VERIFICATION OF PUTATIVE NOVEL WNT/β-CATENIN PATHWAY TARGETS USING ALTERNATIVE EFFECTORS

by

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Aileme ve Ezgim'e...

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ABSTRACT

VERIFICATION OF PUTATIVE NOVEL WNT/β-CATENIN PATHWAY TARGETS USING ALTERNATIVE EFFECTORS

The Wnt/β-catenin pathway plays important roles in embryogenesis, development and tissue homeostasis in adult organisms. Mutations in almost any member of this pathway have been associated with a disease including Alzheimer's disease, schizophrenia and cancer. Therefore understanding the mechanisms through which Wnt/β-catenin pathway acts and identification of new downstream elements carry a major importance in genetic and cancer research, and also diagnosis and treatment of many diseases.

We have previously conducted SAGE (Serial Analysis of Gene Expression) and genome-wide microarray screens and using the results of these experiments as a starting point we have previously identified *BRI3* (Brain Protein I3) and *HSF2* (Heat Shock Factor 2) as novel Wnt/β-catenin pathway targets and verified our findings using various methods such as inhibition of GSK3β activity by lithium treatment, chromatin immunoprecipitatiton and luciferase activity assays. To strengthen our hypothesis the effect of hyper-active Wnt/β-catenin pathway in Snu449 cells on these genes was examined and also several methods that stimulate the pathway were compared.

We have observed increased *BRI3* and *HSF2* mRNA levels in response to lithium treatment in Snu449 cells, and in response to Wnt agonist treatment in both Snu449 cells and Huh7 cells. These findings have supported out hypothesis that *BRI3* and *HSF2* transcriptions are regulated by the Wnt/β-catenin pathway.

ÖZET

WNT/β-KATENİN YOLAĞININ MEFRUZ YENİ HEDEF GENLERININ ALTERNATİF EFEKTÖRLER KULLANILARAK TEYİDİ

Wnt/β-katenin yolağı embriyogenez, gelişme ve doku homeostasisinde önemli roller oynar. Bu yolağın hemen hemen her üyesinde meydana gelen mutasyonlar Alzheimer, şizofreni ve kanser gibi çeşitli hastalıklarla ilişkilendirilmiştir. Bu yüzden Wnt/β-katenin yolağının çalışma mekanizmasının anlaşılması ve bu yolağın kontrol ettiği yeni genlerin tanımlanması genetik ve kanser araştırmalarında ve ayrıca çeşitli birçok hastalığın tanı ve tedavisinde büyük önem taşımaktadır.

Daha önceden gerçekleştirdiğimiz SAGE (Serial Analysis of Gene Expression: Gen Anlatımının Seri Analizi) ve tüm-genom mikroaray çalışmalarının bulgularından yola çıkarak gerçekleştirdiğimiz lityum tuzlarına maruz burakma, kromatin imünopresipitasyon ve lusiferaz aktivite analizleri sonucunda *BRI3* (Brain Protein I3) ve *HSF2* (Heat Shock Factor 2) genlerini Wnt/β-katenin yolağının olası yeni hedef genleri olarak belirlemiştik. Bu savımızı güçlendirmek için, bu çalışmada hiperaktif Wnt/β-katenin yolağının bu genler üzerindeki etkisi Snu449 hücreleri kullanılarak incelenmiştir. Ayrıca yolağı uyaran farklı metodlar uygulanmış ve sonuçları karşılaştırılmıştır.

Deneylerimiz sonucunda *BRI3* ve *HSF2* mRNA seviyelerinin lityum tuzlarına maruz bırakma sonucunda Snu449 hücrelerinde ve Wnt agoniste maruz bırakma sonucunda da hem Snu449 hem de Huh7 hücrelerinde arttığı görülmüştür. Bu bulgular *BRI3* ve *HSF2* genlerinin Wnt/β-katenin yolağı tarafından regüle edildiği savımızı güçlendirmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

S	Serine
Y	Tyrosine
APC	Adenomatous polyposis coli gene product
APS	Ammonium peroxidisulfate
ARM	Armadillo
BPB	Bromophenol blue
cDNA	Complementary deoxyribonucleic acid
$CaCl_2$	Calcium chloride
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
ChIP	Chromatin immunoprecipitation
CK1γ	Casein kinase 1γ
CKIϵ	Casein kinase Iϵ
CO_2	Carbondioxide
CRD	Cysteine rich domain
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DVL	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
FAP	Familial adenomatous polyposis coli
FBS	Fetal bovine serum
FRP	Frizzled-related protein
GFP	Green flourescent protein
GITC	Guanidium isothiocyanate
GSK3β	Glycogen synthase kinase 3 β

HCC	Human hepatocellular carcinoma
KCl	Potassium chloride
LB	Luria-Bertani
LDL	Low density lipoprotein
LEF	Lymphoid enhancer factor
LiCl	Lithium chloride
LRP	Low density lipoprotein receptor-related protein
mRNA	Messenger ribonucleic acid
MgCl2	Magnesium chloride
MgSO4	Magnesium sulphate
MOPS	Morpholino propane sulfonic acid
NaCl	Sodium chloride
NaOAc	Sodium Acetate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NFκB	Nuclear factor κB
NFAT	Nuclear factor of activated T-cells
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonyl fluoride
QRT-PCR	Quantitative reverse transcriptase mediated PCR
RNA	Ribonucleic acid
Rnase	Ribonuclease
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAGE	Serial Analysis of Gene Expression
SDS	Sodium dodecyl sulphate
TAE	Tris-acetic acid EDTA

TCF	T cell factor
TEMED	N,N,N,N-tetramethylethylenediamine
WNT	Wingless-type mouse mammary tumor virus (MMTV) integration site family member

1. INTRODUCTION

1.1. Wnt/β-catenin Signaling Pathway

Wnts are a large family of evolutionarily conserved [1] secreted glycoproteins that participate in many fundamental mechanisms such as cell proliferation, cell fate determination, cell polarity and motility during embryonic development [2–4] as well as tissue homeostasis in adults [5–8] and tumorigenesis [9]. Because of its important roles in development and tumorigenesis, mutations in the Wnt pathways often lead to cancer and other diseases [10], which makes this pathway an appealing research subject to many scientists.

There are 19 mammalian Wnt homologs [11] which induce at least three different intracellular signaling cascades: Wnt/β-catenin pathway (also called canonical Wnt pathway), planar cell polarity (PCP) pathway and Wnt/Ca^{2+} pathway [5, 12]. Wnt/β-catenin pathway is the most established one among these Wnt pathways. Wnt/β-catenin pathway operates in a finely bordered homeostatic range throughout the metazoan's life. When this pathway, either by hyperactivity or hypoactivity, starts to operate outside of the homeostatic range, the organism experiences various diseases [10, 13, 14].

The β-catenin molecule, encoded by *CTNNB1* gene, lies in the heart of the Wnt/β-catenin pathway. It was originally discovered as a molecule that connects cadherin cell adhesion molecules to the actin cytoskeleton and as a binding partner of E-cadherin and α-catenin [15, 16]. It is a member of the Armadillo (ARM) repeat protein superfamily and has 12 ARM repeats in its center forming a superhelix of helices [17–20] and this structure acts as a rigid scaffold and allows β-catenin to bind to various factors such as cadherins, TCFs, Adenomatous Polyposis Coli (APC) and Axin [15, 21–23].

Compared to other signaling pathways, the canonical Wnt pathway follows a

rather unusual mechanism probably due to it's funciton in cell-to-cell adhesion. Instead of typical cascade of phosphorilation events or synthesis of an intracellular messenger, cells continuously synthesize β-catenin regardless of presence or absence of a Wnt signal. In the absence of Wnt ligands, this constantly replenished pool of β-catenin molecules is diminished by continous degradation of cytosolic β-catenin molecules by a large destruction complex [1, 12]. Axin lies at the core of the destruction complex as a scaffolding protein which uses separate domains to interact with the other members of the destruction complex and β-catenin and brings them to close proximity to initiate sequential phosphorilation of β-catenin at serine 45 by casein kinase 1α (CK1α) [1], threonine 41, serine 37 and serine 33 by glycogen synthase kinase 3 β (GSK3β) [24]. Axin also recruits APC to the destruction complex. Apart from the Axin binding domain, APC has two different β-catenin binding sites, three 15 amino acid (aa) repeats and seven 20 aa repeats [24]. After its phosphorilation, β-catenin is ubiquitinated by βTrcp and degraded by the 26S proteasome [12, 24].

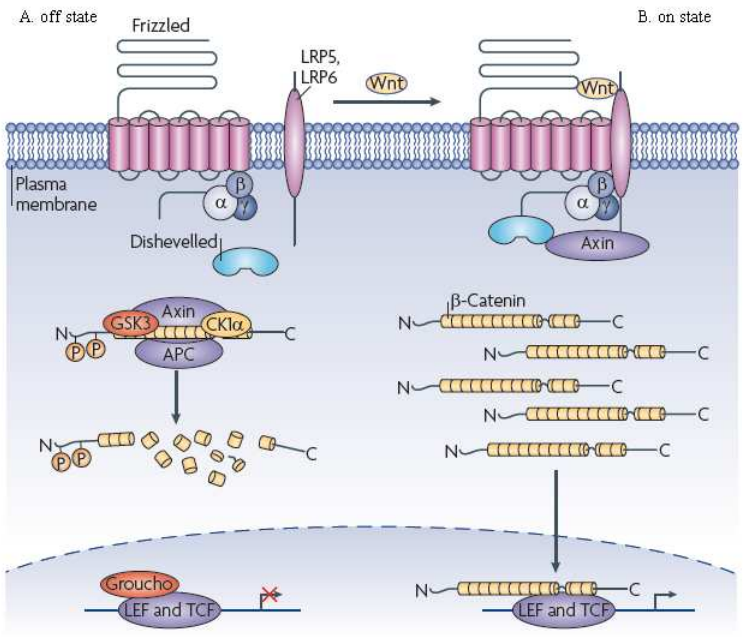

Figure 1.1. The "off" (A) and "on" (B) states of Wnt/β-catenin signaling pathway (Adapted from 14). In its "off" state, cytoplasmic β-catenin is continuously degraded by ubiquitination. In its "on" state, β-catenin accumulates in the cytosol and translocated into the nucleus where it activates TCF/LEF transcription factors.

Wnt signaling is mediated by Frizzled receptors (FZ) and low-density lipoprotein-related protein (LRP) 5/6 [25]. When a Wnt molecule binds to Fz and LRP, dishevelled (DVL) inhibits GSK3β dependent phosphorilation of β-catenin and leads to its accumulaion in the cytoplasm [26–28] and its translocation to nucleus by a not well understood mechanism [29, 30]. In the nucleus it binds to T-cell factor (TCF) and lymphoid enhancer factor (LEF) leading to expression of various downstream genes [31–33].

1.2. Importance of Wnt/β-catenin Pathway in Disease and Cancer

Being such an important pathway in embryonic development and in adult organisms, it is not surprising that mutations in members of the Wnt/β-catenin pathway cause a wide assortment of diseases in humans. Since the discovery of Wnts in 1982 [34] and its first link with cancer revealed in 1990 [35], studies of this pathway in humans as well as other animal models has shed light onto sources and progression of many maladies (Table 1.1). Mutations or changes of expression levels of almost any Wnt genes as well as their receptors or intracellular elements of the pathway have been associated with various pathologies including cancer. Therefore, understanding the mechanisms through which the Wnt/β-catenin pathway acts and identification of new downstream elements carry a major importance in genetic and cancer research and their diagnosis and treatment.

We have focused on identification of novel Wnt/β-catenin pathway targets by analysis of Huh7 cells stably over-expressing degradation-resistant β-catenin using SAGE (Serial Analysis of Gene Expression) and genome-wide microarray screens in our laboratory and have identified Brain Protein I3 (*BRI3*) and Heatshock Factor 2 (*HSF2*) genes as potential candidates.

1.3. Brain Protein I3 (*BRI3*)

BRI3 is a member of the brain integral membrane proteins containing at least three members (*BRI1, BRI2* and *BRI3*) [69] with largely unknown functions. North-

Table 1.1. Wnt signaling components and human genetic diseases caused by their mutations, activity or expression changes (adapted and expanded from 13)

Gene	**Condition/disease**	**Reference**
WNT1	Schizophrenia	36
WNT3	Tetra-amelia	37
WNT4	Intersex	38
	Mullerian-duct regression and virilization	39
	Kidney damage	40, 41
	Polycystic kidney disease	42
	SERKAL syndrome	43
WNT5a	Leukaemia	44
	Metastasis	45
WNT5b	Type II diabetes	46
WNT7a	Fuhrmann syndrome	47
WNT10a	Odonto-onycho-dermal dysplasia	48
WNT10b	Obesity, Split-Hand/Foot Malformation	49, 50
sFRP3	Osteoarthritis	51
FZ4	Familial exudative vitreoretinopathy	52, 53
LRP5	FEVR, low bone mass	54, 55
	High bone mass	56, 57
	Osteoperosis-pseudoglioma Syndrome	56, 57, 58
LRP6	Late onset Alzheimer	59
DSH/DVL	Lung cancer	60
APC	Cancer	61
	Polyposis coli	62, 63
AXIN	Cancer	64
	Caudal duplication	65
AXIN2	Cancer, tooth agenesis	66
β-catenin	Cancer	64
	Aggressive fibromatosis	67
	Pulmonary fibrosis	68

ern analysis of *BRI3*'s expression showed that its expression patterns contrast with the other two members of the family: *BRI3* is expressed at high levels in the brain and at very low levels in other organs whereas *BRI1* is mainly expressed in osteoand chondrogenic tissues and skin at low levels in brain and *BRI2* is expressed in most tissues with high levels in heart, placenta, kidney and pancreas [69]. BRI3 protein has previously been shown to interact with upbeta amyloid converting enzyme (BACE) [70] but a recent study showed that BRI3 only interacts with immature BACE and not mature BACE protein [71]. In the same study it was also shown that BRI3, like BRI2, binds to amyloid precursor protein (APP) but with lower affinity. This interaction with APP inhibits production of β amyloid (Aβ) peptides, the main composition of senile plaques seen in Alzheimer disease [71].

It has been reported that in L929 cells treated with tumor necrosis factor (TNF), the *BRI3* mRNA levels have increased significantly and over-expression of *BRI3* in the same cell line induced apoptosis instead of protecting the cell from TNF induced cell death [72]. Another study has shown reduced *BRI3* expression increases resistance to TNF-induced cell death more than 1000 fold [73]. The results from both studies indicate that BRI3 is a negative checkpoint for TNF-induced cell death. Wu *et al.* also shown that over-expression of sense or anti-sense *BRI3* mRNA is toxic to L929 cells, indicating that endogenous levels of this gene is important for cell survival [73].

1.4. Heatshock Factor 2 (*HSF2*)

HSF2 is a DNA binding transcription factor that belongs to the heat shock factor (HSF) family. In yeast, nematode and fruitfly there is only a single Hsf protein whereas in vertebrates there are four members: HSF1 and HSF2 are common to all vertebrates, HSF3 is only expressed in birds and HSF4 is only seen in mammals [74]. In mammals, even though they are the members of the same family, HSF1, HSF2 and HSF4 has different roles and characteristics. HSF1, for example, is activated in response to stressful conditions such as high temperatures, heavy metals, exposure to oxidants and infections by viruses or bacteria [75, 76] and plays a major role in heat shock responses, whereas HSF2 is known to be involved in spermatogenesis, corticogenesis

and brain development [77, 78] and HSF4 has not yet been shown to take role in stress related functions but various studies have proved its part in lens formation and olfactory epithelium maintenance [79–81].

1.5. Artificial Activation of Wnt/β-catenin Pathway

There are several methods to artificially activate the Wnt/β-catenin pathway. The most direct and comparably natural way is to add Wnt protein in purified form or as conditioned medium. The former method is not feasible because Wnt proteins have poor solubility due to their fatty acyl group which makes them difficult to purify [82]. For the latter method, we have successfully stimulated Huh7 cells by Wnt3a in an autocrine fashion through transfection [83] and there are commercially available L cells that secrete active Wnt proteins. However, it should be noted that these cells will also be exposed to Wnts and activation of the pathway may lead to other secreted factors such as FGFs. Unfortunately, using control cells that do not secrete Wnt proteins as negative control will not suffice to erase the question mark on the observed effects.

Transfection of cells with degradation resistant β-catenin is one of the most commonly used techniques to artificially activate the Wnt/β-catenin pathway. For this purpose, either an amino-terminal truncated form of β-catenin or a complete sequence with silent point mutations on GSK3β phosphorilation sites can be used [84]. In this case degradation resistant β-catenin will accumulate in the cytoplasm and will be translocated to the nucleus to induce transcription of target genes. However this method is not universally applicable since some cell lines cannot be transfected with acceptable efficiencies. Using RNAi to block negative regulators of Wnt signaling is limited due to the same condition.

Using LiCl to inhibit GSK3β activity is another method to activate the Wnt/β-catenin pathway. LiCl is a known inhibitor of GSK3β and has been used to successfully activate the Wnt/β-catenin pathway [85, 86]. However, lithium ions have been used as mood stabilizers and as primary treatment of bipolar disorder [87] showing that lithium ions have other effects on cells which decreases the value of the method.

2. PURPOSE

The aim of this study was to strengthen our hypothesis that *BRI3* (Brain protein I3) and *HSF2* (Heat-shock factor 2) are transcriptional targets of the Wnt/β-catenin pathway by applying different techniques to stimulate the the Wnt/β-catenin pathway in Snu449 and Huh7 hepatocellular carcinoma cell lines. Various techniques were used to activate the Wnt/β-catenin pathway and change in expression levels have been analyzed as well as the efficiencies of these techniques.

We have previously identified *BRI3* and *HSF2* as putative novel targets of Wnt/β-catenin pathway as a result of SAGE and genome-wide microarray screens and strengthened our hypothesis with various techniques including over-activating the Wnt/β-catenin pathway with lithium treatment or transfection of cells with degradation resistant β-catenin and measure candidate expression changes with RT-PCR and QRT-PCR, analysis of promoter activities of these genes in response to β-catenin overexpression and Wnt3a conditioned medium with luciferase activity reporter assay, and chromatin immunoprecipitation to show physical interaction between β-catenin and candidate gene promoters.

3. MATERIALS

3.1. Chemicals

All laboratory chemicals were analytical grade from Sigma (St. Louis, MO, USA), Merck (Schucdarf, Germany) or Apllichem (Darmstadt, Germany) unless stated otherwise in the text. Tissue culture media and solutions were purchased from Invitrogen (San Diego, CA USA) and Biochrom AG (Berlin, Germany). In vitro transfection reagent, Turbofect was purchased from Fermentas (Burlington, Canada). Wnt agonist (catologue no. 681665) and GSK-3β Inhibitor XII, TWS119 (catologue no. 361554) were purchased from Calbiochem (Darmstat, Germany).

3.2. Buffers and Solutions

3.2.1. Electrophoresis and Western Blotting Buffers and Solutions

50X Tris-acetic acid EDTA (TAE)	242 g Tris Base 100 mL of 0.5 M pH 8.0 EDTA 57.1 ml glacial acetic acid Distilled water up to 1 L pH 7.8
Ethidium bromide (EtBr)	1 mg/ml (stock solution) 0.5 μg/ml (working solution)
DNA Loading buffer	6X loading buffer purchased from Fermentas (Burlington, Canada)

12.5 per cent SDS-PAGE gel (running gel)	12.5 per cent acrylamide : bisacrylamide (39:1) 375 mM Tris-HCl (pH 8.8) 0.1 per cent SDS 0.1 per cent Ammonium persulfate (APS) 0.1 per cent TEMED
3.5 per cent SDS-PAGE gel (stacking gel)	3.5 per cent acrylamide : bisacrylamide (39:1) 125 mM Tris-HCl (pH 6.8) 0.1 per cent SDS 0.1 per cent APS 0.1 per cent TEMED
6X SDS-PAGE sample buffer	80 mM Tris-HCl (pH 6.8) 30 per cent glycerol 10 per cent SDS 0.6 M DTT 0.012 per cent bromophenol blue
5X SDS electrophoresis buffer	0.125 M Tris base 0.96 M glycine 0.5 per cent SDS
Western blot lysis buffer	150 mM NaCl 5 mM EDTA 1 per cent Triton X 100 10 mM Tris-HCl pH 7.4 5 mM DTT 0.1 mM PMSF

Transfer buffer	25 mM Tris 200 mM Glycine 10 per cent methanol
Ponceau S staining solution	0.5 per cent Ponceau S (w:v) 1 per cent acetic acid
Tris buffered saline with Tween 20 (TBST)	150 mM NaCl 20 mM Tris-HCL (pH 8.0) 0.1 per cent Tween 20 0.01 per cent (w:v) sodium azide
Blocking solution	5 per cent non-fat milk in TBST

3.2.2. Bacterial Culture Buffers and Solutions

Luria-Beertani medium (LB)	10 g tryptophan 5 g yeast extract 10 g NaCl Distilled water up to 1 L, autoclaved
Luria-Bertani agar	10 g tryptophan 5 g yeast extract 10 g NaCl 15 g agar Distilled water up to 1 L, autoclaved
Ampicillin stock	100 mg/ml in 50 per cent ethanol Sterilized by filtration and stored at -20^oC 100 μg/ml working concentration

Chloramphenicol stock	30 mg/ml in absolute ethanol sterilized by filtration and stored at -20^oC 30 μg/ml working concentration
SOC	20 g tryptone 5 g yeast extract 2 ml of 5M NaCl 2.5 ml of 1M KCl 10 ml of 1M $MgCl_2$ 10 ml of $MgSO_4$ 20 ml of 1 M glucose Distilled water up to 1 L Sterilized by filtration and stored at -20^oC

3.2.3. Immunofluorescence Buffers and Solutions

10X PBS	81.8 g NaCl 2 g KCl 14.2 g Na_2HPO_4 2.45 g KH_2PO_4 Up to 1 L with distilled water
1X PBST	1X PBS 0.5 per cent Triton X-100
20 per cent paraformaldehyde stock	10 g paraformaldehyde 50 μl of 10 M NaOH up to 50 ml with distilled water Heat to 65^{o}C to dissolve Store at -20^{o}C

4 per cent paraformaldehyde in PBS	10 ml 20 per cent paraformaldehyde 5 ml 10X PBS 35 ml distilled water
Blocking solution	3 per cent BSA in PBS
Cell permeabilization solution	1X PBS 0.5 per cent Triton X-100

3.3. Enzymes

Restriction enzymes, Taq DNA Polymerase and Pfu DNA Polymerase were purchased from Fermentas (Burlington, Canada) together with their respective reaction buffers. T4 DNA ligase was purchased from Promega (Madison, WI, USA). Trypsin (0.025 per cent, ready to use) was purchased from Invitrogen (San Diego, CA, USA).

3.4. Antibodies

Mouse anti-β-catenin polyclonal antibody was purchased from BD Biosciences (catalog no: 610154), rabbit pan-actin polyclonal antibody was from Cell Signaling (catalog no: 4968), sheep anti-mouse/HRP polyclonal anyibody and donkey anti-rabbit/HRP polyclonal antibodies were purchased from Amersham (catalog no's: NA931 and NA934, respectively) and goat anti-mouse/AlexaFluor®488 polyclonal antibody was from Invitrogen (catalog no: A-11001).

3.5. Western Blotting Reagents

Hybond-P nitrocellulose membrane and RCL Plus Western Blotting Detection Reagents were purchased from Amersham Biosciences (Upsalla, Sweden). Kaleidoscope pre-stained molecular weight marker was purchased from Biorad (Hercules, CA, USA).

3.6. 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 Õztũrk, Bilkent University. *BRI3* and *HSF2* gene clones in pCMV-SPORT6 were purchased from RZPD gmbH (Berlin, Germany). Full length clones of *BRI3* and *HSF2* and mutated β-catenin (S33Y) were subcloned into pcDNA3 vector (Invitrogen; San Diego, CA USA) in our laboratory. Primers were purchased from Harvard University Mgh Sequencing Core (Boston, USA).

3.7. Bacterial strains and Mammalian Cell Lines

E. Coli bacterial strain TOP10 was used for cloning purposes.

Human hepatocellular carcinoma cell lines Huh7 and Snu449, both of which were kindly provided by Prof. Mehmet Öztürk (Bilkent University), were used in this study.

3.8. Kits

QIAprep Spin Miniprep kit, Endofree Plasmid Maxi Kit, QIAquick Gel Extraction kit and RNeasy Mini Kit were purchased from Qiagen (Hilden, Germany). Dual-glo Luciferase assay kit and ImPromII Reverse Transcription System was purchased from Promega (Madison, WI, USA). LightCycler FastStart DNA Master SYBR Green I kit was 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, Habas, 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^{o}C, Arçelik, TURKEY
	-70^{o}C Freezer, Harris, UK
	-86^{o}C ULT Freezer, ThermoForma, USA
Documentation Systems	Gel Doc XR System, Bio-Doc, ITALY
	Stella Imaging Station, Raytest, GERMANY
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
	Invervet Microscope, Observer.Z1, Zeiss,
	GERMANY

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, 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/) AxioVision, Zeiss, GERMANY
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 *E. coli* and Transformation

Five ml of LB medium with 25 μg/ml streptomycin was inoculated with 100 μl *E. Coli* strain TOP10 glycerol stock and grown overnight at 37^{o}C with shaking at 200 rpm. The next day, 25 ml LB medium was inoculated with 250 μl of the overnight culture and grown until OD 595 reached between 0.4 and 0.6. Cells were centrifuged for 10 minutes at 3000 g at 4^{o}C. Obtained pellet was resuspended in 12.5 ml of ice-cold sterile 50 mM $CaCl_2$ and incubated on ice for 30 minutes. Cells were centrifuged again using the same conditions and pellet was resuspended in 2.5 ml ice-cold sterile 50 mM $CaCl_2$. 50 μl of this preparation was used for transformations. For long term storage, glycerol was added to 10 per cent final concentration and the cells were snap-frozen in liquid nitrogen and stored at -80^{o}C.

For transformation 100 μl of the competent cells were thawed on ice for 15 min and 1 μl of plasmid was added and gently stirred with the help of the micropipette tip. Cells were then incubated on ice for 20 minutes, then placed in 42^{o}C heat-block for 1 minutes and then immediately on ice for 2 minutes. 500 μl of room temperature SOC medium was added and cells were incubated for 1 hour at 37^{o}C with shaking at 200 rpm. 100 μl of the cell suspension was spread on selective plates containing appropriate antibiotic and grown overnight at 37^{o}C.

4.2. Plasmid Purification

All plasmid purifications were done using Qiagen's QIAprep Spin Miniprep Kit, Plasmid Midi Kit and Endofree Plasmid Maxi Kit following manufacturer's protocol. Concentrations and qualities of plasmids were checked by spectrophotometric measurements and by agarose gel electrophoresis. OD 260/280 ratio was between 1.8 and 2.0. Only endotoxin-free preparations of plasmids were used for transfections.

4.3. Cell Culture Techniques

4.3.1. Cell Growth and Handling

Huh7 cell line was grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 per cent FBS, 1 per cent penicillin/streptomycin and 1 per cent non essential amino acids. Snu449 cell line was grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10 per cent FBS, 1 per cent penicillin/streptomycin. All cells were incubated in five per cent CO_2 incubator at 37^oC. Media were kept at 4^oC and warmed to 37^oC in a sterile waterbath before use. Cells were routinely passaged before reaching 90 per cent confluence. For passaging, the medium was aspirated and cells were washed with calcium and magnesium-free PBS. After washing cells were treated with trypsin-EDTA solution (0.025 per cent trypsin, 0.5 mM EDTA) and incubated in 37^oC (Huh7 cells for five minutes and Snu449 cells for 8 to 10 minutes). Five volumes of fresh media were added to inactivate trypsin and the suspension was pipetted with a 10 ml serological pipette to disperse the cells. The cells were transfered to fresh Petri dishes in 1:10 ratio for standard passaging.

4.3.2. Freezing and Thawing

Cells were frozen in their respective complete growth media containing 10 per cent DMSO at about one million cells per mililiter density. Tubes were put in -20^oC freezer for two hours and than transferred to -80^o freezer. For long term storage cells were transferred to liquid nitrogen tank the next day.

4.3.3. Transfection

Transfections were performed using Turbofect transfection reagent (Fermentas; Burlington, Canada) following the manufacturer's procedure and suggested ratios unless stated otherwise in the text.

4.3.4. Wnt Agonist and TWS119 Treatment

For determination of effective working concentrations of Wnt Agonist and TWS119, they were serially diluted from 5 mM stock solutions to 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 15 or 20 μM concentration and to final volume of 10 μl using DMSO. These dilutions were then added to appropriate growth media in sterile tubes (volume of the media varied according to the experiment), vortexed and spun down and added to the cells. Cells were treated with these different concentrations of Wnt Agonist or TWS119. DMSO was used as negative control. If conditioned media were to be added at different time points, they were transferred to cell culture dishes and placed in incubator to eliminate any differences in temperature and concentration changes due to evaporation. For other experiments 0.7 μM Wnt Agonist and 5 μM TWS119 were used unless stated otherwise.

4.3.5. Lithium Treatment

Lithium treatment was done as previously described [86]. Briefly, cells were seeded into 12 or 6 well plates and at indicated time points, but in reverse order, lithium chloride or sodium chloride were added to a final concentration of 25 mM. Cells in all wells were harvested at t=0 time point to eliminate the effect of confluency. For RNA isolation cells were harvested in 350 μl of RLT buffer (provided with RNeasy®Mini Kit, Qiagen) and for western blotting cells were lysed in 100 μl (12 well) or 200 μl (6 well) western blot lysis buffer.

4.4. Luciferase Assay

In a 12-well plate, cells were transfected with 0.5 μg of pGL3-OT plasmid (contains 3 wild-type TCF 4 binding sites) and 50 ng of pRL-TK plasmid (internal control, Renilla Luciferase) using Turbofect reagent (Fermentas) following manufacturer's instructions. Four hours post-transfection cells were treated with varying concentrations of Wnt Agonist, TWS119 or DMSO. Cells were harvested 48 hours pots-transfection using 200 μl 1X PLB (Passive Lysis Buffer, provided with the kit). Lysates were vor-

texed and sput at 16K g for 5 minutes at 4^{o}C to pellet the cell debris. 100 μl of the cleared lysates were transfered to 96-well flat bottomed opaque plates and mixed with 100 μl of Firefly luciferase substrate reagent. Plates were placed into Fluoroskan Ascent FL (Thermo Electron) and incubated for 10 minutes inside the chamber at room temperature. Luminescence reads were taken for 1 second. Next, 100 μl of Renilla luciferase substrate reagent (Stop&Glo^{TM}) which also quenches Firefly luciferase luminescence. After incubating for 10 minutes inside Fluoroskan Ascent FL's chamber luminescence was measured for 1 second. Firefly luciferase reads were normalized to Renilla luciferase reads and graphs were plotted in Microsoft Excel.

4.5. Immunofluorescence Methods

For immunostaining, cells were grown in 48-well plates up to 50 to 60 per cent confluency, washed twice with PBS, fixed in 4 per cent ice-cold paraformaldehyde in PBS for 20 minutes at room temperature, blocked and permeabilized with blocking solution (2 per cent BSA and 0.2 per cent Tween 20 in PBS) for 30 minutes. After blocking cells were incubated with 1:300 dilution of mouse anti-β-catenin antibody (BD Biosciences) in blocking solution overnight at 4^{o}C with mild shaking. Cells were then washed three times with PBS for 5 minutes each with mild shaking. After washing, the cells were incubated with 1:300 dilution of anti-mouse AlexaFluor®488 antibody in blocking solution for 2 hours at room temperature in the dark. Secondary antibody was washed with PBS three times for 10 minutes each. DAPI was added to the first wash to concentration of 1 μg/ml to stain the nuclei. Cells were covered with PBS and observed under Observer.Z1 (Zeiss) inverted fluorescent microscope.

4.6. RNA Isolation and Reverse Transcription

RNA isolation was performed using Qiagen's RNeasy Mini Spin Kit following manufacturer's protocol. Quality of RNA was checked with spectrophotometry (OD260/280 values were between 2.0 and 2.20) and agarose gel electrophoresis. For

cDNA synthesis Promega's ImpProm-II^{TM}Reverse Transcription System was used according to manufacturer's protocol using 1 μg total RNA as starting material. The final solution was diluted to 100 μl with dH_2O.

4.7. Polymerase Chain Reactions

For reverse transcriptase mediated PCR regular PCR 1 μl of cDNA (1 per cent of total cDNA synthesized from 1 μg total RNA) was used as template. The optimum cycle number for each gene was determined by changing the cycle number in the range of 25 to 31. The steps of the reactions were 94^{o}C denaturation for 30 sec., 55^{o}C annealing for 1 min. and 70^{o}C elongation for 1 min. The reactions were started with a hot-start at 94^{o}C for 5 min. and ended with final elongation step at 70^{o}C.

Realtime PCR was dune with Light Cycler Fast Start DNA Master SYBR Green I kit (Roche; Basel, Switzerland) following manufacturer's protocol. The amplification was done for 45 cycles and each cycle had 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. Reactions always started with an initial denaturation step at 95^{o}C for 10 minutes. Amplification was followed by a melting curve step.

4.8. Agarose Gel Electrophoresis

DNA fragments were resolved on standard 1X TAE agarose gels. The percentage was determined by the length of the DNA fragment to be run and was between 1 per cent and 2 per cent. Agarose powder was melted in 1X TAE buffer by heating in a microwave oven and after cooling the solution to room temperature ethidium bromide was added to a final concentration of 0.5 μg/ml. The gels were run in 1X TAE buffer at constant voltage until the bromophenol dye front migrated to desired distance.

4.9. SDS-PAGE Electrophoresis and Western Blotting

Mini-Protean III cell and Mini-Transblot cell (Biorad) were used for SDS-PAGE gel preparation, running and transfer. For western blotting, 10 per cent separating gels were cast up to 3 cm from the top of the gel cassettes and layered with 0.1 per cent SDS in distilled water and left for polymerization. After 30 minutes SDS layer was poured off and rinsed with dH_2O and 3.5 per cent stacking gel was poured. Combs of appropriate width and size were inserted immediately after casting stacking gel and any trapped air bubbles were removed to obtain perfectly shaped wells. Samples were lysed with SDS lysis buffer, centrifuged for 5 minutes at 4^oC to pellet cell debris and mixed with appropriate volume of 6X SDS-PAGE loading dye. After heating at 95^oC for 10 minutes they were vortexed and flash-centrifuged and loaded into the wells. 5 μl of Kaleidoscope pre-stained marker (BioRad) was used as molecular weight estimate. Two gels were run in the same electrophoresis chamber applying 50 V until the BPB front entered the separating gel and the voltage increased to 100 V. Runs were stopped when BPB has left the gel or was about to reach the end of the gel.

For blotting the SDS-PAGE gels were removed from the cassette and stacker gels were discarded. Remaining separating gels, nitrocellulose membranes, sponges and blotting papers were immersed in transfer buffer for 10 minutes. The transfer cassettes were assembled and put in blotting chamber. Transfers were performed in cold room (4^oC) and with ice-block supplied with the Trans-blot cell and with stirring. Proteins were transferred onto nitrocellulose membranes for 1 hr at 100 V. After the transfer the cassettes were disassembled and the transfer efficiency was checked by the complete transfer of the prestained marker onto the membrane and then with Ponceau S staining and TBST was used for destaining. After the membranes were completely destained, 5 per cent non-fat milk in TBST was used to block the blots for 1 hr at room temperature or over night at 4^oC with shaking at about 100 rpm. Blots were then washed with TBST for 5 minutes and incubated with primary antibodies following manufacturer's dilution recommendations. Primary antibodies were washed three times with TBST for 5 minutes each with shaking at 100 rpm and followed by secondary HRP conjugated antibodies following manufacturer's suggested procedures.

Secondary antibodies were washed three times with TBST for 5 minutes. For detection, ECL Plus Western Blotting Detection Reagents (Amersham) were used to develop the blots and analyzed using Stella Imaging Station (Raytest). Images were quantified using ImageJ software (http://rsb.info.nih.gov/ij).

5. RESULTS

5.1. Transient Transfection of Huh7 Cells With Degradation Resistant β-catenin Leads to Accumulation of the Protein But Does Not Activate the Pathway

We have previously performed Serial Analysis of Gene Expression (SAGE) and genome-wide microarray screens in our laboratory using Huh7 cells stably overexpressing S33Y-β-catenin and have identified several candidate novel target genes. For conformation of these findings we have transiently transfected HCC cell lines with degradation resistant β-catenin mutant (S33Y) and conducted various experiments. Colony formation and proliferation experiments performed by Kader Çavuşoğlu showed no significant differences in colony formation ability and proliferation rate between Huh7 cells transfected with pCI-neo/S33Y construct and pCI-neo empty plasmid [88]. Moreover, RT-PCR experiments' results were in contradiction with SAGE and microarray screens. To examine the situation further, we have transiently transfected Huh7 cells and performed luciferase reporter assay and QRT-PCR. The luciferase assay showed no significant differences between cells transfected with pCI-neo/S33Y construct and pCI-neo plasmid and QRT-PCR analysis also showed more than 40 fold increase in *CTNNB1* mRNA levels but no change in *AXIN2* expression (Figure 5.1). As shown by Ayaz Najafov, transient transfection of Huh7 cells also leads to increase in protein levels of β-catenin [89]. These results suggest that transfection of degradation resistant β-catenin indeed leads to accumulation of the protein product but does not always lead to transcription of target genes.

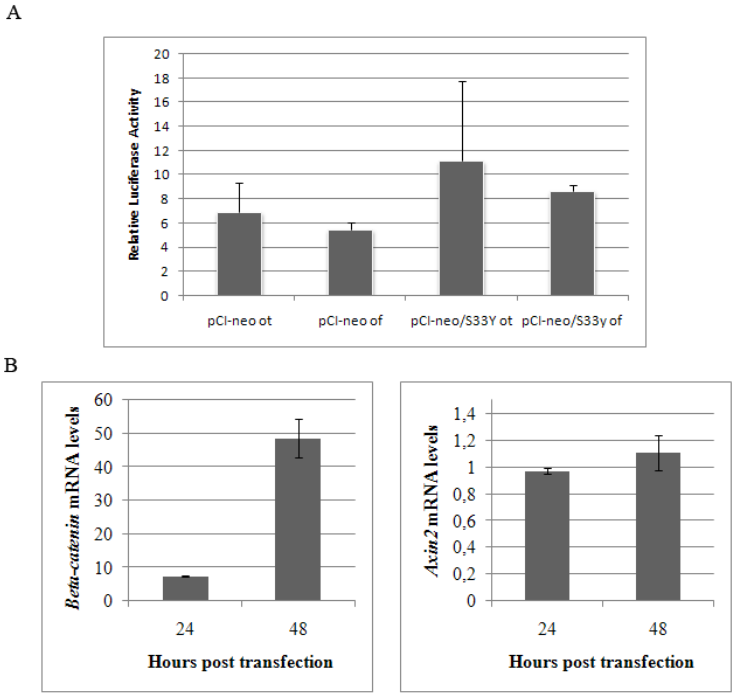

Figure 5.1. (A) Huh7 cells were co-transfected with combinations of degradation resistant β-catenin (pCI-neo/S33Y) or empty plasmids (pCI-neo) and pGL3-OT (ot) or pGL3-OF (of) and luciferase readings were taken (n=3). (B) Transient transfection of the same cell line with pcDNA3/S33Y leads to increase in β-*catenin* mRNA levels (left) but mRNA levels of *AXIN2* remain unchanged (right).

5.2. Lithium Treatment Stimulates the Wnt/β-catenin Pathway in Snu449 Cells But Yields Highly Variable Results

Lithium treatment is another technique widely used to inhibit GSK3 activity and therefore increasing β-catenin levels and activation of transcriptional targets' expression [86, 90] and in our laboratory Ayaz Najafov has shown lithium treatment leads to β-catenin accumulation, translocation into the nuclei and induction of expression the target genes [89]. To support our hypothesis, that *BRI3* and *HSF2* are transcriptional targets of Wnt/β catenin pathway, we treated Snu449 cells with lithium to expand our analysis on different cell lines. Even though we did not experience any difficulties with lithium treatment when using several other cell lines, Snu449 cells proved to yield varying results (Figure 5.2). To investigate the situation we have checked β-catenin

protein levels in the cells and its localization. Western blotting experiments showed accumulation of β-catenin in Snu449 cells at 24 and 48 hours after treatment with 25 mM LiCl compared to cells treated with 25 mM NaCl (Figure 5.3). LiCl treatment also translocated β-catenin into nuclei in Huh7 (Figure 5.4) cells and Snu449 cells (Figure 5.5). These results indicate that lithium treatment successfully stabilizes β-catenin and localize it to the nucleus, however the mRNA levels of transcriptional targets vary greatly between experiments.

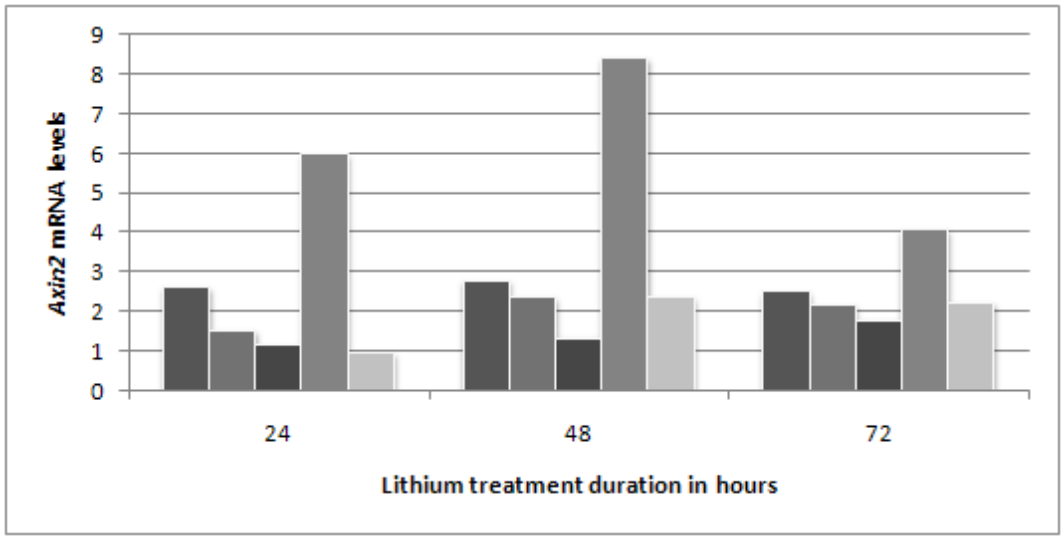

Figure 5.2. Lithium treatment of Snu449 cells does not yield reproducible results. Snu449 cells were treated with 25 mM LiCl for indicated durations and *AXIN2* mRNA levels were measured with QRT-PCR. Each bar represents an independent experiment.

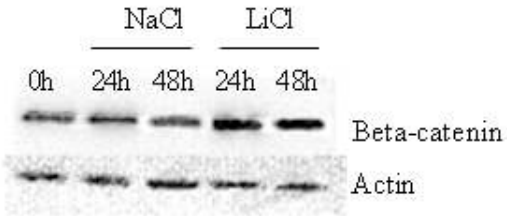

Figure 5.3. Lithium treatment leads to β-catenin protein accumulation in Snu449 cells. Snu449 cells were treated with 25 mM LiCl or 25 mM NaCl for 24 and 48 hours and western blots were performed using whole cell lysates with anti-β-catenin antibody and anti-actin antibody as internal control.

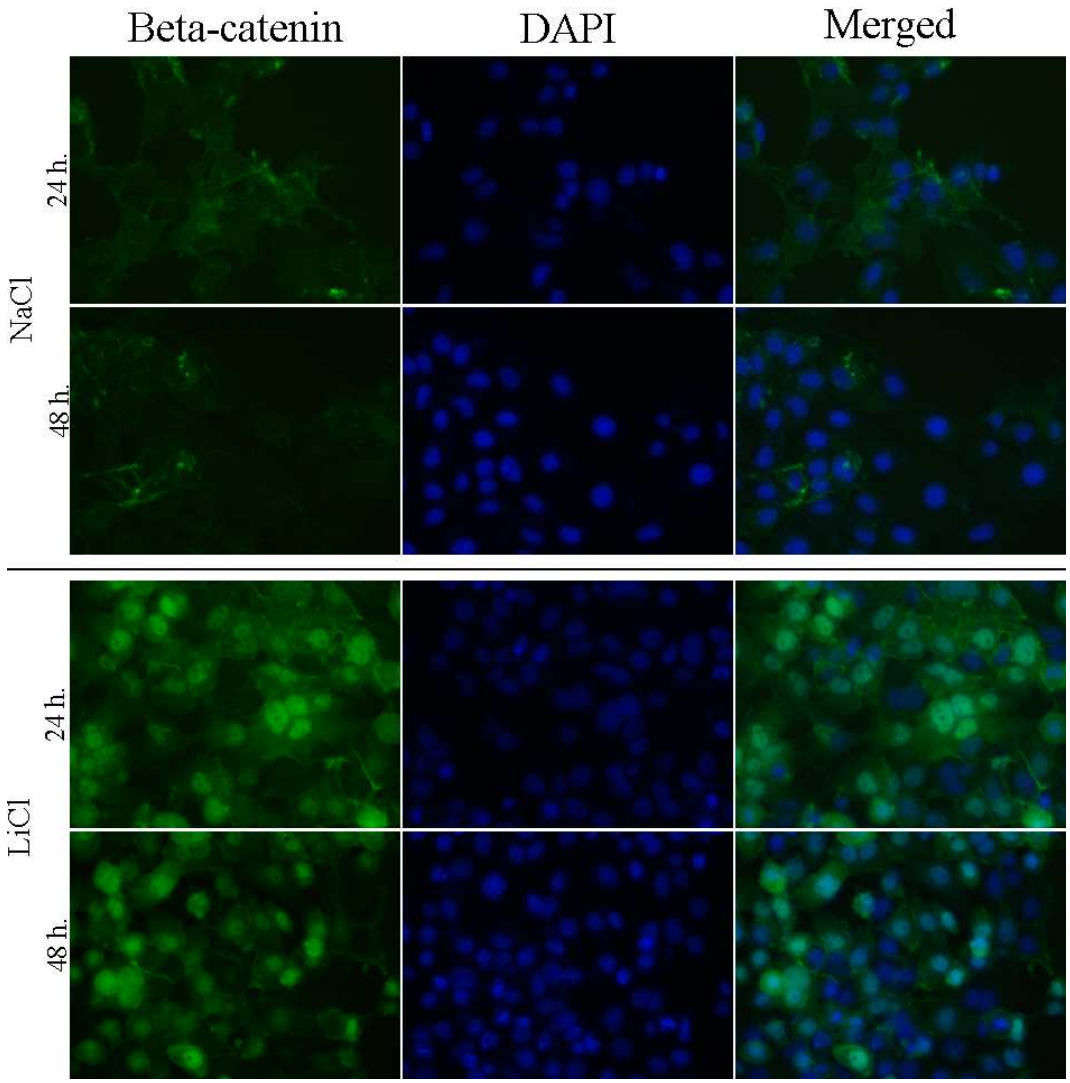

Figure 5.4. Lithium treatment leads to nuclear localization of β-catenin in Huh7 cells. Cells were treated with 25 mM LiCl and NaCl for 24 and 48 hours and localization of β-catenin (green) was determined with immunofluorescence using mouse anti-β-catenin as primary antibody and anti-mouse/AlexaFluor®488 as secondary antibody. Nuclei were stained with DAPI (blue).

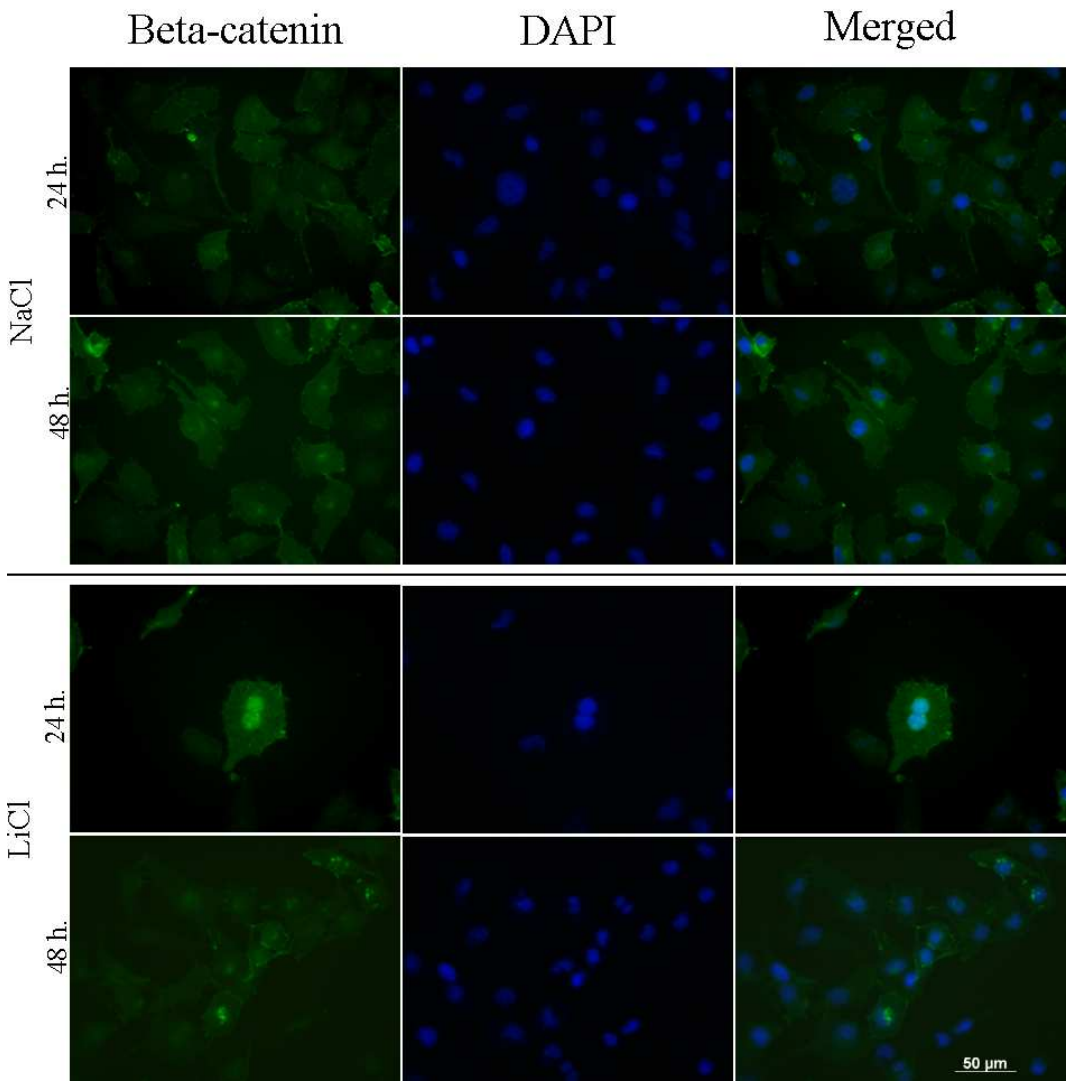

Figure 5.5. Lithium treatment leads to nuclear localization of β-catenin in Snu449 cells. Cells were treated with 25 mM LiCl and NaCl for 24 and 48 hours and localization of β-catenin (green) was determined with immunofluorescence using mouse anti-β-catenin as primary antibody and anti-mouse/AlexaFluor®488 as secondary antibody. Nuclei were stained with DAPI (blue).

5.3. Wnt agonist and TWS119 Treatment of Huh7 and Snu449 Cells

To be able to activate Wnt/β-catenin pathway and obtain consistent results, we also tried commercially available Wnt agonist and GSK3β inhibitor TWS119. For determination of their half maximal effective working concentrations (EC_{50}), Huh7 cells were co-transfected with pGL3-OT and pRL-TK luciferase plasmids and different concentrations of Wnt agonist or TWS119 were added to the media. After 24 hours of treatment luciferase readings were taken (Figure 5.6). EC_{50} for Wnt agonist was calculated to be 0.679212 μM and in accordance with the original paper of the molecule [91] 0.7 uM was used as EC_{50}. TWS119, on the other hand, did not produce the expected curve and therefore it was not possible to calculate EC_{50}. The curve tends not to reach the plateau even at 20 μM but this concentration was very toxic to cells. It has been previously reported that TWS119 was successfully used at 3 μM and 10 μM concentrations on P19 and hepatic stellate cells, respectively [92, 93] but in our hands 10 μM TWS119 proved to be toxic to Huh7 and Snu449 cells, preventing their growth whereas concentration of 5 μM allowed cell growth and did not cause any major morphological changes even after 48 hours of treatment and this concentration was used for experiments, unless stated otherwise.

Wnt agonist showed to be toxic to Huh7 and Snu449 cells at prolonged exposures even at 0.1 μM concentration (Figure 5.7). To check the toxicity of 0.7 μM concentration Huh7 and Snu449 cells were treated with Wnt agonist for 15 min., 30 min., 1 hr., 2 hrs., 4 hrs, 6 hrs., 12 hrs. and 24 hrs. but in reverse order changing the media at t_0-t. This experiment showed that up to 6 hours Snu449 and Huh7 cells can grow without significant differences in terms of cell number and morphology compared to control cells. However longer exposures cause cell death and minor morphological changes in both cell lines (Figures 5.8 and 5.9).

Western blotting, QRT-PCR and immunofluorescence experiments were performed to check the effects of Wnt agonist and TWS119 treatments on Huh7 and Snu449 cells. Immonufluorescence experiments showed that neither Wnt agonist (Figures 5.10 and 5.11) nor TWS119 (Figures 5.12 and 5.13) were able to localize β-catenin

to nuclei. Contrary to these findings western blotting and QRT-PCR experiments showed increased β-catenin protein levels and *AXIN2* levels in Huh7 cells treated with Wnt agonist (Figure 5.14). Snu449 cells, on the other hand, showed a different response to Wnt agonist treatment. Shown both by western blots and QRT-PCR, Snu449 cells gave a quick response to Wnt agonist treatment with β-catenin protein and *AXIN2* mRNA levels peaking at 20 minutes and 15 minutes respectively and subsequently dropping down dramatically (Figure 5.15).

Eventhough TWS119 theoretically activates the Wnt/β-catenin pathway more directly than the Wnt agonist, it did neither in Huh7 cells nor in Snu449 cells. Both western blotting and QRT-PCR experiments showed no increase in β-catenin protein levels or *AXIN2* mRNA levels in treated cells (Figure 5.16).

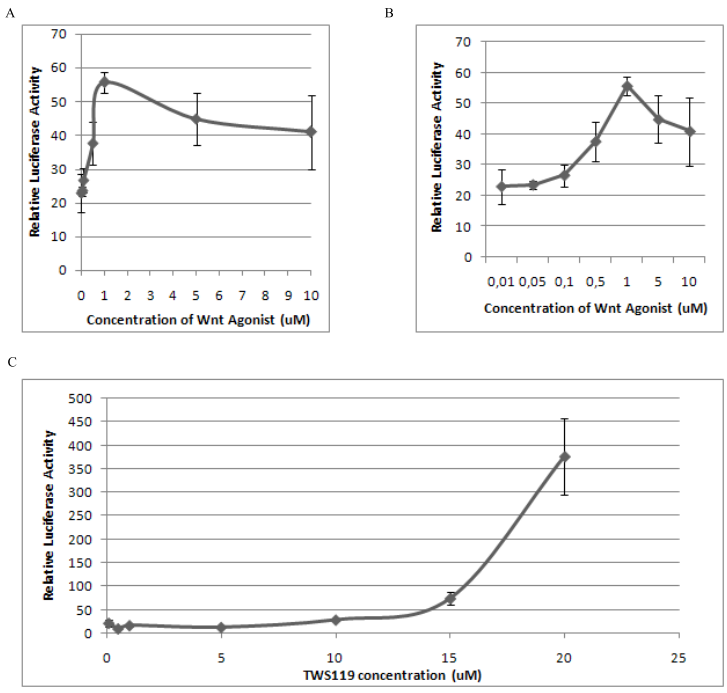

Figure 5.6. Huh7 cells were co-transfected with pGL3-OT and pRL-TK and treated with indicated concentrations of Wnt agonist (A and B) or GSK3β inhibitor TWS119 (C) and (EC_{50}) of each compound were determined using luciferase activity assay (n=3). An "off the scale" (x-axis) graph was also given for Wnt agonist to show the results clearly (B).

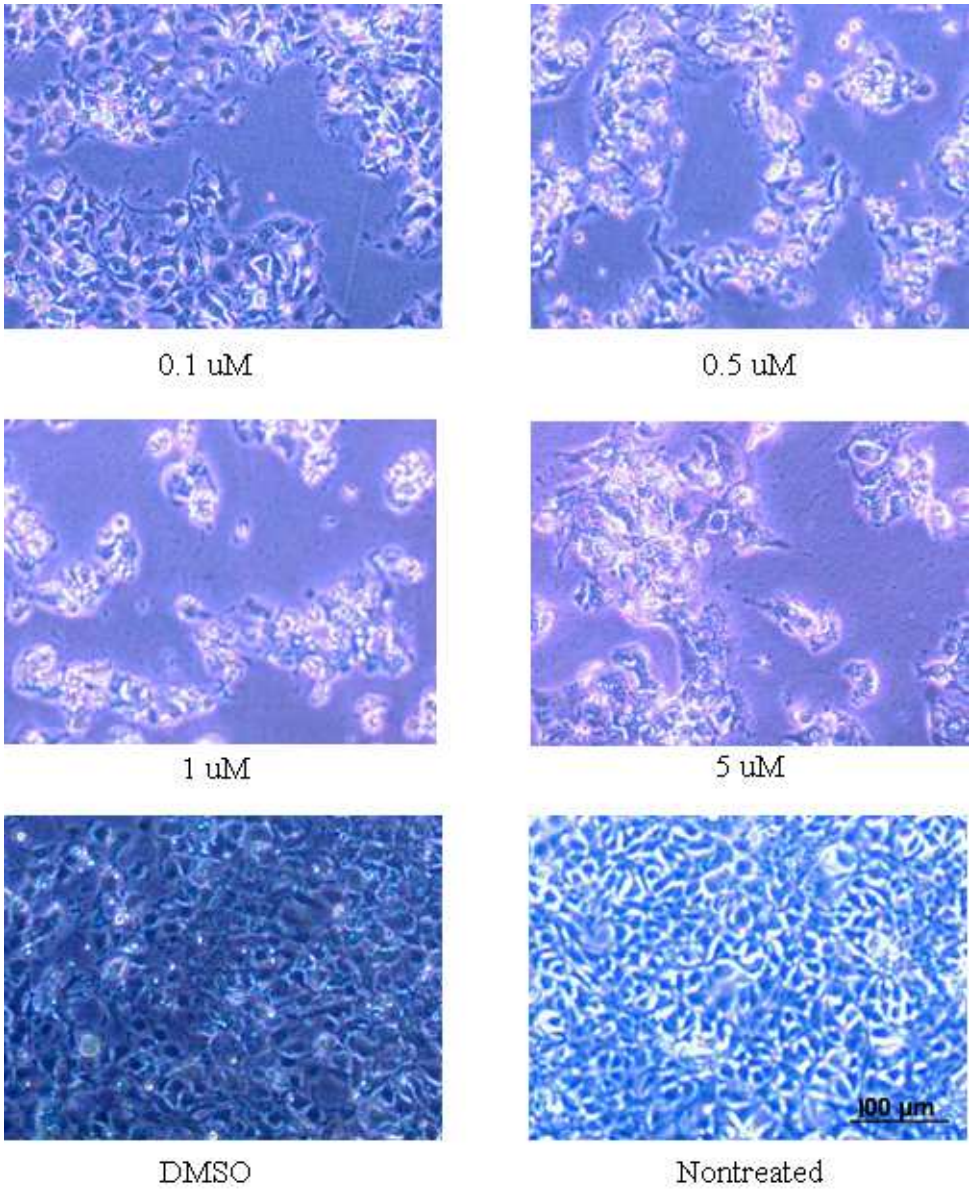

Figure 5.7. Effects of different dosages of Wnt agonist on Huh7 cells after 24 hours of exposure. Huh7 cells were treated with indicated concentrations of Wnt Agonist for 24 hours and photographed by bright field microscopy. DMSO treated and nontreated cells were used as negative controls.

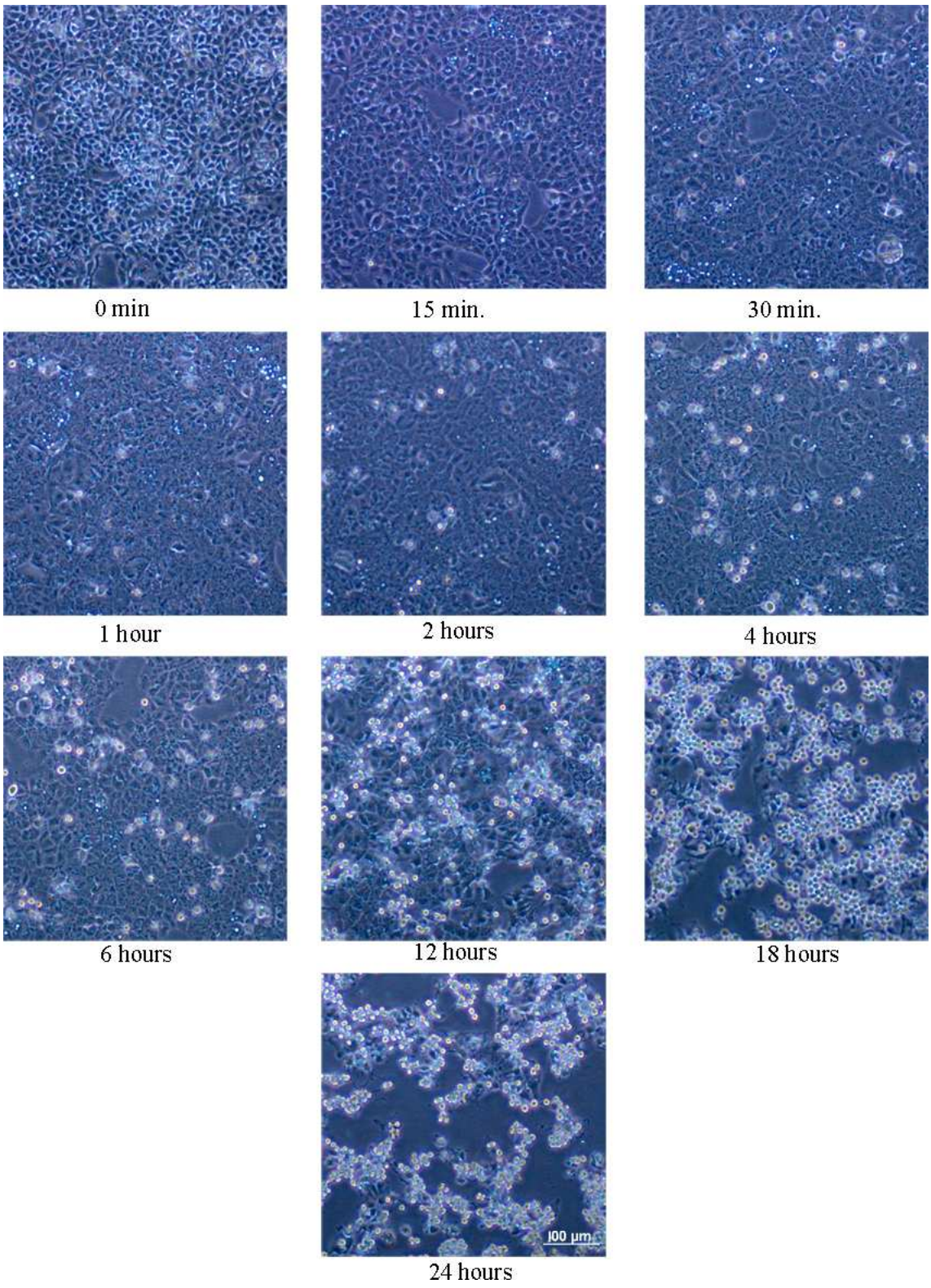

Figure 5.8. Effect of 0.7 μM Wnt agonist on Huh7 cells after indicated durations of exposure. Huh7 cells were treated with 0.7 μM Wnt agonist for 24 hours, but in reverse order, and photographed by bright field microscopy at indicated time points. "0 minute" cells are treated with DMSO for 24 hours.

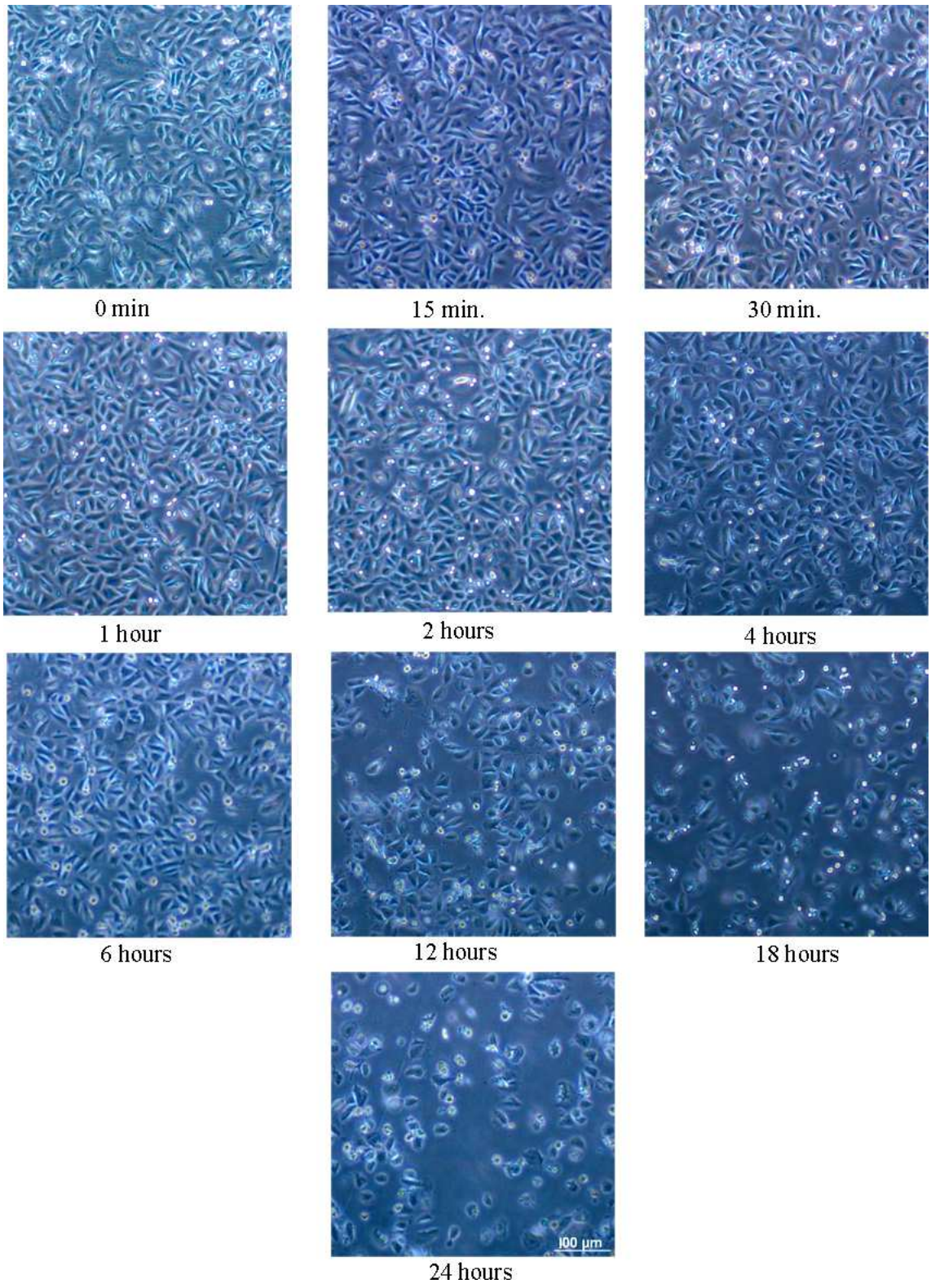

Figure 5.9. Effect of 0.7 μM Wnt agonist on Snu449 cells after indicated durations of exposure. Snu449 cells were treated with 0.7 μM Wnt agonist for 24 hours, but in reverse order, and photographed by bright field microscopy at indicated time points. "0 minute" cells are treated with DMSO for 24 hours.

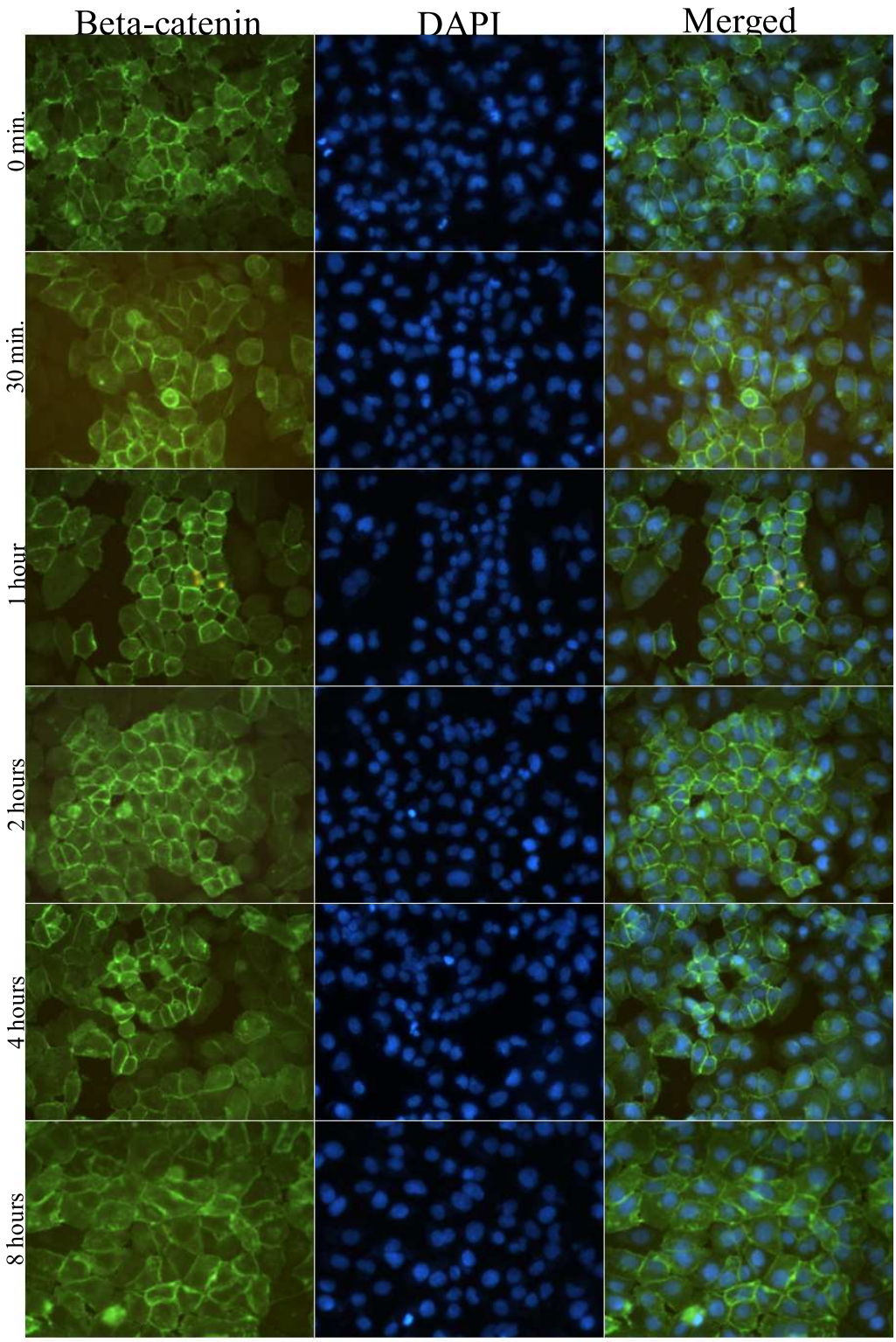

Figure 5.10. Effect of 0.7 μM Wnt agonist on localization of β-catenin in Huh7 cells. Huh7 cells were treated with 0.7 μM Wnt agonist for indicated durations and localization of β-catenin was visualized by immunofluorescence (green). Nuclei were counter-stained with DAPI (blue).

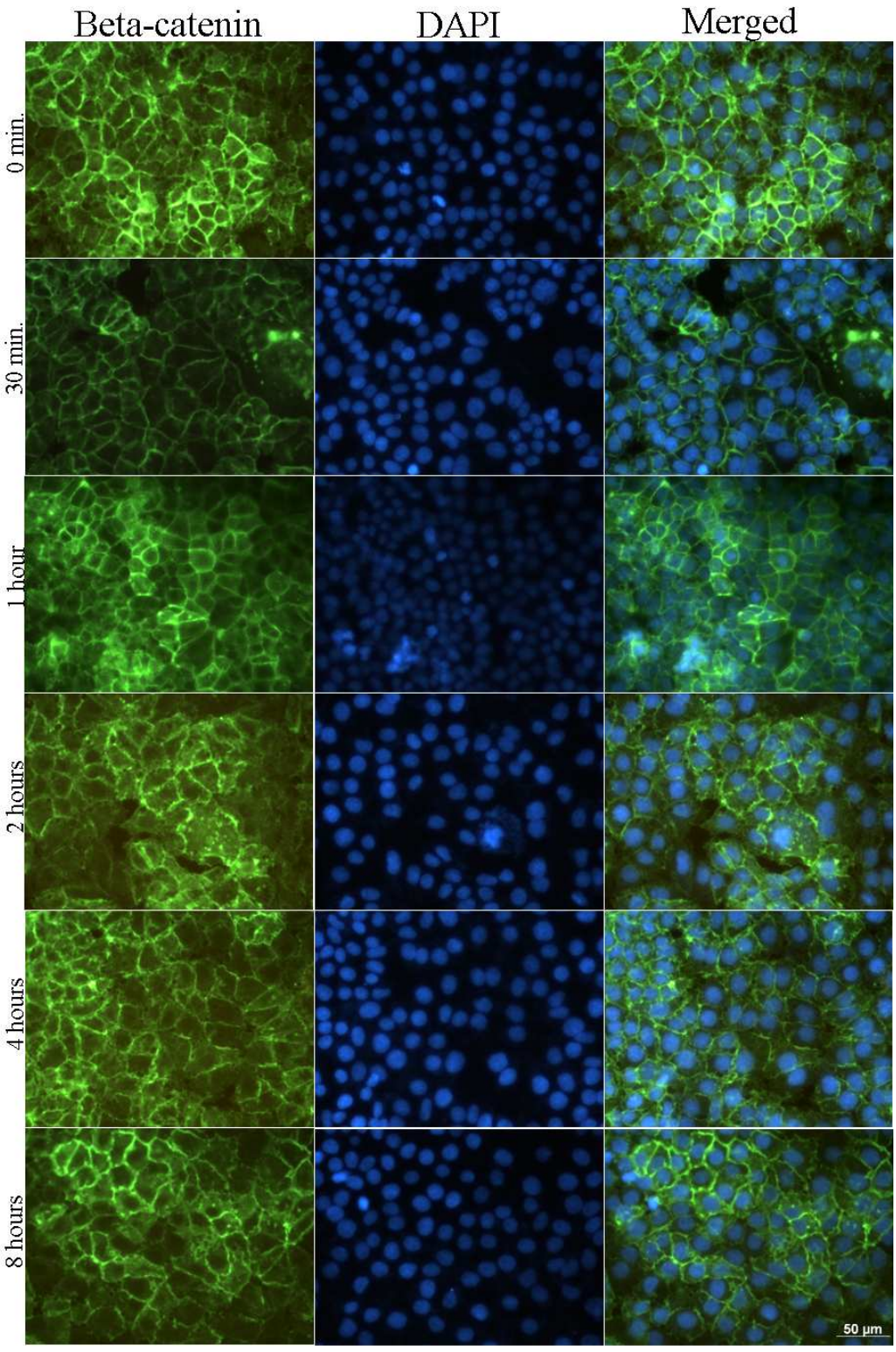

Figure 5.11. Effect of 0.7 μM Wnt agonist on localization of β-catenin in Snu449 cells. Snu449h7 cells were treated with 0.7 μM Wnt agonist for indicated durations and localization of β-catenin was visualized by immunofluorescence (green). Nuclei were counter-stained with DAPI (blue).

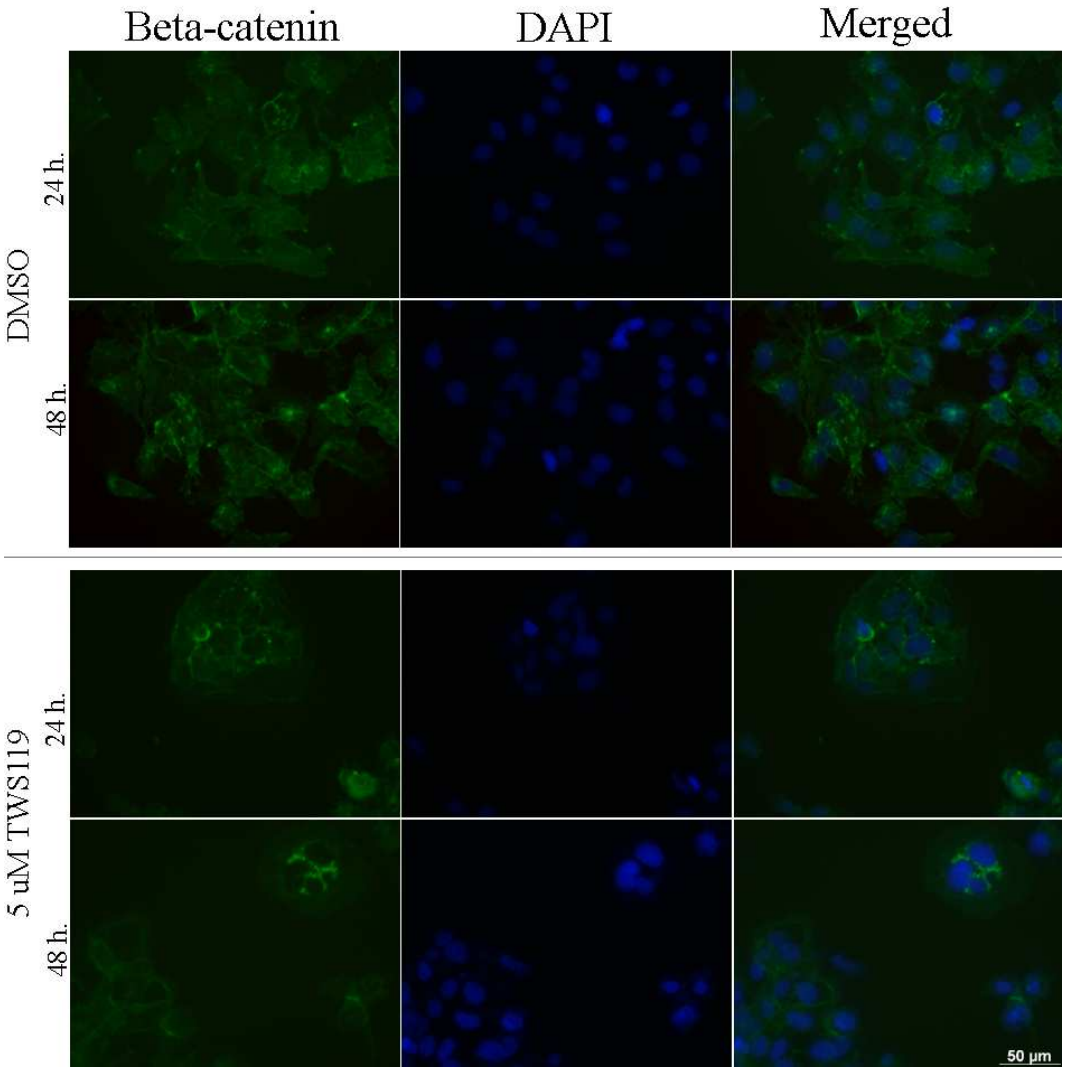

Figure 5.12. Effect of 5 μM TWS119 agonist on localization of β-catenin in Huh7 cells. Huh7 cells were treated with 5 μM TWS119 for indicated durations and localization of β-catenin was visualized by immunofluorescence (green). Nuclei were counter-stained with DAPI (blue).

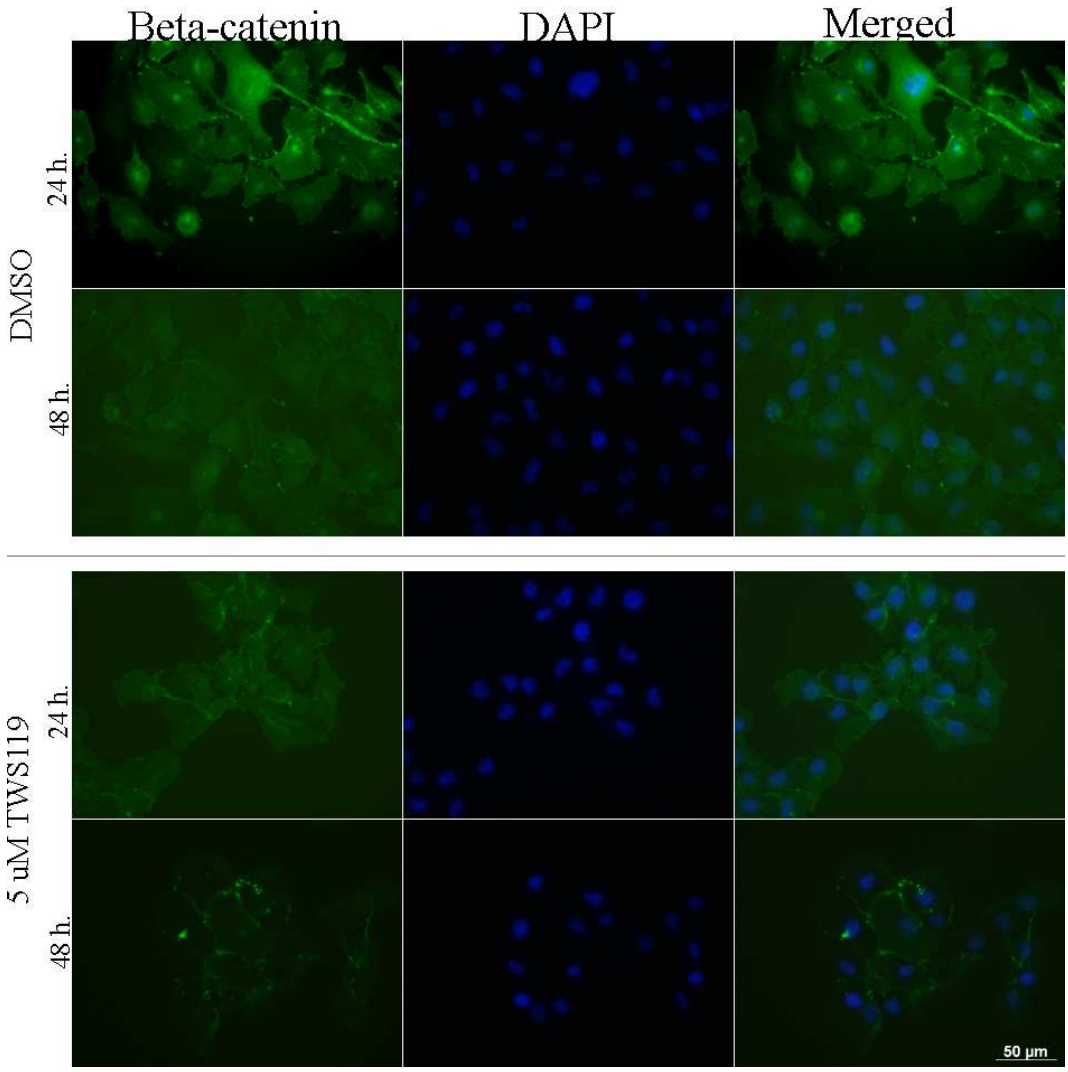

Figure 5.13. Effect of 5 μM TWS119 on localization of β-catenin in Snu449 cells. Snu449 cells were treated with 5 μM TWS119 for indicated durations and localization of β-catenin was visualized by immunofluorescence (green). Nuclei were counter-stained with DAPI (blue).

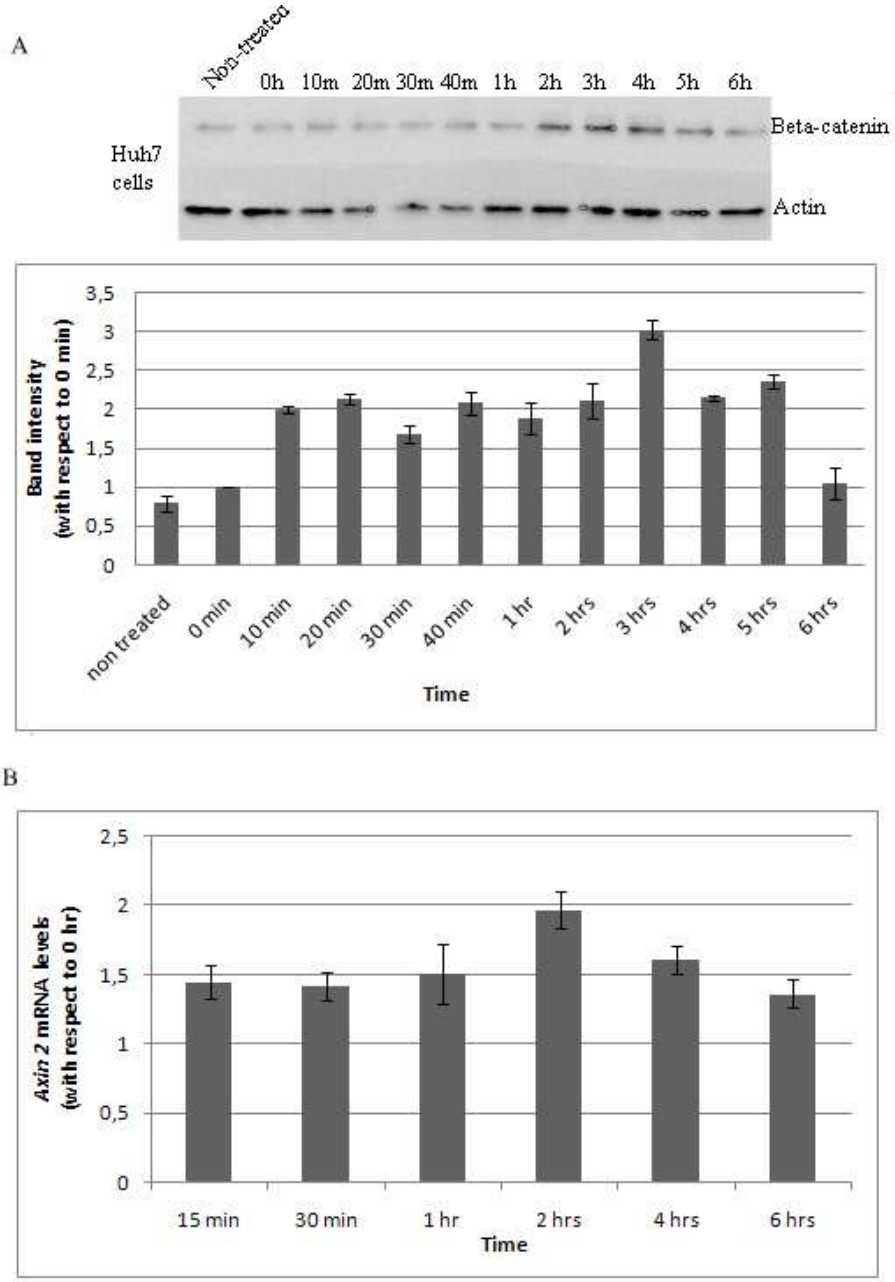

Figure 5.14. Huh7 cells were treated with 0.7 μM Wnt agonist and abundance of β-catenin was determined by western blotting (n=2, with respect to 0 min, normalized to actin) (A) and *AXIN2* by QRT-PCR (n=2, with respect to 0 min, normalized to geometric means of *GAPDH* and *ACTB*).

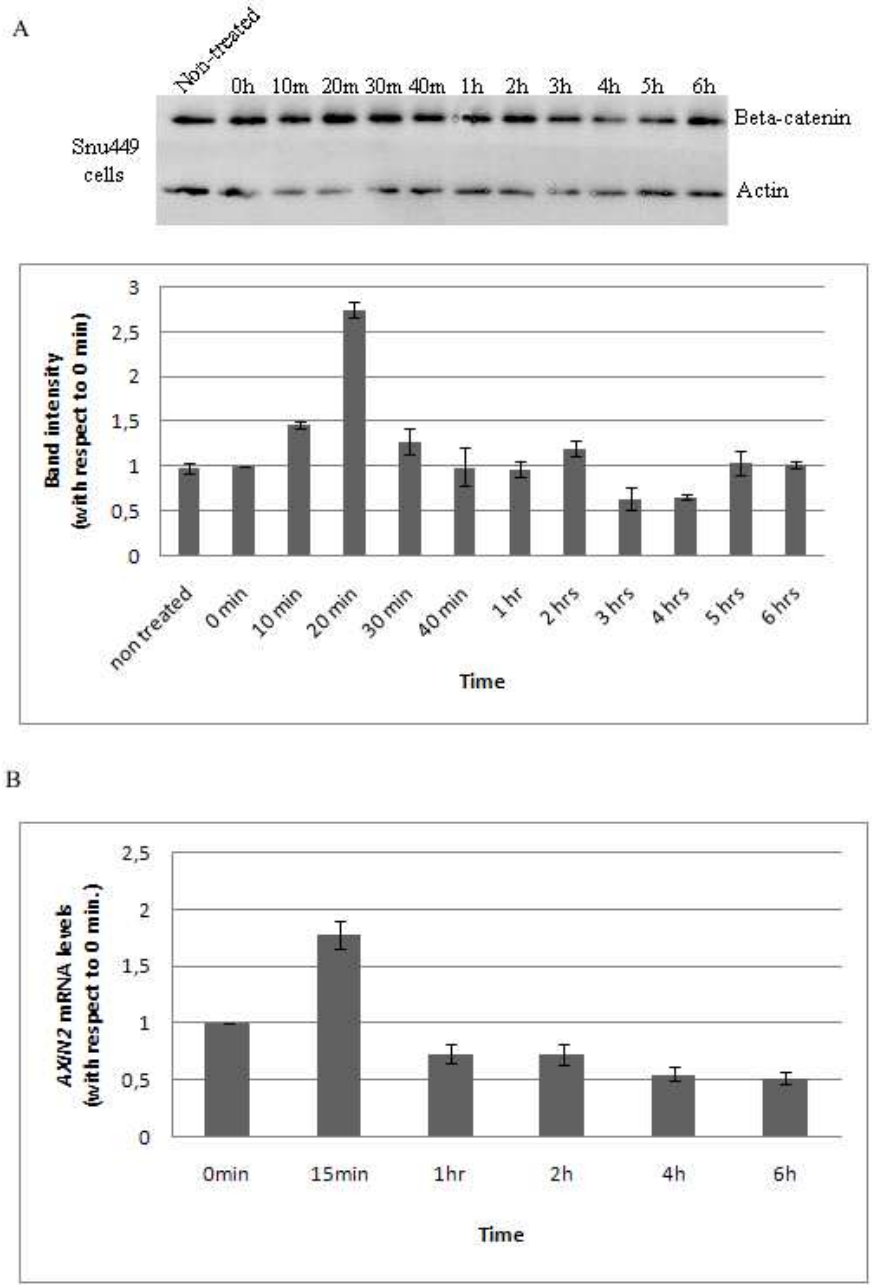

Figure 5.15. Snu449 cells were treated with 0.7 μM Wnt agonist and abundance of β-catenin was determined by western blotting (n=2, with respect to 0 min, normalized to actin) (A) and *AXIN2* by QRT-PCR (n=2, with respect to 0 min, normalized to geometric means of *GAPDH* and *ACTB*).

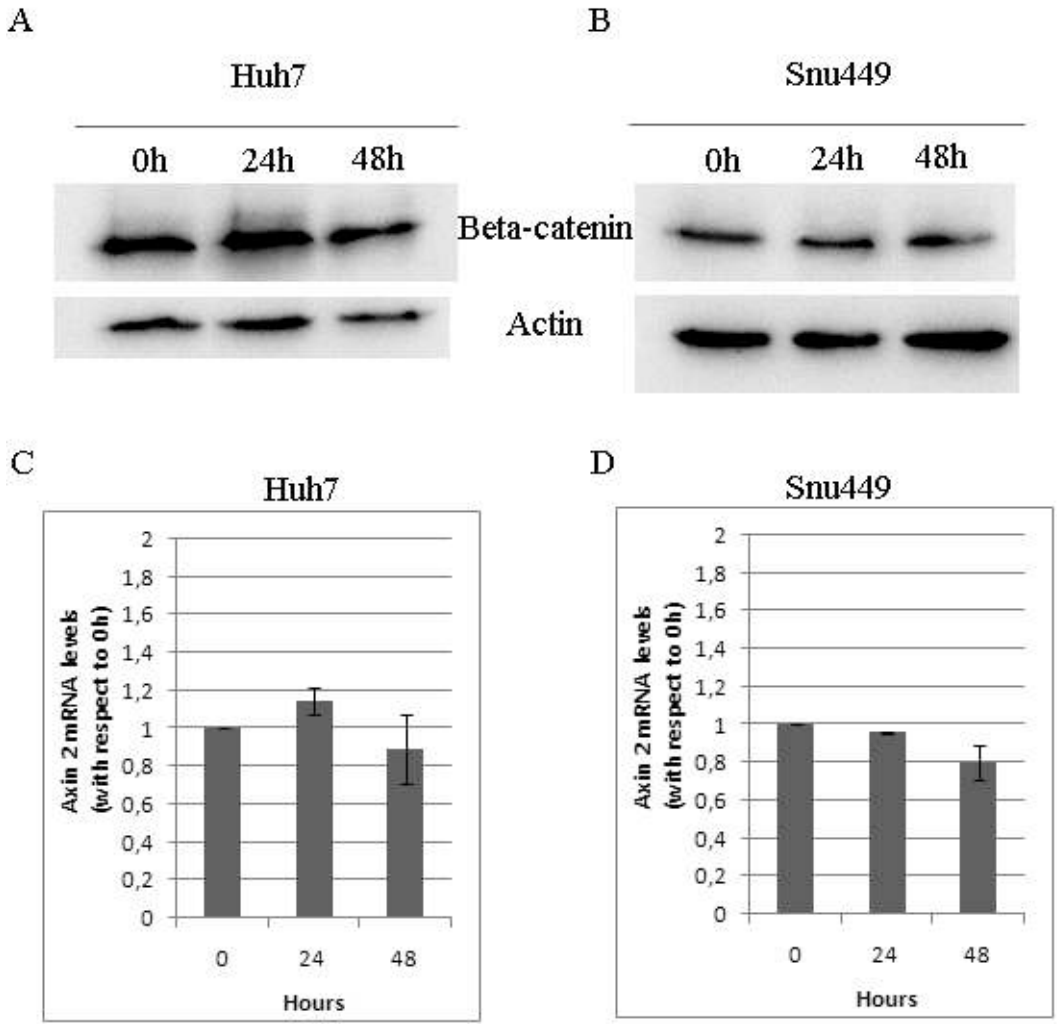

Figure 5.16. Huh7 and Snu449 cells treated with 5 μM TWS119 and β-catenin protein level (A, B) and *AXIN2* transcription (C, D) was checked by western blotting and QRT-PCR (n=2, with respect to 0 min, normalized to geometric means of *GAPDH* and *ACTB*), respectively. Blots are representative of two experiments.

5.4. Wnt Agonist Treatment Leads to Increased *BRI3* and *HSF2* mRNA Levels in Huh7 Cells

It has previously been shown in our laboratory that *BRI3* and *HSF2* mRNA levels increase in Huh7 cells in response to lithium treatment [89]. To verify this finding we have treated Huh7 cells with Wnt agonist and performed QRT-PCR. We observed increase in mRNA levels of both genes with the increase in the *AXIN2* mRNA levels (Figure 5.17).

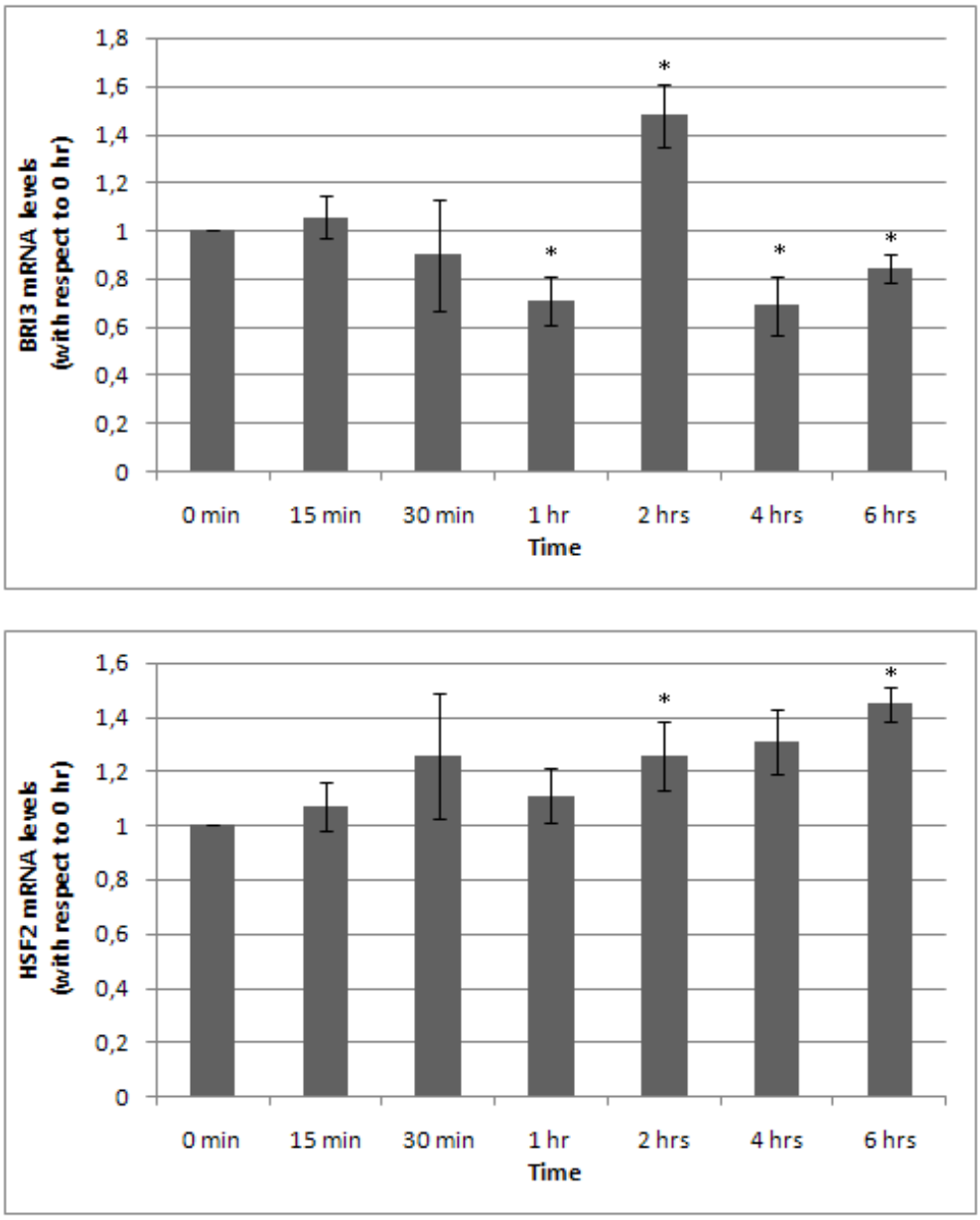

Figure 5.17. Huh7 cells were treated with 0.7 μM Wnt agonist and harvested at indicated time points. Results are normalized to geometric means of *GAPDH* and *ACTB* and fold differences with respect to 0 min. were plotted (n=2, * = $p<0.05$, Student's t-test).

5.5. *BRI3* and *HSF2* mRNA Levels Increase in Response to Increased Wnt/β-catenin Pathway Activity in Snu449 cells

In our laboratory we have previously shown that *BRI3* and *HSF2* transcription are regulated by the Wnt/β-catenin pathway in Huh7 and U373 MG (human

glioblastoma-astrocytoma, epithelial-like cell line) cell lines. To strengthen our hypothesis in the liver context, we aimed to show the same effects on another HCC cell line, namely Snu449. Using the techniques outlined above to artificially activate the Wnt/β-catenin pathway in Snu449 cells we investigated *BRI3* and *HSF2* transcription.

QRT-PCR analysis showed an increase in both *BRI3* and *HSF2* mRNA levels in response to lithium (Figure 5.18) and Wnt agonist treatment. (Figure 5.19).

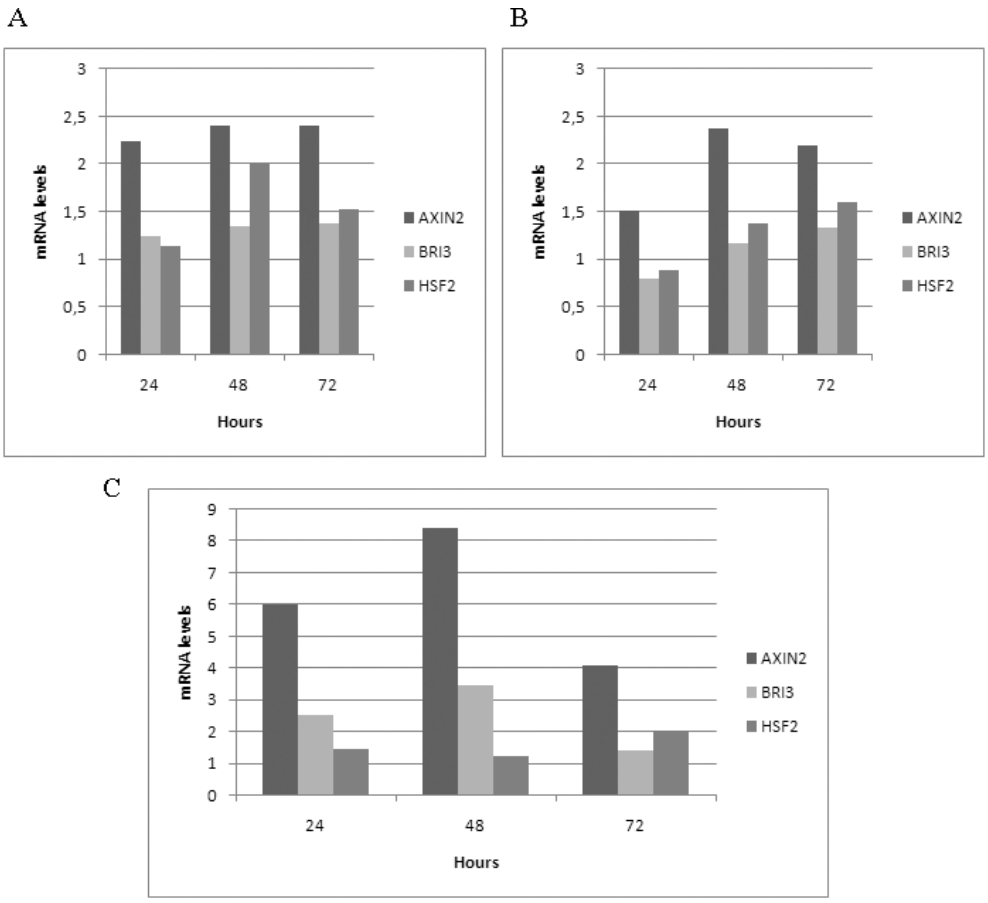

Figure 5.18. Snu449 cells were treated with 25 mM LiCl or NaCl and harvested at indicated time points. A, B and C show different day experiments. Results are normalized to geometric means of *GAPDH* and *ACTB* and fold differences with respect to NaCl values were plotted (n=2).

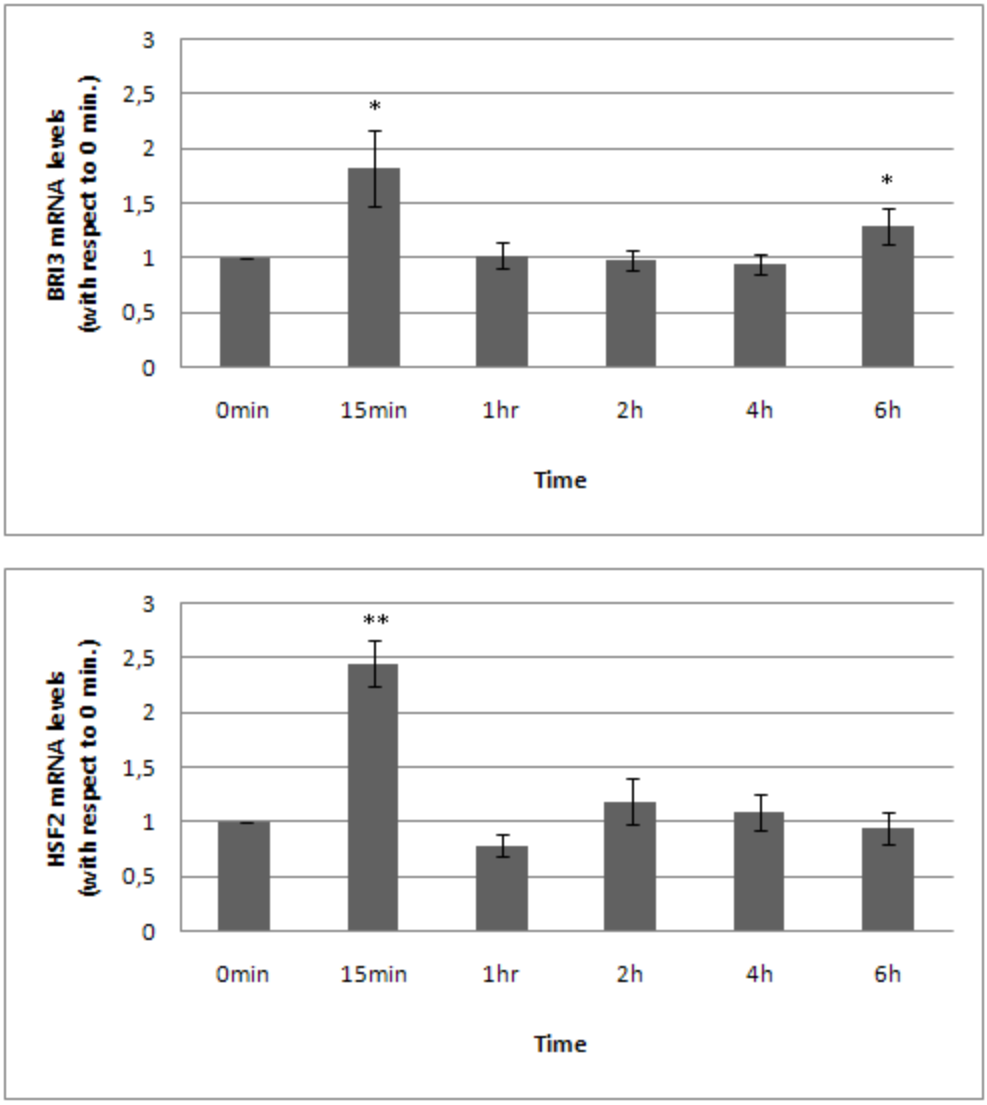

Figure 5.19. Snu449 cells were treated with 0.7 μM Wnt agonist and harvested at indicated time points. Results are normalized to geometric means of *GAPDH* and *ACTB* and fold differences with respect to 0 min. were plotted (n=2, * = p<0.05, ** = p<0.01, Student's t-test).

6. DISCUSSION

In this study, we have tested the effectiveness and reproducibility of various techniques that artificially stimulate the Wnt/β-catenin pathway and strengthened our hypothesis that *BRI3* and *HSF2* transcription is regulated by β-catenin levels in the cell using QRT-PCR, transfection of degradation resistant β-catenin, lithium mediated inhibition of GSK3β and treatment with two commercially available reagents, Wnt agonist and TWS119 as main methods.

Our laboratory has previously identified *BRI3* and *HSF2* as putative targets of the Wnt/β-catenin pathway as a result of SAGE and genome-wide microarray screens [88]. Since then, we have supported our hypothesis with strong evidence *in vitro* including regulation of the target genes' promoter activities by β-catenin via luciferase assay, direct interaction of *BRI3* and *HSF2* promoter regions with β-catenin with chromatin immunoprecipitaion (ChIP) and *in silico* by analysis of promoter regions [89]. Even though these results were strong, most of the experiments were done using Huh7 cells and some of the results were confirmed in U373 MG (human glioblastoma-astrocytoma, epithelial-like cell line) cells. To generalize our results, we decided to repeat the experiments in different cell lines and in this study Snu449 human hepatocellular carcinoma cells were used for that goal. Huh7 cells were also used for the procedures that have not been previously performed in our laboratory, such as Wnt agonist and TWS119 treatment.

BRI3 and *HSF2* genes are only a small fraction of the putative novel candidates of Wnt/β-catenin targets. To harvest the fruits of our SAGE and genome-wide microarray findings, we have and still are working on other candidate genes. Considering the difficulties we have experienced in stimulating the Wnt/β-catenin pathway, assessing a trustworthy method that yields reproducible results is of utmost importance for our studies. Apart from the commercially available Wnt agonist and TWS119 treatment reported in this work, we are working on building a tetracycline inducible system in Huh7 cells. Even though given the fact that one can control the activation of

transcription of the transfected gene in a tet-on/tet-off system, this system might prove inefficient or even inapplicable to cell lines with low susceptibility to transfection with commercially available products. Considering the hazard and required investments to establish a lentiviral system, methods that do not involve gene transfer and are widely applicable rise above others.

Protein phosphatase 2A (PP2A) complex interacts with APC and axin, major scaffolding components of the β-catenin destruction complex, and regulates the activity of the Wnt/β-catenin pathway [94]. Even though it is known that this complex regulates the pathway, there are no solid findings to classify PP2A as a negative or positive regulator of Wnt as there are several contradicting reports that place the complex as a negative regulator [95, 96] or positive regulator [97, 98]. Interestingly, HSF2 is known to interact and inhibit PP2Ac, a catalytic subunit of protein phosphatase 2A complex [99]. This direct interaction of a potential Wnt/β-catenin pathway target sure is appealing. HSF2, under the light of this knowledge, could regulate the Wnt/β-catenin pathway through inhibition of the PP2Ac molecule. This regulation could be positive or negative depending on the role of PP2A complex which is yet to be clarified (Figure 6.1).

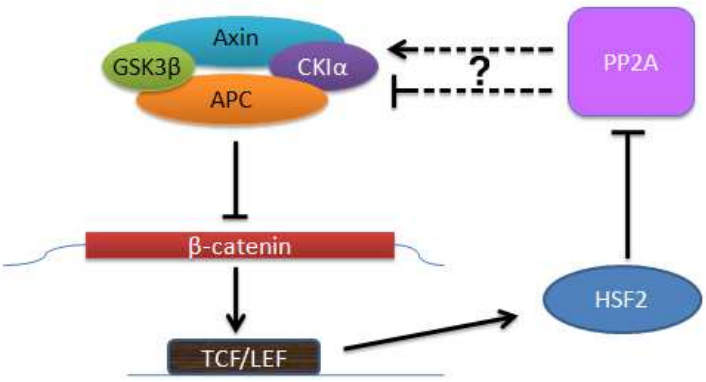

Figure 6.1. A model proposition for the regulation of the β-catenin destruction complex by HSF2, a putative novel Wnt/β-catenin pathway target. HSF2 inhibits the interaction between PP2A and the β-catenin destruction complex. Depending on PP2A's effect on the destruction complex (activation or inhibition) HSF2 can be a negative or positive regulator of β-catenin activity.

BRI3 expression has previously been reported to significantly increase in L929 murine aneuploid fibrosarcoma cells in response to TNF treatment [72]. Interestingly over-expression of *BRI3* in the same cell line induced apoptosis instead of protecting the cell from TNF induced cell death [72]. Another study has shown reduced *BRI3* expression increases resistance to TNF-induced cell death more than 1000 fold indicating BRI3 is a negative checkpoint for TNF-induced cell death [73]. The same study also reported that over-expression of sense or anti-sense *BRI3* RNA is toxic to L929 cells, indicating endogenous levels of this gene are important for cell survival. It is interesting that lithium treatment has also been shown to promote cytotoxic actions of TNFα [100–102]. In our laboratory we have shown that *BRI3* mRNA levels are positively regulated by the Wnt/β-catenin pathway and the β-catenin molecule physically interacts with the *BRI3* promoter region [89]. These findings may indicate a possible role of Wnt/β-catenin pathway on regulation of TNF induced cell death through controlling *BRI3* levels in the cell.

Another interesting point in *BRI3*'s potential candidacy is its possible role in propagation of Alzheimer's disease. BRI3 has previously been shown to interact with upbeta amyloid converting enzyme (BACE) [69] and amyloid precursor protein (APP) inhibiting the production of β amyloid peptides, the main component of senile plaques seen in Alzheimer's disease [71]. A single nucleotide polymorphism (Ile-1062-to-Valin) LRP6 receptor has previously been associated with late onset Alzheimer's disease [59] and to lead to decreased β-catenin signaling in HEK293T cells. To our knowledge, the exact relation between reduced β-catenin signaling and Alzheimer's disease onset is not known yet. Decrease in BRI3 levels due to reduced β-catenin levels caused by the mutation in LRP6 receptor may disturb the inhibition of APP and lead to production of β amyloid peptides. Considering *BRI3* gene is rather newly discovered and that only little is known about its functions this relation can prove to be an interesting line of research.

In response to lithium treatment, *BRI3* and *HSF2* mRNA levels increased in Snu449 cells, peaking at 72 hours (∼1.5 fold) and 48 hours (∼1.8 fold), respectively. Since it is a known target of Wnt/β-catenin pathway, *AXIN2* mRNA levels were

analyzed to confirm activation of the pathway, and they remained at around 2.5 fold compared to control cells at all three time points. *BRI3* has previously been shown to have a late reaction to lithium treatment in Huh7 cells [89], peaking at 72 hours as well. Contradicting this slow response to lithium treatment, *BRI3* and *HSF2* mRNA levels significantly increased to over 2 fold in only 15 minutes in Snu449 cells (Figure 5.19). Response of Huh7 cells, however, was not as swift as that of Snu449 cells. *BRI3* mRNA levels reached its peak in two hours with about 1.5 fold increase. *HSF2* levels showed a steady increase reaching its peak ($\sim$1.5 fold) at sixth hour of treatment. Interestingly, both genes' mRNA levels seem to reach their peak values when the *AXIN2* mRNA levels also peak. These findings, combined with previous results obtained in our laboratory, support our hypothesis that *BRI3* and *HSF2* are transcriptional targets of the Wnt/β-catenin pathway.

TWS119 treatment has been shown to increase β-catenin levels and activity of TCF4 transcription factor (up to 5 fold at 5 μM concentration) in P19 cells [92]. In this study, we have also shown increased TCF4 activity in Huh7 cells via luciferase activity assay but with higher concentrations (Figure 5.6). However concentrations higher than 5 μM have shown to cause cell death both in Huh7 and Snu449 cells (data not shown) and treatment with 5 μM TWS119 did not increase β-catenin levels nor *AXIN2* mRNA levels in Huh7 and Snu449 cells. Thus this compound, when used at this concentration, does not stimulate the stabilization of β-catenin molecule through inhibition of GSK3β in these cell lines and since higher concentrations cause cell death, TWS119 is not suitable to be used with Huh7 and Snu449 cells.

We have failed to observe translocation of β-catenin into the nucleus in response to treatment with Wnt agonist (Figures 5.10 and 5.11) and TWS119 (Figures 5.12 and 5.13) whereas in Huh7 and cells treated with 25 mM LiCl, localization of β-catenin inside the nuclei was clearly seen (Figures 5.4 and 5.5). Since TWS119 treatment did not give positive results on western blotting and QRT-PCR experiments, it is only expected not to see nuclear localization of β-catenin. Wnt agonist treatment, on the other hand, has shown to be activating β-catenin mediated activation of transcription and β-catenin accumulation via luciferase reporter assay, QRT-PCR and western blot-

ting (Figures 5.6, 5.14, 5.15 and 5.17). Even though lithium treatment translocates immense amount of β-catenin into the nucleus, a comparably small amount of the protein, that could not be detected with immunofluorescence, might be sufficient to activate transcription.

Even though canonical Wnt pathway, as well as treatment with lithium salts are known to prevent apoptosis by activating β-catenin/TCF mediated transcription [103–107], in our hands, activation of the β-catenin/TCF mediated transcription in HCC cell lines by lithium treatment showed to cause cell death at long exposure times (data not shown). Along with this observation, considering that Wnt agonist and TWS119 treatment also cause cell death weakens the probability of these molecules being chemically toxic to the HCC cells and points in the direction of an effect induced by them. This hypothesis is supported by several reports showing the opposite effects of GKS3β inhibition by lithium treatment [100, 108] and consequent activation of the Fas death domain-containing receptor [109]. Song *et al.* have also shown other inhibitors of GSK3β, including indirubin-3'-monoxime, kenpaullone and rottlerin have similar effects [109]. Under the light of these reports, it might be safe to assume that treating HCC cells with LiCl, Wnt agonist and TWS119 also leads to apoptosis through a similar mechanisms.

Different techniques were used to artificially activate the Wnt/β-catenin pathway in this study including transfection with degradation resistant β-catenin, treatment with lithium salts and exposure to two commercially available compounds: Wnt agonist and TWS119, a GSK3β inhibitor. Our experience has shown that neither of these techniques is flawless and results should be interpreted with caution to avoid misconclusions.

Over-expression of degradation resistant β-catenin is widely and successfully used but since this technique involve gene transfer, it is limited to cell lines that are liable to transfection. Moreover, in our hands Huh7 cells did not respond well to β-catenin over expression. Even though *CTNNB1* mRNA levels are significantly increased, no activation of target genes or increase in luciferase activity was observed in luciferase

reporter assays and QRT-PCR experiments (Figure 5.1). This finding is in accordance with a previous paper which reported that transfection of degradation resistant β-catenin is not sufficient to activate the TCF4 mediated transcription in Huh7 cells [110]. Moreover, the same study showed that HCC cell lines, including Huh7, that are hepatitis B virus (HBV) negative are refractory to Wnt/β-catenin signaling whereas HBV-positive HCC cells show normal response.

Among these methods inhibition of GSK3β using lithium salts is cheap, easy to establish and widely used [86, 90]. Although this method has proven to work, the exact mechanism of inhibition is still not known, lithium salts are also used for their other effects as mood stabilizers and in treatment of bipolar disorder [87]. Because it is not a very specific assay, activation of Wntβ-catenin pathway should be confirmed to avoid unexpected results due to its other effects on the cell and to that end, we have confirmed lithium treatment leads to accumulation of β-catenin by western blotting in Snu449 cells (Figure 5.3) and its translocation to the nucleus in Huh7 and Snu449 cells (Figures 5.5 and 5.4). Even though this technique has successfully been used in various cell lines in our laboratory, Snu449 cells show an interesting inconsistency in activation of the pathway (Figure 5.2) although β-catenin levels increase significantly (Figure 5.3) followed by translocation to the nucleus (Figure 5.5).

Cells responded to Wnt agonist treatment much quicker than they did to lithium treatment. Snu449 cells responded as fast as 15 minutes (Figure 5.15) but both *AXIN2* mRNA levels and β-catenin levels dropped back to or below normal levels (Figure 5.15). On Huh7 cells, however, β-catenin levels increased steadily starting from 10 minutes until 5 hours of treatment and *AXIN2* mRNA levels stayed roughly around 1.5 fold peaking at 2nd hour with about 2 fold and (Figure 5.14). This indicates, even though they are both HCC cell lines, Snu449 and Huh7 cell lines show different reaction times from each other. Snu449 cells seem to take the Wnt/β-catenin pathway "under control" right after an initial activation whereas Huh7 cells seem to be letting the pathway "loose". This difference of reaction times suggests that treatment with the Wnt agonist might not be universally applicable, even to the cell lines with an intact Wnt/β-catenin pathway. To test this hypothesis, how various cell lines which were

obtained from different tissues as well as cells that did not originate from cancer such as HEK293 cells should be tested for their response to the Wnt agonist treatment. At first glimpse, this difference in response may seem like it could also explain the difficulties we have experienced with these cells in lithium treatment but it should be investigated thoroughly considering the fact that lithium treatment requires much longer exposures.

In conclusion, this study strengthened our hypothesis that *BRI3* and *HSF2* are transcriptional targets of the Wnt/β-catenin pathway and added another evidence that this a general interaction by adding another cell line to the assortment of models we have used. We have also established different methods as means of activation of the pathway and compared their pluses and minuses to ease our work in pursuin the identification of new potential candidates to Wnt/β-catenin pathway.

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