ELUCIDATING THE ROLE OF FOXO TRANSCRIPTION FACTORS ON LITHIUM INDUCED GROWTH ARREST IN HEPATOCELLULAR CARCINOMA CELLS

by

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To my beloved family and to my fiancé…

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ABSTRACT

ELUCIDATING THE ROLE OF FOXO TRANSCRIPTION FACTOR ON LITHIUM INDUCED GROWTH ARREST IN HEPATOCELLULAR CARCINOMA CELLS

Lithium is one of the most effective drugs for the treatment of bipolar disorder for years. Many studies have shown that lithium has neuroprotective effect and has acapacity to inhibit cell proliferation. Specifically, it can inhibit cell growth by inducing G2/M arrest in different cell lines including hepatocellular carcinoma cells. Up to now, many mammalian enzymes have been identified as a target of lithium; however, how lithium exerts these diverse effects is unclear. In this study, we aimed to elucidate the role FOXO transcription factors in lithium induced growth arrest in hepatocellular carcinoma cells. The possible role of FOXO was observed by luciferase reporter assay and real time PCR. There was an increase in FOXO promoter activity along with an increase in lithium induced GADD45a expression. To test the possible molecular mechanisms for FOXO regulation, FOXO regulatory kinases were analyzed by Western Blotting and an increase in MST activity was observed after lithium treatment. The increase in MST activity after lithium treatment suggests probable mechanism that lithium can activate FOXO to induce cell cycle arrest with the help of MST. To determine the FOXO and MST dependency in lithium induced growth arrest, stably FOXO and MST expressed cells were generated. It was found that lithium induces MST activation and MST increases the GADD45a expression through FOXO or another way. It was also found that the role of MST in lithium actions could be independent of its kinase activity. The experimental data and findings obtained from this study possess the potential of uncovering the molecular mechanisms lithium induced cell cycle arrest in hepatocellular carcinoma cells. The exploration of molecular mechanisms involved in lithium mediated HCC growthinhibition may provide new insights for therapy of liver tumors.

ÖZET

FOXO TRANSKRİPSİYON FARTÖRLERİNİN LİTYUMUN İNDÜKLEDİĞİ KARACİĞER KANSER HÜCRELERİNDEKİ BÖLÜNME DURMASINDAKİ ROLÜNÜN SAPTANMASI

Lityum tuzu uzun bir süredir tedavi amaçlı kullanılmasına rağmen, cancer gelişimindeki rolü ancak son zamanlarda fark edilmiştir.Hepatosellüler karsinom hücreleri de dahil olmak üzere farklı hücre hatları üzerinde spesifik olarak hücreleri G2/M fazında durdurarak hücre büyümesini inhibe ettiği görülmüştür. Şimdiye kadar lityumun etkilediği bazı enzimler bulunmuş fakat bunların lityumun indüklediği hücre duraksamasında herhangi bir rolü keşfedilmemiştir.Bu proje kapsamında, lityumun hücre döngüsünde duraksamaya neden olduğu hepatoselüler karsinom hücrelerinde FOXO transkripsiyon faktörlerinin rolünü ortaya koymak amaçlanmıştır. Bu projede, lityum ile muamele edilen karaciğer kanser hücrelerinde FOXO transkripsiyon aktivitesininanlamlı bir şekilde arttığı tespit edilmiştir. Lityumun FOXO'yu hangi mekanizmayla etkilediğine dair araştırmalar yapılmış ve FOXO üzerinde pozitif bir regulasyona sahip olan MST aktivitesinde lityuma bağlı bir artış gözlenmiştir. Bu MST aktivitesindeki artış, lityumun varlığındaki FOXO'nun hücre döngüsü duraksamasındaki olası rolünün MST aracılığıyla olabileceğini ortaya koymaktadır. Daha sonra lityumun tetiklediği büyüme durmasında FOXO ve MST proteinlerine bağımlılığını belirlemek için, istikrarlı bir şekilde FOXO ve MST proteinlerini sentezleyen hücreler oluşturulmuş ve yapılan çeşitli deneylerle, lityumun MST aktivasyonunu tetiklediği ve MST'nin de GADD45a'nın ifadesini doğrudan FOXO aracılığıyla ya da dolaylı olarak arttırabileceği bulunmuştur. Aynı zamanda, MST'nin FOXO ve GADD45a üzerindeki rolünü MST'nin kinaz aktivitesinden bağımsız olduğu gözlenmiştir.Bu çalışmada elde edilen deneysel veriler ve bulgular, hepatosellüler karsinom hücrelerindeki lityum kaynaklı hücre döngüsü duraksamasının moleküler mekanizmasını aydınlatma potansiyeline sahiptir.Lityumtetiklediği hücre duraksamasının moleküler mekanizmaların aydınlatılması karaciğer tümörlerinin tedavisi için yeni bakış açıları sağlayabilir.

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LIST OF SYMBOLS

u	Unit
V	Volt
°C	Centigrade degree
µg	Microgram
µl	Microliter

LIST OF ACRONYMS/ABBREVIATIONS

APS	Ammonium persulfate
bp	Base Pairs
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
cDNA	Complementary DNA
cm	Centimeter
CO_2	Carbon dioxide
DMEM	Dubecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleosidetriphosphate
EDTA	Ethylenediaminetetraacetate
EtOH	Ethanol
FBS	Fetal Bovine Serum
g	Gravity
GFP	Green Fluorescent Protein
gr	Gram
HRP	Horseradish Peroxidase
kDa	Kilodalton
LiCl	Lithium Chloride
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
ng	Nanogram
nm	Nanometer
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PI	Propodium Iodide
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic Acid
rpm	Revolution per minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
SAGE	Serial analysis of gene expression
NaCl	Sodium Chloride
SDS	Sodium dodecyl sulfate
NaF	Sodium Flouride
TEMED	Tetramethylethylenediamine

1. INTRODUCTION

1.1. Lithium

Lithium is a soft metallic element, which is shortly defined by symbol Li on the periodic table. In nature, lithium is typically found in the form of alloys and compounds, since it is extremely reactive. In addition, trace amount of lithium have been observed in the bodies of all organisms but it seems that lithium has no noticeable vital biological function, since animals and plants can survive in good health without lithium.

1.1.1. What is Lithium used for?

Lithium is a monovalent cation, which is used in a range of industries. Two well-known applications of lithium includelithium ion batteries and lithium carbonate tablet for mood stabilization.

Clinically,as a classic mood stabilizer, lithium has been used to treat bipolar disorder for more than half a century (Johnson and Gershon, 1999). Also, recent studies on animals suggest that lithium has beneficial effect on other mental disorders such as brain ischemia, Alzheimer's disease and Huntington's disease(Aghdam and Barger, 2007; Alvarez *et al.*, 2002).The strong anti-suicidal effect of lithium also has been shown recently (Tondo and Baldessarini, 2009).

Therapeutic margin of lithium is very narrow that have been demonstrated to be effective in the treatment of bipolar disorder. It is ranging from 0.6mM to 1.2mM in the serum level(Moscovich, 1993; Speirs and Hirsch, 1978).Lithium does not seem to be carcinogenic or mutagenic but it can cause to kidney and liver damage at prolonged exposures of 2mM or more (American Psychiatric Association, 2002; Gould *et al.*, 2003; Mazlo *et al.*, 1983). In spite of its narrow therapeutic margin and well-known adverse effects, it is safe to use lithium in the therapeutic dose range.

1.1.2. Effects of Lithium at Cellular Level

At systemic level, it was reported that pharmacological doses of lithium cause stabilization of mood disorder, developmental defects and increased neutrophil production (Boggs and Joyce, 1983; Kao and Elinson, 1998). With different studies, it was confirmed that lithium has pleiotropic effects on cell growth, development, differentiation and apoptosis (Rodriguez de la Concepcion *et al.*,2005, Rowe and Chuang, 2004, Takahashi-Yanaga *et al.,* 2003and Umbach *et al.*,2005).

Although lithium has been widely used for years, its cell specific effects have not been completely understood. It has been shown that lithium can affect the cell proliferation either positively or negatively depending on the cell type. *In vitro* and *in vivo* findings showed that lithium can induce survival and proliferation in neurons but it may lead to growth arrest and senescence in epithelium like cells (Smits *et al.,* 1999; Ohteki *et al.,* 2000).

Recent studies at the cellular level showed that lithium induced inhibition of cell proliferation associated with the interference of cell cycle progression. They demonstrated that the effect of lithium was specifically observed on G2/M phase of the cell cycle through the inhibition of cdc2in different cell lines including embryonalcarcinomaand osteosarcoma (Smits *et al.,* 1999). According to their report, cdc2 activation is necessary to complete the cell cycle. In addition, the similar G2/M arrest was observed in bovine aortic endothelial cells after lithium treatment that was associated with the stabilization of p53 (Mao *et al.,* 2001). With another study, the inhibition of the astrocytes proliferation bylithium additionwas also accompanied by G2/M transition block (Yarden *et al.*,2002). All these evidences indicate that G2/M checkpoint of the cell cycle progression is a significant event in lithium actions of cell proliferation.

1.1.3. Mechanisms of Lithium's Actions

Although there is a great investigation about lithium, the action mechanisms of its effects are not completely clear. It can be thought that lithium has effects on electrolytes and ion transport because it is closely related to sodium in its properties.Lithiumion can also exert biochemical and molecular effects on signal transduction cascades, ion transport,

and neurotransmitter receptor-mediated signaling and gene expression (Manji and Lenox, 2000) since lithium has a very low density that can allow it to travel in the cell freely.

Independent studies show that lithium exerts its effects on a group of molecular targets. Most of them are the members of metal-dependent enzymes that includes glycogen synthesis kinase 3 (GSK-3) (Cohen and Goedert, 2004;Klein and Melton, 1996; Mora A, *et al.,* 2002), protein kinase B (PKB) (Mora A, *et al.,* 2002), inositol monophosphatase (IMPase) (Quiroz, 2004)and bisphosphate 3′-nucleotidase (BPNT1) (Spiegelberg, 2005).It was reported that lithium induces its acts via direct competition with Mg^{2+} (Freland and Beaulieu, 2012).

GSK-3 and IMPase are two well-documented targets of lithium in mammalian cells.Lithium can directly inhibit GSK-3activity by competing with Mg^{2+}, which isnecessary for kinase activity (Klein and Melton, 1996; Ryves and Harwood, 2001). GSK-3 has role in the signaling of the Wnt pathway and it is assumed to have role in the effects of embryonic development (Hedgepeth *et al.,* 1997; Sinha *et al.,* 2005). In addition, lithium has been proposed to delay G-protein-coupled signaling through the depletion of intracellular inositol by direct inhibition of IMPase (Hallcher and Sherman, 1980). However, until now, whether the inhibition of GSK-3 and IMPase is related to the cellcycle arrest induced by lithium is not known.

1.1.4. Lithium and Hepatocellular Carcinoma

Although lithium is mainly used to treat mental disorders, it targets not only the nerve cells. Lithium also induced attenuation of cell proliferation in normal or neoplastic cells (Huot *et al.,* 1972).Moreover, the possible preventive nature of lithium in human cancer development was also reported recently (Cohen *et al.,* 1998). In that study, they revealed that mental patients with lithium treatment have lower cancer prevalence than the general population. In fact, it was proposed that the observed lower cancer prevalence in mental patients is probably due to the advantages of lithium. Later studies on cancer cells have shown that lithium salts are also effective for inhibiting glioma cellproliferation (Nowicki *et al.*,2008), colorectal cancer cell (Vidal *et al.*,2011), medulloblastoma cell (Ronchi *et al.*,2011), and also hepatocellular carcinoma cell (Erdal *et al.*,2005).At the molecular level, lithium has been reported to inhibit the growth or tumorigenicity of cancer cells by

modulating the biologic activities of many cancer related genes such as STAT3 (Zhu et *al.,* 2011), β-catenin/WNT (Vidal *et al.,* 2011), (Ronchi *et al.,* 2011), TNF (Beyaert *et al.,* 1989),Fas-L (Kaufmann*et al.,* 2011) and p53 (Mao*et al.,* 2001).

Independent studies support the growth inhibitory effect of lithiumonhepatocellular carcinoma cells. Lithium has been shown to inhibit the hepatocellular cell proliferation by inducing G2/M arrest through Chk1 that is independent of GSK-3 repression and inositol depletion (Wang *et al.,* 2008). Another study, with different kinds of hepatocellular cell lines also indicates the growth inhibitory effect of lithium through the depletion of both AKT and cyclin E proteins(Erdal *et al.,*2005).This data also suggests that lithium induces G2/M arrest via p53 independent pathway.

With this project, it was aimed to investigate the role of FOXO transcription factors in lithium induced growth arrest in hepatocellular carcinoma cells. One of the supporting evidence for FOXO transcription factors, as a candidate key molecule in this event, is the observation of AKT inactivation in lithium treated hepatocellular carcinoma cells according to previous report. AKT is a kinase protein that phosphorylates FOXO protein in conserved residues, leads to its nuclear exclusion and inactivation of FOXO dependent transcription. In addition to this, an increase in the expression of GADD45a, which is one of the transcriptional target molecules of FOXO and has a role in cellular growth arrest, is observed in lithium treated cells via our preliminary data. Increased expression of GADD45a was observed in different studies as an inducer for G2/M arrest, similar to the effect of lithium. Thus, it can be speculated that lithium induced cell senescence or growth arrest may be mediated by FOXO through GADD45a up-regulation.

1.2. FOX Family

FOX family constitutes structurally related Forkhead family of transcription factors that have been identified in all eukaryotes ranging from *Saccharomyces cerevisiae*to human. Forkhead transcription factors are characterized by a conserved about 100 amino-acid DNA-binding domain (the ‘Forkhead box’ or FOX). The three-dimensional structure of Forkheaddomain consists of three major α helices and two large wing-like loops (Lehmann *et al.,*2003).

The first member of the Forkhead family was identified as a *forkhead* gene in *Drosophilamelanogaster,* whose mutation results in the development of a Forkhead-like appearance (Wiegel *et al.,* 1989). To date, more than 100 members of the Forkhead family have been identified and they are classified from FOXA to FOXR on the basis amino acid sequence of their conserved Forkhead domains(Myatt and Lam, 2007).

Comparative genome studiessuggest that there is a relation between the number of Forkhead transcription factors and evolution. A greater number of FOX proteins have been identified in vertebrates than in invertebrates. It was reported that, there were 4 FOX genes in *S.cerevisiae*, 15 in *C.elegans*, 20 in *D.melanogaster* and 43 inhumans (Mazet *et al.,*2003). These FOX transcriptional regulators play various roles during development, from organogenesis to language acquisition. For example, FOXD1 (Hatini *et al.,* 1996) and FOXE3 (Blixt *et al.,* 2007) play an important role in proper eye development. In addition, FOX factors are very critical for cell proliferation, differentiation and tumorigenesis. For instance, FOXC1 (Zhou *et al.,* 2002), FOXK2 (Li *et al.,* 1992) and FOXO family members(Medema *et al.,* 2000) are thought as candidate tumor suppressor genes.

1.3. FOXO Family

FOXO proteins are the members of the most divergent (“O” ther) subclass of the FOX family. They have a unique five amino acid (GDSNS) insertion within the forkhead domain, which is not found in other subfamily of FOX transcription factors (Arden, 2006). FOXO proteins are also different from other subclasses by the presence of highly conserved sites for phosphorylation by the survival kinase AKT (a downstream target of PI3K signaling) within and nearby their Forkhead domains.

1.3.1. Classification of FOXO Proteins

There is only one FOXO gene in invertebrates, termed *daf-16* in the worm and *dFOXO* in the fly. In mammals, there are four functional FOXO genes, FOXO1, 3A, 4, and 6 (Table 1.1).

Table 1.1.FOXO family members in mammals (Adapted and modified from Greer and Brunet, 2005).

GENE NAME	LOCATION	CANCER TYPE	EXPRESSION PATTERN	KNOCKOUT PHENOTYPE
FOXO1	13q14.1	Alveolar rhabdomyosarcomas	Ubiquitous. Highest in He, Sp, Ad, Ki, Br	E10.5 lethality Angiogenesis defects
FOXO3a	6q21	Secondary acute myeblastic leukemia	Ubiquitous. Highest in He, Sp, Lu, Ki, Br, Ad, Ov	Female sterility, anemia, glucose uptake defects, increased neutrophil apoptosis
FOXO4	Xq13.1	Acute leukemias	Ubiquitous. Highest in He, Br, Sp, Lu	Viable
FOXO6	1p34.1	-	Br, Th, Ki	Not done

He, heart; Sp, spleen; Ad, adipose tissue; Ki, kidney; Br, brain; Lu, lung; Ov, ovaries; Th, thymus.

Three members of the FOXO family (FOXO1, FOXO3A and FOXO4) were found at chromosomal translocations in human tumors, suggesting that they might play a critical role in tumor development. FOXO1 gene was found in rhabdomyosarcoma, FOXO3A in secondary acutemyeloblastic leukemia andFOXO4 gene inacute lymphocytic leukemia.

FOXO1, FOXO3A and FOXO4 mRNA are ubiquitously expressed to varying degrees in mammalian tissues. FOXO1 is the most abundantFOXO isoform in insulin-responsive tissues such as liver, kidney, spleen and brain (Armoni *et al.*, 2006). FOXO3A is highly expressed in theheart, spleen, lung, kidney, ovary, adipose tissue and brain (Zhu *et al.*, 2004). FOXO4 is most abundant in the lungs, brain,heart and skeletal muscle and kidneys (Nakae *et al.*, 2001).FOXO6, as a latest member of FOXO family, is predominantly expressed in the developing brain at embryonic stages. In the adult animal, FOXO6 is alsoexpressed in kidney and thymus (Jacobs *et al.*, 2003).

The knockout studies of FOXO proteins showed that embryos of FOXO1 homozygous knockoutmice were smaller and die at embryonic day 10.5 due to several embryonic defects(Nakae *et al.*, 2002). FOXO3Aknockout mice had hematological abnormalities and decreased glucose uptake (Castrillon *et al.*, 2003). FOXO4 knockout mice showedno

obvious abnormalities (Hosaka *et al.,* 2004).The phenotype of FOXO6 deficient animals is not reported, but due to its specific expression in the developing brain, it was reported that itmay play a role in embryologic development of the central nervous system (Hoekman *etal.,* 2006). In fact, the four mammalian isoforms may have both different functions together with overlapping functions; therefore, compensation of one FOXO member by another may mask the function of individual FOXO.

1.3.2. Domain Structure of FOXO

From N-terminus to C-terminus, FOXO protein contains a proline-rich domain, a forkhead domain, nuclear localization signal (NLS), nuclear export signal (NES), aLxxLL motif and an activation domain (Figure 1.1). The proline rich domain stabilizes the interaction between FOXO factors and other proteins. The Forkhead domain is responsible for binding to target gene promoters. At central region of FOXO, NLS and NES are responsible for cellular localization of FOXO. The LxxLL motif is reported to have role in binding to SIRT1 and has an important role in regulating its transcriptional activity (Nakae *et al.,* 2006). The C-terminus of FOXO contains the activation domain, which stimulates the promoter activity.

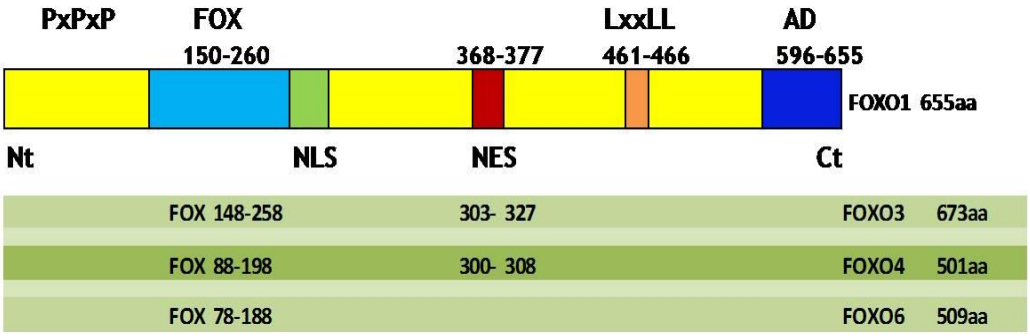

Figure 1.1. Domain structures of human FOXO proteins.

1.3.3. Functions of FOXO Factors

Studies in *C. elegans* showed that direct activation of daf-16 results in extension of lifespan, stress resistance and arrest at the dauer diapause stage (Ogg *et al.,* 1997). Besides, daf-16 is activated in starvation, heat and oxidative stress conditions whereas it is deactivated when the nutrient is rich in the environment. The unique FOXO homologue in *Dro-*

sophila, dFOXO, seems to play similar roles, which are negatively controlled by the insulin-PI3K-PKB signaling cascade and nutrients (Junger *et al.*, 2003; Kramer *et al.*, 2003; Puig *et al.*, 2003). dFOXO-knockout flies are viable and normal size but they are more vulnerable to the oxidative stress suggesting that dFOXO provides protection against oxidative stress.

The role of mammalian FOXO transcription factors is diverse which was confirmed by loss and gain of function experiments in transgenic and knockout mice.It was announced that the critical role of FOXO on cell fate decisions depends on thebalance between growth factor stimulation versus cellular stress and damage. They can regulate cell fate by modulating the expression of genes involved in apoptosis, cell cycle transition, DNA repair, oxidative stress and energy homeostasis and glucose metabolism (Figure 1.2).

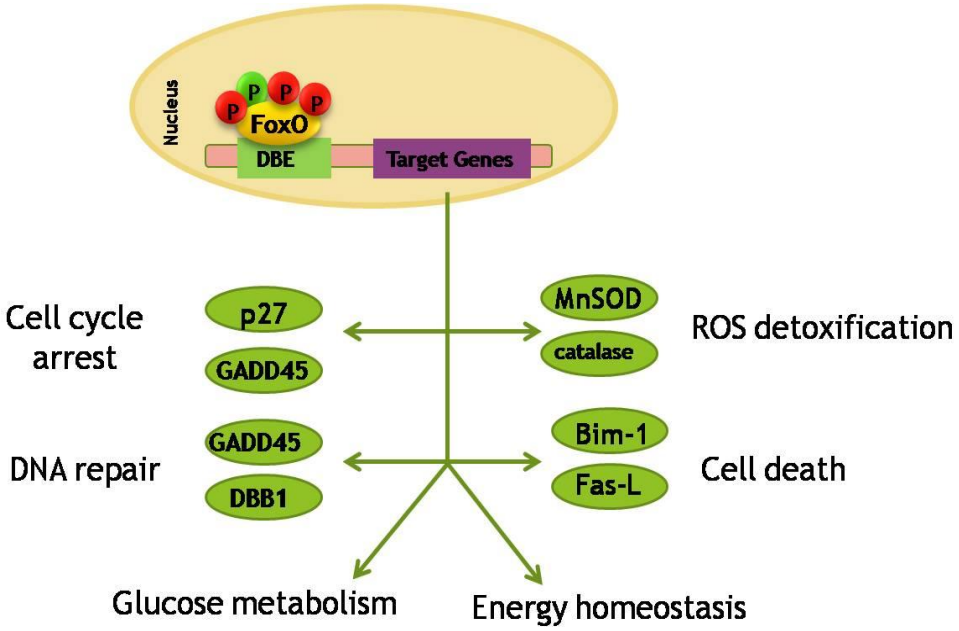

Figure 1.2. Crucial cellular processes carried out by FOXO.

The possible role of FOXO factors in cancer was implicated in different studies.It was shown that in mammalian cancer cells the overexpression of FOXO1, FOXO3, and/or FOXO4 induces cell cycle arrest and apoptosis (Burgering and Kops, 2002).In addition, nuclear exclusion of FOXO3A inprimary breast tumors was correlated with PI3K activation and reduced survival of the patients(Hu *et al.*, 2004). In nude mice, FOXO factors reduced tumorigenicty (Hu *et al.*, 2004;Ramaswamy *et al.*, 2002). Furthermore, the inte-

raction of FOXO proteins with many oncogenessuch as beta-catenin (Essers *et al.,* 2005) or tumor suppressors such as p53 (Brunet *et al.*,2004) was reported.

1.3.3.1. FOXO has role in Cell Cycle Check Point.The cell cycle of eukaryotic cells are divided into four phases: M phase (mitosis), in which the nucleus and the cytoplasm divide; S phase, in which the DNA in the nucleus is replicated, and two gap phases, G1 and G2.The G1 phase is also involved in the control of DNA and G2 phase checks the completion of DNA replication andgenomic integrity before the cell division starts.A major cell cycle restriction point (R) is located at the end of the G1 phase.The transition from one phase of the cell cycle is driven by the actions of CDKs(cyclin-dependent kinase) and their activating cyclin subunits.Progression through the mitotic CDK activity is suppressed through interactions with two main families of inhibitory proteins (CDK inhibitors). CDKs remain at a constant number throughout the cycle whereas cyclins change.

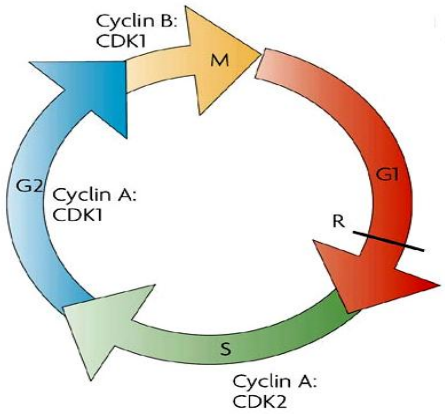

Figure 1.3. Cell cycle checkpoints.

Cell cycle checkpoints are control mechanisms that ensure all the processes required to replicate the genome and cytoplasm and then divide them equally between two daughter cells during the cell cycle by coordinating internal and external signals.G1/S and G2/M boundaries are two checkpoints that are regulated in response to DNA damage (Hartwell and Weinert, 1989). If there is no DNA damage in G1, then there will be enough cyclins (D cyclins)produced to bind to the CDKs, which allows the cell to enter S phase (DNA replication). The G2/M checkpoint ensures there is no DNA damage, and also that the chromosomes have successfully replicated. If everything is in order, then the M phase cyclins(B cyclins) will be abundant enough to bind to the CDKs. At the final stages, during

mitosis, sister chromosomes are separated and mitotic cyclin B is degraded. If mitotic cyclin B is not degraded, cells are stacked at G2 phase. If damage is found, the checkpoint controls the signal mechanism to stall cell division until the problem is eliminated. Defects in these steps may result in a mutated phenotype that is associated with tumorigenesis.

FOXO transcription factors play a major role in G1 arrest by upregulating cell cycle inhibitors such as p21 (Hauck *et al.*,2007; Lawlor and Rotwein, 2000), p27 (Medema *et al.,* 2000; Stahl *et al.,* 2002) and p130 (Chen *et al.,* 2006) or by repressing cell cycles activators cyclin D1 and D2 (Schmidt *et al.,* 2002).p21 is a cyclin-dependent kinase inhibitor. Loss of p21 expression has beendescribed as a poor prognostic factor and as an independent predictor of bladder cancerprogression in muscle invasive cancer (Stein *et al*., 1998). p27 is a protein that binds tocyclins and cdk to block the entry into S phase.

GADD45 is known as DNA damage-inducible protein 45. It was identified depending on its rapid transcriptional induction after UV irradiation (Fornace *et al.,* 1989) and it can induce G2/M arrest (Wang *et al.,* 1999). Microarray analysis allowed to the identification of GADD45a and cyclin G2 as thedownstream target genes of FOXO3A for G2/M arrest and DNA repair (Tran *et al.,*2002). *In vivo* experiment showed that FOXO3A and FOXO4 activate GADD45a promoter directlyinteracting with the DBE. It was observed that oxidative stress activates the GADD45a promoter in aFOXO-dependent manner, resulting in increased level of GADD45a mRNA and proteins as well as G2 arrest (Furukawa-Hibi *et al.,* 2002). Also, in response to glucose starvation, FOXO1 also affects GADD45a mRNA level in islets (Martinez *et al.,* 2006).

1.3.4. Regulations of FOXO Proteins

As described above, FOXO proteins have diverse cellular functions by acting as transcription factors. The activity of FOXO proteins can be regulated by posttranslational modifications including phosphorylation, acetylation and ubiquitination.

1.3.4.1. Phosphorylation of FOXO.In the cells, FOXO proteins are tightly regulated to respond specifically to the environmental condition. A major form of regulation is AKT mediated phosphorylation of FOXO in response to growth factors. Phosphorylation is a process wherein phosphate groups are added to proteins by protein kinases or removed

from proteins by protein phosphates. Thephosphorylation of FOXO at three conserved residue results in the export of FOXO from nucleus to cytoplasm thereby inhibiting FOXO dependent transcription.FOXO proteins also phosphorylated by other kinases including MST, which phosphorylate FOXO under condition of oxidative stress.This phosphorylation keeps the FOXO in the nucleus in its active state (Figure 1.4).

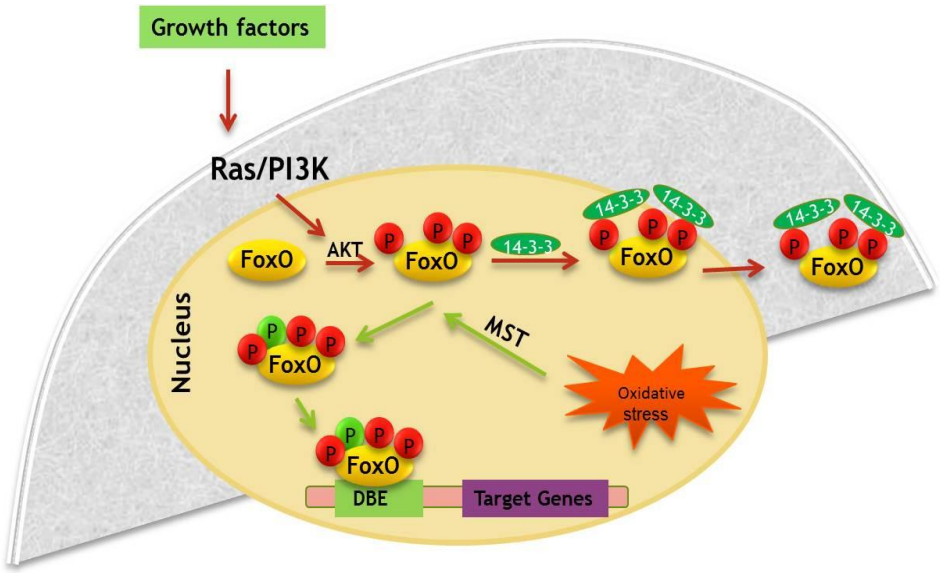

Figure 1.4. Posttranslational regulation of FOXO by phosphorylation.

Genetic studies in *C.elegans* have demonstrated that activation of the PI3K/AKT pathway by insulin or insulin-like growth factor suppresses activity of the daf-16 forkhead transcription factor, the nematode ortholog of mammalian FOXO proteins (Kimura *et al.*, 1997;Lin *et al.*, 1997; Ogg *et al.*, 1997). Analysis of the DAF-16 sequence reveals three consensus AKT sites [RXRXX(S/T)] (Alessi *et al.*, 1996; Brunet *et al.*, 1999). These sites have been found conserved in all the members of the mammalian FOXO family, which include FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6 in humans (Biggs, 3rd *et al.*, 1999; Brunet *et al.*, 1999; Jacobs *et al.*, 2003; Kops *et al.*, 1999; Rena *et al.*, 1999; Tang *et al.*, 1999). AKT-phosphorylated FOXO proteins bind to 14-3-3 chaperone proteins and become sequestered in the cytoplasm, where they are unable to regulate gene expression.

MST1 is a serine/threonine protein kinase that mediates cell death induced by oxidative stress in primary mammalian neurons through direct activation of FOXO transcription factors. MST1 phosphorylates FOXO proteins at a conserved site within the FOX domain (Ser207) disrupting their interaction with 14-3-3 proteins, promoting FOXO nuclear translocation, and thereby inducing cell death in neurons under stress conditions such as hydrogen peroxide treatment. Knockdown of the *C. elegans*MST1 ortholog CST-1 shortens lifespan and accelerates tissue aging, while overexpression of *cst-1*promotes lifespan and delays aging. The *cst-1*-induced lifespan extension requires *daf-16* (Lehtinen *et al*., 2006).

2. PURPOSE

Lithium has been used clinically for years but the possible protective nature of lithium in human cancer development was reported only fifteen years ago. Studies have shown that lithium is effective for inhibiting development of other cancer cells including hepatocellular carcinoma cell. Different studies withhepatocellular carcinoma support that lithium inhibits the hepatocellular cell proliferation by inducing G2/M arrest; however, the underlying molecular mechanisms involving in this is not well explained.

With this study, it was aimed to investigate the role of FOXO transcription factors in lithium induced growth arrest in hepatocellular carcinoma cells. FOXO was picked as one of the affected target, based on previous report that lithium causes depletion at the level of AKT, which has regulatory role in FOXO transcription factors. In addition, evidence that its target, GADD45a, expression was increased after lithium treatment. Those findings suggest that lithium induced growth arrest may be mediated by FOXO through GADD45aupregulation.In this regard, FOXO and GADD45a expression were quantified by real time PCR and their activities were measured by luciferase assay after lithium treatment. Furthermore, to clarify FOXO regulation in the presence of lithium, the activity of FOXO regulatory kinases, AKT and MST activity was measured by Western Blotting. Consequently, stably FOXO and MST expressing cells were generated and whether the action of lithium on cancer cells depends on those proteins by expression analysis, luciferase assay and cell cycle analysis.

3. MATERIALS

3.1. General Chemicals, Kits and Reagents

All chemicals used in this study were analytical grade from Merck (Schucdarf, Germany) Sigma (St. Louis, MO, USA), and unless declaredin the Table 3.1.

Table 3.1. List of some chemicals used in this work.

Bovine Serum Albumin (BSA)	AppliChem, Germany
Ethanol	Emsure, Germany
Isopropanol	Emsure, Germany
Methanol	Emsure, Germany
N, N, N', N'-tetramethylethylenediamine (TEMED)	AppliChem, Germany
N,N'-Methylenebisacrylamide	Sigma-Aldrich, USA
NP-40	Roche, Switzerland
Phosphate Saline Buffer (PBS) - Mol. Biology Grade	Gibco, UK
Sodium Chloride (NaCl)	Fisher Scientific, USA
Sodium Dodecyl Sulfate (SDS)	AppliChem, Germany
Tris-Base	AppliChem, Germany
Tris-Cl	AppliChem, Germany

Table 3.2. List of kits and reagents used in this work.

BCA Protein Assay Kit	Pierce, USA
Protein Molecular Weight Marker	PageRuler Prestained Protein Ladder, Fermentas, USA
GeneJET Plasmid Midiperp Kit	Thermo Scientific, USA
High Pure Plasmid Isolation Kit	Roche, Switzerland
ImProm-II Reverse Transcription System	Promega, USA
Light Cycler Fast Start DNA Master SYBR Green I kit	Roche, Switzerland
Dual-Glo Luciferase Assay System	Promega, USA
Western Blotting Luminol Reagent	Santa Cruz, USA

3.2. Western Blotting Buffer and Solutions

Table 3.3. Buffer and solutions used in this work.

4X Protein Loading Dye	200 mM Tris-Cl (pH: 6.8) 8% SDS 40% Glycerol 4% β-mercaptoethanol 50 mM EDTA 0.8% Bromophenol Blue
10X SDS Running Buffer	1% SDS 1.92M Glycine 250 mM Tris-Base
10X Transfer Buffer	1.92M Glycine 250 mM Tris-Base
1X Transfer Buffer	10% 10X Transfer Buffer 20% Methanol
Cell Lysis Buffer	137 mM NaCl 20 mM Tris-Cl (pH: 7.4) 2 mM EDTA 0.2 % NP-40 5 mM NaF
Propodium Iodide Staining Solution	20 μg/ml Propodium Iodide 0.1% Triton X-100dissolved in 1X PBS
TBS-T	50 mM Tris-Base (pH: 7.4) 150 mM NaCl 0.1% Tween 20

3.3. Biological Materials

3.3.1. Plasmids

pcDNA3 (Invitrogen, CA, USA), pJ3H-Mst1 (Addgene, USA) and pJ3M-Mst1 K59R (Addgene, USA) plasmids were commercially obtained and cloned to pcDNA3. FOXO3A in pcDNA3 and 8 × DBE-Luciferase were kindly gifted by Prof. Dr. Jürgen Behrens, University of Erlangen. FOXO ΔC plasmid was generated from FOXO3A in pcDNA3 by deleting the C terminus of FOXO3A. GADD45a in pGL3 plasmid was purchased from (Addgene, USA).

3.3.2. Cell lines

Human Hepatocellular Carcinoma (HCC) derived Huh7 and Hep3B cell lines were kindly provided by Dr. Mehmet Öztürk, Bilkent University.

3.3.3. Cell Culture Reagents

Table 3.4. Cell culture reagents used in this work.

DMEM	HyClone, USA
FBS	HyClone, USA
Penicillin/Streptomycin	HyClone, USA
Phosphate Saline Buffer (PBS) - Cell Culture Grade	HyClone, USA
Trypsin	HyClone, USA
Turbofect	Fermentas, USA
G418, Geneticin	Sigma-Aldrich, USA
Sodium Chloride (NaCl)	Fisher Scientific, USA
Lithium Chloride (LiCl)	Fisher Scientific, USA

3.3.4. Primers

Table 3.5. Primers used in this work.

Primer ID	Sequence	Application
GADD45a_2R	CACAACACCACGTTATCGGG	Q-RT-PCR
GADD45a_1F	GAGAGCAGAAGACCGAAAGGA	Q-RT-PCR
18S_1F	CTGAAACTTAAAGGAATTGACGGA	Q-RT-PCR
18S_2R	GTTATCGGAATTAACCAGACAAATC	Q-RT-PCR
GADD45a_F	AAGCTAGCAAGCTTAGGGCATATCG	Promoter Cloning
GADD45a_R	AACTCGAGCCTCCCAGCCACTGCCT	Promoter Cloning

3.4. Antibodies

Table 3.6. Antibodies used in western blotting.

Name	Species	Dilution	Source
Anti-phospho-Akt(Ser473) (#4060)	Rabbit	1/2000	Cell Signaling
Anti-phospho-Akt(Thr308) (#2965)	Rabbit	1/1000	Cell Signaling
Anti-Akt (pan) (#4691)	Rabbit	1/1000	Cell Signaling
Anti-GADD45a (#3518)	Rabbit	1/1000	Cell Signaling
Anti-Mst1 (#3682)	Rabbit	1/1000	Cell Signaling

Table 3.6. Antibodies used in western blotting (cont.).

Anti-Phospho-Mst1(Thr183)/(Thr180) (#3681)	Rabbit	1/1000	Cell Signaling
Anti-Phospho-Cyclin B1 (Ser147) (#4131)	Rabbit	1/1000	Cell Signaling
Anti-Cyclin B1 (#4138)	Rabbit	1/1000	Cell Signaling
Anti-Phospho-FoxO1 (Thr24)/FoxO3a (Thr32) (#9464)	Rabbit	1/1000	Cell Signaling
Anti-Phospho-FoxO1 (Thr24)/FoxO3a (Thr32)/FoxO4 (Thr28) (#2599)	Rabbit	1/1000	Cell Signaling
Anti-Phospho-FoxO3a (Ser318/321) (#9465)	Rabbit	1/1000	Cell Signaling
Anti-FOXO3 (Ser207)/FOXO1(Ser212) (#1789)	Rabbit	1/1000	Invitrogen
Anti-beta actin (#4970)	Rabbit	1/1000	Cell Signaling

3.5. Disposable Labware

Table 3.7.List of labwaresused in this work.

Cell culture plates (10 cm, 145mm, 6- well, 96- well)	Thermo, USA
Cell scraper	TPP, Switzerland
Cryo tubes	Greiner Bio One, UK
Filtered tips and normal tips	Axygen ,USA
Insulin syringes	Set Medikal, Turkey
Test tubes(1.5 ml, 2ml, 15ml, 50ml)	Axygen ,USA

3.6. Equipments

Table 3.8. List of equipments used in this work.

Autoclave	Midas 55,Prior Clave, UK
Carbon dioxide Tank	2091, Habaş, Turkey
Cell Culture Incubator	Hepa Class 100, Thermo, USA
Centrifuges	5415R, Eppendorf, USA
	Allegra X-22, Beckman Coulter, USA
Deepfreezers	−20°C, Arçelik, Turkey
	−80°C ULT Freezer, ThermoForma, USA
Flow Cytometer	FACSCalibur , Becton Dickinson, USA
Heat Blocks	DRI-Block DB-2A, Techne, UK
Hemocytometer	Improved Neubauer, Weber Scientific International Ltd. UK
Laminal Flow Cabinet	Labcaire BH18, UK
Luminometer	Fluoroskan Ascent FL, Thermo Electron, USA

Table 3.8. List of equipments used in this work (cont.).

Micropipettes	Finnpipette, Thermo, USA
Microplate Reader	680, Biorad, USA
Microscopes	Inverted Microscope, CKX41, Olympus, JAPAN
	Fluoroscence Microscope, Observer.Z1, Zeiss, Germany
PCR Machine	Gene Amp. PCR System 2700, Applied Biosystems, USA
pH meter	WTW, GERMANY
Pipettor	Pipetus-akku, Hirschmann Labogerate, GERMANY
Power Supply	Biorad, USA
Real Time PCR	LightCycler 1.5, Roche, SWITZERLAND
Refrigerators	2082C, Arçelik, TURKEY 4030T, Arçelik, TURKEY
SDS Gel Electrophoresis	Biorad, USA
Shaker	VIB Orbital Shaker, InterMed, DENMARK
Spectrophotometer	NanoDrop 1000, USA
Stella	Raytest, Germany
Vortex	Vortexmixer VM20, Chiltern Scientific, UK
Water purification	WA-TECH Ultra-Pure Water Purification System, GERMANY

4. METHODS

4.1. SDS/PAGE and Western Blotting

4.1.1. Cell Lysis and Protein Extraction

To extract total protein from the cells, cells were grown in 10 cm or 145 mm plates and treated either sodium or lithium. Then, medium of the cells were aspired and cells were washed twice with 1X PBS. Ice-cold lysis buffer with 1XPMSF was added onto the cells (about 300µl for 10 cm plate and 800µl for 145 mm plate) and the plate was immediately placed on ice. After 10-15 min, cells were scraped and collected in 1.5 ml micro centrifuge tubes. Tubes were left on ice for one hour for lysis and to prevent protein degradation during the lysis. Then, with insulin syringes, lysates were homogenizedby disrupting genomic DNA. Cell lysates were centrifuged at 14000g for 15 min at 4°C. Supernatant including the proteins were transferred into clean tubes and kept on ice.

4.1.2. BCA Assay

After obtaining protein lysates, protein quantification was performed by BCA Protein Assay Kit (Pierce). BSA standards with different concentrations (125, 250, 500, 750, 1000, 1500 and 2000 µg/ml) was prepared via serial dilution with 1X PBS. For assay, protein samples were diluted (commonly 1/5) with lysis buffer in a clean tube. According to assay protocol, 50 parts Reagent A was mixed 1 parts Reagent B and 200 µl of the mixture was added to 96 -well plate which was sitting on ice. Next, 5 µl of the dilutions (triplicate) and BSA standards (duplicate) were added onto each well containing BCA mixture and then plate was incubated at 37°C for 30 min after shaking. After incubation, the absorbance of samples was measured at 562 nm on the plate reader. To calculate the concentration of samples, a standard curve was prepared by plotting blank corrected BSA measurements vs. their corresponding concentrations in µg/ml. By using this standard curve, concentration of each sample was calculated according to average absorbance.

4.1.3. Preparation of Cell Lysates for Western Blotting

To load equal amount of protein to wells, concentrations of each protein were adjusted according to the lowest amount of protein sample by addition of cell lysis buffer. 4X Protein Loading Dye was added into each sample and denatured at 95°C for 5 min.

4.1.4. SDS/PAGE

SDS-PAGE gels were cast, run and transferred using Mini-Protean Tetra cell and Mini Trans-blot cell (BioRad). In this study, 10 % stacking gels (with 37.5:1, acrylamide: bis-acrylamide ratio) were cast first,isoproponal was added on top of the gel to remove the bubbles and make the top of the gel linear. After 30 min of polymerization, isoproponal was removed and gels were washed with distill water and dried. On the top of the stacking gel, 5 % stacker gel (with 37.5:1, acrylamide:bis-acrylamide ratio) was cast and left for polymerization about 30 min. SDS/PAGE gel was prepared with the reagent as indicated below.

Table 4.1. Contents of SDS-PAGE gels.

	Resolving Gel	Stacking Gel
ddH_2O	3.65 ml	1.825 ml
1M Tris-Cl (pH:6.8)	-	313 µl
1.5M Tris-Cl (pH:8.8)	2.25 ml	-
Acrylamide:bis-acrylamide (30%/0.8% w/v)	3 ml	335 µl
SDS (20% w/v)	45 µl	13 µl
APS (10% w/v)	56.5 µl	21 µl
TEMED	13.5µl	6 µl

4.1.5. Western Blotting

Samples were heated at 95ºC for 5 min, vortexed, centrifuged and loaded into the wells on the stacker gel. About 30-35 µg protein (it can be more or less based on the antibody efficiency) and 4 µl of protein ladder were loaded to each well. Gels were run with 1X running buffer at 100Vabout 1 hour. After running was completed, proteins were trans-

ferred from gel to PVDF membrane. SDS/PAGE was placed onto transfer cassettes and PVDF membrane was first activated in methanol for 45 seconds andtransferred into ddH_2O and then placed onto SDS/PAGE. Transfer was done for about 1 hour with 1X transfer buffer including 20% methanol in the cold room. Ice-block supplied with the Trans-blot cell was used routinely during transferring. When transfer was completed, blots were blocked with 5% nonfat dry milk in TBST for 1 hour at RT while shaking at about 100rmp. After blocking, the blots were washed three times with TBST for 5 min each. Primary antibodies were prepared in or 5% BSA in TBST. Blots were incubated with primary antibodies overnight at 4°C. Next day, blots were washed with TBST, three times, for 5 min while shaking. Secondary antibody, HRP-conjugated anti-rabbit IgG was prepared in 5% non-fat dry milk as 1:2000 dilutions. Incubation with secondary antibody was performed at RT for 1 hour. Finally, blots were washed 3 times with TBST. To visualize the proteins on the blots, Luminol Reagent (Santa Cruz) was used by mixing Solution A and Solution B (1:1) as1ml for one blot. Blots were analyzed using Stella Imaging Station (Raytest) and Xstella image acquisition software (Raytest), according to manufacturer's manual.

4.2. Molecular Techniques

4.2.1. Extraction of Total RNA from Tissue Culture Cells

To obtain total RNA from the cells, cells in 6-wellplate was washed with 1X PBS and then collected with trypsin. Cells were collected in a micro centrifuge tube and High Pure RNA Isolation Kit (Roche) was used according to manufacturer's protocol.

4.2.2. Reverse Transcription and cDNA Synthesis

After RNA isolation, reverse transcription and cDNA synthesis was done by Promega, ImProm-II Reverse Transcription System according to manufacturer's protocol. 1 µg total RNA was used as starting material and it was incubated with 1 µl of oligo-dT primersat 70°C in total 5 µl of mixture completed by nuclease free water. Then, the protocol was applied without any changes.

4.2.3. Real Time PCR

Real Time PCR was done with the Light Cycler Fast Start DNA Master SYBRGreen I kit (Roche; Basel, Switzerland) according to the manufacturer's protocol. In order to calculate the efficiency of the PCR, standard curveswere plotted for each primer pairs and cDNAs. Results were analyzed with Light Cycler 4.0 Analysis Software (Roche; Basel, Switzerland). PCR reaction was started with initial denaturation at 95°C for 10 min. Amplification cycles continued witha denaturation step at 95°C for10 seconds, an annealing step at 57°C for 5 seconds and an elongation step at 72°C for 10 seconds. It was repeated for 45 cycles and finished by a melting curve.

4.2.4. Luciferase Assay

In order to determine any change in promoter activity, luciferase assay was performed according to Dual-Glo Luciferase Assay System (Promega) protocol. For the assay, cells were co-transfected with 3 μg (per well of a 6-well plate) plasmid of a pGL3-promoter plasmid or FOXO 8XDBE and200ng of Renilla luciferase (internal control). About 48hours post-transfection, cells were collected by trypsin, and then washed with 1X PBS. Pellet was completely resuspended in 100μl 1X PBS and put into 96-well plate. Then, 100μlof Firefly luciferasesubstrate reagent added on to the lysates, and after 10min incubation at room temperature in the dark,measurements were taken using Fluoroskan Ascent FL (Thermo Electron). Next, 100 μl ofRenilla luciferase substrate reagent (Stop&GloTM) that also quenches the Firefly luciferaseluminescence was added and after 10min incubation at room temperature in the dark, measurements were taken. Luminescence reads were taken 1-5 seconds. Firefly luciferase readswere normalized to Renilla luciferase reads and graphs were plotted in Microsoft Excel.

4.2.5. Cell Cycle Analysis

Cells from 10 cm plate were washed with 1X PBS, trypsinized and then collected into 15 ml falcon tubes. In order to remove trypsin, cells were centrifuged for 5 min at 2000rpm. Supernatant was aspired and cells in the pellet were resuspended with growth medium and washed with 1X PBS. Again, cells were centrifuged and resuspended in 100 μl PBS. For fixation, 5 ml of 70% ice-cold EtOH was added onto cells centrifugedfor 5 min at 2500rpm and washed with PBS and centrifuged again to remove ethanol.For staining, 1 ml of PI stain including 200 μg/ml of RNAse A was added onto cells and tubes were covered with aluminum foil to prevent direct light exposure. Cells were incubated with PI

at 37°C for 45 min and then washed with 1X PBS and centrifugedfor 5 min at 2500rpm. Finally, cells were transferred into flow cytometer tubes and FACS analysis was performed.

4.3. Cell Culture Techniques

4.3.1. Growth Conditions of Cells and Handling

Cell lines used in cell culture were grown in DMEM containing High Glucose, 10% FBS and 1% penicillin/streptomycin (complete DMEM) in an incubator at 37ºC, with 5% CO_2 and 95%air. Growth medium was kept at 4°C and warmed to 37°C in a heater before use. Prior to each use, all materials and containers were wiped with 70%EtOH to prevent any contamination.

4.3.2. Passaging

Cells were passaged routinely when they reach to about 90% confluency. For passaging, the growth medium of the cells was first removed and cells were washed once with 1X PBS. In order to remove the cells from plate surface, trypsin-EDTA solution (0.025% trypsin, 0.5mM EDTA) were added onto the cells and incubated at 37°C for 3-5 min. Next, about 4 volumes of growth medium were added to inactivate trypsin. The suspension was collected in a 15ml falcon tube and centrifuged at 1600 rpm for 4 min. Supernatant was discarded and the pellet was resuspended in 4mlmedium via gently vortex. 1/5 of the cells were transferred to fresh Petri dishes based on the experiments.

4.3.3. Cryopreservation

In order to keep the cells for years, trypsinized cells were resuspended with growth medium including 10% DMSO and split into screw capped-cyrotubes. Confluent 10 cm plates were split into each 4 tubes containing 1 ml of cells solution. Cyrotubes were placed into NALGENE Cyro 1°C Freezing Container which was filled with isopropanol, placed into -80°C freezer. This container allows cells to freeze -1°C/min. Next day, tubes were transferred to -150°C freezer.

4.3.4. Thawing

Frozen stock cells (one or two vials) were taken from freezer and placed intoincubator at 37°C. As soon as cells were thawed, they were transferred into a 15 ml tube and 5 ml of growth medium was added. Cells were centrifuged at 1600 rpm for 4 min. The pellet was resuspended with growth medium and seed into a Petri dish.

4.3.5. Transient Transfection of Cells

Transient transfection of the cells was done in 6 well plates by using*in vitro* Turbofect (Fermentas) reagent. The amount of plasmid and corresponding amount of transfectionreagent was determined according to the manufacturer's protocol. Cells wereseeded one the day before the transfection to obtain 50-60% confluency at the day oftransfection. Next day, the medium was exchanged with certain amount of growth medium and transfection mix was added on to cell drop wise and incubated at37°C. After 4-5hours, transfection media were replaced with fresh growth medium and keep in incubator until the day of experiment.

4.3.6. Generation of Stable Cell Lines

For the stable transfection, cells were seeded to 10 cm plates one the day before the transfection to obtain 50-60% confluency at the day of transfection. Next day, the medium of the cell were replaced with fresh medium, again, the amount of plasmid and corresponding amount of transfectionreagent was determined according to the manufacturer's protocol and added onto cells. After4-5hours, transfection media were changed with fresh growth medium. Next day, the medium was changed with DMEM that includes low glucose, 2% FBS and 1% penicillin/streptomycin and the antibiotics that is specific for the used plasmid. It was 2% G418 (Geneticin). The amount of the G418 was determined by controlling the selection rate. Selection continued during one week. If the transfected plasmid was integrated to the host genome, the cells could stay alive in the presence of antibiotic G418. Next, viable cells were trypsinized and seeded to a new Petri dish with normal growth medium including 1% G418. Whenever it was necessary, cell were passaged and the generation of stable cell continued about 3 weeks.

4.3.7. Lithium Treatment Assay

Cells were seeded into 10 cm culture dishes or 6-well plates based on the experiments. Following day, cells were treated with the indicated concentrations oflithium chloride (LiCl)or sodium chloride (NaCl) to a final concentrationof 25 mM. If the transfected cells were treated, treatment was performed 6 hours post transfection. For the analysis, cells were collected with trypsinafter addition of lithium and sodium as indicated times in the result part.

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5. RESULTS

5.1. Confirmation of Lithium Growth Inhibitory Effect on HCC

5.1.1. Visualizing Cells under Light Microscope

It was previously reported that lithium inhibits the proliferation of hepatocellular carcinoma cells by inducing G2/M arrest. In order to confirm the growth inhibitory effect of lithium on Huh7, cells were seeded into 10 cm plate with the same confluency. Then, the following day, they were incubated with either 25mM NaCl (control) or 25mM LiCl for 48 hours. For this study, the concentration of lithium was determined according to previous reports. After 48h, cells were visualized under the lightmicroscope. The result of this cell growth experiment was shown that lithium is an efficient growth inhibitor of Huh7 cells (Figure 5.1).

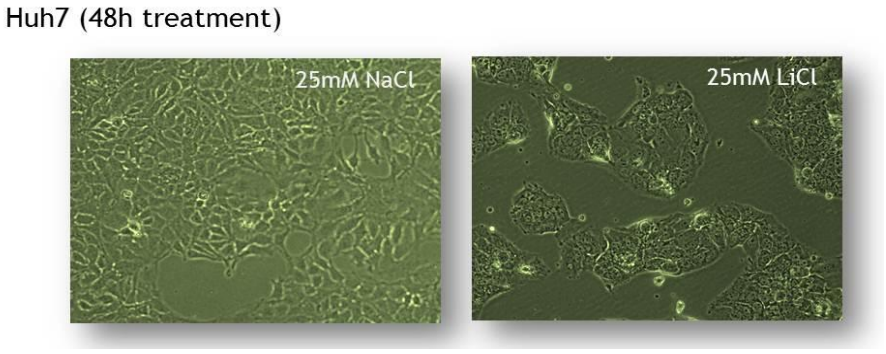

Figure 5.1. Lithium inhibits the proliferation of Huh7 cells.

5.1.2. Cell Cycle Analysis

To further study growth inhibitory effects of lithium on Huh7, FACS analysis was performed. FACS analysis alsoallowed validating which phase of the cell cycle was affected after lithium treatment. For the analysis, Huh7 cells were incubated either withNaCl or LiCl for 48hours, fixed, stained with PI including RNAse, subjected to flow cytometer and cell cycle profiles were visualized. Flow cytometric analysis distributes cells according

to DNA contents and result showed that lithium treated cells accumulatesat G2/M phase of the cell cycle (Figure 5.2).

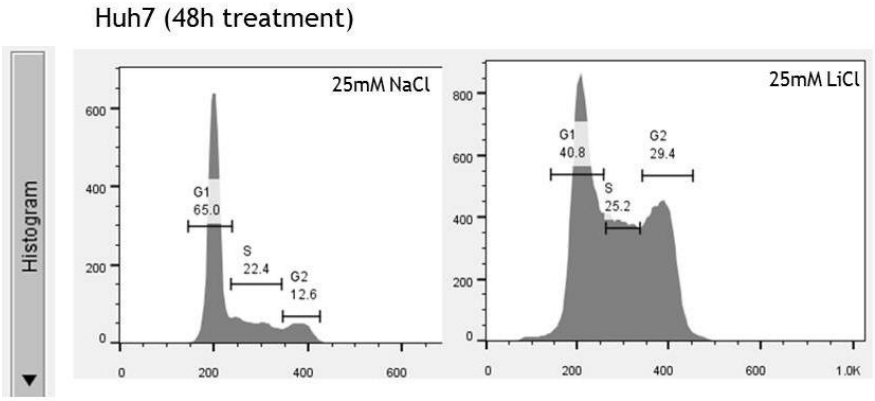

Figure 5.2. Lithium induced G2/M cell cycle arrest in Huh7. Asynchronous cells were treated with NaCl or LiCl and cell cycle profiles were monitored by flow cytometric analysis of DNA content.

5.1.3. Reversible Effect of Lithium Chloride

It was confirmed that if there is lithium in the medium of the cells, cells stop or decrease the proliferation. In order to examine whether lithiumtreated cells can resume growth following lithium removal, cells were seeded with same confluency and treatment was performed in the following day.Two groups of the cells were treated only sodium or lithium as a control and the lithium in the testing groups were replaced with sodium at different time points(Figure 5.3).

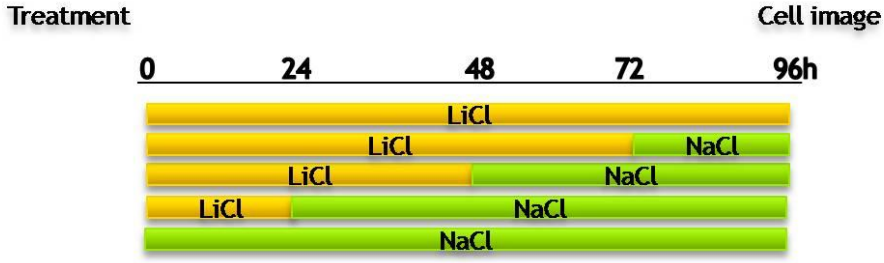

Figure 5.3. Experimental setup for the testing the effect of lithium recovery. “0” indicates when treatment start, “24, 48, 72” are time points when lithium in the medium was replaced with sodium and “96” indicates the time when images were taken.

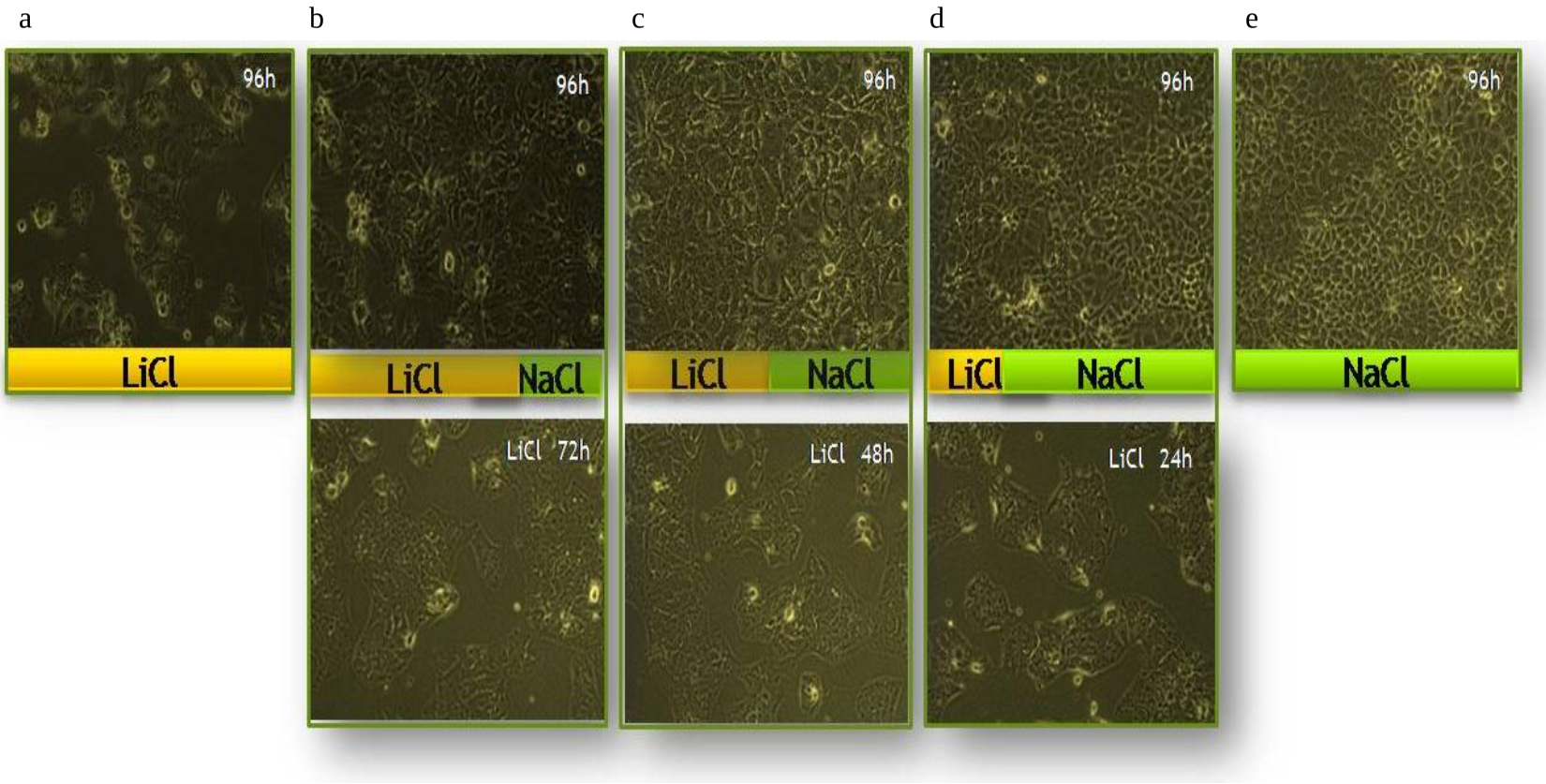

Figure 5.4. Growth inhibitory effect of lithium chloride is reversible. Light microscope images (x10 magnification) of live Huh7 for 96 hours total treatment. (a) Cells were treated for 96 hours with 25 mM LiCl. (b), (c), (d); (lower pictures) cells were first treated with 25 mM LiCl for 72 hours, 48 hours, 24 hours, respectively; (upper pictures) 25 mM LiCl in the medium of the cell were replaced by 25 mM NaCl.

5.2. Analysis of FOXO Activity in Lithium Treated Cells

Lithium induced growth arrest has been observed in different cancer cell lines but its transduction pathway has not been elucidated yet. With this study, the possible role of FOXO transcription factors was sought in lithium treated hepatocellular carcinoma cells. As it was mentioned before FOXO transcription factors were chosen as a candidate since there was an increase in the expression of FOXO target, GADD45a which is an inducer for G2/M arrest, after lithium treatment and it was reported that FOXO regulatory kinase, AKT activity was affected with lithium. Therefore, to investigate the role of FOXO in lithium induced growth arrest, the activity of FOXO transcription factors was tested by both checking the FOXO promoter activity and expression level of FOXO target gene, GADD45a involve in the control of cell cycle progression.

5.2.1. FOXO Promoter Activity

Luciferase reporter assay is a widely employed method for studying promoter activity. In order to analyze FOXO promoter activity in lithium treated cells, cells were seeded to 6-well plate one day before the transfection and next day, transfected with luciferase reporter construct driven by 8 tandem repeats of DAF-16/FOXO binding elements (8 × DBE-Luciferase). Cells were treated with the different concentrationslithium chloride (LiCl) to a final concentration of 25 mM. In all experiments, cells were also co-transfected with internal control plasmid (Renilla) to be used for normalization to eliminate variations in transfection efficiencies and cell number between samples.The experiments were performed using Dual-GloTM Luciferase Assay system (Promega). Luciferase activity was measured after 24 hours of lithium treatment. Cells treated with higher concentrations of LiCl produced higher luminescence signal. There was a significant increase in the FOXO promoter activity with 25 mM lithium addition (Figure 5.5a). Another hepatocellular carcinoma cell line, Hep3B, was affected with similar way for FOXO promoter activity (Figure 5.5b).

To further validate FOXO promoter activity depends on the LiCl, FOXO promoter activity was measured after lithium recovery. For this purpose, two groups of cells were incubated either 25 mM NaCl or LiCl for 48 hours, another group was treated for first 24

hours with LiCl and then, LiCl was replaced with NaCl for another 24 hours. It was observed that the increased in the FOXO promoter activity caused by LiCl was reduced by the removal of LiCl (Figure 5.6). Taken together, these luciferase reporter assays strongly suggest that FOXO promoter is activated by lithium.

a b

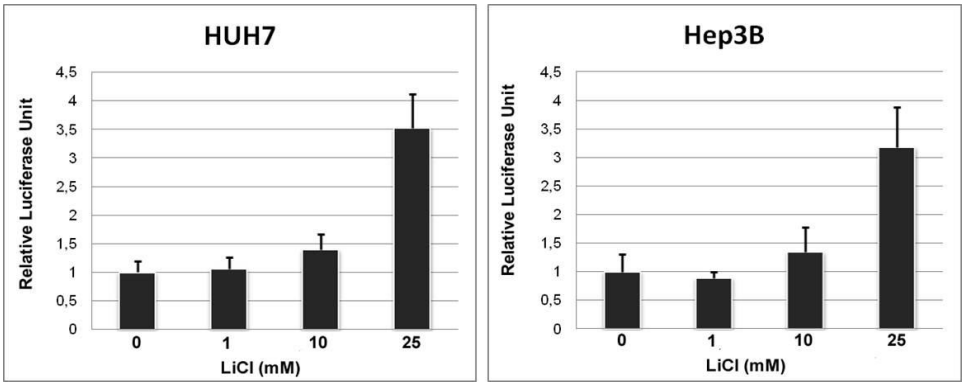

Figure 5.5. Lithium treatment leads to increase in on FOXO promoter activity in (a) Huh7 (b) Hep3B.Luciferase reading was normalized to Renilla luciferase activity used as internal control. Thegraphs indicate the results of at least two independent experiments. Error bars represent standard error.

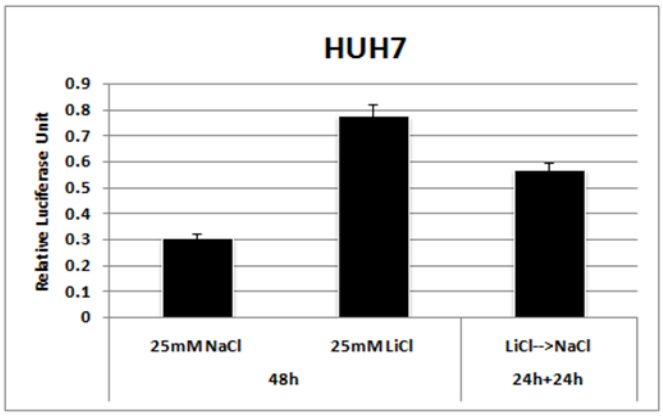

Figure 5.6. Lithium induced FOXO promoter activity decreased after LiCl recovery.Cells were co-transfected with 8 × DBE-Luciferase plasmid and Renilla and luciferase assay was performed 48 hours after treatment. Thegraphs indicate the results of two independent experiments.Error bars represent standard error.

5.2.2. Transcriptional Effect of Lithium on GADD45a

In order to support the hypothesis that lithium activates FOXO transcription factors, the expression of GADD45a, transcriptional target of FOXO, was analyzed. For this purpose, cells were seeded to 6-well plate and following day, the medium was supplemented with different doses of (1-25 mM) LiCl and NaCl. After 48 hours, cells were lysed and GADD45a mRNA level were quantified by Real Time PCR. It was observed that there was a dose dependent increase in GADD45a expression and 25 mM LiCl led to a significant increase in GADD45a expression (Figure 5.7).

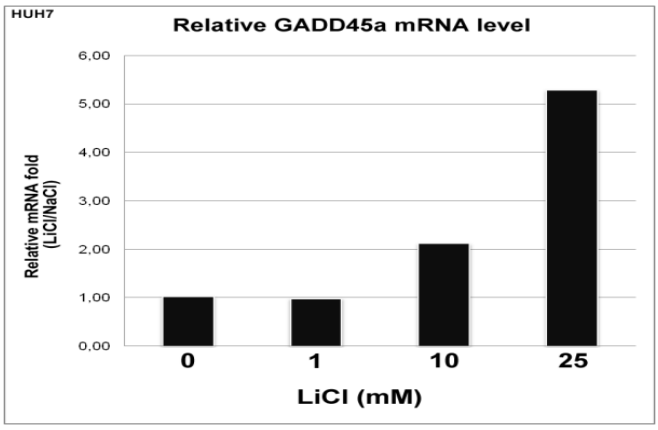

Figure 5.7. Lithium treatment leads to increase in GADD45a mRNA level in Huh7. mRNA levels were analyzed by Real Time PCR.18S was used as internal controls for normalization.

In the next step, the expression of GADD45a was analyzed in a time dependent manner in the presence of 25 mM LiCl. Cells were treated either with 25 mM LiCl or NaCl at the same time points and they were lysed at different time points after treatment. It was observed that the increase in expression of GADD45a was at most for 72 hours (Figure 5.8).

In order to validate lithium dependency in GADD45a expression, expression profile was analyzed after lithium replacement with sodium. The increase in lithiuminduced GADD45a expression was decreased after lithium removal (Figure 5.9). These results suggest that GADD45a as a check point regulator could have role in lithium induced G2/M arrest.

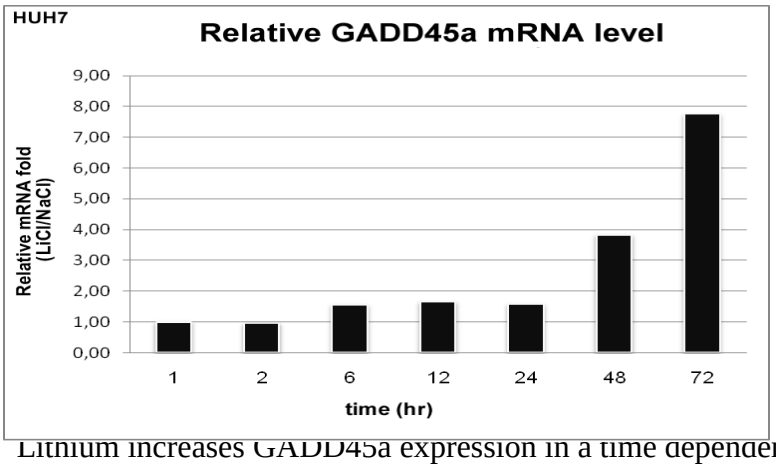

Figure 5.8. Lithium increases GADD45a expression in a time dependent manner. Relative GADD45a mRNA level was calculated by normalizing both GADD45a mRNA level in lithium treated cells to sodium treated cells and 18S mRNA levels.

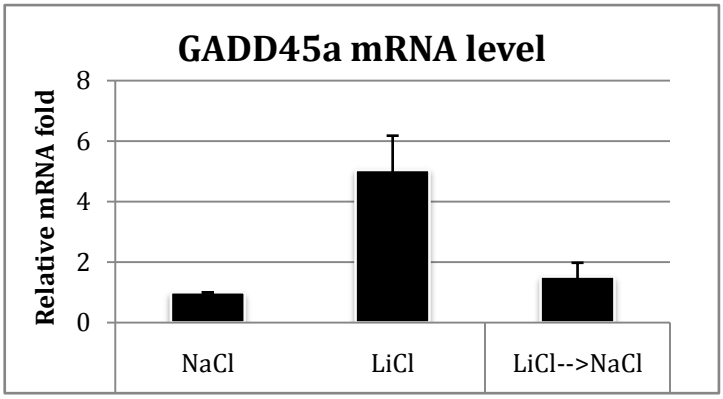

Figure 5.9. Lithium induced GADD45a expression was decreased after lithium recovery. Cells were treated for 48 hours with either 25 mM LiCl or NaCl, another group was treated for 24 hours with LiCl and then, LiCl was replaced with NaCl for another 24 hours and mRNA levels were analyzed by Real Time PCR. 18S was used as internal control for normalization.

5.3. Analysis of Protein Levels and Regulatory Kinases of FOXO

In the cells, the activity of FOXO transcription factors is regulated by different pathways including the phosphorylation of FOXO. Phosphorylation of FOXO with different proteins changes its activity. It means if FOXO proteins are phosphorylated by AKT, they are inactivated whereas MST add phosphor group to another residue that ac-

tivates FOXO. Previously, it was reported that AKT is inactivated by lithium treatment. Therefore, to investigate the mechanismswhether lithium exerts its effect on FOXO activity through AKT, the phospho-FOXO levels in the treated cells along with the protein level of kinases that have role in FOXO regulation was checked by Western Blot analysis.

5.3.1. Phospho-FOXO Levels

In order to control phospho-FOXO levels in the cells, they were treated with 25 mM lithium in a time dependent manner and also with sodium as a control. At different time points (0, 1, 3, 6, 12, 24 hours), cells were prepared for western blotting. About 35 µg of protein were used from each sample. Protein bands were visualized by anti p-FOXOs that detected FOXO proteins specifically phosphorylated by AKT proteins. If there is a decrease in AKT activity after lithium treatment, it was expected to observe a decrease in FOXO phosphorylation. However,there was a time dependent increase in p-FOXO levels, and an observable difference in p-FOXO levels when compared to sodium for 24hours(Figure 5.10).

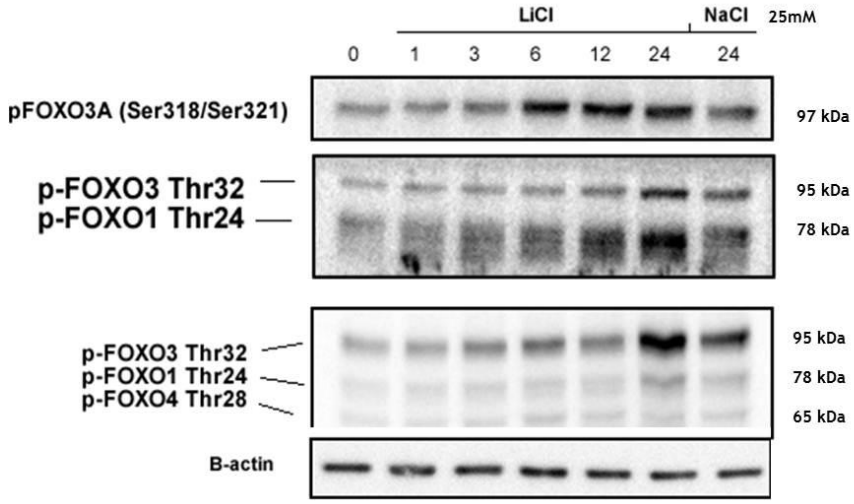

Figure 5.10. Lithium treatment leads to AKT dependent FOXO phosphorylation. Western Blotting using polyclonal p-FOXO antibodies and anti-beta actin antibodies as internal control. Each image is a representative of at least two independent experiments.

5.3.2. Total AKT and phospho-AKT Level

The increase in FOXO levels phosphorylated by AKT after lithium treatment is an indicator of AKT activation and this result was not consistent with the previous report.

Therefore, the activity of AKT protein was investigated again at the protein level. To understand the activity of AKT, two different phospho states, (Ser473, Thr308)which was necessary for its kinase activity was analyzed. For this purpose, cells were treated with different concentrations (0, 1, 10, 25 mM) of lithium for 24 hours along with 25mM lithium with different time points. Contrary to the previous report, there was an increase in AKT activity in the presence of 25mM LiCl. In addition, there was a time-dependent increase activity and a significant difference when the bands of LiCl and NaCl treated cells for 24 hours were compared (Figure 5.11).

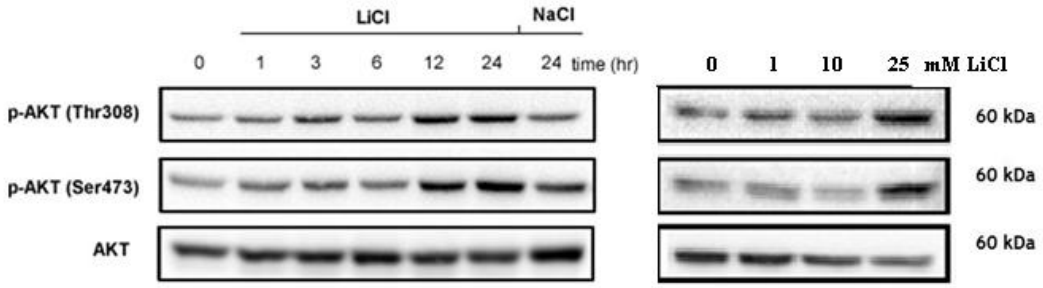

Figure 5.11. Lithium treatment leads to AKT activation. Western Blotting using polyclonal p-AKT antibodies and AKT antibodies as activation control. Each image is a representative of at least two independent experiments.

5.3.3. Total MST and phospho-MST Levels

The increase in FOXO activity after lithium treatment could not be AKT dependent since the increased in AKT activity was not correlated with increased FOXO activity caused by lithium because AKT protein inactivates FOXO by adding phosphor groups. Therefore, effects of lithium on another FOXO regulatory kinase, MST, were investigated by Western blotting. The activity of MST protein was investigated at the protein level by specific antibodies that recognize active form of MST (p-MST). Before analysis, cells were treated with 25 mM lithium with different time point. After lithium treatment, it was observed that there was an increase in MST activation level (p-MST) compared to sodium (Figure 5.12).The effect of lithium on MST activation was confirmed after lithium recovery. It was observed that lithium induced MST activation decreased with removal of lithium (Figure 5.13).

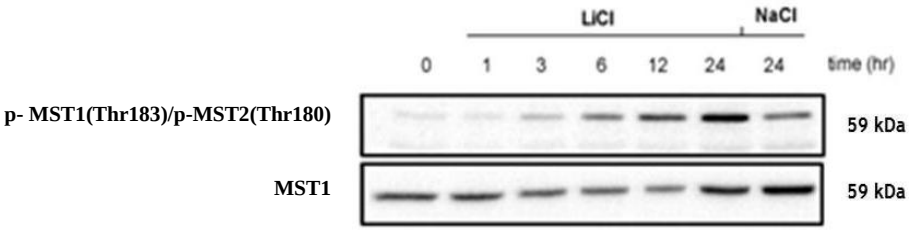

Figure 5.12. Lithium treatment leads to MST activation.Proteins were visualized by polyclonal p-MST1 and MST1 antibodies. Each image is a representative of at least two independent experiments.

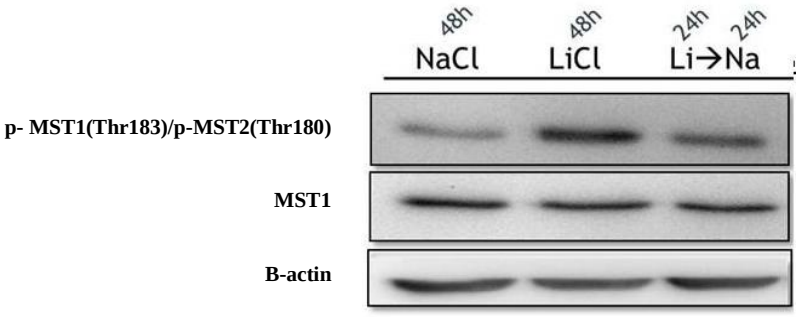

Figure 5.13. Lithium removal leads to decrease in MST activation. Cells were treated with either 25mM LiCl or NaCl for 48 hours and another group was treated for 24 hours with LiCl and then LiCl was replaced with NaCl for another 24 hours.

5.4. Analysis of FOXO Dependency in Lithium Treated Cells

In order to determine whether the effect of lithium on cell proliferation depends on FOXO activity, FOXO3A and FOXO ΔC (deletion from C-terminus of FOXO3A) overexpressing stable cell lines were generated.Since C-terminus of FOXO contains DNA binding domain, the form of FOXO ΔC cannot bind to DNA and it was expectedFOXO ΔC to decrease FOXO dependent transcription. Huh7 cells were transfected with either FOXO3A or FOXO ΔC in pcDNA3 and empty pcDNA3 as a transfection control. Huh7 stable clones overexpressing FOXO3A full-length and FOXO ΔC compared with the control vector was confirmed by Western Blotting (Figure 5.14).After generating of stable cell lines, FOXO dependency in lithium effect was investigated by analyzing GADD45a expression level, GADD45a promoter activity along with the cell cycle analysis.

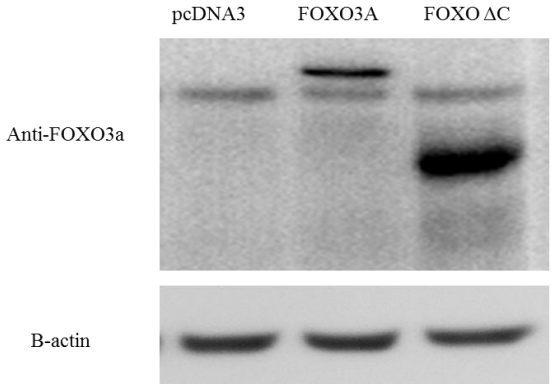

Figure 5.14. Confirmation of stable overexpression of FOXO3A and FOXO ΔC (denoted by “FOXO 3A” and "FOXO ΔC", respectively) in Huh7 cells. Whole cell lysates were analyzed by Western Blotting using polyclonal FOXO3Aantibody and anti-beta-actin antibody as internal control.

5.4.1. GADD45a Promoter Activity

In order to test FOXO dependency in lithium actions, stable cell lines, overexpressing FOXO3A, FOXO ΔC and control cell line were seeded to 6-well plate. The following day, each group of cells was co-transfected with GADD45a in pGL3 and internal control Renilla.About 6 hours post-transfection, cells were treated either 25 mM NaCl or LiCl and 48 hours after treatment, luciferase activity was measured. It was observed that lithium induced GADD45a promoter activity was enhanced by FOXO3A overexpression. However, the deleted version of FOXO ΔC was not able to decrease the lithium induced GADD45a promoter activity (Figure 5.15).

5.4.2. GADD45a Transcription Level

To further validate FOXO dependency in lithium actions, Huh7 cells stably expressing FOXO3A, FOXO ΔC or empty vector (pcDNA3) were established and treated either 25 mM NaCl or LiCl for 48 hours. Cells were lysed and GADD45a mRNA level was quantified by Real Time PCR. It was observed that lithium induced GADD45a expression further increased in the presence of stably expressed FOXO3A. However, the deleted version of FOXO ΔC was not able to decrease the lithium induced GADD45a expression (Figure 5.16).

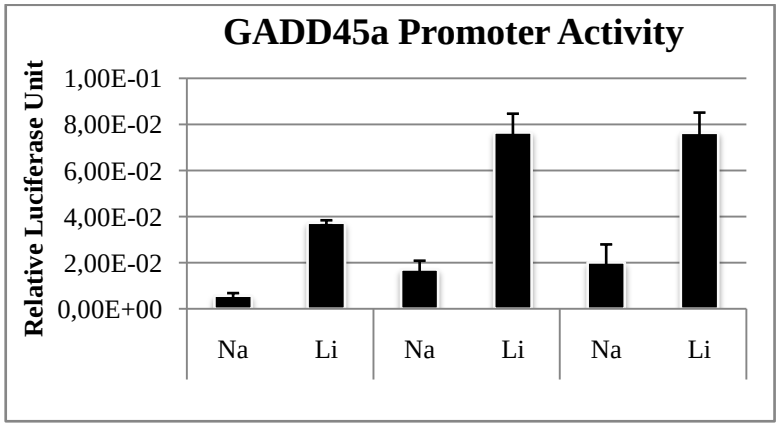

Figure 5.15. Stably expressed FOXO3A increases lithium induced GADD45a promoter activity. The graphs indicate the results of two independent experiments. Error bars represent standard error.

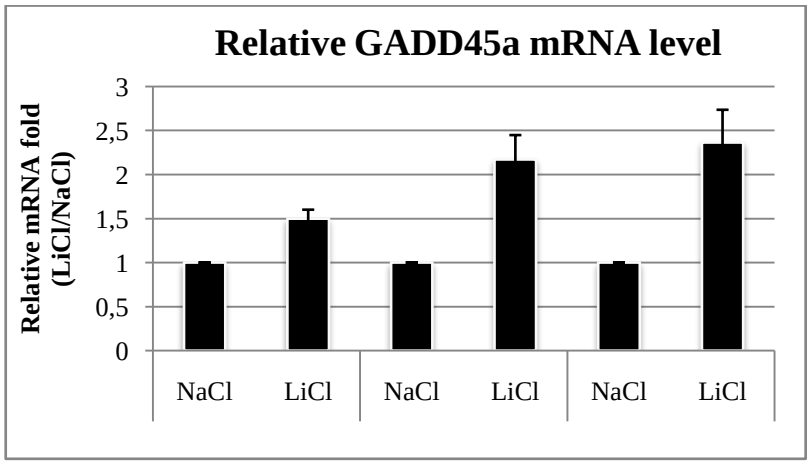

Figure 5.16. Stably expressed FOXO3A increases lithium induced GADD45a expression.18S was used as internal controls for normalization. The graphs indicate theresults of two independent experiments. Error bars represent standard error.

5.4.3. Cell Cycle Analysis

To characterize the role of FOXO in lithium induced G2/M arrest, cell cycle profiles of Huh7 cells stably expressing FOXO3A, FOXO ΔC or empty vector (control) was monitored by flow cytometry. Cell cycle analysis was performed as indicated in Section 4.2.5. As shown in Figure 5.17A, a 48 hours exposure of cells to 25 mM LiCl resulted in an increase in G2/M phase cell. Upregulation of FOXO slightly amplified the lithium induced G2/M arrest compared to control (from 32.5% to 42%) but downregulation of

FOXO could not weaken the effect of basal FOXO (Figure 5.17).These results support the hypothesis that FOXO may have role in G2/M arrest induced by lithium.

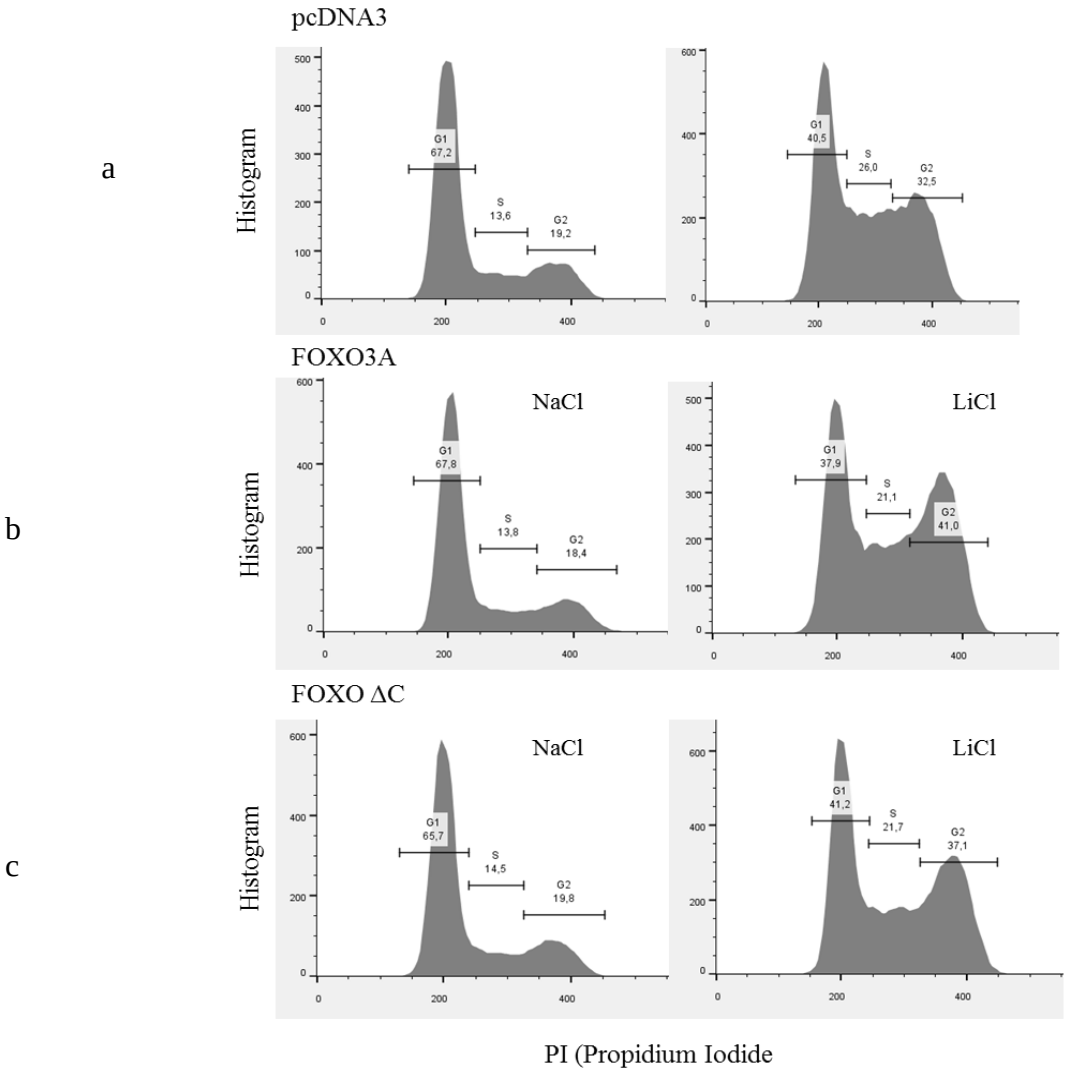

Figure 5.17. The effect of lithium on cell cycle was partially increased by FOXO upregulation.(a) Cell cycle profile of control cell line (b) Cell cycle profile of FOXO overexpressing stable cell line (c) Cell cycle profile of FOXO ΔC stable cell line.

Table 5.1. The percentage of cells in each phase of cell cycle in Figure 5.17.

Stable cells overexpressing	pcDNA3		FOXO3A		FOXO ΔC	
Treatment	NaCl	LiCl	NaCl	LiCl	NaCl	LiCl
G1 (%)	67.2	40.5	67.8	37.9	65.7	41.2
S (%)	13.6	26	13.8	21.1	14.5	21.7
G2/M (%)	19.2	32.5	18.4	41	19.8	37.1

5.5. Analysis of MST Dependency in Lithium Treated Cells

In the previous results, it was observed that upregulation of FOXO furtherincreased the effect of lithium action on Huh7 cells. In addition, an increase in the activity of MST protein was reported after lithium treatment. In order to investigate whether the possible role of FOXO in lithium actions depend on MST protein, stable cell lines were generated to sustained expression of MST and MSTkinase mutant “MST-K59R”. Again, pcDNA3, which is anempty vector,was used as a control. The confirmation of overexpression of MST and MST-K59R was seen in Figure 5.18.

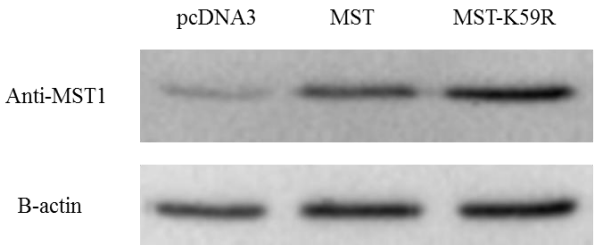

Figure 5.18. Confirmation of stable overexpression of MST and MST-K59R (denoted by“MST” and “MST-K59R”, respectively) in Huh7 cells. Whole cell lysates were analyzedby Western Blotting using polyclonal MST1 anti-B-actin as internal control.

5.5.1. FOXO Promoter Activity

To investigate the role of MST in FOXO activity induced by the lithium, FOXO promoter activity was analyzed at first step. Huh7 cells stably expressing normal and mutant form of MSTwere seeded to 6-well plate and next day, transfected with FOXO luciferase reporter construct (8 × DBE-Luciferase). Cells were treated with25mM NaCl or LiCl and luciferase activity was measured after 48 hours. The increase inFOXO promoter activity with lithium was 2-fold increased after MSTupregulation. It was seen that MST plays role in FOXO activity but the overexpression of kinase dead mutant form of MST could not abrogate the lithium induced FOXO promoter activity (Figure 5.19)

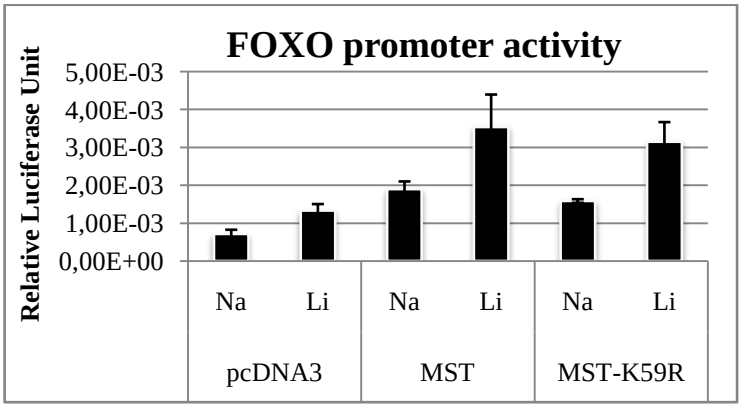

Figure 5.19.Stably expressed MST increases lithium induced FOXO promoter activity. The graphs indicate the results of two independent experiments.
Error bars represent standard error.

5.5.2. GADD45a Promoter Activity

In the next step, to validate the role MST in FOXO activity, GADD45a promoter activity was measured. For analysis, each group of cells was co-transfected with GADD45a in pGL3 and internal control Renilla. Cells were treated either 25 mM NaCl or LiCl and 48 hours after treatment, luciferase activity was measured. Sustain expression of MST further increased the effect of lithium on GADD45a promoter activity; however, the mutant MST for its kinase activity could not abrogate and even increased the effect of lithium on GADD45a promoter activity (Figure 5.20).

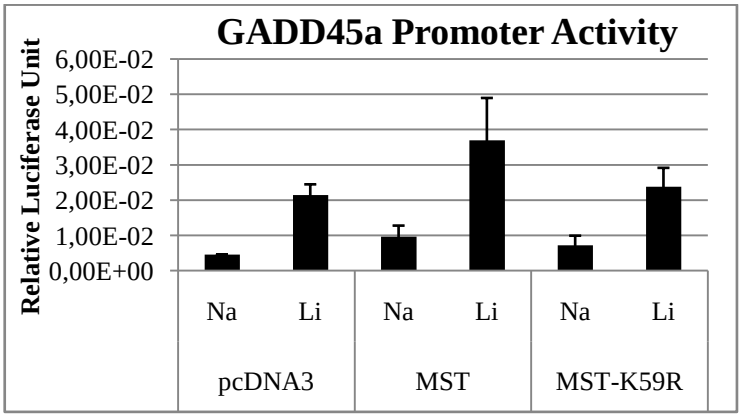

Figure 5.20. Stably expressed MST increases lithium induced GADD45a promoter activity. The graphs indicate the results of two independent experiments. Error bars represent standard error.

5.5.3. GADD45a Expression Level

To further validate the role MST in FOXO activity, GADD45a expression level was measured. Stable cell lines were incubated with 25 mM NaCl or LiCl and after 48 hours, total RNA was isolated and by Real Time PCR, GADD45a mRNA level was measured. It was seen that lithium induced GADD45a expression enhanced in the presence of stably expressed MST. However, the mutant MST for its kinase activity could not weaken the effect of lithium on GADD45a expression (Figure 5.21).

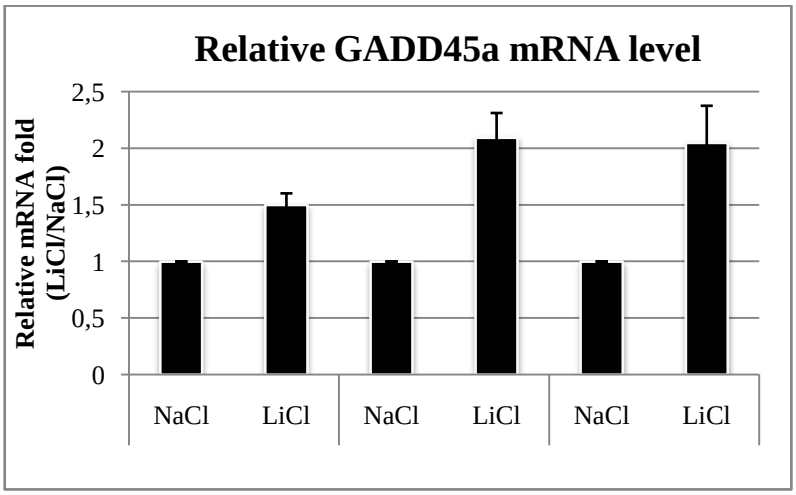

Figure 5.21. Stably expressed MST increases lithium induced GADD45a expression. The graphs indicate the results of two independent experiments.
Error bars represent standard error.

5.5.4. Cell Cycle Analysis

In the last step, cell cycle analysis was performed to confirm the role of MST in lithium induced G2/M arrest. Huh7 cells stably expressing MST, MST-K59R or empty vector (control) were incubated with either sodium or lithium for 48 hours and then subjected to flow cytometer. As in Figure 5.23, the increase in the G2/M phase cells by lithium further increased after MST upregulation. The overexpression of mutant MST did not block the increase of G2/M phase by lithium (Figure 5.22).

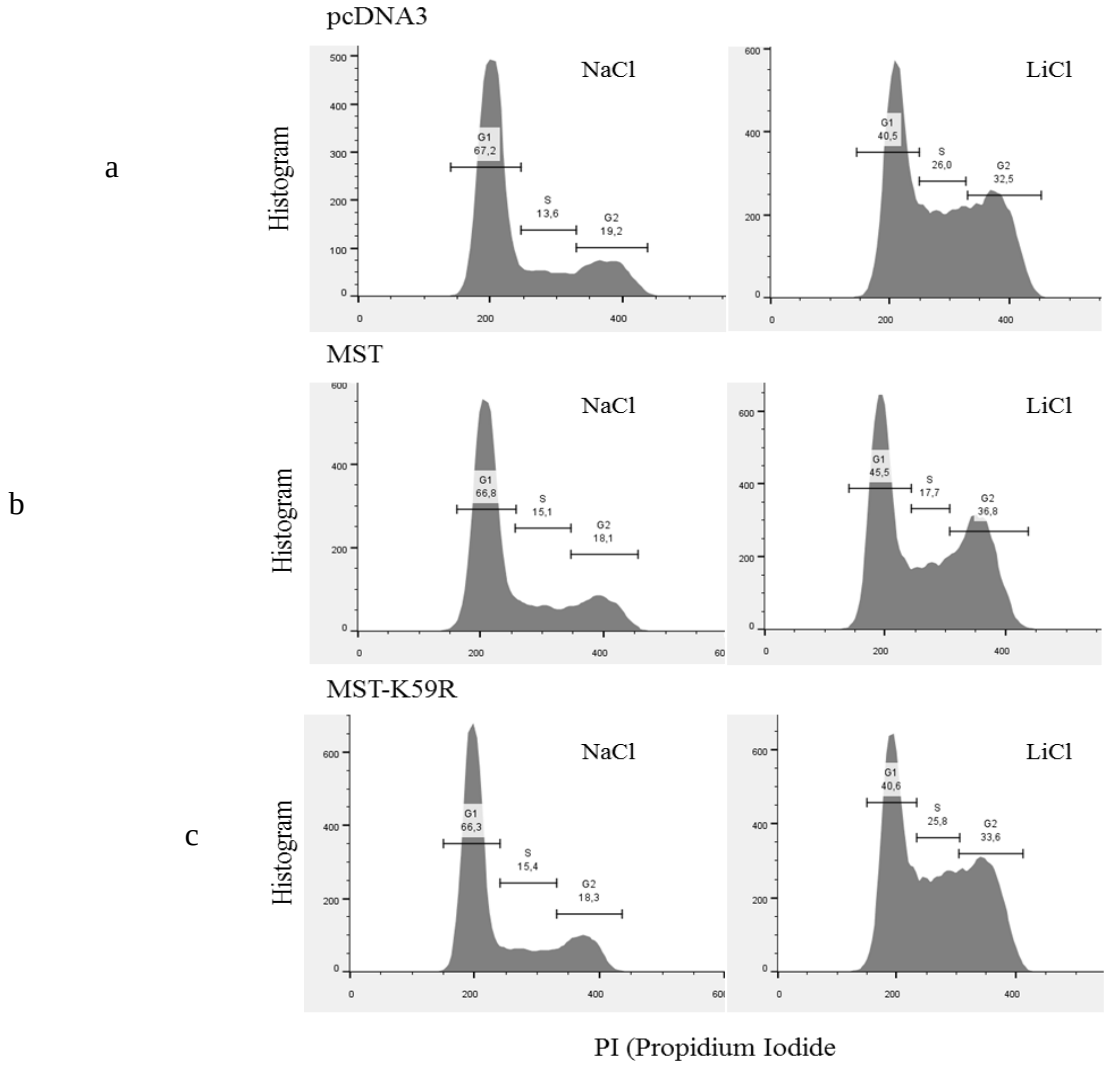

Figure 5.22. Stably expressed MST increases the G2/M phase cells.
(a) Cell cycle profile of control cell line (b) Cell cycle profile of MST overexpressing stable cell line (c) Cell cycle profile of MST-K59R stable cell line.

Table 5.2. The percentage of cells in each phase of cell cycle in Figure 5.18.

Stable cells overexpressing	pcDNA3		MST		MST-K59R	
Treatment	NaCl	LiCl	NaCl	LiCl	NaCl	LiCl
G1 (%)	67.2	40.5	66.8	45.5	66.3	40.6
S (%)	13.6	26	15.1	17.7	15.4	25.8
G2/M (%)	19.2	32.5	18.1	36.8	18.3	33.6

6. DISCUSSION

Lithium is a therapeutic agent used to treat several diseases such as bipolar mood disorders and Parkinson's disease. Although lithium has been widely used for years, its cell specific diverse effects have not been well understood yet. *In vitro* and *in vivo* findings showed that lithium can induce survival and proliferation in neurons. On the other hand, it was reported that lithium can lead to growth arrest and senescence inepithelium-like cells.However, to date, underlying mechanisms and the molecular targets of lithium action have been established neither during development nor during therapy.

The possible protective nature of lithium in human cancer development was also reported recently. It was observed that mental patients with lithium treatment have lower cancer prevalence than the general population. Later studies have shown that lithium salts are also effective for inhibiting development of other cancer cells including hepatocellular carcinoma cell. Different studies on HCC support that lithium inhibits the hepatocellular cell proliferation by inducing G2/M arrest however the underlying molecular mechanisms involving in this is not well explained. With this study, it was aimed to investigate the role of FOXO transcription factors in lithium induced growth arrest in hepatocellular carcinoma cells. The reasons of focusing FOXO transcription factors were evidence from literature and our preliminary data. One of the supporting evidence was the observation of AKT inactivation in lithium treated hepatocellular carcinoma cells according to previous reports. Another was the increased GADD45a expression after lithium treatment via our preliminary data.In addition, increased GADD45a was observed in different studies as an inducer for G2/M arrest, similar to the effect oflithium.Those findings suggest that lithium induced growth arrest may be mediated by FOXO through GADD45a upregulation.

During this study, as a hepatocellular carcinoma cell line, Huh7 cell line was used. The concentration of LiCl was determined according to dose dependent experiment and 25 mM was found as most effective concentration in lithium actions. As a control, NaCl was used since there was no obvious effect on cell growth that was also used as a control in previous reports. For dose dependent experiments, 1 mM, 10 mM and 25 mM LiCl were

used and completed to total 25mM by NaCl and unless specified otherwise, 25mM LiCl for 48 hours was used in experiments because lithium was most effective in those conditions.

At first,the effects of lithium treatment on morphology and growth properties of Huh7 cells were studied. Huh7 cells were incubated with lithium for 48 hours and the morphology of the cells was visualized under the microscope. These cells responded to lithium treatment with the formation of clustered cells. These morphological changes were specific to lithium, as NaCl treated cells had no morphological changes (Figure 5.1). Lithium induced morphological changes were not associated with programmed cell death which was previously reported(Erdal *et al.*, 2005).

To further characterize the inhibitory effect of lithium on cell proliferation, the cell cycle profiles of cells were monitored by flow cytometry.As it was reported before, lithium induced growth arrest by inducing G2/M arrest.25mM LiCl resulted in an increase of G2/M phase cells, which was accompanied by a decrease in G1 phase cells.

In the next step, to find out whether lithium growth inhibitory effect wasreversible, lithium in the medium of the cell were replaced with sodium at different time points. The effect of lithium on cell proliferation was obvious at all time points (24, 48, 72 hours) and recovery of lithium allowed the cells to continue to growth (Figure 5.4). Since cells can regain their proliferation ability after lithium recovery, it can be said that cells did not enter senescence with lithium; it was only temporary growth arrest.

As first step towards the elucidation of the role FOXO in lithium actions on HCC, FOXO promoter activity and expression level of FOXO target gene, GADD45a which has role in cell cycle arrest were analyzed.For Huh7, lithium was able to induce FOXO promoter activity in a dose dependent manner. The similar pattern was observed in another hepatocellular cell line, Hep3B. To confirm whether the increase in FOXO activity depends on the lithium, the promoter activity was measure after lithium recovery and it was observed that the increased in the FOXO activity by lithium was reduced by the removal of lithium. For further characterization of FOXO activity, target expression was measured and the increase in GADD45a expression was confirmed in the presence of lithium. GADD45a

expression was highest with 25mM LiCl for 72 hours. Again, lithium dependency in GADD45a was validated after lithium replacement with sodium. According to these result, it can be speculated that GADD45a as a G2/M check point regulator could have a critical role in lithium induced growth arrest and increased FOXO activity might stimulate the expression of GADD45a.

After determining that possible role of FOXO in lithium actions, the molecular mechanism for FOXO activation was investigated. Since the activity of FOXO transcription factors is regulated by post transcriptional modifications including the phosphorylation of FOXO, the phospho states of FOXO were analyzed by Western Blotting.If FOXO proteins are phosphorylated by AKT, they are kept in cytoplasm as an inactive state, whereas MST add phosphor group to another residue that keeps FOXO in nucleus in its active state.

As a first step for FOXO activation, the p-FOXO levels which were generated by AKT were analyzed at the protein level.As seen in Figure 5.10, there was an increase in the p-FOXOlevels after lithium treatment. Interestingly, the increase in the level of p-FOXO generated by AKT after lithium treatment suggested that AKT was activated by lithium. From that point, the effect of lithium on AKT activity was analyzed at the protein level and the increase in AKT activity was confirmed by increased p-AKT level. To understand the activity of AKT, two different phospho states (Ser473, Thr308) of it, which was necessary for its kinase activity was analyzed. Opposite to the previousreport, there was an increase in AKT activity in the presence of 25 mM LiCl compared to 25 mM NaCl treated cells. These findings suggested that the increase in FOXO activity after lithium treatment could not be AKT dependent. In the next step,effects of lithium on MST activity were investigated by Western blotting. While there was a decrease in total MST level that might be associated with protein synthesis, the MST activity represented by p-MST was increased after lithium treatment (Figure 5.12). The effect of lithium on MST activation was also confirmed after lithium recovery. It was observed that lithium induced MST activation decreased with removal of lithium (Figure 5.13). The effects of lithium induced MST activation on FOXO activity can be verified by an antibody specifically recognize FOXO proteins when phosphorylated at the conserved MST site. According to those results, it can be said that in the presence of lithium, MST might have role in lithium action on cell proliferation through FOXO or directly by GADD45a.

In order to reveal the FOXO dependency in lithium induced growth arrest, stable cell lines were generated that overexpress FOXO3A, mutant form of FOXO (FOXO ΔC) and empty pcDNA3 as a control.Stable cell lines were used to sustained expression of FOXO for longer time and eliminate the differences caused by transfection.At first,in stable cell lines, the overexpression of proteins was confirmed by Western Blotting (Figure 5.14). Then, FOXO dependency in lithium actions was analyzed by GADD45a expression level, GADD45a promoter activity along with the cell cycle analysis. It was seen that lithium induced GADD45a expression further increased in the presence of stably expressed FOXO3A. On the other hand, the mutant form of FOXO was not able to decrease the lithium induced GADD45a expression (Figure 5.16).The similar effects were observed on GADD45a promoter activity. While lithium induced GADD45apromoter activity further increased with stably expressed FOXO3A, FOXO ΔC was not able to decrease the lithium induced GADD45a promoter activity compared to empty pcDNA3 (Figure 5.15). To further characterize FOXO dependency in lithium induced G2/M arrest, cell cycle profiles of stably FOXO3A, FOXO ΔCexpressing cells were analyzed by flow cytometry. Upregulation of FOXO amplified the percent of lithium induced G2/M phase cells compared to control (from 32.5% to 41%); however, downregulation of FOXO could not decrease the arrested cells and slightly increased the G2/M phase cells (from 32.5% to 37.1 %). These results partially support the hypothesis that FOXO may have role in G2/M arrest induced by lithium. In fact, the mutant form of FOXO3A might be compensated by other forms of FOXO in the cells or the deleted version of FOXO might indirectly increase the GADD45a activity.

In the previous results, an increase in the activity of MST protein was observed after lithium treatment. To clarify whether the possible role of FOXO in lithium actions depend on MST activity, stable cell lines were generated to sustained expression of MST and MST kinase mutant “MST-K59R”in which the ATP binding site was mutated. pcDNA3, which is an empty vector, was used as a control. Overexpression of MST and MST-K59R was confirmed at first step (Figure 5.18). Next, MSTdependency was analyzed in lithium actions on cell cycle arrest. As a first step of analysis, FOXO promoter activity was measured andit was seen that stably expression ofMST increased lithium induced FOXO promoter activity about 2-fold (Figure 5.20). To confirm the role MST in FOXO activity,

GADD45a promoter activity was analyzed by luciferase assay and it was observed that stably expressed MST increased the lithium induced GADD45a promoter activity (Figure 5.21). Along with the promoter activity, lithium induced expression level of GADD45a enhanced in the presence of stably expressed MST(Figure 5.22). Sustain expression of MST further increased the effect of lithium however the mutant MST for its kinase activity could not abrogate the effect of lithium. In the last step, MST dependency in lithium induced G2/M arrest was investigated by cell cycle analysis. The upregulation of MST further increased the G2/M phase cells by lithium (from 32.5% to 36.8%); however, the overexpression of kinase-dead MST did not block the increase of G2/M phase by lithium (from 32.5% (control) to 33.6%). These results suggest that MST at least is partially involved in G2/M arrest induced by lithium.Interestingly, the increase of FOXO and GADD45a activity by lithium seems to be independent of the kinase activity of MST because the overexpression of kinase-dead MST could not block the increase in FOXO and GADD45a promoter activity and GADD45a mRNA level. In addition, the increase in G2/M phase cell could not be blocked by kinase dead MST. Therefore, it can be thought that the effect of MST might be independent of MST kinase activity and it behaved like normal MST. In addition, MST protein is a stress related kinase, the activity of it might be increase by overexpression of any protein and so it might not be possible to see the effect of kinase dead MST.

In this study, as a first time, lithium induced GADD45a expression was observed. If the FOXO transcription factors have role in lithium induced growth arrest, whether the increase in GADD45a expression caused by FOXOpromotes this growth arrest was investigated. Stably expression of FOXO partially confirms the FOXO dependency in lithium induced GADD45a activity and growth arrest.

With regard to the previous literature, AKT was considered as a negatively regulated by lithium and conversely, a positive regulation on AKT was observed in this study. In addition, relating to the FOXO activation, MST activation was reported in the presence of lithium in this study. The identification of MST regulation in the presence of lithium will be a first step for the determination of the exact action mechanism of lithium, both as being a novel target of the lithium on HCC and playing an important role in lithium induced growth arrest.MST dependency in lithium actions was investigated by stably ex-

pressing MST and MST kinase dead mutant and the results suggested that lithium induced MST activates FOXO independent of its kinase activity.

To sum up, the results, which are obtained in this study, suggest a possible molecular mechanism for lithium induced growth arrest in Huh7. In this mechanism, lithium induces MST activation and active MST can increases the activity of FOXO transcription factors and finally FOXO increases the GADD45a expression which inhibits G2 to M transition (Figure 5.23).

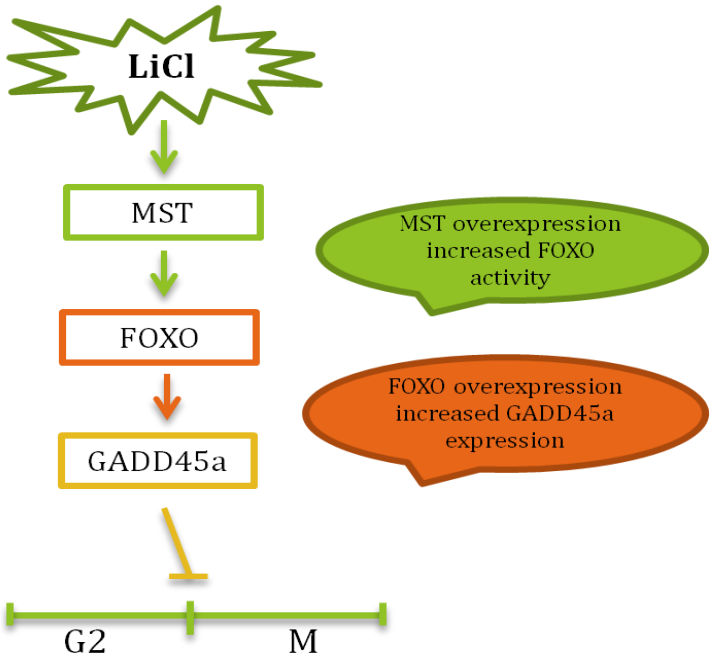

Figure 6.1. Possible mechanism for lithium induced growth arrest in Huh7.

For the future prospects, those potential interactions need to be further investigated and verification should be done accordingly, by means of RNA interference. Since overexpression studies bring many questions and doubts such as overexpression of a protein may allow unspecific events and protein level may reach toxic level,it will be better to examine effect of siRNA or shRNA mediated FOXO knock-down on GADD45a expression and growth arrest. In addition, GADD45a siRNA should be used to confirm GADD45a is key molecule play role lithium induced G2/arrest. For the further characterization of MST and its confirmed activation,it will be better to examine effect of siRNA or shRNA mediated MST knock-down on FOXO,GADD45a expression and growth arrest. For future, it is worth to expand the studies with *in vivo* xenograft experiments on nude mice in order to confirm *in vitro* resultson hepatocellular carcinoma cells.

During this study, we observed that 25 mM is the most effective dose of LiCl which is necessary for growth inhibition in Huh7. However, it was reported that lithium has toxic effect on human metabolism if it exceeds 2 mM in the serum level. If the molecular mechanism is understood in lithium action, the effective dosage of lithium can be reduced by combining other chemicals which have synergetic effects on the same pathway. After reducing the effective dose in normal level, lithium can be used for treatment of liver tumors in future.

In summary, this proposed project is aimed to study the effects of lithium treatment on the hepatocellular carcinoma cells thoroughly at the molecular level by means of various approaches and the molecules being handled. During the evaluation stage, the main features of this molecular mechanism will be depicted and the roles of relevant molecules will be elucidated. The experimental data and findings obtained from this study possess the potential of giving rise to novel strategies in the therapy of various diseases through lithium treatment.

REFERENCES

1. Aghdam, S. Y. and S. W., Barger, "Glycogen Synthase Kinase-3 in Neurodegeneration and Neuroprotection", *Current Alzheimer Research*, Vol. 4, pp. 21–31, 2007.

2. Alessi, D. R., F. B., Caudwell, M., Andjelkovic, B. A., Hemmings and P., Cohen, "Molecular Basis for the Substrate Specificity of Protein Kinase B; Comparison with MAPKAP Kinase-1 and p70 S6 Kinase", *Federation of European Biochemical SocietiesLetters*, Vol. 399, pp. 333-338, 1996.

3. Alvarez, G., JR., Muñoz-Montaño, J., Satrústegui, J., Avila, E., Bogónez and J., Díaz-Nido, "Regulation of Tau Phosphorylation andProtection againstBeta-Amyloid Induced Neurodegeneration by Lithium. Possible implications for Alzheimer's disease", *Bipolar Disorders*, Vol. 4, pp. 153–165, 2002.

4. American Psychiatric Association, "Practice Guideline for the Treatment of Patients with Bipolar Disorder (revision)", *The American Journal of Psychiatry*, Vol. 159, pp. 1−50, 2002.

5. Arden, K.C., "Multiple Roles of FOXO Transcription Factors in Mammalian Cells Point to Multiple Roles in Cancer", *Experimental Gerontology*, Vol. 41, pp. 709-717, 2006.

6. Armoni, M., C., Harel, S., Karni, H., Chen, F., Bar-Yoseph, M.R., Ver, M.J.,Quon and E., Karnieli, "FOXO1 Represses Peroxisome Proliferator ActivateReceptor Gamma1 and Gamma2 Gene Promoters in Primary Adipocytes. A Novel Paradigm to Increase Insulin Sensitivity", *The Journal of Biological Chemistry*, Vol. 281, pp. 19881-19891, 2006.

7. Beyaert, R., B., Vanhaesebroeck, P., Suffys, F.,Van Roy and W., Fiers, "Lithium Chloride Potentiates Tumor Necrosis Factor-mediated Cytotoxicity in Vitro and in Vivo",

Proceedings of the National Academy of Sciences of the United States of America, Vol. 86, pp. 9494–9498, 1989.

8. Biggs, W. H., J., Meisenhelder, T., Hunter, W. K., Cavenee and K. C., Arden, "Protein Kinase B/Akt-mediated Phosphorylation Promotes Nuclear Exclusion of the Winged Helix Transcription Factor FKHR1", *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 96, pp. 7421-7426, 1999.

9. Blixt, A., H., Landgren, B.R.,Johansson and P., Carlsson, "Foxe3 is Required for Morphogenesis and Differentiation of the Anterior Segment of the Eye and is Sensitive to-Pax6 Gene Dosage", *Developmental Biology,* Vol. 302, pp. 218-229, 2007.

10. Boggs, D. R. and R. A., Joyce, "The Hematopoietic Effects of Lithium", *Seminars in Hematology,* Vol. 20, pp. 129–138, 1983.

11. Brunet, A., A., Bonni, M. J., Zigmond, M. Z., Lin, P., Juo, L. S., Hu, M. J., Anderson, K. C., Arden, J., Blenis and M. E., Greenberg, "Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor", *Cell,* Vol. 96, pp. 857-868, 1999.

12. Brunet, A., L.B., Sweeney, J.F., Sturgill, K.F., Chua, P.L., Greer, Y., Lin, H., Tran, S.E., Ross, R., Mostoslavsky, H.Y., Cohen, L.S., Hu, H.L., Cheng, M.P., Jedrychowski, S.P., Gygi, D.A., Sinclair, F.W.,Alt and M.E., Greenberg, "Stress-dependent Regulation of FOXO Transcription Factors by the SIRT1 Deacetylase", *Science,* Vol. 303, pp. 2011-2015, 2004.

13. Burgering, B.M. and G.J., Kops, "Cell Cycle and Death Control: Long Live Forkheads", *Trends in Biochemical Sciences*, Vol. 27, pp. 352–360, 2002.

14. Castrillon, D.H., L., Miao, R., Kollipara, J.W., Horner and R.A., DePinho, "Suppression of Ovarian Follicle Activation in Mice by the Transcription Factor Foxo3a", *Science,* Vol. 301, pp. 215-218, 2003.

15. Chen, J., I., Yusuf, H.M., Andersen and D.A., Fruman, "FOXO Transcription Factors Cooperate with Delta EF1 to Activate Growth Suppressive Genes in B Lymphocytes",*The Journal of Immunology*, Vol. 176, pp. 2711-272, 2006.

16. Cohen, P. and M., Goedert, "GSK3 Inhibitors: Development and Therapeutic Potential", *Nature Reviews Drug Discovery*, Vol. 3, pp. 479–487, 2004.

17. Cohen, Y., A., Chetrit, Y., Cohen, P.,Sirota and B., Modan, "Cancer Morbidity in Psychiatric Patients: Influence of Lithium Carbonate Treatment", *Medical Oncology*, Vol. 15, pp. 32–36,1998.

18. Erdal, E., N., Ozturk, T., Cagatay, E., Eksioglu-Demiralp and M., Ozturk, "Lithium-mediated Downregulation of PKB/Akt and Cyclin E with GrowthInhibition in Hepatocellular Carcinoma Cells", *International Journal of Cancer*, Vol. 115, pp. 903–910, 2005.

19. Essers, M.A., L.M., de Vries-Smits, N., Barker, P.E., Polderman, B.M.,Burgering and H.C., Korswagen, "Functional Interaction between Beta-catenin and FOXO in Oxidative Stress Signaling", *Science*, Vol. 308, pp. 1181-1184,2005.

20. Fornace, A.J., D.W., Nebert, M.C., Hollander, J.D., Luethy, M., Papathanasiou, J., Fargnoli and N.J., Holbrook, "Mammalian Genes Coordinately Regulated by Growth Arrest Signals and DNA-damaging Agents", Molecular *and Cellular Biology*, Vol. 9, pp. 4196-4203, 1989.

21. Freland, L. and J.M., Beaulieu, "Inhibition of GSK3 by Lithium, from Single Molecules to Signaling Networks", *Frontiers in Molecular Neuroscience*, Vol. 5, pp. 1-4, 2012.

22. Furukawa-Hibi, Y., K., Yoshida-Araki, T., Ohta, K., Ikeda and N., Motoyama, "FOXO Transcription Factors Induce G(2)-M Checkpoint in Response to Oxidative Stress", *The Journal of Biological Chemistry*, Vol. 277, pp. 26729-26732, 2002.

23. Gould, T. D., N. A.,Gray and H. K., Manji, “Effects of a Glycogen Synthase Kinase-3 Inhibitor, Lithium, in Adenomatous Polyposiscoli Mutant Mice”, *Pharmacological Research,* Vol. 48, pp. 49−53, 2003.

24. Greer, E.L. and A., Brunet, “FOXO Transcription Factors at the Interface between Longevity and Tumor Suppression”, *Oncogene,* Vol. 24, pp. 7410-7425,2005.

25. Hallcher, L.M. and W.R., Sherman, “The Effects of Lithium Ion and Other Agents on the Activity of Myo-inositol-1-phosphatase from Bovine Brain”, *The Journal of Biological Chemistry,* Vol. 255, pp. 10896–10901, 1980.

26. Hartwell, L.H. and T.A., Weinert, “Checkpoints: Controls that Ensure the Order of Cell Cycle Events”, *Science,* Vol. 246, pp. 629-634,1989.

27. Hatini, V., S.O., Huh, D., Herzlinger, V.C.,Soares and E., Lai, “Essential Role of-Stromal Mesenchyme in Kidney Morphogenesis Revealed by Targeted Disruption of-Winged Helix Transcription Factor BF-2”, *Genes and Development,* Vol.10, pp. 1467-1478, 1996.

28. Hauck, L., C., Harms, D., Grothe, J., An, K., Gertz, G., Kronenberg, R., Dietz, M., Endres and R., von Harsdorf, “Critical Role for FoxO3a-dependent Regulation of p21CIP1/WAF1 in Response to Statin Signaling in Cardiac Myocytes”, *Circulation Research,* Vol. 100, pp. 50-60,2007.

29. Hedgepeth, C.M., L.J., Conrad, J., Zhang, H.C., Huang, V.M.,Lee and P.S., Klein, “Activation of the Wnt Signaling Pathway: A Molecular Mechanism for Lithium Action”, *Developmental Biology,* Vol. 185, pp. 82–91, 1997.

30. Hoekman, M.F., F.M., Jacobs, M.P.,Smidt and J.P., Burbach, “Spatial and Temporal Expression of FoxO Transcription Factors in the Developing and Adult Murine Brain”, *Gene Expression Patterns,* Vol. 6, pp. 134-140, 2006.

31. Hosaka, T., W.H., Biggs, D., Tieu, A.D., Boyer, N.M., Varki, W.K., Cavenee and K.C., Arden, "Disruption of Forkhead Transcription Factor (FOXO) Family Members in Mice Reveals Their Functional Diversification", *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 101, pp. 2975-2980,2004.

32. Hu, M.C., D.F., Lee, W., Xia, L.S., Golfman, F., Ou-Yang, J.Y., Yang, Y., Zou, S., Bao, N., Hanada, H., Saso, R., Kobayashi and M.C., Hung, "IkappaB Kinase Promotes Tumorigenesis through Inhibition of Forkhead FOXO3a", *Cell,* Vol. 117, pp. 225-237, 2004.

33. Huot, J., G.,Nosal and S., Radoucu-Thomas, "Effects of Lithium Chloride on Normal and Neoplastic cells in Vitro", *Experientia,* Vol. 28, pp. 456–457, 1972.

34. Jacobs, F. M., L. P., van der Heide, P. J., Wijchers, J. P., Burbach, M. F.,Hoekman, and M. P., Smidt, "FoxO6, a Novel Member of the FoxO Class of Transcription Factors with Distinct Shuttling Dynamics", *The Journal of Biological Chemistry,* Vol. 278, pp. 35959-35967,2003.

35. Johnson, G. and S., Gershon, "Early North American Research on Lithium", *Australian and New Zealand Journal of Psychiatry,* Vol. 33, pp. 48–53, 1999.

36. Junger, M.A., F., Rintelen, H., Stocker, J.D., Wasserman, M., Vegh, T., Radimerski, M.E.,Greenberg and E., Hafen, "The Drosophila Forkhead Transcription Factor FOXO Mediates the Reduction in Cell Number Associated with Reduced Insulin Signaling", *Journal of Biology,* Vol. 2, No. 20, 2003.

37. Kaestner, K. H., W., Knochel and D. E., Martinez, "Unified Nomenclature for the Winged-helix/Forkhead Transcription Factors", *Genes and Development,* Vol. 14, pp. 142-146, 2000.

38. Kao, K.R. and R.P., Elinson, "The Legacy of Lithium Effects on Development", *Biology of the Cell,* Vol. 90, pp. 585–589, 1998.

39. Kaufmann, L., G., Marinescu, I., Nazarenko, W., Thiele, C., Oberle, *et al*., "LiCl Induces TNF-alpha and FasL Production, thereby Stimulating Apoptosis in Cancer Cells", *Cell Communication and Signaling,* Vol. 9, pp. 15, 2011.

40. Kimura, K. D., H. A., Tissenbaum, Y., Liu and G., Ruvkun, "daf-2, an InsulinReceptor-like Gene that Regulates Longevity and Diapause in CaenorhabditisElegans", *Science,* Vol. 277, pp. 942-946, 1997.

41. Klein, P. S., and D. A., Melton, "A Molecular Mechanism for the Effect of Lithium on Development", *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 93, pp. 8455–8459,1996.

42. Kops, G. J., N. D., De Ruiter, A. M., De Vries-Smits, D. R., Powell, J. L.,Bosand B. M., Burgering, "Direct Control of the Forkhead Transcription Factor AFX by Protein Kinase B", *Nature,* Vol. 398, pp. 630-634,1999.

43. Kramer, J.M., J.T., Davidge, J.M.,Lockyer and B.E., Staveley, "Expression of Drosophila FOXO Regulates Growth and can Phenocopy Starvation", *BMC Developmental Biology,* Vol.3, No.5, 2003.

44. Lawlor, M.A. and P., Rotwein, "Insulin-like Growth Factor-mediated Muscle Cell Survival: Central Roles for Akt and Cyclin-dependent Kinase Inhibitor p21", *Molecular and Cellular Biology,* Vol. 20, pp. 8983-8995, 2000.

45. Lehmann, O.J., J.C., Sowden, P., Carlsson, T., Jordan and S.S., Bhattacharya,"Fox's in Development and Disease", *Trends in Genetics,* Vol. 19, pp. 339-344, 2003.

46. Lehtinen, M.K., Z., Yuan, P.R., Boag, Y., Yang, J., Villen, E.B., Becker, S., DiBacco, N., De la Iglesia, S., Gygi, T.K.,Blackwell and Bonni, A., "A Conserved MST-FOXO Signaling Pathway MediatesOxidative-stress Responses and Extends Life Span", *Cell,* Vol. 125, pp. 987-1001, 2006.

47. Li, C., A.J., Lusis, R., Sparkes, S.M.,Tran and R., Gaynor, "Characterization and Chromosomal Mapping of the Gene Encoding the Cellular DNA Binding Protein HTLF",*Genomics*, Vol. 13, pp. 658-664, 1992.

48. Lin, K., J. B., Dorman, A., Rodan and C., Kenyon, "daf-16: An HNF-3/Forkhead Family Member that can Function to Double the Life Span of Caenorhabditis Elegans", *Science*, Vol. 278, pp. 1319-1322,1997.

49. Manji, H.K. and R.H.,Lenox, "Signaling: Cellular Insights into the Pathophysiology of Bipolar Disorder", *Biological Psychiatry*, Vol. 48, pp. 518–530, 2000.

50. Mao, C.D., P., Hoang and P.E., DiCorleto, "Lithium Inhibits Cell Cycle Progression and Induces Stabilization of p53 in Bovine Aortic Endothelial Cells", *The Journal of Biological Chemistry*, Vol. 276, pp. 26180–26188,2001.

51. Martinez, S.C., C., Cras-Meneur, E., Bernal-Mizrachi and M.A., Permutt, "Glucose Regulates Foxo1 throughInsulin Receptor Signaling in the Pancreatic Islet Beta-Cell", *Diabetes*, Vol. 55, pp. 1581-1591, 2006.

52. Mazet, F., J.K., Yu, D.A., Liberles, L.Z.,Holland and S.M., Shimeld, "Phylogenetic Relationships of the Fox (Forkhead) Gene Family in the Bilateria", *Gene*, Vol. 316, pp. 79-89, 2003.

53. Mazlo, M., I., Patakyand R., Szucs, "Morphological Study of the Kidneys of Lithium Treated Rats", *Acta Morphologica Hungarica,* Vol. 31, pp. 309−314, 1983.

54. Medema, R.H., G.J., Kops, J.L.,Bos and Burgering, B.M., "AFX-like Forkhead Transcription Factors Mediate Cell-Cycle Regulation by Ras and PKB through p27kip1", *Nature*, Vol. 404, pp. 782-787, 2000.

55. Mora, A., G., Sabio, A.M., Risco, A., Cuenda, J.C., Alonso, *et al*., "Lithium Blocks the PKB and GSK3 Dephosphorylation Induced by Ceramide through Protein Phosphatase-2A", *Cell Signaling*, Vol. 14, pp. 557–562, 2002.

56. Moscovich, D. G., “Lithium Neurotoxicity at Normal Therapeutic Levels”, *TheBritish Journal of Psychiatry,* Vol. 163, pp. 410−412, 1993.

57. Myatt, S.S. and E.W., Lam, “The Emerging Roles of Forkhead box (Fox) Proteins in Cancer”, *Nature Reviews Cancer,*Vol. 7, pp. 847-859,2007.

58. Nakae, J., W.H., Biggs, T., Kitamura, W.K., Cavenee, C.V., Wright, K.C.,Arden and D., Accili, “Regulation of Insulin Action and Pancreatic Beta-Cell Function by Mutated Alleles of the Gene Encoding Forkhead Transcription Factor Foxo1”, *Nature Genetics,* Vol. 32, pp. 245-253,2002.

59. Nakae, J., Y., Cao, H., Daitoku, A., Fukamizu, W., Ogawa, Y., Yano and Y., Hayashi, “The LXXLL Motif of Murine Forkhead Transcription Factor FoxOMediates Sirt1-Dependent Transcriptional Activity”, *The Journal of Clinical Investigation,* Vol. 116, pp. 2473-2483, 2006.

60. Nakae, J., T., Kitamura, D.L.,Silver and D., Accili, “The Forkhead Transcription Factor Foxo1 (Fkhr) Confers Insulin Sensitivity onto Glucose-6-phosphatase Expression”, The *Journal of Clinical Investigation,* Vol. 108, pp. 1359-1367,2001.

61. Nowicki, M.O., N., Dmitrieva, A.M., Stein, J.L., Cutter, J., Godlewski, *et al*., “Lithium Inhibits Invasion of Glioma Cells; Possible Involvement of Glycogen Synthase Kinase-3”, *Journal of Neuro-Oncology,* Vol. 10, pp. 690–699, 2008.

62. Ogg, S., S., Paradis, S., Gottlieb, G. I., Patterson, L., Lee, H. A.,Tissenbaum and G., Ruvkun, “The Forkhead Transcription Factor DAF-16 Transduce Insulin-like Metabolic and Longevity Signals in C. elegans”, *Nature,* Vol. 389, pp. 994-999, 1997.

63. Ohteki, T., M., Parsons, A., Zakarian, RG.,Jones, LT., Nguyen, J.R.,Woodgett and P.S., Ohashi, “Negative Regulation of T Cell Proliferation and Interleukin-2 Production by the Serine Threonine Kinase GSK-3”, *The Journal of Experimental Medicine,* Vol. 192, pp. 99–104, 2000.

64. Puig, O., M.T., Marr, M.L.,Ruhf and R., Tjian, "Control of Cell Number by Drosophila FOXO: Downstream and Feedback Regulation of the Insulin Receptor Pathway", *Genes and Development*, Vol. 17, pp. 2006-2020, 2003.

65. Quiroz, J.A., T.D.,Gould and H.K., Manji, "Molecular Effects of Lithium", *Molecular Interventions*, Vol. 4, pp. 259–272, 2004.

66. Ramaswamy, S., N., Nakamura, I., Sansal, L.,Bergeron and W.R., Sellers, "A Novel Mechanism of Gene Regulation and Tumor Suppression by the Transcription Factor FKHR", *Cancer Cell*, Vol. 2, pp. 81-91, 2002.

67. Rena, G., S., Guo, S. C., Cichy, T. G.,Unterman and P., Cohen, "Phosphorylation of the Transcription Factor Forkhead Family Member FKHR by Protein Kinase B", *The Journal of Biological Chemistry*, Vol. 274, pp. 17179-17183,1999.

68. Rodriguez de la Concepcion, M.L., P., Yubero, R., Iglesias, M., Giralt and F., Villarroya, "Lithium Inhibits Brown Adipocyte Differentiation", *Federation of European Biochemical Societies Letters*, Vol. 579, pp. 1670–1674,2005.

69. Ronchi, A., R., Salaroli, S., Rivetti, E., Della Bella, T., Di Tomaso, *et al*., "Lithium Induces Mortality in Medulloblastoma Cell Lines", *International Journal of Oncology*, Vol. 37, pp. 745–752,2011.

70. Rowe, M. K. and D. M., Chuang, "Lithium Neuroprotection: Molecular Mechanisms and Clinical Implications", *Expert Reviews in Molecular Medicine*, Vol. 6, pp. 1−18,2004.

71. Ryves, W. J. and A. J., Harwood, "Lithium Inhibits Glycogen Synthase Kinase-3 by Competition for Magnesium", *Biochemical and Biophysical Research Communications*, Vol. 280, pp. 720–725,2001.

72. Schmidt, M., S., Fernandez de Mattos, A., van der Horst, R., Klompmaker, G.J., Kops, E.W., Lam, B.M.,Burgering and R.H., Medema, "Cell Cycle Inhibition by FoxO Fork-

head Transcription Factors Involves Downregulation of Cyclin D", *Molecular and Cellular Biology,* Vol. 22, pp. 7842-7852,2002.

73. Sinha, D., Z., Wang, K.L., Ruchalski, J.S., Levine, S., Krishnan, W., Lieberthal, J.H. Schwartz and S.C., Borkan, "Lithium Activates the Wnt and Phosphatidylinositol 3-Kinase Akt Signaling Pathways to Promote Cell Survival in the Absence of Soluble Survival Factors", *American Journal of Renal Physiology,* Vol. 288, pp. 703–713,2005.

74. Smits, V.A., M.A., Essers, D.S., Loomans, R., Klompmaker, G., Rijksen and R.H., Medema, "Inhibition of Cell Proliferation by Lithium is Associated with Interference in Cdc2 Activation", *Federation of European Biochemical Societies Letters,*Vol. 457, pp. 23–27, 1999.

75. Speirs, J. and S. R., Hirsch, "Severe Lithium Toxicity with 'Normal' Serum Concentrations", *British Medical Journal,* Vol. 1, pp. 815−816,1978.

76. Spiegelberg, B.D., "Alteration of Lithium Pharmacology through Manipulation of Phosphoadenosine Phosphate Metabolism",*The Journal of Biological Chemistry,* Vol. 280, pp. 5400–5405,2005.

77. Stahl, M., P.F., Dijkers, G.J., Kops, S.M., Lens, P.J., Coffer, B.M.,Burgering andR.H., Medema, "The Forkhead Transcription Factor FoxO Regulates Transcription of p27-Kip1 and Bim in Response to IL-2", *The Journal of Immunology,* Vol. 168, pp. 5024-5031, 2002.

78. Stein, J.P., D.A., Ginsberg, G.D., Grossfeld, S.J., Chatterjee, D., Esrig, M.G., Dickinson, S., Groshen, C.R., Taylor, P.A., Jones, D.G.,Skinner and R.J., Cote, "Effect of p21WAF1/CIP1 Expression on Tumor Progression in Bladder Cancer", *Journal of the National Cancer Institute,* Vol. 90, pp. 1072-1079,1998.

79. Takahashi-Yanaga, F., Y., Taba, Y., Miwa, Y., Kubohara, Y., Watanabe, M., Hirata *et al*., "Dictyostelium Differentiation-Inducing Factor-3 Activates Glycogen Synthase

Kinase-3beta and Degrades Cyclin D1 in Mammalian Cells", *TheJournal of Biological Chemistry*, Vol. 278, pp. 9663–70, 2003.

80. Tang, E. D., G., Nunez, F. G.,Barr and K. L., Guan, "Negative Regulation of the Forkhead Transcription Factor FKHR by Akt", *The Journal of Biological Chemistry*, Vol. 274, pp. 16741-16746, 1999.

81. Thomas, J.H., "Chemosensory Regulation of Development in C.elegans", *Bioessays*, Vol. 15, pp. 791-797, 1993.

82. Tondo, L. and R. J., Baldessarini, "Long-term Lithium Treatment in the Prevention of Suicidal Behavior in Bipolar Disorder Patients", *Epidemiologia e Psichiatria Sociale*, Vol.18, pp. 179−183,2009.

83. Tran, H., A., Brunet, J.M., Grenier, S.R., Datta, A.J., Fornace, P.S., DiStefano, L.W.,Chiang and Greenberg, M.E., "DNA Repair Pathway Stimulated by the Forkhead Transcription Factor FOXO3a through the Gadd45 Protein", *Science*, Vol. 296, pp. 530- 534, 2002.

84. Umbach, J.A., Y., Zhao and C.B., Gundersen, "Lithium Enhances Secretion from Large Dense-core Vesicles in Nerve Growth Factor-Differentiated PC12 Cells", *Journal of Neurochemistry*, Vol. 94, pp. 1306–14,2005.

85. Vidal, F., W.M., de Araujo, A.L., Cruz, M.N., Tanaka, J.P., Viola, *et al*., "Lithium Reduces Tumorigenic Potential in Response to EGF Signaling in Human Colorectal Cancer Cells", *International Journal of Oncology*, Vol. 38, pp. 1365–1373,2011.

86. Wang, X.M., J., Li, X.C., Feng, Q., Wang, D.Y.,Guan and Z.H., Shen, "Involment of the Role of Chk1 in Lithium Induced G2/M Phase Cell Cycle Arrest in Hepatocellular Carcinoma Cells", *Journal of Cellular Biochemistry*, Vol. 104, pp. 1181-1191, 2008.

87. Wang, X.W., Q., Zhan, J.D., Coursen, M.A., Khan, H.U., Kontny, L., Yu, M.C., Hollander, P.M., O'Connor, A.J., Fornaceand C.C., Harris, "GADD45 Induction of a G2/M

Cell Cycle Checkpoint", *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 96, pp. 3706-3711, 1999.

88. Weigel, D., G., Jurgens, F., Kuttner, E., Seifert and H., Jackle, "The Homeotic Gene Forkhead Encodes a Nuclear Protein and is Expressed in the Terminal Regions of the Drosophila Embryo", *Cell,* Vol. 7, pp. 645-658,1989.

89. Yarden, R.I., S., Pardo-Reoyo, M., Sgagias, K.H.,Cowan and L.C., Brody, "BRCA1 Regulates the G2/M Checkpoint by Activating Chk1 Kinase upon DNA Damage", *Nature Genetics,* Vol. 30, pp. 285–289,2002.

90. Zhou, Y., H., Kato, K., Asanoma, H., Kondo, T., Arima, K., Kato, T., Matsuda and Wake,N., "Identification of FOXC1 as a TGF-beta1 Responsive Gene and its Involvementin Negative Regulation of Cell Growth", *Genomics,* Vol. 80, pp. 465-472,2002.

91. Zhu, W., G.N., Bijur, N.A. Styles and X., Li, "Regulation of FOXO3a by Brain Derived Neurotrophic Factor in Differentiated Human SH-SY5Y Neuroblastoma Cells", *Brain Research,* Vol. 126, pp. 45-56,2004.

92. Zhu, Z., P., Kremer, I., Tadmori, Y., Ren, D., Sun, *et al*., "Lithium Suppresses Astrogliogenesis by Neural Stem and Progenitor Cells by Inhibiting STAT3Pathway Independently of Glycogen Synthase Kinase-3 Beta", *Public Library of Science One,* Vol. 6, e23341, 2011.