, Canada)

3.2. Buffers and Solutions

3.2.1. DNA Gel Electrophoresis

50X Tris-acetic acid EDTA (TAE)	2 M Tris-acetate 50 mM ethylenediaminetetraacetic acid pH 8.5
Ethidium bromide (EtBr)	10 mg/ml (stock solution) 30 ng/ml (working solution)
10X Tris Base EDTA (TBE)	108 g Tris base. 55 g Boric acid 9.3 g EDTA UV treated distilled water up to 1 L
Loading buffer	6X loading buffer purchased from Fermentas (Burlington, Canada)

3.2.2. RNA Gel Electrophoresis

Diethylpyrocarbonate treated water (DPH)	1 per cent (v/v) Diethylpyrocarbonate
10X Morpholino Propane Sulfonic Acid (MOPS)	41.8 g. MOPS 20 ml 0.5 M EDTA 16.8 ml 3 M NaOAc DPH upto 1 L. pH 7.00
EtBr RNA loading buffer	0.72 ml formamide 0.16 ml 10X MOPS 0.26 ml formaldehyde 0.18 ml DEPC treated water (DPH) 0.1 ml 80 per cent glycerol 0.08 ml Bromophenol blue 50 µg EtBr
3M Sodium Acetate (NaOAC)	246.1 g NaOAc DPH upto 1 L pH 5.2
0,5M EDTA	37.2 g Na-EDTA 10 ml 10 N NaOH pH 8.0

3.2.3. Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide stock solution	40 per cent polyacrylamide (19:1 or 29:1 acrylamide:bisacrylamide) in distilled water
Ammonium Peroxidisulfate (APS)	10 per cent Ammonium Peroxidisulfate

TEMED	N,N,N,N-tetramethylethylenediamine
10 X Sample Buffer	95 per cent formamide, 20 mM EDTA 0.05 per cent xylene cyanol 0.05 per cent bromophenol blue

3.2.4. RNA Isolation Solutions

Guanidium Isothiocyanate (GITC)	20 mM NaOAc 4 M Guanidum Isothiocyanate 0.5 per cent (v/v) N-lauryl sarcosine 1.54 mg Dithiothreitol (DTT) UV treated distilled water up to 100 ml pH 5.5
5.7 M Cesium Chloride (CsCl)	96 g CsCl in 90 ml 10 mM EDTA
Sodium dodecyl sulphate (SDS)	10 per cent (m/v) SDS in DPH
Tris-EDTA Sodium Chloride (TES)	5 ml of 1 M Tris pH 8.0 2 ml of 0.25 M EDTA 0.3 g NaCl Distilled water up to 100 ml
N-Lauryl Sarcosine	10 per cent (m/v) N-Lauryl Sarcosine in DPH
70 per cent Ethanol (EtOH)	70 per cent (v/v) EtOH in DPH

3.3. Microbiological Media and Antibiotics

Luria-Bertani medium (LB)	10 g tryptophan 5 g yeast extract 10 g NaCl Distilled water up to 1 L pH 7, autoclaved
Luria-Bertani Agar	10 g tryptophan 5 g yeast extract 5 g NaCl 15 g Agar Distilled water up to 1.L pH 7.5, autoclaved
Chlorampenicol stock	30 mg Chlorampenicol in 1 ml absolute ethanol 30 ng/ml (working concentration)
Ampicillin stock	100 mg/ml in double distilled water sterilized by filtration and stored at -20 °C 100 µg/ml (working concentration)
SOB	20 g Tryptone 5 g Yeast Extract 2 ml of 5 M NaCl 2.5 ml of 1 M KCl 10 ml of 1 M $MgCl_2$ 10 ml of 1 M $MgSO_4$ Distilled water up to 1 L
SOC	20 g Tryptone 5 g Yeast Extract

2 ml of 5 M NaCl.

2.5 ml of 1 M KCl.

10 ml of 1 M $MgCl_2$

10 ml of 1 M $MgSO_4$

20 ml of 1 M glucose

Distilled water up to 1 L

3.4. Enzymes

Amplification grade DNase and the appropriate buffer was purchased from Invitrogen (San Diego, CA USA). Taq Polymerase was purchased from Fermentas (Burlington, Canada) together with the $MgCl_2$ (25 mM) and the 10X reaction buffer. Trypsin (0.025 per cent, ready to use) was purchased from Invitrogen (San Diego, CA USA).

3.5. Nucleic Acids

DNA molecular weight markers and deoxyribonucleotides were purchased from Fermentas (Burlington, Canada). pCI-Neo and pCI-S33Y plasmids were kindly provided by Mehmet Öztürk, Bilkent University. Gene 6 and Gene 3 clones in pCMV-SPORT6 were purchased from RZPD GmbH (Berlin, Germany). The full length cDNA clones were subcloned into pcDNA3 vector (Invitrogen; San Diego, CA USA) in our laboratory. Primers used in polymerase chain reaction and sequencing were purchased from Harvard University Mgh Sequencing Core (Boston, USA).

3.6. Bacterial Strain

Bacterial strain used in the study is *E. coli* HT115.

3.7. Cell Lines

HCC derived Huh7 cell line and Huh7 derived stable clones expressing PCI-S33Y and PCI-Neo vectors were used in this study. All cells were kindly provided by Mehmet Öztürk, Bilkent University.

3.8. Kits

3.8.1. I-SAGE Kit

I-SAGE kit was used for the construction of SAGE libraries. It was purchased from Invitrogen (San Diego, CA USA). Contents of the kit are listed below.

- cDNA Synthesis Module (1A)
- cDNA Synthesis Module (2A)
- Cleavage Module
- Ditag Formation Module
- Ditag PCR Module
- Concatemer Module
- *Nla* III Module
- Performance Check Module
- Zero Background Cloning Kit
- One Shot TOP10 Electrocomp *E.coli*
- S.N.A.P. Columns, Collection Tubes, and Magnetic Stand

3.8.2. Sequence and Polymerase Chain Reaction Clean-up Kits

Montage, SEQ 96 Sequence Reaction Clean-up kits and Montage, Polymerase Chain Reaction Clean-up kits were purchased from Millipore (Billerica, MA USA).

3.8.3. Plasmid Purification Kits

Qiagen Plasmid Purification Kits (Hilden, Germany); mini, midi and maxi were used for purifying plasmids from overnight grown *E.coli* cultures.

3.8.4. cDNA Synthesis Kit

ImPromII Reverse Transcription System from Promega (Medison, USA) was used for cDNA synthesis prior to RT-PCR and QRT-PCR.

3.8.5. Proliferation Assay Kits

Cell Titer 96 Aqueous One Solution Proliferation Assay and Cell Titer 96 Non-Radioactive Proliferation Assay were purchased from Promega (Medison, USA).

3.8.6. Total RNA Isolation Kit

Total RNA was isolated from cell lines with RNeasy Mini Kit (Qiagen; Hilden, Germany).

3.8.7. Quantitative Real Time PCR Kit

Light Cycler Fast Start DNA Master SYBR Green I kit was purchased from Roche (Basel, Switzerland).

3.9. Equipment

Autoclave	Midas 55, Prior Clave, UK
Balances	DTBH 210, Sartorius, GERMANY
	Electronic Balance VA 124, Gec Avery, UK
Carbon dioxide tank	2091, Habaş, TURKEY
Cell culture incubator	Hepa Class 100, Thermo, USA
Centrifuges	Ultracentrifuge J2MC, Beckman Coulter, USA
	Mini Centrifuge 17307-05, Cole Parmer, USA
	Centrifuge 5415R, Eppendorf, USA
	Centrifuge, Allegra X-22, Beckman Coulter, USA
Deepfreezers	-20°C, Arçelik, TURKEY
	-70°C Freezer, Harris, UK

	-86°C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio-Doc, ITALY
Electrophoresis Systems	Mini-sub Cell GT, Biorad, USA
	Mini-Protean III Cell, Bio-Rad, ITALY
Hand tally counter	Milky Way Counter, TAIWAN
Heat blocks	DRI-Block DB-2A, Techne, UK
Hemocytometer	Improved Neubauer, Weber Scientific International Ltd, UK
Laminal flow cabinet	Labcaire BH18, UK
Magnetic Stirrers	M221 Elektro-mag, TURKEY
	Clifton Hotplate Magnetic Stirrer, HS31, UK
Micropipettes	Finnpipette, Thermo, USA
Microplate Reader	680, Biorad, USA
Microscope	Inverted Microscope, CKX41, Olympus, JAPAN
Microwave oven	M1733N, Samsung, MALAYSIA
pH meter	WTW, GERMANY
Pipettor	Pipetus-akku, Hirscmann Labogerate, GERMANY
Power Supply	Biorad, USA
Real Time PCR	LightCycler 1.5, Roche Diagnostics, SWITZERLAND
Refrigerators	2082C, Arçelik, TURKEY
	4030T, Arçelik, TURKEY
Shakers	VIB Orbital Shaker, InterMed, DENMARK
	Lab-Line Universal Oscillating Shaker, USA
Software	Quantity One, Bio-Rad, ITALY
	Light Cycler 4.0 Analysis Software, Roche Diagnostics, SWITZERLAND
	ImageJ, Image Analysis Software, (http://rsb.info.nih.gov/ij/)
	iSAGE, SAGE Analysis Software, Invitrogen, USA
Spectrophotometer	Agilent 8453, USA
	NanoDrop 1000, USA
Syringe	701N 26S/51/2, Hamilton, SWITZERLAND
Thermocyclers	Gene Amp. PCR System 2700, Applied Biosystems, USA
Vacuum pump	KNF Neuberger, USA
Vortex	Vortexmixer VM20, Chiltern Scientific, UK

Water baths	TE-10A, Techne, UK
Water purification	WA-TECH ultra pure water purification system, GERMANY

4. METHODS

4.1. Preparation of Chemically Competent Cells by Calcium Chloride Method

Two to five ml LB was inoculated with E.coli strain HT115 and grown overnight. Next day, 25 ml LB was inoculated with 250 µl of the grown culture to make 1:100 dilutions. Cells were grown in the shaker till OD 595 reaches 0,4. Cells were spinned for 10 min. at 3000 revolutions per minute (rpm) at 4 ^{o}C. Pellet was resuspended in 12.5 ml of ice-cold sterile 50 mM $CaCl_2$ and incubated on ice for 30 min. Cells were spinned again with same conditions and pellet was resuspended in 2.5 ml ice-cold sterile 50 mM $CaCl_2$. 50-200 µL of this preparation was used for transformations. For long term storage at -80 ^{0}C, glycerol was added to yield 10 per cent final concentration and cells were rapid-frozen in dry ice/ethanol or liquid nitrogen.

4.2. Transformation of the Chemically Competent HT115

A vial of competent cells was thawed on ice for 15 min. and 1 µl of plasmid was added. Following incubations were ice/water bath for 10 min, 42 ^{o}C water bath for 1 min, ice/water bath for 2 min. 1 ml SOC medium was added and incubated for 1 hr. at 37 ^{o}C with shaking. Ten µl, 100 µl and 250 µl were plated on antibiotic resistance plates and grown overnight at 37 ^{o}C.

4.3. Plasmid Purification

All plasmid purifications were carried out with Qiagen plasmid kits, mini, midi and maxi according to the manufacturer's protocol. Plasmids that would be used in transfections were purified with midi or maxi gravity flow kits to yield transfection grade plasmids. Plasmids were further purified in QIAprep mini spin colons according to the manufacturer's protocol when required. Quality of plasmids was checked by spectrophotometric measurements and agarose gel electrophoresis. OD 260/280 was between 1.8-2.00.

4.4. Gel Electrophoresis

4.4.1. Horizontal Agarose Gels of DNA Samples

DNA fragments were fractionated by horizontal electrophoresis by using standard buffers and solutions. DNA fragments were generally separated on one per cent to four per cent agarose gel depending on the size of the fragments and purpose of fractionation. Agarose gels were completely dissolved in 1X TAE or 1X TBE electrophoresis buffer to required percentage in microwave and ethidium bromide was added to final concentration of 30 µg/ml. The DNA samples were mixed with 6X loading buffer in a certain amount to get 1X concentration. Water was added if required. The gel was run in 1X TAE or 1X TBE at different voltage and time depending on the size of the fragments at room temperature.

4.4.2. Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was used to fractionate DNA fragments with standard buffers and solutions. 12 per cent PAGE with 19:1 acrylamide: bisacrylamide ratio was used for fractionating PCR products and SAGE ditags. eight per cent PAGE with 29:1 acrylamide: bisacrylamide ratio was used for fractionating SAGE concatemers. One per cent APS and TEMED was used to maintain polymerization. Standard polymerization time after the gel was poured was about 30 min. Running buffer was either 1X TAE buffer or 1X TBE buffer.

4.4.3. Total RNA Gel Electrophoresis

Total RNA was fractionated by 1 per cent agarose gels containing formaldehyde to disrupt hydrogen bonds. 0.4 g. agarose was dissolved in 35 ml DEPC treated water and after cooling to about 60 ^{o}C, 4 ml 10X MOPS buffer and 1.2 ml 37 per cent formaldehyde was added to the mixture. One to five µg of RNA was mixed with 20µls of ERLB. Samples were kept at 95 ^{o}C for 2 min., spinned and chilled on ice for at least 1 min. Gel was run in 1X MOPS buffer.

4.5. Cell Culture Techniques

4.5.1. Growth Conditions of Cells

HCC derived cell line Huh7 was grown in DMEM containing 10 per cent FBS, 1 per cent penicillin/streptomycin and 1 per cent nonessential amino acids. G418 (200 µl/ml) was added to the medium to grow Huh7 derived stable clones expressing PCI-S33Y and PCI-Neo vectors. All cells were incubated in 5 per cent CO2 incubator at 37 ^{o}C. Media were kept at 4 °C and warmed to 37 °C in a sterile waterbath before use.

4.5.2. Passaging

The cells were passaged before reaching confluence. The growth medium was aspirated and the cells were washed once with 1X calcium and phosphate-free PBS. In order to remove the monolayer cells from the surface, trypsin (0.025 per cent, ready to use) was added to the petri dish and cells were incubated at 37 ^{o}C for 3-5 min. Ten volumes of fresh medium was added to inactivate trypsin and the suspension was pipetted gently to disperse the cells. The cells were transferred to fresh petri dishes in a 1:10 ratio for standard passaging.

4.5.3. Thawing

One vial of frozen cell line was taken from -80 ^{o}C freezer and thawed under 40 ^{o}C (at most) running tap water. Immediately after cells are thawed, they were resuspended in proper amount of medium. Cells were then transferred to fresh petri dishes.

4.5.4. Cryopreservation of Cell Lines

Cells were harvested by trypsinization and neutralized with 10 volumes of growth medium. The cells were counted with haemocytometer and precipitated at 1500 rpm for 5 min. The pellet was suspended in freezing solution (10 per cent DMSO, 20 per cent FCS and 70 per cent DMEM). 1.5 ml of this solution was placed into 2 ml screw capped-cryotubes. The tubes were left at -20 °C for 2 hours and transferred to -80 ^{o}C freezer. Next

day, main stocks were transferred into the liquid nitrogen storage tank. Number of cells frozen in a single vial was between one to three millions.

4.5.5. Extraction of Total RNA From Tissue Culture Cells

4.5.5.1. Extraction of total RNA with Qiagen RNAeasy kit. RNA extraction from six and 12 well tissue culture plates was done according to manufacturer's protocol.

4.5.5.2. Large scale RNA isolation with CsCl method. Before reaching confluency, culture medium of 15 cm plate was aspirated and rinsed with 5 ml 1X PBS twice in order to get rid of remaining media. 4 ml GITC was distributed over the plate and immediately cells were lysed with a cell scraper. The lysate was passed through a 23 gauge needle four to five times in order to shear DNA. The lysate was then transferred to silanized and autoclaved SW41 tubes (Beckman Coulter; Fullerton, CA USA) containing 5 ml CsCl. Tubes were spinned overnight at 18 ^{0}C, 32000rpm, breaking down to 800 rpm. Next day, supernatant was aspirated with water suction, slowly descending from top, circling around tube walls to dry them with extreme care not to detach the pellet. Tube was cut 2 cm away from bottom and pellet was washed with 300 µl 70 per cent EtOH at room temperature. Tube was reverted and let air-dry. Pellet was resuspended in 300 µl TES buffer and transferred to 2 ml eppendorf tube (Axygen Scientific, Union City, CA USA). RNA was precipitated with 30 µl NaOAc and 900 µl absolute EtOH (-20 ^{0}C) by incubating 30 min at -20 ^{0}C and spinning 10 min at 4^{0} C at maximum speed. Supernatant was removed and 1 ml 70 per cent EtOH (-20 ^{0}C) was added and spinned at maximum speed at 4 ^{0}C in order to remove remaining supernatant. Pellet was than air-dried and redissolved in appropriate amount of DPH.

Quality of RNA preparations was checked with RNA gel electrophoresis and spectrophotometer. The concentration was also determined by spectrophotometry.

4.5.5.3. DNase treatment of total RNA preparations. Amplification grade DNaseI (Invitrogen; San Diego, CA USA) was used. 2 µg total RNA was put into reaction with 1 unit of enzyme and proper amount of buffer. Reaction was incubated at room temperature for 15 min. and then 25 mM EDTA was added. Additional incubation at 65 ^{o}C for 15 min

was done to heat-inactivate DNaseI. Then the reaction was put on ice for 1 min and the reaction mixture was collected by a brief centrifugation. Half of the mixture was loaded on agarose gel to check the efficiency and the other half was used directly for reverse transcription.

4.5.6. Proliferation Assay

Two different proliferation assay kits (Cell Titer 96 Aqueous One Solution Proliferation Assay, Cell Titer 96 Non-Radioactive Proliferation Assay) were used for proliferation studies. Both assays are based on the cellular conversion of a tetrazolium salt into a formazan product that is easily detected using a 96-well plate reader (Bio-Rad,USA). The tetrazolium salts and the products are different (MTS and MTT respectively), and the readings were taken in 490 nm and 570 nm, respectively. Cells were trypsinized, counted and seeded in 96 well plates (1000 per well) and the first reading was taken next day. Medium was changed every two days. Readings were taken for 8 days. The linearity range of the assay was determined by seeding different number of cells and taking the readings next day (Table A.1).

4.5.7. Colony Formation Assay

Cells were trypsinized, counted with haemocytometer and seeded on six well plates, 1500 per well. Medium was changed next day and changed every three days. After day seven, plates were checked for the formation of colonies. When the colonies become visible to the eye, the media were aspirated and the plates were washed twice with 1X PBS. Then Giemsa staining was done. Giemsa solution, ready to use was added to the wells, 1.5-2 ml for each well. Plates were incubated in hood for three hours, and then washed with 50 per cent methanol once and 25 per cent methanol twice. Plates were left in the hood to air-dry. Colonies were counted in Gel-Doc documentation system.

4.5.8. Transient Transfection of Cells

Transfections were carried out in six, 12 or 24 well plates with in vitro polyethylenimine transfection reagent, Exgen. Optimization for transfection efficiency was

done with GFP expression vector transfections. Two parameters were tested for optimizing the efficiency; DNA amount (0.5-2 µg) and number of equivalents of Exgen. Optimum transfection conditions are stated in Table 4.1. Cells were seeded the day before transfection to obtain 50-60 per cent confluency at the day of transfection. Next day medium was changed with certain amount of antibiotic-free medium. Later in the day, transfection was done according to manufacturer's protocol. 24 hours after transfection, transfection medium was removed and fresh medium was added.

Table 4.1. Optimized transfection conditions for different multi-well plates

Multiwell plates	Growth area(cm^2/well)	Seeding volume (ml)	Cell # to seed the day before transfection	Amount of DNA	# of Exgen Equivalents	Volume of Exgen (µl)
6 well	9.5	2	$20x10^4$	1.5 µg in 200 µl	7	5.7
12 well	4	1	$10x10^4$	1 µg in 100 µl	7	3.8
24 well	2	1	$5x10^4$	0.5 µg in 100 µl	7	1.9

4.6. Construction of SAGE Libraries

SAGE libraries were constructed with I-SAGE kit (Invitrogen, San Diego, CA USA). Kit manual was followed until preparation of 100 bp tags and EMBO's SAGE manual (EMBO SAGE Congress, 2005) was followed there after. 5 µg of total high quality RNA was used as input material for each SAGE library. Construction of a SAGE library up to sequencing of the clones is a nine day procedure containing general molecular biology techniques such as RNA isolation, cDNA synthesis, DNA ligations, E. coli transformations, restriction enzyme analysis, polyacrylamide gel electrophoresis. Table 4.2. outlines SAGE procedure day by day. The anchoring enzyme was *Nla* III and the tagging enzyme was *BsmF* I. Before step 9 on Table 4.2, ditag PCR was optimized by changing three parameters; template amount, enzyme amount and cycle number. 1:20 dilution of the ditag sample was used as the template and amplified by 1 unit of enzyme per reaction for 27 cycles. 196 PCR reactions were done to amplify the ditags at step 10.

At step 16, the concatemers were purified as three different fragments; 1st fragment (1000-500 bp), 2nd fragment (500-300 bp) and 3rd fragment (300-80 bp). Analysis of the transformants by colony PCR and respective agarose gel electrophoresis (step 20, Table 4.2) revealed that the 1st fragment gives the best insert size for sequencing (400-800 bp).

Sequencing was done with M13 forward primer (step 21, Table 4.2) from the colonies transformed with the 1st fragment concatamers. Colony PCR products were purified with the Millipore, Montage 96 PCR reaction clean up kits (Millipore; Billerica, USA). For each colony PCR, a replicate 96 well glycerol stock was constructed for longer preservation at -80 ^{o}C. Sequencing reactions were done with Amersham 96 capillary (Piscataway, USA) in Sabancı University. 41898 and 42126 tags were counted from PCI and S33Y libraries, respectively. SAGE libraries were analysed by normalizing to the lower count. Since SAGE is based on high through-put sequencing, many colony PCR reactions and respective sequencing reactions were done in our labarotary by the help of many graduate and undergraduate students. I-SAGE analysis program, which identifies the anchoring enzyme sites and extracts the two tags flanked by *Nla*III site was used to analyse the SAGE libraries. Extracted tag sequences were analysed with sagExplore, a tool developed by Erşen Kavak in our laboratory (http://aklab.boun.edu.tr/sagexplore/). Gene identity and UniGene cluster assignment of each SAGE tag was obtained by using the tag-to-gene "reliable" map, from SAGEmap NCBI site. Duplicate ditags and multiple matches to a UniGene ID are eliminated from the data. Duplicate ditags are propably PCR artifacts and multiple matches to a UniGene ID actually come from different transcripts. The tags extracted were uploaded to SAGEmap and corresponding accession numbers were retrieved using the *H. sapiens* NCBI-GenBank database.

Table 4.2. Experimental overview of SAGE (I-SAGE Kit, Version E)

Day	Step	Action
1	1	Isolate total RNA or mRNA using a method of choice and check the quality of your RNA preparation.
	2	Prepare oligo (dT) beads and bind your RNA sample to the beads.
	3	Synthesize cDNA from the mRNA bound on the beads.
	4	Cleave the cDNA with an anchoring enzyme, *Nla* III.
	5	Divide the cDNA into two tubes, and ligate Adapters A and B to the cDNA.
2	6	Cleave the cDNA with a tagging enzyme, *Bsm*F I, to release the adapter with a short piece of DNA.
	7	Perform a Klenow reaction to fill in 5′ overhangs left after *Bsm*F I digestion.
	8	Ligate the adapter-linked cDNA with T4 DNA ligase to form 100-bp ditags.
3–4	9	Amplify the 100-bp ditags using PCR.
	10	Perform **Scale-up PCR** (200–600 PCR reactions) of the 100-bp ditag.
5	11	Purify the 100-bp PCR product after scale up using polyacrylamide gel electrophoresis.
	12	Excise the 100-bp ditags from the polyacrylamide gel and purify the DNA using spin columns.
6	13	Digest the purified 100-bp ditags with *Nla* III to yield 26-bp ditags.
	14	Purify the 26-bp ditags on a polyacrylamide gel.
7	15	Ligate the 26-bp ditags to form concatemers.
	16	Purify the concatemers by polyacrylamide gel electrophoresis.
	17	Digest pZErO®-1 with *Sph* I.
	18	Clone gel-purified concatemers into pZErO®-1 digested with *Sph* I.
	19	Transform the ligation reaction into One Shot® TOP10 Electrocomp™ *E. coli* and select transformants on low salt LB plates containing 50 μg/ml Zeocin™.
8	20	Analyze transformants by restriction enzyme digestion or colony PCR.
9	21	Sequence the selected clones using M13 forward sequencing primers.
	22	Analyze sequences using the SAGE™ analysis software.

4.7. Semi-Quantitative and Quantitative Reverse Transcriptase Mediated PCR (RT and QRT-PCR)

4.7.1. cDNA Synthesis

cDNA synthesis was performed with ImPromII Reverse Transcription System kit according to manufacturer's protocol. 1 μg total RNA was used as starting material. The reaction volume was doubled because the DNase treated RNA was directly used in the reverse transcription, without any purification step. Appropriate controls were included as described in the kit manual.

4.7.2. Primer Design

Primer design for the RT-PCR and Q-RT PCR was done with the program, Perl Primer (http://perlprimer.sourceforge.net/). Primers were designed to encompass an intron to see any genomic contamination, and the amplicon size was usually between 100-300 bp.

4.7.3. Polymerase Chain Reaction

One per cent of cDNA synthesized from 1 μg total RNA was used as template in PCR reactions. The optimum cycle number for each gene was determined by changing the cycle number in the range of 25-31. Cycle number of the house keeping control gene actin was always kept at 25 since above that actin exceeds its linear range. The PCR reaction starts with an initial denaturation step at 94 ^{o}C for 2 min. A PCR cycle has three steps; a denaturation step at 94 ^{o}C for 30 sec., an annealing step at 55 ^{o}C for 1 min. and an elongation step at 70 ^{o}C for 1 min. PCR reaction ends with a final elongation step at 70 ^{o}C for 5 min.

4.7.4. Real Time Polymerase Chain Reaction

Real Time PCR was done with the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche; Basel, Switzerland) according to manufacturer's protocol. Standard curves were constructed for each primer pair and cDNA sample in order to calculate the

efficiency. Experiments were carried out in triplicate. Results were analysed with Light Cycler 4.0 Analysis Software (Roche; Basel, Switzerland). Reaction starts with an initial denaturation step at 95 ^{0}C for 10 min. Amplification cycle consists three steps; a denaturation step at 95 ^{o}C for 10 sec., an annealing step at 57 ^{o}C for 5 sec. and an elongation step at 72 ^{o}C for 10 sec. 45 cycles are followed by melting curve step.

5. RESULTS

5.1. High β-catenin/ TCF Activity Does Not Lead to Higher Proliferation Rate in Huh7 Cells

Based on the tumor formation assay results done in Bilkent University, it was suspected that the high β-catenin/TCF activity might have an affect on the growth rate of cells. In order to test this possibility, proliferation assay was done with PCI-S33Y, PCI-Neo stable cells and Huh7 cells. Raw data are given in Table B.1. There is not a significant difference between the growth rate of S33Y and PCI-Neo stables, Figure 5.1. Neither did colony formation assay on plastic testing the clonogenic survival of cells give a significant difference (Figure 5.2. and Table 5.1.).

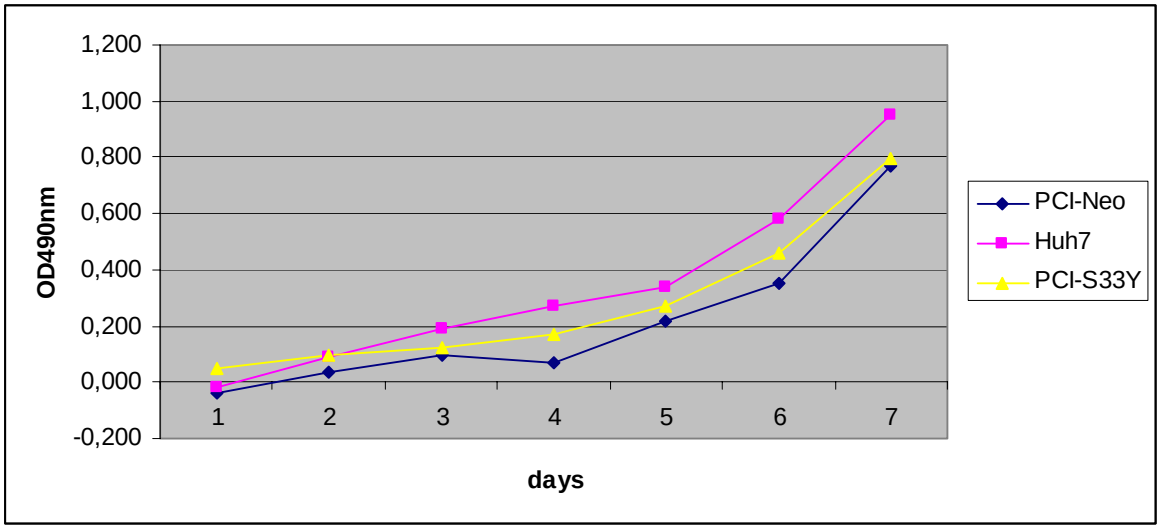


Figure 5.1. Growth curves of Huh7, PCI-Neo and PCI-S33Y stables. Experiments were done in triplicate, given values are the median values of three measurements. Experiments were started with 1000 cells per well.

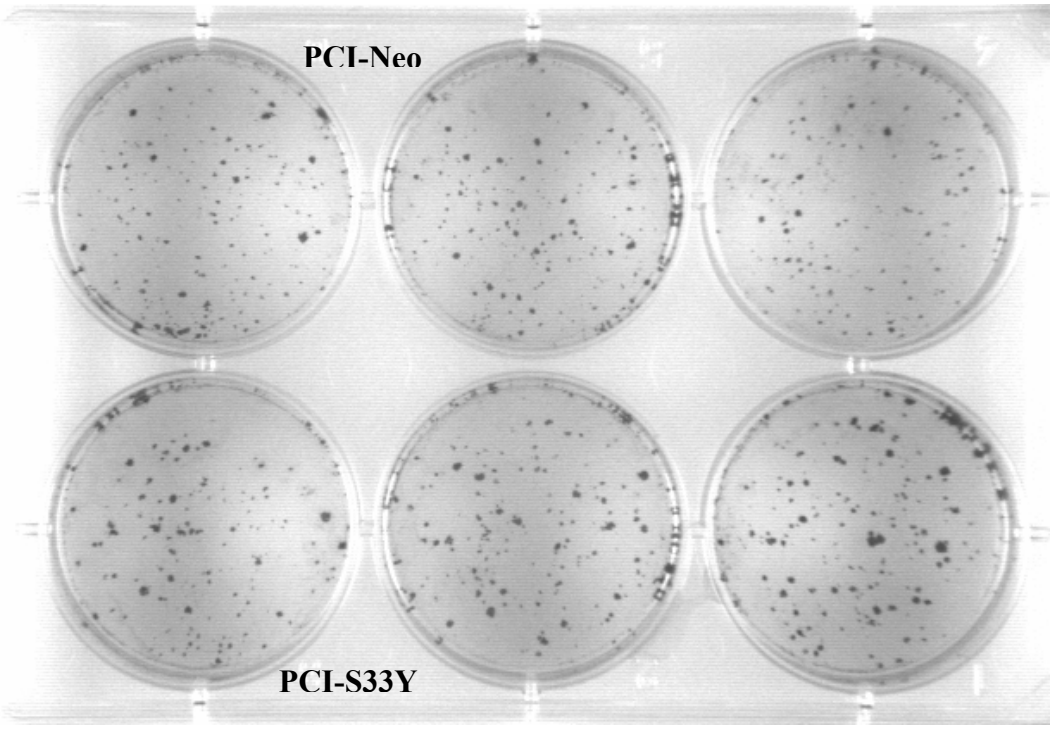


Figure 5.2. Colony formation sssay. Upper row shows PCI-Neo as indicated, lower row shows S33Y cells

Table 5.1 . Colony formation assay colony counts

	Colony Numbers		
PCI-S33Y	122	132	111
PCI-Neo	118	128	121

(Colonies were counted with Gel-Doc documentation system. Student's t-test was used to compare data and it showed that there was not a significant difference (p=0,92).)

5.2. Two SAGE Libraries were Constructed and Analysed from Huh7 Cells with High and Low β-catenin/ TCF Activity

Procedure was started with 5 µg RNA from each sample (Figure 5.3.).

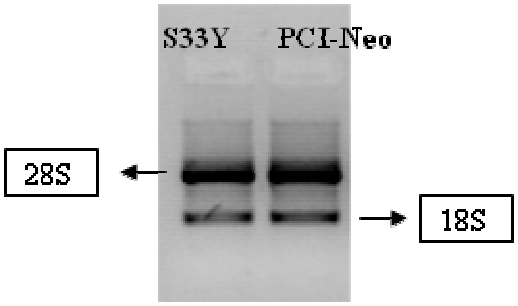


Figure 5.3. RNA samples used in SAGE protocol. First lane shows the S33Y RNA and second lane shows the PCI-Neo RNA. 1 µg of RNA isolated by GITC method was loaded from each sample. 28 S and 18 S ribosomal RNAs can be seen.

After making the 100 bp ditags, they were amplified by scale-up PCR, pooled together and loaded on PAGE to be purified from gel (Figure 5.4). 100 bp ditags were carefully separated from the 80 bp contaminants that do not contain the ditag sequence but just the adaptor sequences.

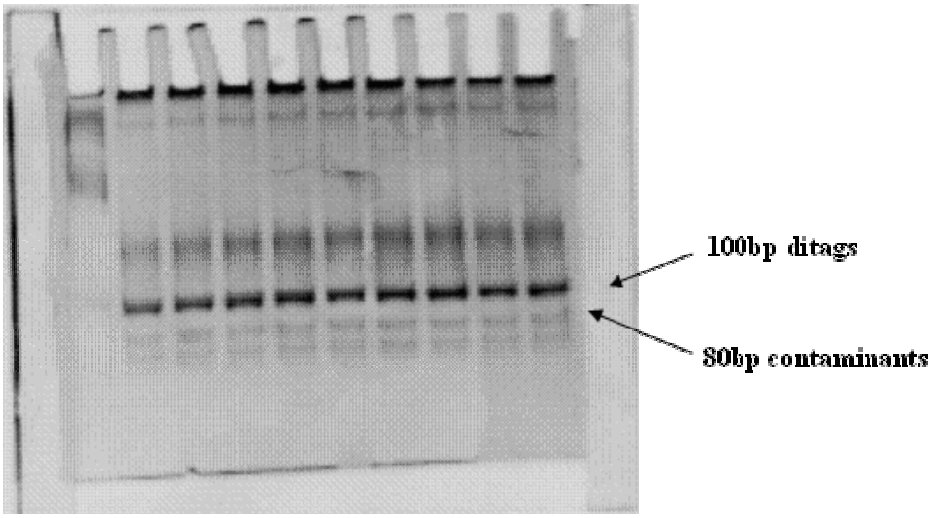


Figure 5.4. 100 bp ditags. Amplified by scale-up PCR

Purified 100 bp ditags were digested with anchoring enzyme to get the 26 bp ditags that contain the tag sequences from two different transcripts and the flanking four bp CATG overhang. 26 bp ditags were also purified from PAGE (Figure 5.5.).

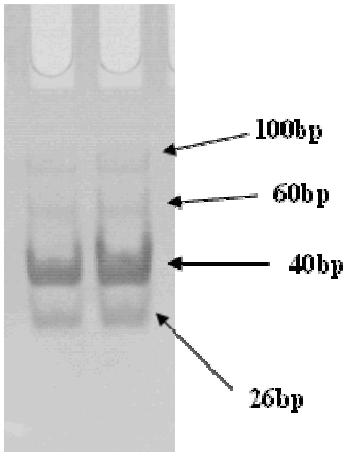


Figure 5.5. 26 bp ditags. 26 bp ditags are seen as faint bands under the 40 bp bands. 100 bp and 60bp bands are results of incomplete *Nla* III digestion. 40 bp bands are the adaptor sequences and the 26 bp bands contain tag sequences from 2 different transcripts.

26 bp ditags were ligated to produce concatemers. Concatemers were loaded on PAGE and purified from gel as three different fragments (Figure 5.6).

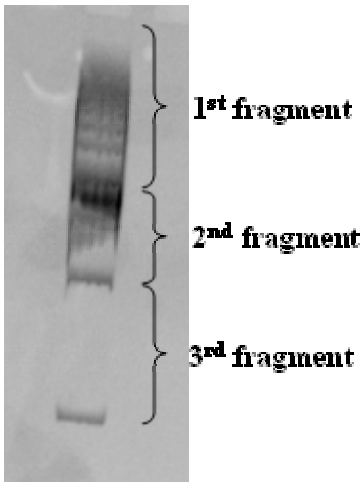


Figure 5.6 . Concatemers. Ligated ditags form different lengths of concatemers. Concatemers were purified from gel as three fragments; 1st fragment starts with the longest fragment and goes down to 500 bp, 2nd fragment is between 500 and 300 bp, 3rd fragment starts with the 300 bp and goes down to 80 bp.

All fragments were cloned into pZErO-1 vector and transformed into electrocompetent *E.coli* cells. Colony PCR analysis of three fragments were done and the 1st fragment gave the range of 400-800 bp which is the optimum range for counting highest number of tags per a sequencing reaction (Figure 5.7). 2nd fragment and 3rd fragment were also analysed and gave the ranges of 300-600 bp and 200-500 bp, respectively.

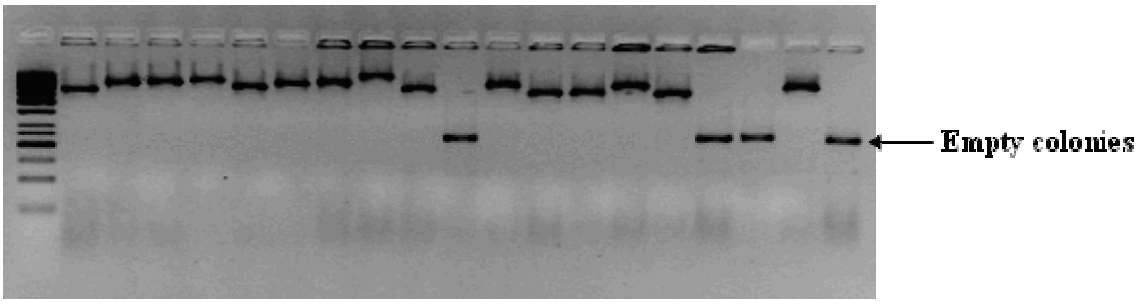


Figure 5.7. Colony PCR of 1st Fragment. Lane 1 is the 50 bp DNA ladder. The 200 bp bands are the empty colonies. Other lanes show inserts in the 400-800 bp range.

First fragment was used for the following sequencing reactions. Sequence data was analysed with the I-SAGE analysis program and the resulting data is exemplified in Table 5.2. 41898 and 42126 tags were counted from PCI and S33Y libraries, respectively. 431 tags were differentially expressed (p value < 0.05), 102 of which are increased in the experiment and the remaining 329 tags are decreased.

Table 5.2. Sequence data analysed with i-SAGE software. Tags and counts given are not the real results of this experiment. They are random examples of the data.

TAG	COUNT	TAG	COUNT	TAG	COUNT
CCCATCGTCC	1286	CACTACTCAC	245	TTCACTGTGA	150
CCTCCAGCTA	715	ACTAACACCC	229	ACGCAGGGAG	142
CTAAGACTTC	559	AGCCCTACAA	222	TGCTCCTACC	140
GCCCAGGTCA	519	ACTTTTTCAA	217	CAAACCATCC	140
CACCTAATTG	469	GCCGGGTGGG	207	CCCCCTGGAT	136
CCTGTAATCC	448	GACATCAAGT	198	ATTGGAGTGC	136
TTCATACACC	400	ATCGTGGCGG	193	GCAGGGCCTC	128
ACATTGGGTG	377	GACCCAAGAT	190	CCGCTGCACT	127
GTGAAACCCC	359	GTGAAACCCT	188	GGAAAACAGA	119
CCACTGCACT	359	CTGGCCCTCG	186	TCACCGGTCA	118
TGATTTCACT	358	GCTTTATTTG	185	GTGCACTGAG	118

Results of SAGE were verified in our laboratory with Q-RT and RT experiments by Erşen Kavak.

5.3. Overexpression of β-catenin in Huh7 Cell Line and Respective Expression Analysis by Semi-Quantitative RT-PCR

In order to compare acute versus stabilized effects of the S33Y mutant of β-catenin , it was also overexpressed in Huh7 cell line with transient transfection of PCI-S33Y vector. An increase in the mRNA level of β-catenin was clearly detected with RT-PCR (Figure 5.8). In order to rule out the possibility that the PCR products might be the results of vector DNA amplification, total RNA used for the reverse transcription was DNase treated. The RNA samples before and after DNase treatment can be seen in Figure 5.9.

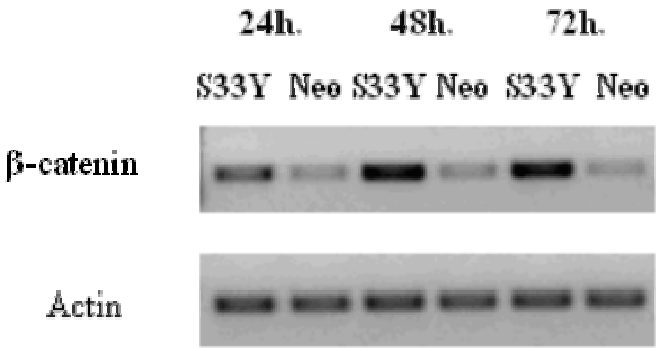


Figure 5.8. β-catenin overexpression in time course. Cells transfected with PCI-S33Y and PCI-Neo vectors were harvested at time points 24h., 48h. and 72h. RT-PCR results shows the higher mRNA levels of β-catenin in S33Y transfected cells. Actin is the control gene.

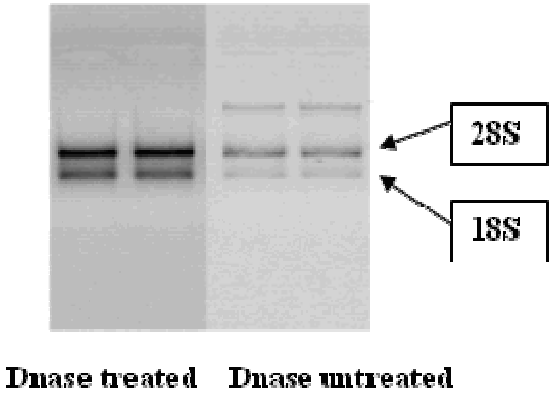


Figure 5.9. RNA samples before and after DNase treatment. Genomic DNA band seen in DNase untreated RNA samples is not seen in DNase treated RNA samples.

The mRNA expression levels of 10 genes, 2 of which are canonical Wnt pathway targets and rest are differentially expressed in cells with high β-catenin/TCF activity, were analysed at 72h. after β-catenin overexpression. TCF binding sites on the promoter regions were determined with the free web-based software TFExplorer (Kim *et al*., 2005). Genes are listed in Table 5.3. and the SAGE results are also given.

Table 5.3. Genes selected for expression analysis

	Tag numbers				# of TCF binding sites		
Gene	PCI-Neo	PCI-S33Y	p value	S33Y/Neo	-500bp 0bp	-1000bp 0bp	-5000bp +5000bp
CNND1	9	6	0,31	0,7	1	1	6
MYC	-	-	-	-	1	1	9
Gene 3	35	7	0	0,2	-	-	8
Gene 5	11	26	0,01	2,4	1	1	5
Gene 6	10	25	0,01	2,5	-	-	3
Gene 7	32	19	0,04	0,6	1	1	10
Gene 13	9	4	0,14	0,4	-	2	16
Gene 16	2	11	0,01	5,5	-	2	12
Gene 21	11	2	0,01	0,2	-	1	8
Gene 32	12	4	0,04	0,3	-	-	-

(Gene annotations are given as internal codes due to patent application formalities.)

Among those genes, mRNA levels of two genes, Gene3 and Gene6 were seen to be changed in β-catenin transfected cells whereas mRNA levels of other genes did not change

in 72 h.(Figure 5.10.). Results of triplicate RT experiments from two different cDNA samples are given in Figure 5.11.

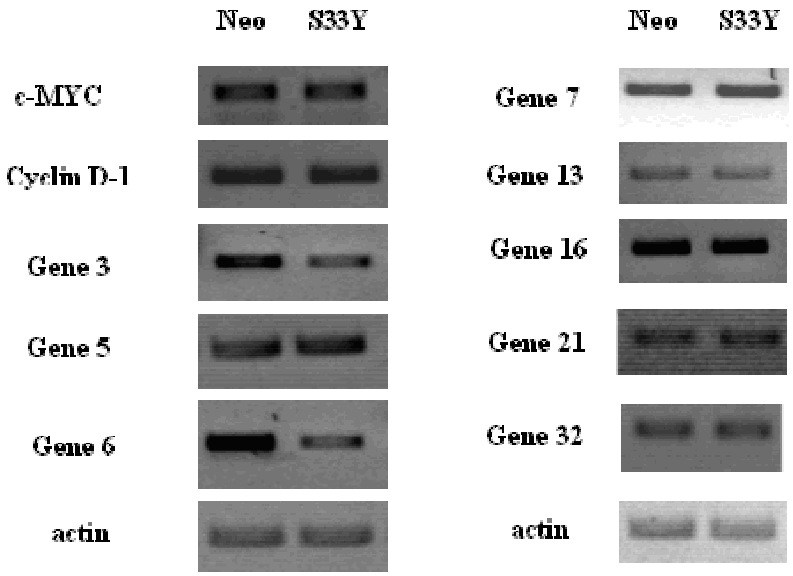


Figure 5.10. mRNA levels of 10 genes in β-catenin transfected cells (S33Y) and empty vector transfected control cells (Neo) mRNA levels of Gene 3 & 6 are significantly reduced. Canonical Wnt targets c-MYC and Cyclin D-1 mRNA levels are same. Actin was used as control.

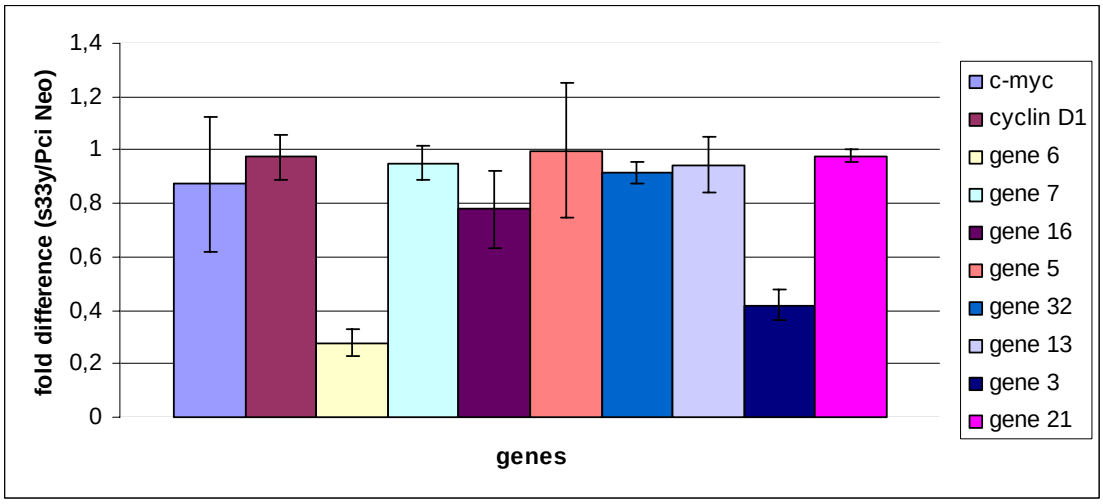


Figure 5.11. mRNA levels as quantified with image analysis software and normalized to Actin levels. All RT-PCR reactions were done in triplicate and the fold difference (S33Y/Neo) is given. Given values are the means of three measurements and the standard deviation is set as the y-error bars.

Gene 3 is repressed upon β-catenin overexpression with the S33Y7/Neo ratio of 0.4, meaning a 2.5 fold decrease in the mRNA level. This result is consistent with the SAGE data; the S33Y7/Neo ratio is 0.2 in SAGE results meaning a five fold decrease in the mRNA level.

Gene 6 is also highly repressed upon β-catenin overexpression. mRNA level has decreased 3.6 fold in S33Y transfected cells. Interestingly SAGE data of Gene 6 is opposite of this finding. Gene 6 is 2.5 fold increased in S33Y stable cells according to verified SAGE data. Gene 6 shows a different expression profile in transient and stable transfections of β-catenin. mRNA level of Gene 6 was also analysed in time course upon β-catenin transfection (Figure 5.12).

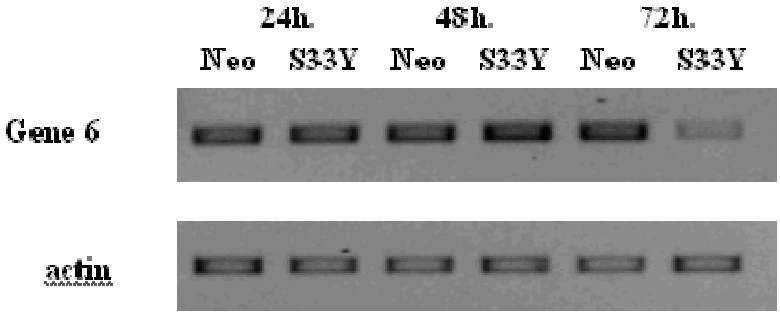


Figure 5.12. Time course analysis of Gene 6 mRNA levels in β-catenin transfected cells (S33Y) and control cells (Neo) RNA levels of Gene 6 does not change after 24 h. or 48 h. of transfection but decreases significantly after 72 h. of transfection. Actin was used as the control gene.

Gene 5 was highly suspected to be a target of β-catenin based on experiments done in our laboratory by Ayaz Najafov. Chromatin Immunoprecipitation (Chip) showed that β-catenin/TCF complex binds to the promoter region of Gene 5. In order to be sure that there is not a change in the mRNA level, Q-RT PCR was performed for this gene and results are similar with the RT-PCR results with the fold 1.16 (S33Y/Neo). The affect of β-catenin overexpression could be at earlier times on this gene, so RT-PCR was done in time course but there was no change at the mRNA levels at any given time point (Figure 5.13)

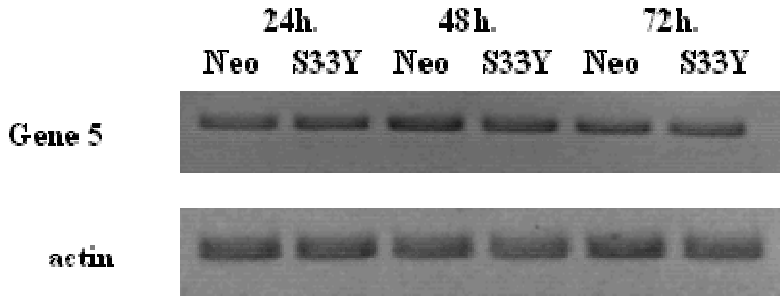


Figure 5.13. Time course analysis of Gene 5 mRNA levels in β-catenin transfected cells (S33Y) and control cells (Neo). No significant change was observed in any time point. Actin was used as the control gene.

5.4. Effect of Gene 6 Overexpression in Proliferation of Huh7 cells

Gene 6 showing a different expression profile in transient and stable transfection of β-catenin with a decrease and increase, respectively was interesting enough for further studies. The effect of overexpression of Gene 5 on proliferation rate of Huh7 cells was investigated with proliferation assay. Cells transfected with Gene6-PCDNA3 and PCDNA3 (as control) were harvested after 48 hours and proliferation assay was started. Results of the 8 day assay are seen in Figure 5.14.

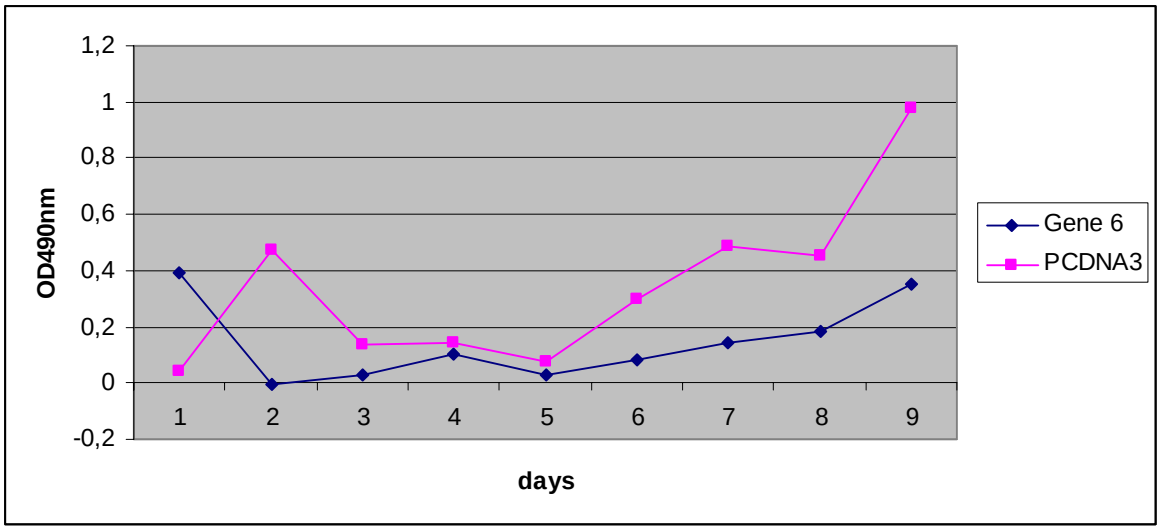


Figure 5.14. Growth curves of Gene 6 transfected and empty vector transfected Huh7 cells

The data of first five days indicate a lag phase for both experimental and control group. After five days cells began to show a linear increase so the slope of the curves after five days was calculated; 0.1963 for control and 0.741 for Gene 6 transfected cells. These results indicate a 2.65 fold decrease in the rate of proliferation calculated from the slopes of OD versus day curves.

This initial finding raised some important questions. First of all this decrease could be due to a toxic affect of the Gene 6 plasmid batch used in the experiment. Second empty vector could lead to an increase in the proliferation of the cells and any other vector could give a lower proliferation rate. In order to rule out these possibilities, experiment was repeated twice at different times with different batches of plasmids and adding other controls; cells transfected with Gene14 (one of the genes that have decreased in the SAGE results; 10:2 tag number, Neo and S33Y, respectively), Huh7 cells and noDNA controls (Figure 5.15)

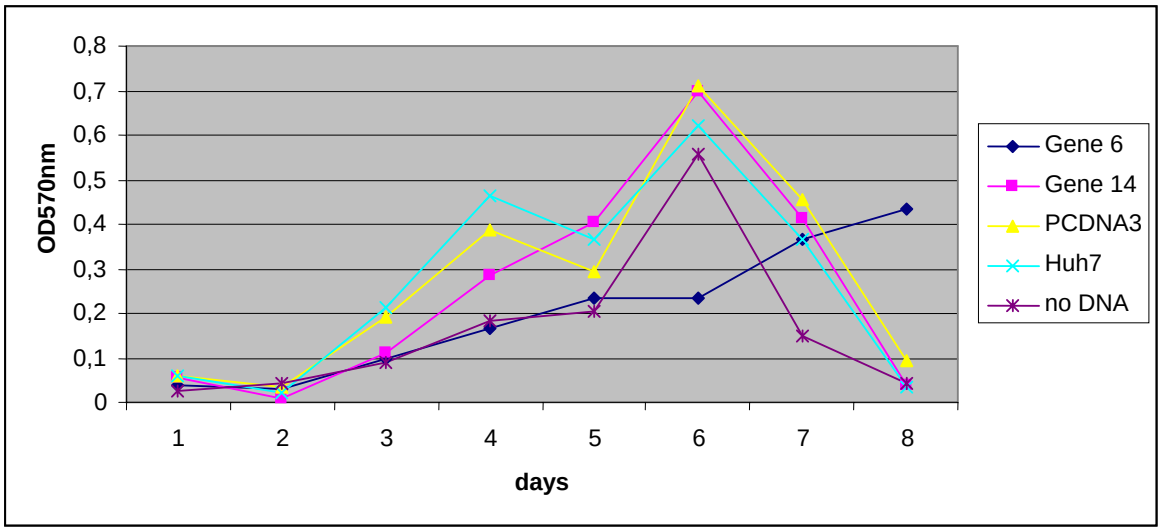


Figure 5.15. Growth curves of Gene6, Gene 14 transfected cells and the appropriate controls

All experimental groups showed the same pattern after day 2 instead of cells overexpressing Gene 6. Based on three different experiments with three different batches of plasmids, slopes of the curves are calculated as described above and the results are given in Figure 5.16.

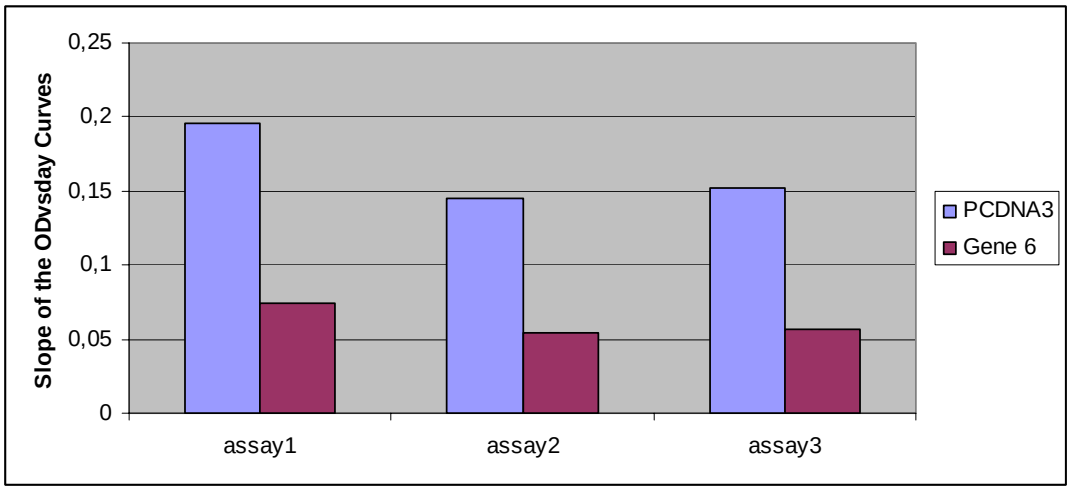


Figure.5.16. Results of three proliferation assays for Gene 6 and PCDNA3 transfected cells

Gene 6 transfected cells always give a smaller slope than the empty vector transfected cells. The mean of the slopes with the standard deviations are given in Figure 5.17.

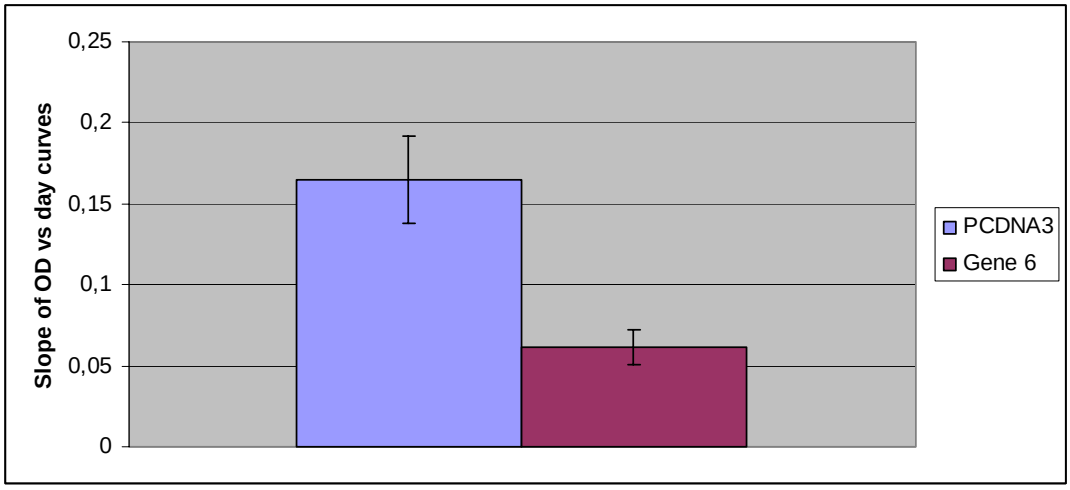


Figure 5.17. Mean of slopes from three proliferation assays

There is a 2.67 fold difference between slopes of Gene 6 and PCDNA3 transfected cells. This decrease can be an indicator of proliferation repression upon Gene 6 overexpression. Raw data of all three proliferation assays are given in Table B.2-4.

For each of the three experiments increase in absorbance as percentage values was also calculated. Increase as percentage in 24 h, 48 h. and 96 h. after absorbance values

began to increase are shown in Table 5.4. These are the same absorbance values used for calculating slope values given above. Seventy two hour values are excluded because control cells show a drop in that time point in each of three experiments. Percentage increase is also low in cells transfected with Gene6 (Figure 5.18).

Table 5.4. Increase in OD values as percentage values

		per cent increase in OD values (%)		
		24h.	**48h.**	**96h.**
assay1	Gene 6	164.5	361.3	1032.3
	PCDNA3	273.4	518.9	1141.7
assay2	Gene 6	241.4	472.4	713.8
	PCDNA3	458.8	1035.3	1988.2
assay3	Gene 6	400	650	1095
	PCDNA3	440.5	1035.1	1927

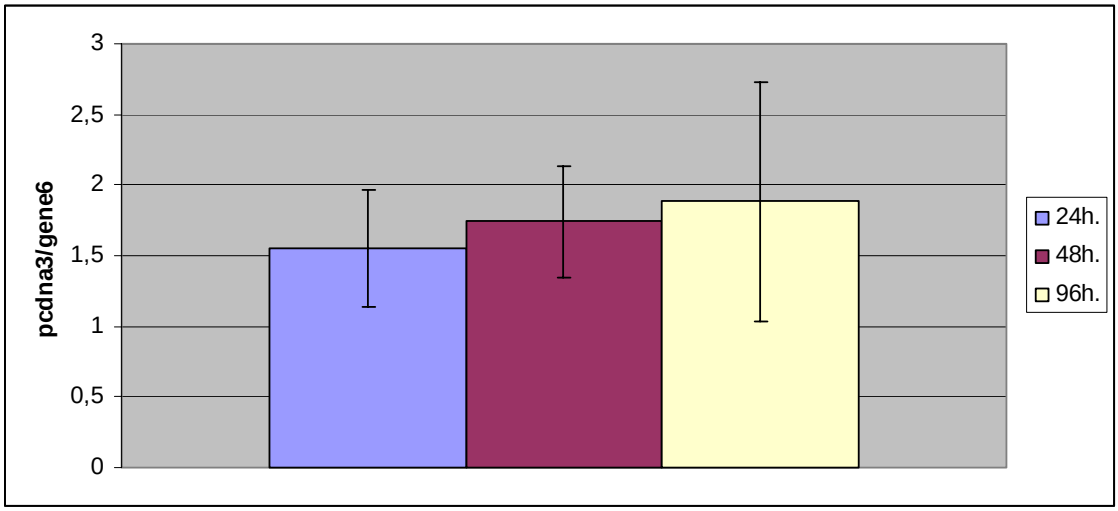


Figure 5.18. Increase as percentage values. Shown are the mean of fold (PCDNA3/Gene6) from three different experiments. Standard deviations are set as Y error bars. At every time point Gene 6 transfected cells show a lower percentage increasecompared to control cells from time point 0.

5. DISCUSSION

The aim of this study was to identify genes that are differentially expressed in high β-catenin/TCF activity cells and investigate their involvement in Wnt/β-catenin pathway. Identifying novel targets of this pathway is very important because the aberrant activation of the pathway is indicated frequently in tumorigenesis.

Highly accepted canonical Wnt/β-catenin targets such as; c-Myc, cyclin D-1 and MMP7 were either not detected or not changed in SAGE results. Two among these targets (c-Myc and cyclinD-1) were also shown not to be up-regulated upon overexpression of β-catenin. Only one Wnt target, Inhibitor of DNA binding 2 (ID2), was upregulated according to SAGE results. The conventional Wnt targets are mainly identified in colorectal cancer studies and the pathway targets in hepatocellular carcinoma (HCC) cell lines may be different. There are consistent (Prange *et al*., 2003) and inconsistent (Lee *et al*., 2006;) findings reported previously regarding the expression of those genes in HCC. It should also be taken into consideration that while much of the observations on the canonical pathways in the literature are based on normal and cancer cell comparisons, our study involves further perturbations of the Wnt pathway in tumor cells.

Recent studies have identified some Wnt target genes in HCCs. Glutamine synthetase (GS), ornithine aminotransferase (OAT) and the glutamate transporter 1 (GLT-1) were identified as targets of Wnt pathway (Cadoret *et al*., 2002). Expression level of OAT and GLT-1 did not change in our experiment whereas there is a 2 fold increase in the expression level of GS. Interleukin-8 (IL-8) was also reported to be a target of Wnt/β-catenin pathway in HCC (Levy *et al*., 2002).

All models explaining transcriptional activity of β-catenin/TCF complex suggest that β-catenin through interaction with co-activators turn TCF into a transcriptional activator (for references see Introduction). Direct transcriptional repression through β-catenin/TCF complex is not reported to date. Our results show that expression levels of some genes have significantly reduced upon overexpression of β-catenin. Transient overexpression of

β-catenin also resulted in down regulation of two genes, one consistent with SAGE data and one inconsistent. This observation might well be a result of indirect regulation of these genes by β-catenin/TCF complex as well as an indication for a novel direct repression role of the complex. In order to test these two possibilities, several experiments could be done. Chromatin immunoprecipitation (Chip), electro mobility shift assay (EMSA) can be applied to observe the direct interaction of β-catenin/TCF complex with the promoter regions of these genes. Reporter gene assays such as luciferase assay can be used to show the response of promoter activity of those genes in response to β-catenin. Gene 3 and Gene 6 which are both down-regulated by overexpressing mutant stable β-catenin in Huh 7 cells and containing TCF binding sites on their promoter regions might be candidate targets of Wnt/β-catenin pathway.

Besides, Gene 6 showed a different expression profile in stable and transient transfection of mutant β-catenin. While the up regulation of Gene6 mRNA in mutant β-catenin expressing stables was shown by verified SAGE results, mRNA level was down regulated after 72 hours of overexpression of β-catenin. This observation brings the possibility that Gene 6 is first down regulated by Wnt/ β-catenin signaling and then up regulated as a defence mechanism of the cells to suppress effects of high β-catenin/TCF activity.

Results of proliferation assays following overexpression of Gene6 support the possibility stated above. Gene 6 overexpression resulted in suppression of proliferation in Huh7 cells. Proliferation assay used in this study was a metabolic assay based on conversion of a tetrazolium salt into a formazan product. Although absorbance was shown to be directly proportional to cell number, it is still an indirect method to estimate cell number. Moreover, it doesn't differentiate between dividing and non-dividing cells. The effect of Gene 6 on proliferation might be due to increased cell death, decreased metabolic activity or a decreased doubling time of cells. Additional experiments, such as apoptosis assays, oxygen consumption assays and BrdU incorporation assays can be done to investigate these possibilities. Gene 6 is a novel gene that hasn't been studied at all and there is no data about the protein it encodes. The full length cDNA clone of this gene was used to transfect cells and increase in the expression level was shown with RT-PCR but data concerning the protein level is missing.

In addition to effect of Gene 6 on proliferation of Huh7 cells, the mechanism which leads to up regulation of this gene is an important question to be answered. Negative feed back loops or cross-talk between pathways might give the answer. Axin, a major component of β-catenin destruction complex was shown to be up regulated in response to high Wnt/ β-catenin activity (Lustig *et al*., 2002). Axin was also shown to be up regulated by the pRb/E2F signaling pathway leading to increased cell death (Hughes and Brady, 2005). Cross talk between nuclear factor κB (NF-κB) and Wnt pathway was also shown. NF-κB signaling pathway plays important roles in immune response, inflammation and apoptosis. NF-κB signalling was shown to suppress the Wnt/β-catenin mediated transcription even in the case of cells expressing mutant stable forms of β-catenin (Masui *et al*., 2002). Another safeguard response of cells to high β-catenin levels is the accumulation of p53 which is a well characterized tumor suppressor (Damalas *et al.*, 1999). Wnt/Ca^{+2} pathway is also known to inhibit the canonical Wnt pathway. Additional analysis of Gene 6 expression levels should be done in mutant β-catenin transfected cells in time course, and in subsequent passages in stables in order to detect timing of the increase in the mRNA level. Parallel analysis of other genes, such as Axin and p53 should be done to be able to explain the mechanism underlying Gene 6 expression regulation.

At Bilkent University, they showed that mutant β-catenin expressing cells formed more aggressive tumors when injected to nude mice. In order to explain this observation, proliferation assay and colony formation assay were done but no significant difference was observed in the proliferation and clonogenic survival of cells. Wnt/β-catenin target genes identified in colorectal carcinoma cells regulate growth control and cell cycling (c-Myc, cyclin D1, c-Jun, fra-1, gastrin, WISP-1, ITF-2), cell survival (Id2, MDR1, COX2), or invasion and tumor dissemination (matrilysin, laminin) (Levy et al., 2002). In case of HCC, there is conflicting data concerning effect of β-catenin on proliferation rate. Stabilized β-catenin was shown to enhance proliferation in HCC (Shang *et al.*, 2004) and in another study it was shown that there was no significant correlation between β-catenin accumulation and proliferation (Prange *et al.*, 2003). In order to explain the oncogenic behaviour of β-catenin, additional assays to detect the anchorage independent growth or contact inhibition resistant growth state of cells can be done.

As the transcriptome analysis method, Serial Analysis of Gene Expression (SAGE) was used in this study. The major advantage of this method is that it gives the opportunity to identify novel genes. That is because SAGE does not require any prior sequence knowledge, unlike microarray system which is based on probes of known sequences. The major disadvantage of SAGE is that it is based on high throughput sequencing. Sequencing increases the cost of the study and also prolongs the time required to get the whole data. In order to get results that are statistically reliable, 40000 tags should be counted from a single SAGE library. That also increases the effort put on the experiment. Construction of a SAGE library up to sequencing is also a challenging procedure. It is a 9 day procedure with an average 10 hour full time work each day. Besides, it contains several optimization steps and check points that increase the time and effort spent. Analysis and interpretation of the huge data of SAGE is also hard to deal with. In our case, a web based free tool designed by Erşen Kavak was used to analyse data. It gives the chance to filter your results based on several different parameters and also you can see the results of other SAGE experiments and search any gene of interest in those results.

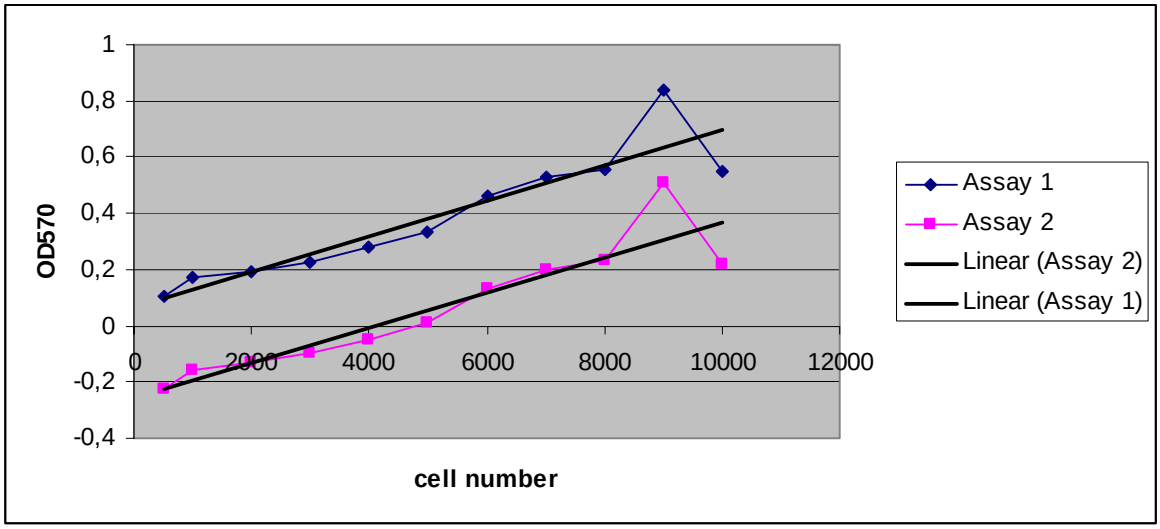
To sum up, results of this study suggests two candidate targets Gene3 and Gene 6, neither of which is studied and implicated in cancer, one of which might play a role in safeguard response of cells to high β-catenin levels regulating cell proliferation.

APPENDIX A: LINEAR RANGE OF PROLIFERATION ASSAY

Table A.1. Linear range of proliferation assay

Cell #	500	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
Assay 1	0.105	0.17	0.194	0.229	0.279	0.337	0.46	0.529	0.559	0.838	0.547
Assay 2	-0.223	-0.158	-0.134	-0.099	-0.049	0.009	0.132	0.201	0.231	0.51	0.219

Figure A.1. Linearity of proliferation assay



APPENDIX B: RAW DATA OF PROLIFERATION ASSAYS

Table B.1. Proliferation assay of Huh7, PCI-Neo and PCI-S33Y stables

	PCI-Neo			PCI-S33Y			Huh7		
Days	**1000 cells**			**1000 cells**			**1000 cells**		
1	-0.171	-0.037	-0.039	0.063	0.048	0.09	-0.007	-0.015	-0.034
2	0.376	-0.009	0.033	0.078	0.094	0.105	0.089	0.065	0.112
3	0.061	0.120	0.094	0.049	0.121	0.306	0.171	0.314	0.189
4	0.008	0.118	0.068	0.114	0.168	0.215	0.21	0.269	0.326
5	-0.076	0.220	0.350	0.137	0.272	0.62	0.339	0.3	0.839
6	0.043	0.355	0.545	-0.195	0.457	0.662	0.079	0.582	1.090
7	0.635	0.766	1.063	0.478	0.799	0.92	0.743	0.952	1.027

Table B.2. Proliferation assay of Gene 6 and empty vector transfected Huh7 cells

Day #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Gene 6	0.392	-0.007	0.031	0.1	0.031	0.082	0.143	0.183	0.351
PCDNA3	0.041	0.473	0.136	0.143	0.079	0.295	0.489	0.454	0.981

Table B.3. Proliferation assay of Gene6, Gene 14 transfected cells and the appropriate controls

Day #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Gene 6	0.039	0.029	0.099	0.166	0.232	0.236	0.367	0.435
Gene 14	0.057	0.008	0.11	0.286	0.404	0.699	0.411	0.037
PCDNA3	0.058	0.034	0.19	0.386	0.294	0.71	0.455	0.095
Huh7	0.058	0.022	0.211	0.465	0.368	0.623	0.368	0.035
No DNA	0.026	0.043	0.089	0.185	0.206	0.558	0.149	0.043

Table B.4. Replicate of proliferation assay of Gene6, Gene 14 transfected cells and the appropriate controls

Day #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Gene 6	0.034	0.02	0.1	0.15	0.237	0.239	0.4	0.345
Gene 14	1	0.012	0.11	0.29	0.35	0.7	0.52	0.025
PCDNA3	0.055	0.037	0.2	0.42	0.312	0.75	0.398	0.075
Huh7	0.06	0.019	0.25	0.5	0.394	0.632	0.35	0.29
No DNA	0.027	0.05	0.0778	0.2	0.21	0.6	0.13	0.04

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