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**REGULATION OF FOXC2 EXPRESSION AND FUNCTION DURING  
EPITHELIAL-MESENCHYMAL TRANSITION BY GSK-3 $\beta$**

by

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**REGULATION OF FOXC2 EXPRESSION AND FUNCTION DURING  
EPITHELIAL-MESENCHYMAL TRANSITION BY GSK-3 $\beta$**

A  
THESIS

Presented to the Faculty of  
The University of Texas  
Health Sciences Center at Houston  
and  
The University of Texas  
M.D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment  
of the Requirements  
for the Degree of

MASTER OF SCIENCE

by

Maryam Shariati, BS

Houston, Texas

August 2012

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I would like to thank my advisor, Dr. Sendurai Mani, for his guidance and help through the course of my research. I am grateful for all the time and dedication he put into the project. I would also like to appreciate my committee members, Dr. Sue-Hwa Lin, Dr. Pierre McCrea, Dr. Kapil Mehta and Dr. Naoto Ueno for the advice and insights they provided during my thesiswork. In addition, I would like to express my gratitude to Dr. Nathalie Sphyris for taking time to provide editing inputs on my thesis. Last, but not least, to my husband for his love, support and encouragement.

## **ABSTRACT**

### **Regulation of FOXC2 Expression and Function during Epithelial-Mesenchymal Transition by GSK-3 $\beta$**

Metastasis is the ultimate cause for the majority of cancer-related deaths. The forkhead box transcription factor FOXC2 is known to be involved in regulating metastasis as well as a variety of developmental processes, including the formation of lymphatic and cardiovascular systems. Previous studies have shown that FOXC2 protein is localized either in the nucleus and/or in the cytoplasm of human breast tumor cells. This pattern of localization is similar to that of another forkhead family member, FOXO3a. Additionally, localization of FOXO3a is known to be differentially regulated by upstream kinase AKT. Therefore, I investigated whether FOXC2 localization could also be regulated by upstream kinases. Analysis of FOXC2 protein sequence revealed two potential phosphorylation sites for GSK-3 $\beta$ . Furthermore, inhibition of GSK-3 $\beta$  significantly reduces FOXC2 protein. In addition, exposure of HMLE Twist cells expressing endogenous FOXC2 to the GSK-3 $\beta$  inhibitor, TWS119, results in accumulation of FOXC2 protein in the cytoplasm with concomitant decrease in the nucleus in a time-dependent manner. Furthermore, continued treatment with TWS119 eventually induces epithelial morphology and decreased stem cell properties including sphere formation in these cells. Further characterization of FOXC2- GSK-3 $\beta$  interaction and the associated signaling cascade are necessary to determine the effect of FOXC2 phosphorylation by GSK-3 $\beta$  on EMT and metastasis.

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## **CHAPTER 1 INTRODUCTION**

### **1.1 Background**

Despite recent advances that are used to treat and often eliminate the primary tumor, metastatic disease usually results in a fatal prognosis for breast cancer patients. Unfortunately, due to the absence of better early diagnostic methods, metastases are not detectable until cancers progress to advanced stages. Moreover, due to the complex nature and the dearth of basic understanding of the metastatic process, there are only limited therapeutic agents available to treat metastasis. Therefore, this lack of effective anti-metastatic treatments necessitates an improved understanding of the molecular mechanisms driving cancer progression.

Metastasis or the spreading of cancer from one organ site to another is facilitated through bloodstream or lymphatic system. Transformation of a normal cells results in uncontrolled cell division and, as a result, these cells forma transformed mass of cells or primary tumor (1). Once the tumor mass reaches more than 1-2 mm, the tumor mass secrets angiogenic factors and

become highly vascularized. The tumor also secretes a number of proteases, including matrix metalloproteinases (MMP), which target the surrounding tissues during tumor advancement (2, 3).

The growth factors from the neighboring stroma (4) as well as the hypoxic environment (5) have been shown to induce EMT in cancer cells. The tumor cells that underwent EMT gain motility, disseminate from its original site, enter capillaries and circulate through the bloodstream to reach other sites in the body (6). Circulating cancer cells that are able to evade the host immune system respond to specific microenvironmental factors present at the distant site and either start to proliferate upon arriving at the secondary site or remain quiescent until the environment favors growth (7). During extravasation at the distant site, the tumor cells traverse the vessel walls and invade the surrounding tissues at the secondary organ eventually forming a metastatic tumor (8).

Many signaling pathways are thought to regulate the complex metastatic process; therefore, identifying the signaling pathways governing tumor progression will facilitate the development of novel therapeutic approaches. EMT which is normally active during embryonic development and latent in most adult tissues is known to be reactivated during cancer progression, thus facilitating metastasis (9). It has also been shown that EMT is not only critical for the motility of metastatic cells, but also responsible for the recurrence of tumor (10).

During EMT, epithelial cells which are known to be confined locally due to the continued expression of epithelial cell-cell adhesion molecules, lose their epithelial markers and acquire phenotypic and behavioral properties characteristic of mesenchymal cells including migratory

and invasive capabilities (11). E-cadherin protein plays an important role in establishing cell-cell adhesion complex and has been known function as a tumor suppressor in human cancer (12).

Downregulation of E-cadherin is one of the important alterations that reduces epithelial morphology and increases cellular motility during EMT (13). Down regulation of E-cadherin could be achieved through multiple mechanisms including transcriptional repression, promoter methylation as well as proteosomal degradation of E-cadherin (14). It has been known that Twist, Goosecoid and Snail1 act as transcriptional repressors of E-cadherin (15).

In addition to EMT, several recent studies demonstrated that cancer stem cells (CSCs), a small population of cancer cells present within tumor is also capable of facilitating both primary tumor formation as well as tumor metastasis (16). CSCs are able to undergo self-renewal and give rise to CSCs as well as differentiated cancer cells (17). CSCs are associated with initiating and maintaining invasive tumor growth (18). In addition, accumulating evidences show that CSCs assist other cancer cells to survive better and therefore, cause relapse and metastasis (19). Most importantly, the EMT process has been shown to facilitate the generation of CSCs within tumor (20), thus generating more invasive and metastasis prone cancer cells within primary tumor. Therefore, the EMT program and the associated signaling pathways serve as a window for targeting the CSCs. Dissection of the EMT program revealed that it is, in part, driven by the expression of some transcription factors capable of promoting migration, invasion, and metastatic process (21). Among the multiple EMT inducing factors, one of the transcription factor called forkhead box C2 (FOXC2) is capable of inducing EMT. Likewise, FOXC2 expression is induced following EMT in response to the overexpression of other EMT-inducing factors. FOXC2 expression has been found to be essential for ocular, cardiovascular, and

lymphatic development during embryogenesis. Besides its role during embryonic development, FOXC2 has recently been shown to be responsible for cancer progression and metastasis (22).

Furthermore, FOXC2 has been suggested as a prognostic factor in esophageal cancer and is an important factor during tumor angiogenesis (23, 24). Additionally, nuclear FOXC2 and the expression of GLI1, a Hedgehog (Hh) signaling effector, are correlated with basal-like breast cancers (25).

Since FOXC2 has been implicated to play important roles during embryo development and in cancer progression, and the FOXC2 protein has been detected in either the cytoplasm or the nucleus and in some instances, both in the cytoplasm and the nucleus, I investigated the regulation of FOXC2 localization in response to upstream signaling molecules.

## **CHAPTER 2 HYPOTHESIS**

Recent evidences indicated that several members of the forkhead box transcription factor family, such as FOXM1 and FOXO3, are involved in cancer progression (26, 27). Moreover, many of these forkhead family members are proposed to be regulated at the post-translational level by upstream signaling kinases. For example, the phosphorylation of FOXO3a by AKT results in FOXO3a export from the nucleus, accumulation in the cytoplasm and eventual degradation of the protein by the proteasome (28). Since the expression, localization and function of several forkhead family transcription factors is known to be regulated post translationally by upstream signaling kinases, I hypothesized that the differential localization of FOXC2 protein observed in our studies is due to the regulation of its localization by upstream kinases.

## **CHAPTER 3 MATERIALS AND METHODS**

### **Cell Culture**

Immortalized human mammary epithelial cells (HMLE) overexpressing Twist (HMLE-Twist) were cultured in a mixture of DMEM: F12 and MEGM (1:1) supplemented with 10mg/ml insulin, 5 $\mu$ g/ml hEGF, 0.5 mg/ml hydrocortisone, 1% penicillin/streptomycin and bovine pituitary extract (BPE). Human breast cancer cell lines SUM159 were cultured using Ham's F-12 supplemented with 5% fetal bovine serum (FBS), 5  $\mu$ g/ml insulin and 1  $\mu$ g/ml hydrocortisone.

### **GSK-3 $\beta$ Inhibitor**

GSK-3 $\beta$  inhibitor (Cat. No. 361554) was purchased from Calbiochem and was dissolved in dimethyl sulfoxide (DMSO) and used at the indicated concentrations.

### **MTS Assay**

HMLE-Twist and SUM159 cells were cultured in 96-well plate (approximately 5000 cells in each well for each cell line). The next day the cells were treated with different concentrations of GSK-3 $\beta$  inhibitor (0.1 $\mu$ M, 0.2 $\mu$ M, 0.5 $\mu$ M, 1 $\mu$ M, 2 $\mu$ M and 5 $\mu$ M) and DMSO as the control for 24 hours. Each treatment condition was in triplicate. After the drug treatment, 20 $\mu$ l of Cell-Titer 96 Aqueous One Solution Reagent was added into each well of the 96-well assay plate containing the samples in 100 $\mu$ l of cultured medium. The plate was incubated at 37°C for 3 hours in a humidified 5% CO<sub>2</sub> atmosphere. To measure the amount of soluble formazan produced by cellular reduction of MTS, the absorbance was recorded at 490nm using a 96-well plate reader.

## **Quantitative RT-PCR**

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) and converted to cDNA using MMLV Reverse Transcriptase enzyme (Invitrogen) and RNasin (Promega). Real-time PCR was performed with TaqMan probes from Applied Biosystems and  $\beta$ -Actin as the control.

## **FOXC2 Mutant Constructs**

pBabe containing FOXC2 sequence tagged with HA on 3' was used to remove HA-tagged FOXC2 through digestion with EcoR1 and BamH1 restriction enzymes. The FOXC2-HA was then cloned into pMIG vector containing GFP. Site-directed mutagenesis was performed to generate T247E, T247A, S363E and S363A mutants. The FOXC2 sequence containing the mutation of interest was amplified using primers designed for each introduced mutation and FOXC2-HA through PCR. Each mutant was then subcloned into the pMIG vector. All the sequences were verified by automated sequencing. Cells overexpressing the FOXC2-mutants were sorted by flow cytometry on the basis of GFP fluorescence.

Primers used for the creation of the FOXC2 mutants are as follows:

FOXC2-HA forward primer: 5'-CGG ATT TCTCAAGCG TAG TCTGGGACG-3'

FOXC2-HA reverse primer: 5'-GCCGGATCCATG CAG GCGCGCTACTCC-3'

T247E forward primer: 5'-AGCGCGGCCTCCGAA CCC GCCGGCTCC CCC-3'

T247E reverse primer: 3'-GGGGGAGCCGGCGGGTTCGGAGGCCGCGCT-5'

T247A forward primer: 5'-AGCGCGGCCTCCGCA CCC GCCGGCTCC CCC-3'

T247A reverse primer: 5'-GGGGGAGCCGGCGGGTGCGGAGGCCGCGCT 3'

S363E forward primer: 5'-CCGAGCGGCCCCACGGAGCCCCTGAGCGCT-3'



S363E reverse primer: 5'-AGCGCTCAGGGGCTCCGTGGGGCCGCTCGG-3'

S363A forward primer: 5'-TCGGACCACCCGGCAGGC CCC ACGTCG CCC-3'

S363A reverse primer: 3'-GGGCGACGTGGGGCCTGCCGGGTGGTCCGA-5'

### **Immunoprecipitation and Western Blot**

Cells were lysed in RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton x-100, 1% Sodium deoxycholate, 0.1% SDS, 1mM EDTA) supplemented with protease and phosphatase inhibitors (phospho-stop; Roche). The lysates were cleared through centrifugation at 4°C and the supernatants incubated with anti-Flag conjugated agarose beads (Sigma) with rotation at 4°C overnight. The next day the beads were washed five times with the RIPA buffer. Immunoblotting was performed using anti-myc antibody (Santa Cruz).

### **Mammosphere Assay**

HMLE-Twist and SUM159 cells (1000 cells/well of a 96-well plate) were suspended in 100µl of MEGM supplemented with 20mg/ml FGF, 10ng/ml EGF, 4µg/ml heparin and 1% methyl cellulose. They were seeded into a 96-well low attachment plate. After 3 days an additional 100µl of mammosphere media containing 1µM of TWS119 or DMSO as the control were added to each well. Every three days half of the media was replaced with 100µl of fresh mammosphere media containing TWS119 or DMSO. The mammospheres were counted after two weeks using a microscope at 10X magnification.

## **CHAPTER 4 RESULTS**

### **4.1 Identification of potential phosphorylation sites in FOXC2**

#### **4.1.1 Identification of consensus GSK-3 $\beta$ phosphorylation sites in FOXC2 primary amino acid sequence**

As a first step towards a better understanding of the mechanism of FOXC2 regulation, I investigated the potential kinases that might phosphorylate FOXC2 using Scansite 2.0, a motif scan analysis program. This analysis revealed two potential phosphorylation sites for the serine/threonine regulatory kinase GSK-3 $\beta$  within the FOXC2 amino acid sequence (Fig. 1.) at threonine 247 and serine 363. GSK-3 $\beta$  is a key component of the Wnt signaling pathway which has been implicated to play an important role in both embryo development and tumorigenesis (Behrens et al, 2004). A recent study demonstrated that GSK-3 $\beta$  promotes the export of Snail, another EMT-inducing transcription factor, from the nucleus to the cytoplasm and primes the protein for beta-Trcp-mediated ubiquitination and proteosomal degradation. Therefore, it is possible that GSK-3 $\beta$  could regulate FOXC2 protein in a similar fashion.

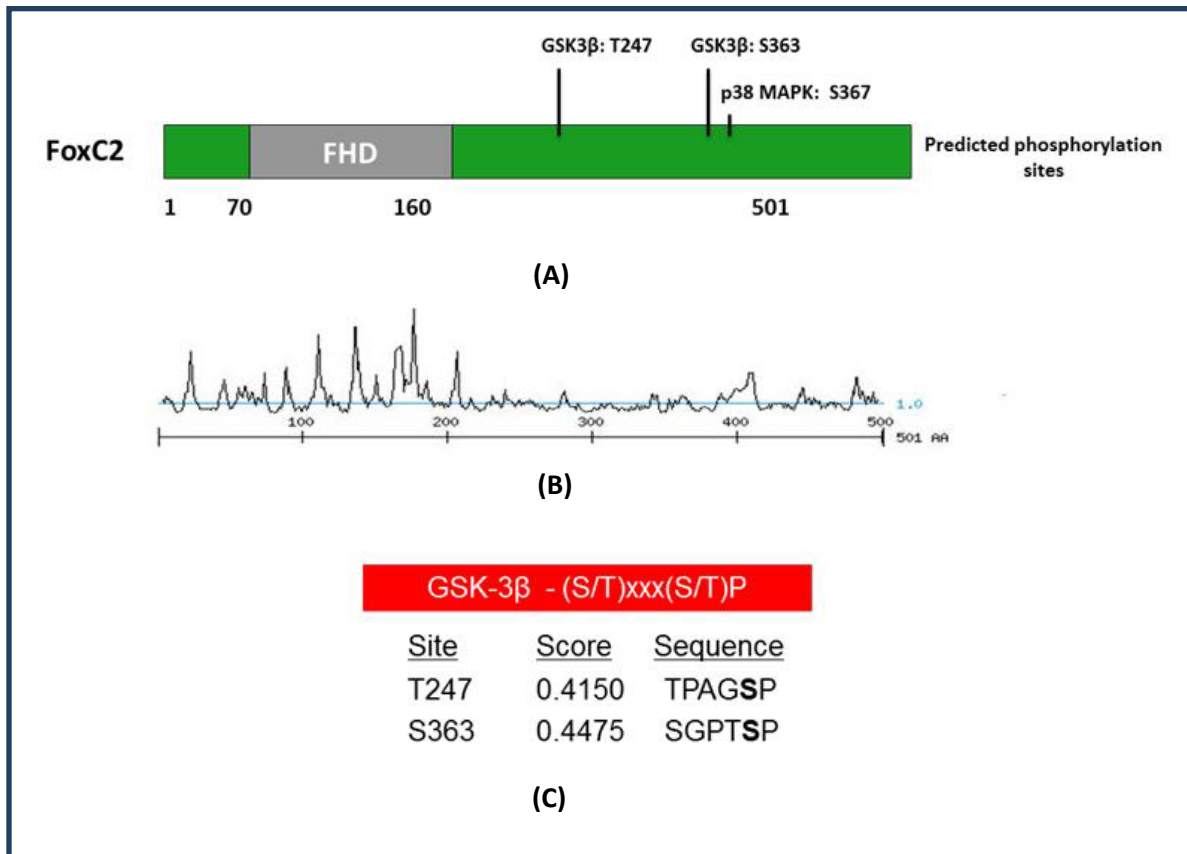


Fig. 1. Potential phosphorylation sites prediction for FOXC2 using Scansite Motif Scan analysis program. A) Predicted phosphorylation sites for GSK-3β. B) The surface accessibility map for FOXC2. C) The candidate sequence for GSK-3β as well as the location of the potential GSK-3β phosphorylation site within the FOXC2 amino acid sequence.

#### **4.1.2 GSK-3 $\beta$ and FOXC2 physically interact with each other.**

In order to characterize the sites identified for GSK-3 $\beta$  using Scansite and to further investigate the role of GSK-3 $\beta$  on the regulation of FOXC2 through phosphorylation, I performed co-immunoprecipitation (Co-IP) assay, because the GSK-3 $\beta$  has to physically interact with FOXC2. For this, I co-transfected HEK293T cells with pMIG plasmid coding for myc-tagged FOXC2 along with flag-tagged either wild type or constitutively active GSK-3 $\beta$  in pMX vector in addition to the control empty pMX vector. I made total extract and performed western for flag and myc to confirm the overexpression (Fig. 2A). Using the total cell protein extract, I precipitated the GSK-3 $\beta$  using anti-flag agarose conjugated beads and analyzed by western immunoblotting using anti-myc antibody. I found that the constitutively active form of GSK-3 $\beta$  physically interact with FOXC2 (Fig. 2B).

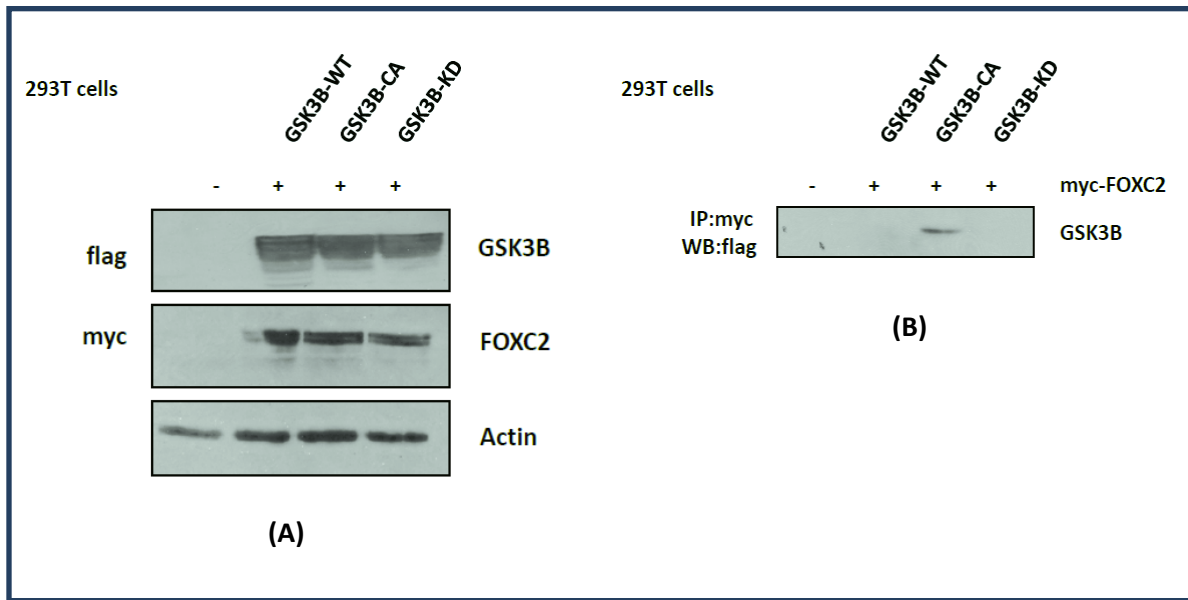


Fig. 2. The physical interaction between FOXC2 and GSK-3 $\beta$ . (A) Western blot showing the overexpression of flag-GSK-3 $\beta$  and myc-FOXC2. Actin is used as a loading control. (B) Immunoprecipitation using myc (myc FOXC2) antibody and western blot using flag (flag-GSK-3 $\beta$ ) antibody. Lane 1 control vector, lane 2 WT-GSK-3 $\beta$ , Lane 3 catalytically active GSK-3 $\beta$  and lane 4 dominant negative GSK-3 $\beta$  co-transfected with myc-tagged FOXC2 in HEK293T cells.

## **4.2 Investigation of the effect of GSK-3 $\beta$ inhibition on FOXC2 expression and localization.**

### **4.2.1 Identification of an optimal concentration of GSK-3 $\beta$ inhibitor to avoid off-target effects**

In order to inhibit GSK-3 $\beta$ , I used the pharmacological inhibitor TWS119, a cell-permeable pyrrolopyrimidine compound that binds to GSK-3 $\beta$  with high-affinity and acts as an inhibitor. To identify the minimum concentration of the inhibitor, capable of affecting the level of FOXC2 protein without affecting the cell viability, I treated HMLE Twist and SUM159 cells expressing endogenous FOXC2 with six different concentrations of TWS119 for 24 hours. The samples were collected after 24 hours for protein extraction and performed western immunoblotting using anti-FOXC2 antibody. I expected to see an increase in FOXC2 protein following exposure to GSK-3 $\beta$  similar to Snail (Zhou et al, 2004); however, I found an opposite effect. The FOXC2 protein started to decrease in response to the exposure of increasing concentrations of TWS119 in a dose dependent manner. In fact, the effect was prominent even starting with 1 $\mu$ M concentration of TWS119 (Fig. 3, lane 5 from panel A and B).

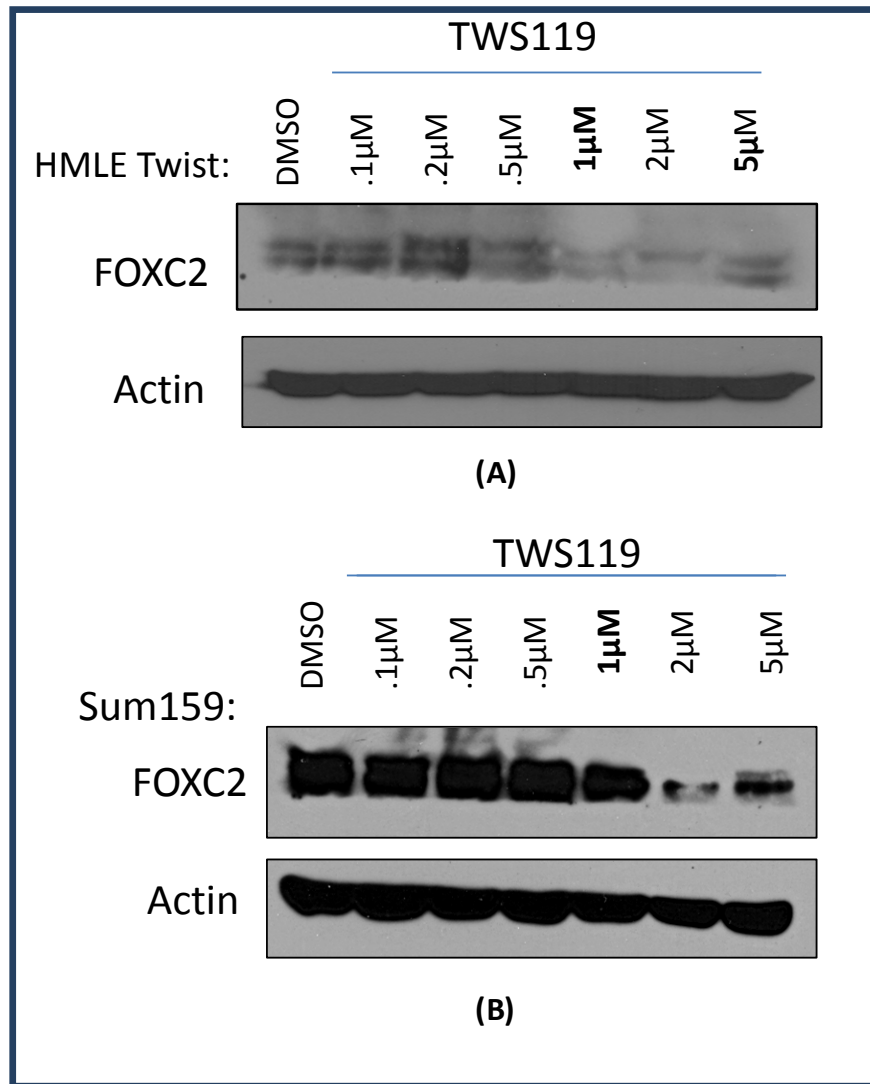


Fig. 3. TWS119 reduces FOXC2 protein level starting at 1μM. Western blot analysis of the expression of FOXC2 protein in HMLE Twist (A) and SUM159 (B) in response to increasing concentration of TWS119. Actin is used as a loading control.

#### **4.2.2 Determine the effect of TWS119 on cell viability.**

In order to ascertain the reduction in FOXC2 is due to post translational modification and not due to increase in cell death at higher concentrations of TWS119, I performed MTS assay by exposing HMLE Twist and SUM159 cells to increasing concentrations of TWS119. There was a little effect on cell viability (Fig. 4) relative to cells treated with DMSO at higher concentration. However, at 1 $\mu$ M, the concentration at which I observed a significant reduction in FOXC2 protein level, there was only very little effect on cell viability (Fig. 4) relative to cells treated with DMSO indicating that the reduction in FOXC2 is not due to selective killing of FOXC2 expressing cells.



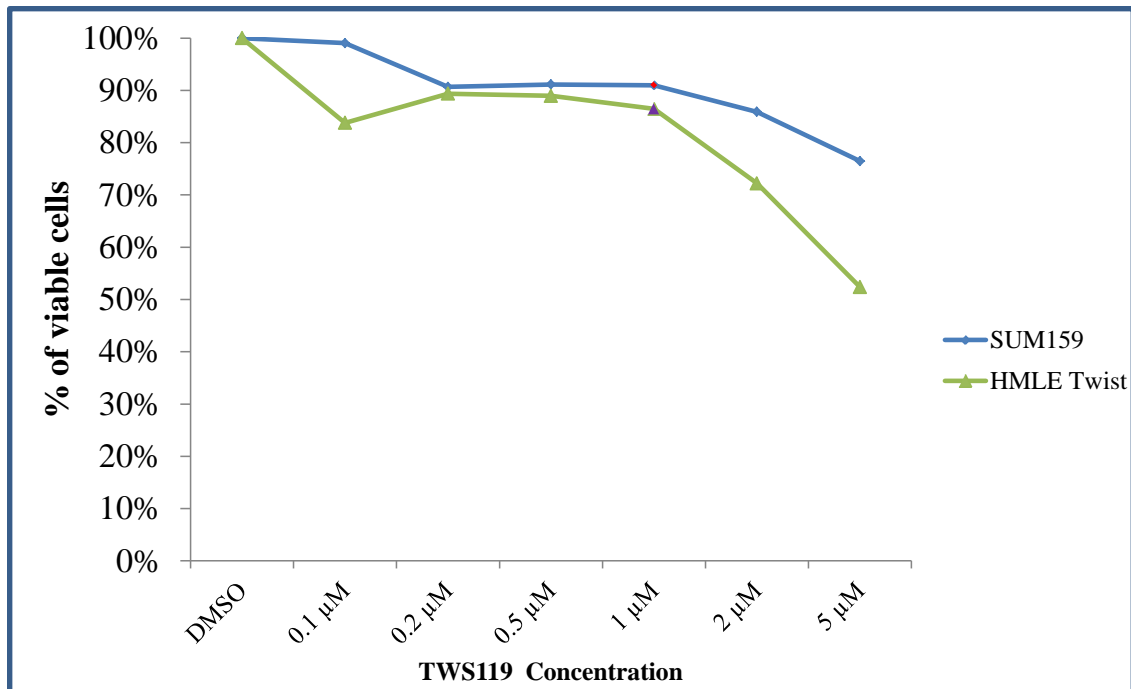


Fig. 4. Cell viability assay using TWS119. Percentage of viable cells after 0.1, 0.2, 0.5, 1, 2, 5  $\mu\text{M}$  of TWS119 exposure for 24 hours using HMLE Twist and SUM159 cells relative to DMSO. FOXC2 mRNA level does not change significantly upon treatment with TWS119.

#### 4.2.3 FOXC2 mRNA level does not change significantly upon treatment with 1 $\mu\text{M}$ TWS119.

To determine whether the reduction in FOXC2 protein is due to the inhibitory effect of GSK-3 $\beta$  on FOXC2 protein or mRNA, I measured the FOXC2 mRNA level using RNAs isolated from HMLE Twist and SUM159 cells that were treated with increasing concentrations of TWS119 over a period of 24 hours. There was no significant change in the FOXC2 mRNA in

HMLE Twist and SUM159 cells (Fig. 5). This suggests that the GSK-3 $\beta$  effect on FOXC2 is mostly at the protein level.

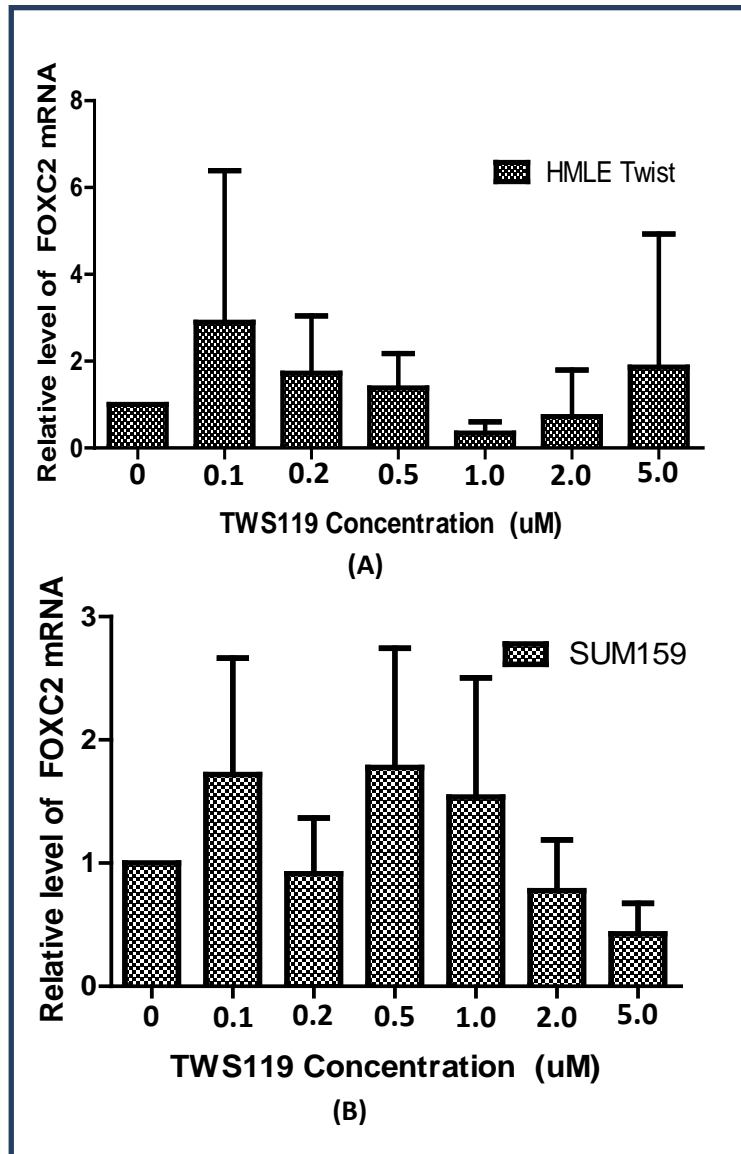


Fig. 5 Relative FOXC2 mRNA levels at different concentrations of TWS119 treatment for 24 hours measured by real-time RT-PCR the mRNA level for each sample was measured relative to DMSO treated cells (0 $\mu$ M TWS119) and actin was used to normalize the loading.

#### 4.2.4 Inhibition of GSK-3 $\beta$ decreases the stability of FOXC2 protein.

To further understand the effect of GSK-3 $\beta$  on the stability of FOXC2 protein, I exposed HMLE Twist cells for up to 24 hours and SUM159 cells for up to 52 hours with previously standardized 1 $\mu$ M of TWS119. Inhibition of GSK-3 $\beta$  using TWS119 resulted in reduction of FOXC2 within 8 hours in HMLE Twist cells (Fig. 6A). However, in SUM159 cells FOXC2 protein level started to decrease only after twenty four hours of TWS119 treatment (Fig. 6B).

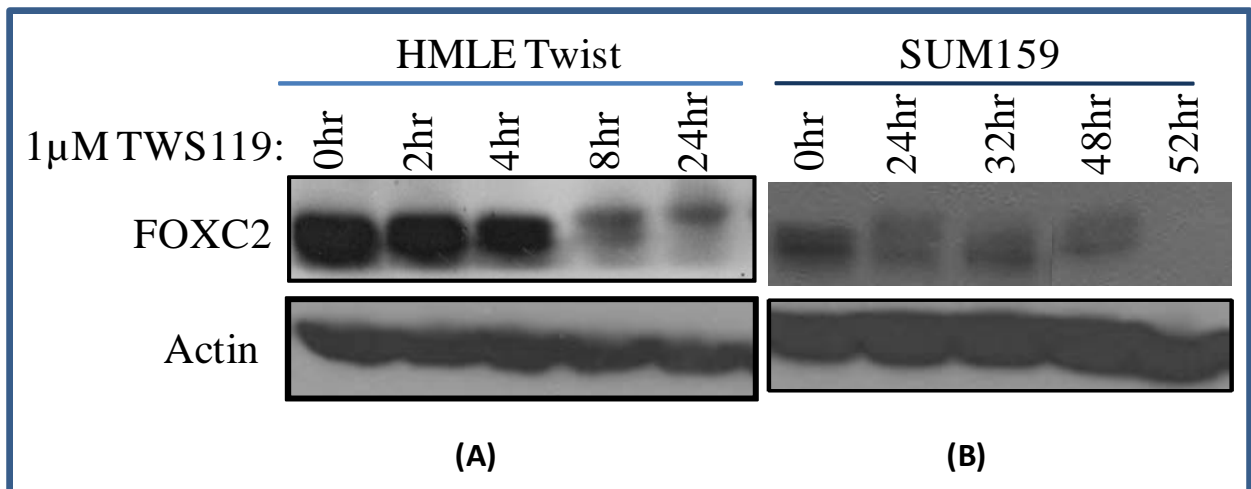


Fig. 6. Decrease in stability of FOXC2 protein upon treatment with TWS119. Western blot analysis for the expression of FOXC2 protein using proteins extracted from HMLE Twist cells after 0, 2, 4, 8 and 24 hours (A) and SUM159 cells after 0, 24, 32, 48 and 52 hours (B) treated with 1 $\mu$ M concentration of TWS119.

#### **4.2.5 Effect of inhibition of GSK-3 $\beta$ using TWS119 on FOXC2 mRNA**

To determine whether the treatment of TWS119 affects the FOXC2 expression at the mRNA level, I measured the level of FOXC2 transcripts. RNA isolated using semi-quantitative qPCR from HMLE Twist and SUM159 cells that were treated with 1 $\mu$ M of TWS119 over a period of 24 and 52 hours, respectively. Preliminary analysis shows that there is a significant change in the FOXC2 mRNA level in HMLE Twist and SUM159 cells (Fig. 7). However, this experiment needs to be repeated to further confirm the findings. These results suggest that the inhibition of GSK-3 $\beta$  either directly or indirectly influences the transcription level of FOXC2. This suggests that the regulatory effect of GSK-3 $\beta$  on FOXC2 expression might be post-translational.

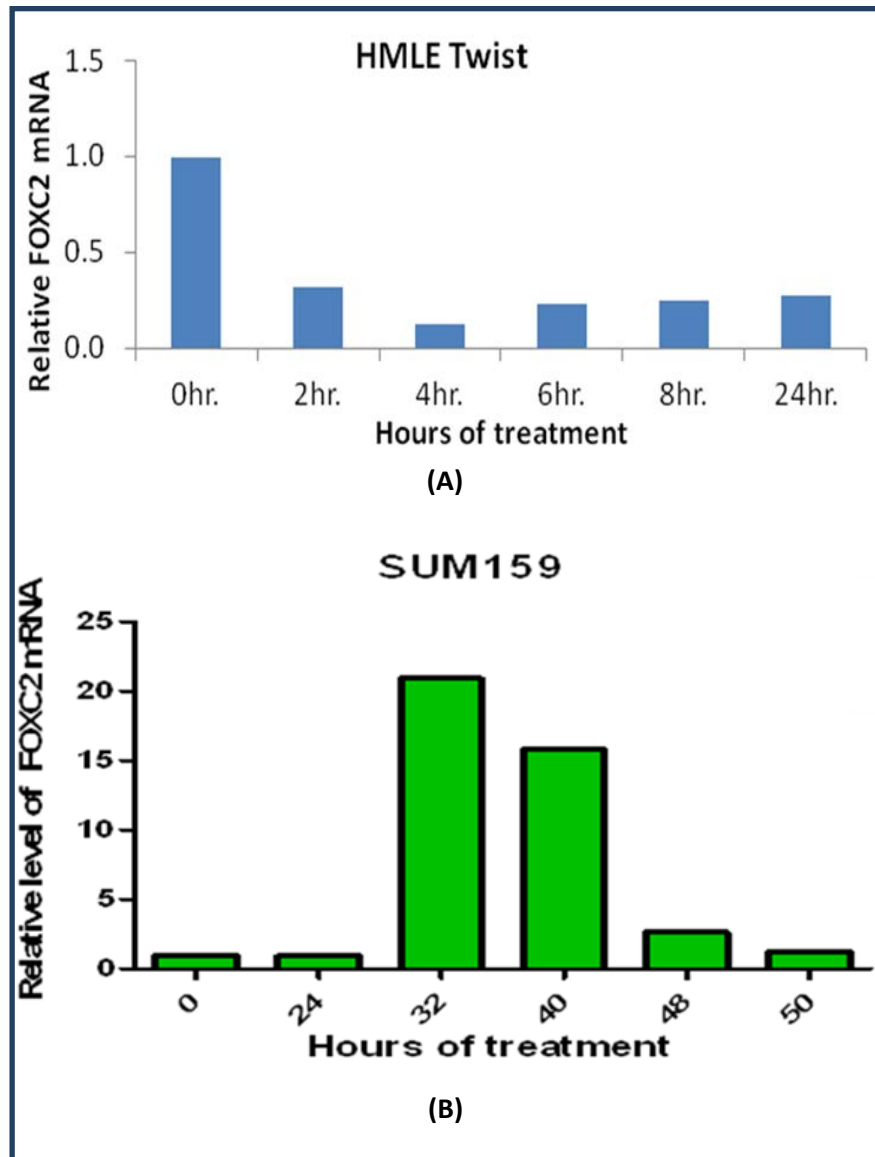


Fig. 7. Effect of GSK-3 $\beta$  on FOXC2 mRNA levels upon treatment with 1 $\mu$ M TWS119. A) Expression of FOXC2 mRNA was measured using RNA isolated from HMLE Twist treated with 1 $\mu$ M of TWS119 for 0, 2, 4, 6, 8 and 24 hours. B) Expression of FOXC2 mRNA was measured using RNA isolated from SUM159 cells treated with 1 $\mu$ M of TWS119 for 0, 24, 32, 40, 48 and 52 hours. Actin mRNA was used to normalize the loading.

#### **4.2.6 Inhibition of GSK-3 $\beta$ results in decrease of nuclear FOXC2 and increase in cytoplasmic FOXC2.**

Since FOXC2 functions as a transcription factor; its intracellular localization could profoundly affect its function. Moreover, phosphorylation of Snail or b-catenin by GSK-3 $\beta$  is known to influence their function, localization and stability. Therefore, I investigated the effect of exposure of HMLE Twist cells to GSK-3 $\beta$  inhibitor on the localization of FOXC2. For this, I treated HMLE Twist cells with 1 $\mu$ M of TWS119 for 24 hours and collected samples after 0, 2, 4, 6, 8 and 24 hours for nuclear and cytoplasmic protein extraction. Analysis of these samples using western blot showed that the FOXC2 protein decreased in the nucleus in a time dependent manner and concomitantly the FOXC2 protein level increased in the cytoplasm (Fig. 8). Because I previously found that the overall FOXC2 protein level decreased following 24 hours of exposure to GSK-3 $\beta$  inhibitor (Fig 6), it is possible that the FOXC2 protein could get phosphorylated in the nucleus and move to the cytoplasm, where it gets targeted for degradation.

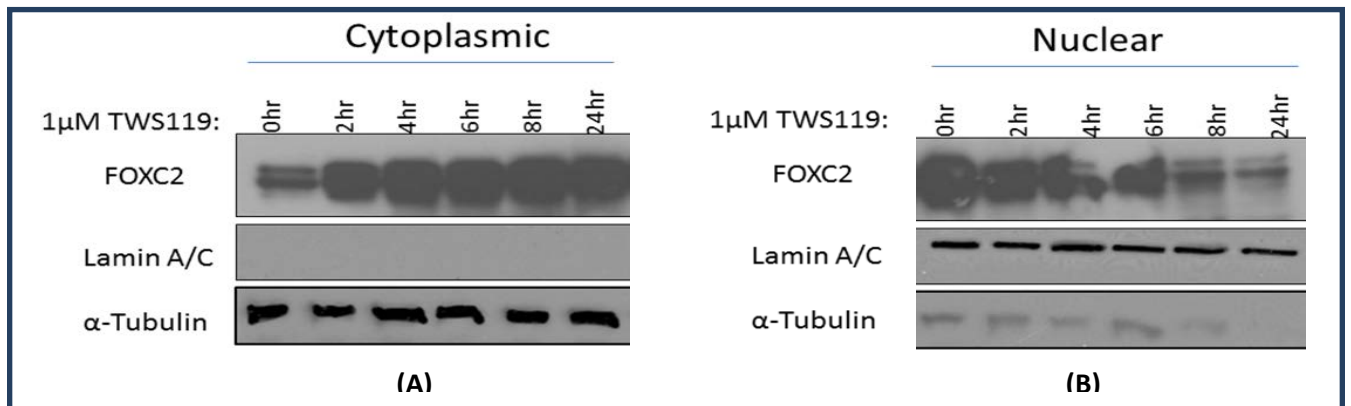


Fig. 8. Inhibition of GSK-3 $\beta$  using 1 $\mu$ M TWS119 results in decrease of nuclear FOXC2 with concomitant increase in cytoplasmic FOXC2. Lamin A/C and  $\alpha$ -Tubulin were used as the loading controls for nuclear and cytoplasmic proteins respectively.

#### 4.2.7 HMLE Twist cells change their morphology in response to TWS119.

HMLE Twist cells express endogenous FOXC2 and appear mesenchymal. I observed that inhibition of GSK-3 $\beta$  decreased FOXC2 protein levels and, strikingly, the morphology of HMLE Twist cells was also changed (Fig. 9). However, I did not notice similar changes in the morphology of SUM159 cells after TWS119 treatment.

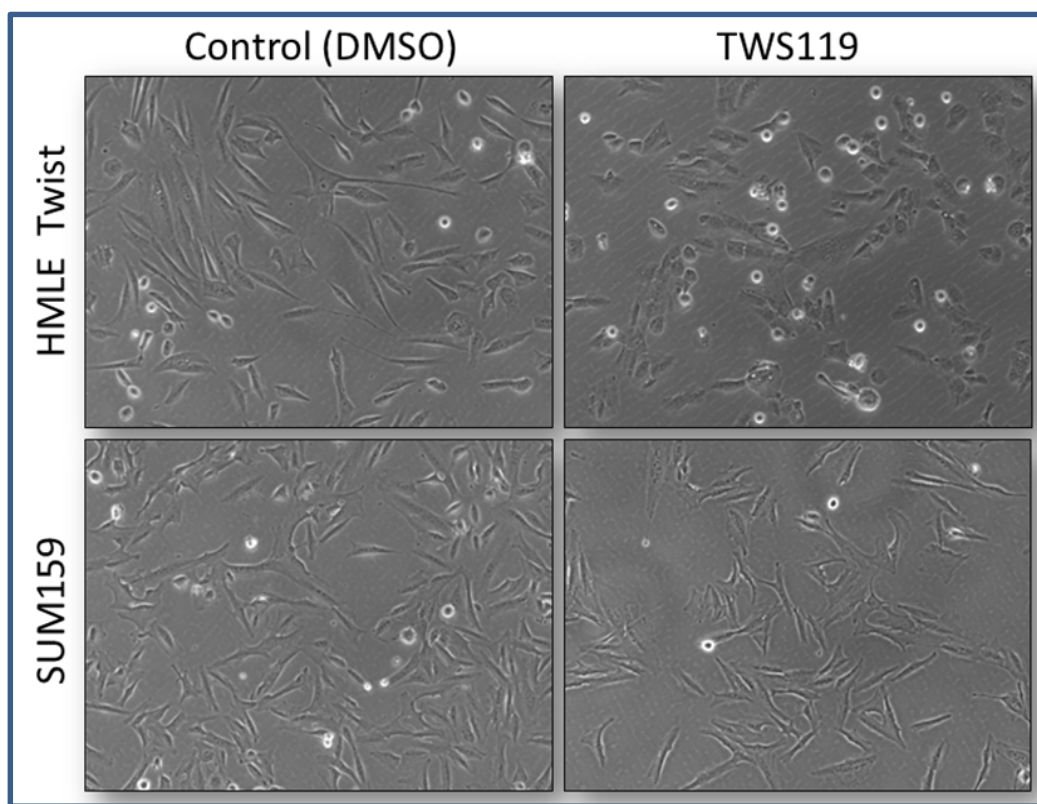


Fig. 9. HMLE Twist cells undergo a morphological change in response to 24 hr treatment with 1 $\mu$ M TWS119 (A&B) but not SUM159 cells (C&D). The control cells were treated with DMSO.



#### 4.2.8 Treatment with TWS119 decreases sphere formation.

HMLE Twist and SUM159 cells are known to form mammospheres, a surrogate assay for stem cell properties. Since inhibition of GSK-3 $\beta$  reduces FOXC2 protein in HMLE Twist and SUM159 cells, I investigated the result of TWS119 treatment on mammosphere formation. Exposure of these cells to TWS119 and DMSO for every three days for two weeks significantly affected the sphere formation compared with untreated cells (DMSO) (Fig. 10A). Sphere formation in SUM159 cells was not influenced by TWS119 treatment (Fig. 10 B). This suggests that the morphology and sphere forming ability might be regulated differently through the function of some other genes in SUM159 cells.

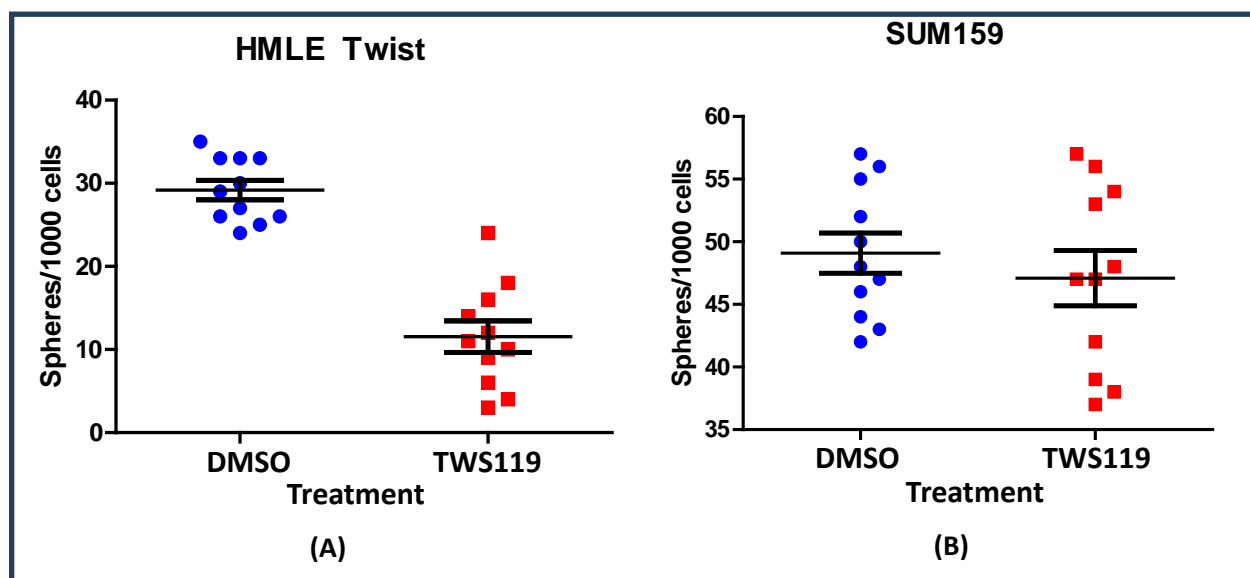


Fig. 10. Decrease in sphere formation in response to TWS119 treatment. HMLE Twist (A) and SUM159 (B) cells plated in low density culture plate and the number of spheres counted after two weeks of culture either presence of DMSO or 1 $\mu$ M TWS119.

### **4.3 Functional characterization of potential GSK-3 $\beta$ phosphorylation sites in FOXC2.**

#### **4.3.1 Generation of phosphomimetic and non-phosphorylatable mutants of FOXC2.**

In order to test whether the GSK-3 $\beta$  phosphorylation has any effect on FOXC2 expression, I mutated the predicted phosphorylation sites using in vitro site directed mutagenesis. To generate phosphomimetic mutants I mutated FOXC2T247 and FOXC2S363 to FOXC2E247 and FOXC2E363 respectively. In addition T247 and S363 were mutated to A247 and A363 to create non-phosphorylatable forms of FOXC2 (at these amino acid locations). Since the FOXC2 antibody may not recognize the mutated protein, I tagged these constructs with HA to monitor and detect the mutant FOXC2 protein. In order to test the stability and function of the mutant FOXC2 protein, I overexpressed the mutant constructs in transformed human mammary epithelial cells (HMLER), capable of undergoing EMT in response to the overexpression of wild type FOXC2. Interestingly, I observed that FOXC2 mutant protein expression varies in these cells. In particular, the phosphomimetic FOXC2 mutants, S363E and T247E protein levels are higher than the wild type FOXC2. FOXC2S363A mutant induced the protein expression at the same level as FOXC2S363E; however, there is a reduction in FOXC2 expression in T247A in comparison with T247E (Fig. 11). The variation in FOXC2 protein stability of these mutants indicates that the phosphorylation of the two amino acids by GSK-3 $\beta$  might have different roles in FOXC2 regulation and stability.

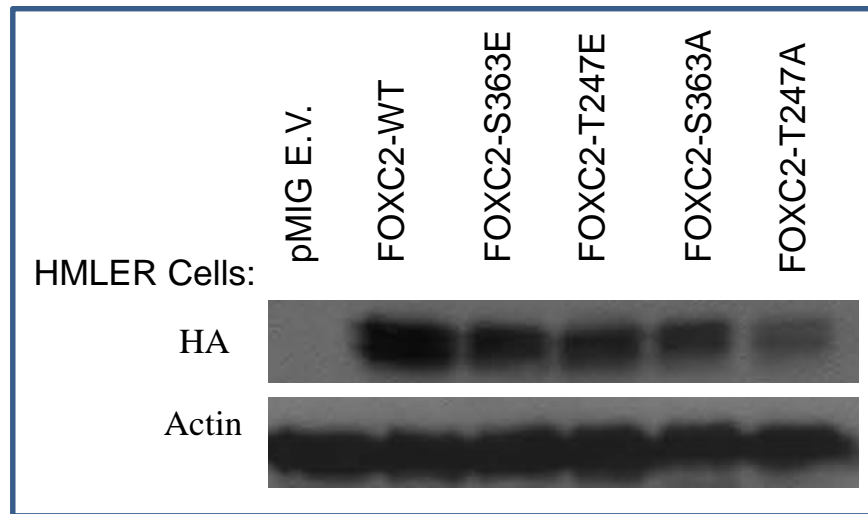


Fig. 11. Western blot analysis of the FOXC2 protein from HMLER cells expressing various FOXC2 mutants. HMLER cells transduced with vector containing FOXC2S363A, FOXC2S363E, FOXC2T247A and FOXC2T247E mutants in addition to wild type FOXC2 and empty vector. All the mutants and wild type FOXC2 were HA-tagged and therefore probed with anti-HA antibody.

## CHAPTER 5 DISCUSSION

The transcription factor FOXC2 is emerging as a potential tumor promoting gene. Accordingly, FOXC2 mRNA and protein levels are elevated in a number of human cancers including breast, colon, and gastric cancers. The facts that FOXC2 functions as a crucial regulator of EMT, stem cell properties and cancer cell invasion, contribute to its potential oncogenic impact during tumor progression and metastasis. Analysis of the FOXC2 amino acid sequence, using Scansite motif analysis software, revealed two potential phosphorylation sites for protein kinase GSK-3 $\beta$ , a well-known kinase that functions downstream of many signaling pathways implicated in cancer. GSK-3 $\beta$  is known to regulate Snail and b-catenin negatively. However, in this study my preliminary data suggest that GSK-3 $\beta$  has a positive role in the stability of FOXC2. However, additional experiments are necessary to further test, whether the increase in the stability of FOXC2 also correlates with its increased activity as a transcription factor.

Using sub cellular fractionation techniques, I further demonstrated a dramatic decrease in the level of FOXC2 protein present in the nucleus of cells treated with GSK-3 $\beta$  inhibitor and a concomitant increase in the level of cytoplasmic FOXC2. These results suggest that FOXC2 relocates from the nucleus to the cytoplasm upon GSK-3 $\beta$  inhibition. Further experiments are needed to determine whether dephosphorylation of both GSK-3 $\beta$  sites or each of them is sufficient for the export of FOXC2 to the cytoplasm. Moreover, it is essential to establish whether FOXC2 localizes to the cytoplasm by itself or through transfer proteins. In my preliminary results, the FOXC2 transcript levels are moderately altered following GSK-3 $\beta$

inhibition. This supports that most likely the effects of GSK-3 $\beta$  on FOXC2 occur primarily post-translationally. Collectively, these findings are consistent with the notion that GSK-3 $\beta$  functions as a kinase to phosphorylate FOXC2 and thereby influences its protein stability, subcellular localization and activity.

Consistent with the results from the GSK-3 $\beta$  inhibitor studies, silencing GSK-3 $\beta$  gene expression through shRNA had also a negative impact on FOXC2 protein levels. In addition, the phosphomimetic mutants induced EMT similar to wild type FOXC2 while the non-phosphorylatable mutants failed to promote a mesenchymal morphology. It is necessary to examine the role of these non-phosphorylatable mutants for their ability to inhibit the function of endogenous wild type FOXC2 using additional experiments.

Whereas my initial studies indicate that GSK-3 $\beta$  could potentially phosphorylate and regulate FOXC2 expression and function, additional experiments are required to conclude the outcome of phosphorylation and dephosphorylation. Furthermore, the contribution of each GSK-3 $\beta$  phosphorylation site in the regulation of FOXC2 protein stability and function is not clear. FOXC2 seems to be excluded and degraded upon inhibiting GSK-3 $\beta$ ; therefore, there could be a potential phosphatase involved in dephosphorylating FOXC2 and regulating its function. In addition, the subcellular localization, protein stability (+/- cycloheximide) and transcriptional activity of the mutants (FOXC2 reporter assays) might provide a better insight for understanding the functional consequences of GSK-3 $\beta$  and FOXC2 interaction.

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