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**Transcriptional Regulation of *TET* genes in Breast  
Cancer**

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# **Transcriptional Regulation of *TET* genes in Breast Cancer**

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**Transcriptional Regulation of *TET* genes in Breast Cancer**

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## **Dedication**

To mom and Dad, who always picked me up on time and encouraged me to go on every adventure, especially this one.

Abrar Ali Daoud Jamous

## **Declaration**

I certify that this thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledges, and that this thesis (or any part of the same) has not been submitted for the higher degree to any other university or institute.

Signed.....

Abrar Ali Jamous

Date: 12/5/2018

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## List of abbreviations

°C	Celsius
µl	Microliter
Min	Minutes
H	Hours
Nm	Nanometer
Ng	Nano gram
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
PBS	Phosphate buffer saline
RPM	Round per minute
SFM	Serum free media
ml	Milliliter
cDNA	Complementary DNA
RT-PCR	Real time- polymerase chain reaction
TF	Transcription factor
CpG	Cytosine phosphate guanine
EMT	Epithelial mesenchymal transition
AN	Accession Number
TSS	Transcription starting site
GnRH	Gonadotropin releasing hormone

## Abstract

Cancer transformation and development is associated with aberrant gene expression, which is acquired through genetic mutations and epigenetic mechanisms. One of the main epigenetic mechanisms altered in cancer is DNA methylation. While the mechanisms responsible for DNA methylation are well established and well-studied, DNA demethylation was thought to be a passive process until recent discovery of the Ten-Eleven Translocation (TET) family which actively demethylate DNA by hydroxylation of methylated cytosine (5hmC). TET family members are reduced in a variety of human malignancies, suggesting a tumor suppressor function of these proteins. Thus our hypothesis was that reduced activity of these enzymes is suggested to be responsible for hypermethylation and aberrant gene expression in breast cancer. In this project we wanted to study the regulation mechanisms of TET enzyme in breast cancer .through the assessment of some regulatory mechanisms impact on the expression of TET enzymes in breast cancer cells, including hormonal signaling and their downstream signaling transduction pathways. We also tested the presence of alternative transcription start sites for the *TET1* gene. And its promoter methylation as a possible regulatory mechanism. Our results show that TET enzymes are regulated by hormonal action and some kinases, well established like PKA, PKC, PI3k and CaMKs, that are involved in the hormonal regulation of the TET enzymes. In addition, our results show that *TET1* have different isoforms with different expression patterns in breast cancer cell lines. Interestingly, these isoforms are differentially regulated by hormonal activities. We also showed that expression of loner isoform of *TET1* is suppressed by promoter methylation. Overall, our finding demonstrate that *TET* genes expression is regulated, in part, in breast cancer at the transcriptional level and that *TET1* has two isoforms of the enzyme that are differentially expressed and regulated. All together, our data suggests that TET enzymes are important in breast tumorigenesis and that further research is needed to elucidate more detailed molecular pathways that are involved in TET enzyme regulation in the context of breast cancer development and progression.



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# Chapter 1

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## 1. Introduction

According to WHO, cancer is the second leading cause of death worldwide (Global Burden of Disease Cancer et al., 2017). This disease is defined as uncontrolled cell division that leads to sustained proliferation. In addition to its continuous cell division, cancer cells acquire different properties that support their survival and progression. These phenotypes including resistance to apoptosis, inducing angiogenesis, and evading the immune system are called the hallmarks of cancer (reviewed in; Hanahan & Weinberg, 2011). Acquiring these characteristic phenotypes through cancer transformation and development is associated with aberrant gene expression that disrupts the balance between oncogenes and tumor suppressor genes as well as other genes that maintain normal cell identity.

In light of the fact that all cells in the human body contain the same genetic information, gene expression regulation is needed for proper cell development and differentiation, and more important, for the response to environmental changes and stimuli. Regulation of gene expression is achieved through different mechanisms at different levels, including; transcription, RNA splicing, RNA degradation, translational regulation, posttranslational modifications, protein localization, allosteric regulation, and protein degradation. The most important mechanism that determines the expression status of genes is transcription initiation, which depends on regulatory sequences in gene promoter, where RNA polymerase and transcription factors assemble to start transcription. Another type of regulatory sequences are enhancers and repressors which are considered as gene-distal regulatory elements (Lee & Young, 2013), in addition to epigenetic regulation level.

Epigenetics literally means “above” genetics and it is defined as all the processes that cause changes in gene expression without affecting DNA sequence. It’s important for normal

cellular development, differentiation and homeostasis. Epigenetic mechanisms involved in gene expression regulation include, but not limited to, chromatin remodeling and histone modification. DNA is wrapped around an octamer structure of histones that contain two copies of four histone proteins; H2B, H2A, H3, and H4, to form a nucleosome. Condensation of the nucleosome to form the chromatin is the basis for chromosome folding. Chromatin structure which could be tight (heterochromatin) or relaxed (euchromatin) changes the accessibility of TF to promoter sequences, which will affect transcription of the respected genes. In heterochromatin, genes are most likely silenced while in euchromatin they are actively transcribed. Chromatin structure compaction is affected by active modification of the histones body and tail. These modifications include; methylation, phosphorylation and acetylation. For example acetylation of histones by adding an acetyl group by acetyltransferases open the chromatin structure and moreover recruits transcription factors to the promoter of a target gene. On the other hand, deacetylation by histone deacetylases closes chromatin structure and inhibits expression. In addition, different studies have shown an association between histone marks and genomic features like promoters and enhancers. For example, H3K4me3 is found to be present in active promoters, while H3K27me3 H3K9me3 are present in inactive and silenced genes. Also, H3K27Ac indicates the presence of active enhancers and promoters (Rivera & Ren, 2013).

Another mechanism involved in epigenetic gene expression regulation is DNA methylation. It involves the addition of methyl group at position 5 of cytosine. This process is catalyzed by DNA methyltransferase enzyme family members; DNMT3a, DNMT3b and DNMT1. This gene expression regulatory mechanism is an extremely important mechanism and defects in this process were found to have fatal outcomes. For example, DNMT1 knockout in mouse model led to embryonic lethality (E. Li, Bestor, & Jaenisch, 1992), while repression of DNMT1 using CRISPR/cas9 in embryonic stem cells led to rapid and massive loss in the DNA methylation which resulted in cell death (Liao et al., 2015)Methylation occurs at CpG dinucleotide regions, which are called CpG Islands. These CpG Islands are usually found at the centromeric tandem repeat units and in the promoter area of many genes. Methylation of the promoter sequence is associated with long-term transcriptional repression. Gene

expression inhibition of methylated genes is believed to be through preventing the binding of transcriptional activators. Moreover, the binding of Methyl-CpG-binding proteins to methylated regions recruits histone deacetylase and other transcriptional repressors to modify chromatin and further repress gene expression (Klose & Bird, 2006).

DNA methylation is involved in many cellular processes like X chromatin inactivation, through maintaining the repression of genes on the inactivated X chromosome (Schübeler, 2015), genome imprinting and silencing of the repetitive element of DNA. Since methylation is very important for proper embryogenesis and normal development, cellular differentiation and reprogramming, aberrations in DNA methylation are associated with many human diseases including cancer. Alteration in DNA methylation pattern is one of the most common events in cancer, which is characterized by global hypomethylation and regional hypermethylation of tumor suppressor genes that leads to genomic instability, which has great role in cancer initiation and progression (Herman, 1999; Robertson, 2005). Moreover, methylation pattern in cancer cells is different from normal cells, which makes it a good biomarker for cancer detection and predicting the progression of the disease (Mikeska & Craig, 2014).

While DNA methylation mechanisms are well understood and documented, mechanisms responsible for counterbalancing and reversing methylation are not very well established in both normal and cancer tissues. The only known demethylation process was thought to be through passive dilution of methylated cytosine during cell division due to the loss of DNMT function. Thus, the exact active mechanism(s) of DNA demethylation was elusive for many years until the discovery of Ten Eleven Translocation proteins (TETs)(Kohli & Zhang, 2013).

The TET enzyme family consists of three members, TET1, TET2 and TET3. TET enzymes structure consists of catalytic domain at the C terminus that is conserved for the three enzymes TET1, TET2, and TET3. It is composed of cysteine-rich region and double strand



beta sheet at the C-terminus (DSBH). This CD contains also binding sites for the cofactors Fe and  $\alpha$ -Ketoglutarate, which are involved in the oxidation reaction of the methylated cytosine, as explained below. TET1 and TET3 have CXXC zinc finger domain in the N-terminus, which is responsible for the recognition of CpG islands and the binding to DNA (fig 1). In one study, it was shown that overexpression the TET1 catalytic domain causes global DNA demethylation, while overexpression of the full-length TET1 didn't lead to the same effect, which reveals that the CXXC domain of TET1 is important for targeting of CpG islands. Moreover, TET1 CXXC domain was found to bind unmethylated CpG sequences which indicates that TET1 is mainly involved in maintaining hypomethylation signal (C. Jin et al., 2014). Another study found that localization of TET1 to the heterochromatin is determined through the guidance of Methyl-CpG binding domain protein 1 (Mbd1) through its CXXC domain (P. Zhang et al., 2017). TET3, CXXC domain mutagenesis revealed that this domain has higher affinity for 5caC and the other derivatives of the cytosine (S. G. Jin et al., 2016). Although TET2 doesn't have CXXC domain (Fig 1.1), it still has the ability to bind DNA through the help of IDAX, which has the same structure as the CXXC domain (Ko et al., 2013).

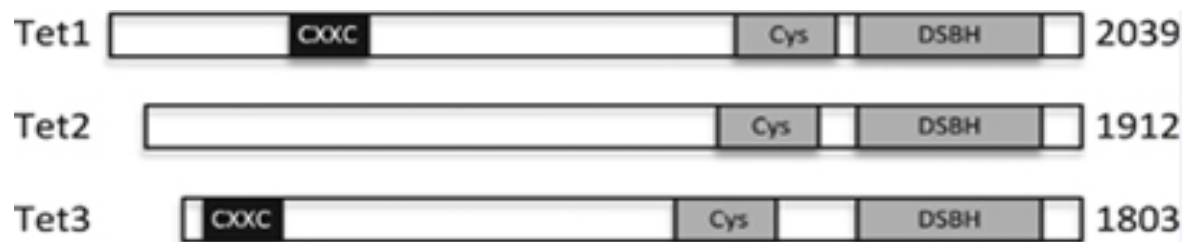


Figure 1.1 Schematic illustration of TET1, TET2, TET3 enzyme structure(Zhao & Chen, 2013).

TET enzymes are hydroxymethylase family of enzymes. These enzymes catalyze DNA demethylation in stepwise manner oxidation reactions. TET enzymes convert 5-methylcytosine to 5-hydroxymethylcytosine which was found to act as a stable epigenetic marker that prevents further methylation of DNA by reducing the binding affinity of DNMT to CpGs that will contribute to passive demethylation of DNA (Hashimoto et al., 2012). In addition, TET enzymes can catalyze active demethylation through further oxidation of 5-hydroxymethylcytosine to form 5-formylcytosine and 5-carboxylcytosine, which can be

converted to free cytosine through the base excision repair system by thymine DNA glycosylase TDG enzyme (Fig1.2) .

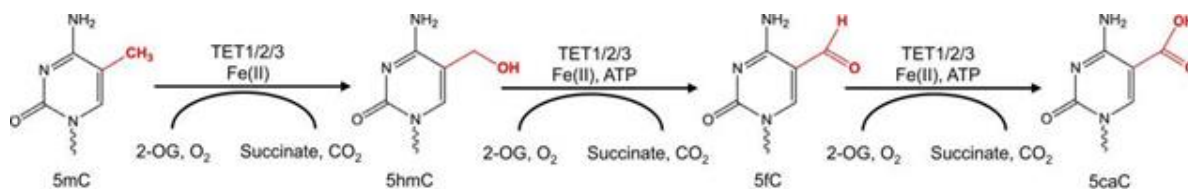


Figure 1.2 DNA demethylation catalyzed by TET enzymes. TET enzymes convert methyl cytosine to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) through serial oxidation reaction (Tan & Shi, 2012)

TET enzymes convert alpha ketoglutarate to succinate and CO<sub>2</sub> in the oxidation conversion of 5-methylcytosine to its products. It was found that TET enzyme activity is inhibited by the accumulation of oncometabolites like; succinate, fumarate and 2-hydroxyglutarate. These alpha ketoglutarate analogues compete with TET enzyme substrate for binding to their catalytic domains (Laukka et al., 2016). Ascorbic acid (vitamin c), an antioxidant known to enhance the  $\alpha$ -KG/Fe dependent dioxygenase enzymatic activity was found to directly interact with the catalytic domain in the C terminus of TET enzyme and induce its dioxygenase activity to convert 5mc to its oxidation products (Yin et al., 2013).

Different physiological functions were assigned to the different TET enzymes. TET enzymes might have common as well as distinct functions in embryonic development, stem cell biology, cell differentiation as well as neuronal cell biology (Rasmussen & Helin, 2016).

TET enzymes have been shown to have different expression pattern in different physiological processes, which indicate that these enzymes have different roles in different biological processes. For example, knockout mouse model for TET3 leads to neonatal lethality, while TET2 knockout did not affect embryonic development but instead it leads to the development of myeloid malignancies (Z. Li et al., 2011). TET1 is highly expressed in embryonic stem cells, which is associated with high level of 5hmc. While TET1 knockout “ES cells show

reduction in 5hmc level, it preserve it's pluripotency properties. Moreover, *TET1* knockout mice produce viable fertile mice (Dawlaty et al., 2011).

*TET1* was found to have two isoforms that are expressed differently in different tissues. For example, the long full length isoform of *TET1* is expressed in mouse embryonic stem cells, and throughout differentiation, the expression of this long isoform is replaced by a shorter isoform, which becomes the dominant isoform in adult tissues (W. Zhang et al., 2016).

The highest levels of 5hmc was found to be concentrated in brain tissue , which is due to the overexpression of TET1 in brain tissue which is very important for the demethylation of very essential genes in the nervous system like fibroblast growth factor 1 FGF1 and brain-derived neurotrophic factor BDNF (Guo, Su, Zhong, Ming, & Song, 2011).

Some studies reported that TET enzymes are important for proper differentiation of embryonic stem cells. In this context TET1,TET2 and TET3 triple knockout mouse has aberrant promoter hypermethylation that results in deregulation of developmental gene expression that leads to impairments in the differentiation of ES (Dawlaty et al., 2014).

In cancer, it has been found that the expression pattern of TET enzymes is altered in both liquid and solid tumors. In liquid cancers, recently, in a mouse model, it was found that loss of function of *TET2* and *TET3* genes cause complete loss of 5hmc which leads to initiating aggressive myeloid cancers (An et al., 2015). Moreover, mutations in *TET2*, for example, were found to be the most common genetic alteration among hematological malignancies (Scourzic, Mouly, & Bernard, 2015). In solid tumors, different studies demonstrated an association between loss of 5-hydroxymethylcytosine and low expression of *TET* genes in different human tumor types including; liver, lung, pancreatic, prostate and breast cancer (H. Yang et al., 2013).

In a liver cancer mouse model, it was found that reprogramming of DNA methylation occurs and is characterized by loss of 5hmc in CGI, an event that is directly associated with hyper methylation and transcriptional silencing of CGI in tumors. Moreover, in *TET1* knockout mice, the liver shows low level of 5hmc , which indicates that the loss of 5hmc during tumorigenesis is, in part, due to the downregulation of TET1 (Thomson et al., 2016). In another study which compared the level of TET1, TET2, and TET3 in cervical cancer (CC) tissue to normal cervical tissue samples, the level of TET1 expression was significantly decreased in cervical cancer patient compared to normal subjects. Also, TET2 and TET3 were shown to be reduced in CC patients (Bronowicka-Klys et al., 2017). Recently, in a study conducted on gastric cancer samples, immunohistochemistry results showed decreased levels of 5hmc in gastric cancer tissues compared to the adjacent normal tissue. This reduction was associated with low expression of the TET1 enzyme (K. C. Wang et al., 2018).

TET2 loss of function in different types of cancer was found to result from mutations or epigenetic silencing and was shown to be the most common genetic alteration among hematological malignancies (Scourzic et al., 2015), however in parathyroid carcinoma, TET2 expression was significantly reduced in cancer tissue compared to normal tissue as a result of heavy TET2 promoter methylation (Barazeghi et al., 2017). Moreover, TET3 was found to inhibit epithelial-mesenchymal transition in ovarian cancer by demethylating the tumor suppressor microRNA mir-30d which directly targets TGF- $\beta$ 1 one of EMT inducers. Thus, it was shown that down regulation of TET3 leads to the activation of TGF- $\beta$ 1-induced EMT and progression of ovarian cancer (Ye et al., 2016)

### **1.1.TET enzymes and Breast cancer**

Breast cancer is the second most common leading cause of death among women (Siegel, Miller, & Jemal, 2017). Like other types of cancer, breast cancer is characterized by heterogeneity which means that cancer tissue contains different cell subtypes that are diverse in their cellular and molecular features. Breast cancer is classified according to the expression of three main types of receptors: estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2). Basal-like tumors which are triple-negative are ER, PR, and HER2 negative and it's high histological grade indicates bad prognosis. While Luminal A tumor are low

grade breast cancer cells that are positive for ER, PR and HER2, luminal B are HER2-enriched(Guiu et al., 2012).

Regarding their role in breast cancer development and progression, it was shown that TET enzymes expression was reduced in breast cancer samples. (L. Yang, Yu, Hong, Yang, & Shao, 2015). In addition, it was found that low *TET1* expression level correlated with a more aggressive cancer and lymph node metastasis. (Sang, Cheng, Tang, Zhang, & Lv, 2015). Another study has found that TET1 and TET2 down regulation is associated with 5hmC levels, and that miss-localization of TET1 contributes to the loss of 5hmC which correlates with bad prognosis of breast cancer (Tsai et al., 2015). Moreover, TET3 was found to be downregulated in metastatic breast cancer cell lines compared to non-tumorigenic ones. Also, TET3 was found to inhibit the proliferation of breast cancer cell with the help of BRAC1 to co-repress EZH2 gene which promotes the tumorigenesis and metastasis of breast cancer (M. Wang et al., 2016).

Regarding the mechanisms behind the reduction of TET enzymes expression of these enzymes at the transcriptional level, many studies showed that the low level of TET1 in many types of cancer is due to the hypermethylation of *TET1* promoter (Li et al., 2016). In breast cancer, it was found that the level of *TET1* promoter hypermethylation in metastatic cells is higher than cells of primary site. Since methylation of the *TET1* promoter was frequently found in breast cancer, it is considered as a diagnostic marker for breast cancer (Sang, Cheng, Tang, Zhang & Lv, 2015). In hypoxia in breast cancer, HIF1 $\alpha$  was found to induce the transcription of *TET1* and *TET3* (Wu et al., 2015).

One of the post-transcription level regulation mechanism of TET1 in breast cancer is by microRNA Mir29a, which is overexpressed in breast cancer was shown to directly target TET1 and reduce its expression. TET1 downregulation was found to be involved in promoting cell proliferation and also epithelial-mesenchymal transition (Pei, Lei & Liu, 2016) Postnatal mammary gland development is dependent on hormones like estrogen and

other female reproductive hormones including; progesterone, prolactin and GnRH (Briskin & O'Malley, 2010). During puberty estrogen is the major mitogenic signal that stimulates the growth of mammary gland cells, and also breast cancer (LaMarca & Rosen, 2007).

Breast cancer is considered as hormone-dependent cancer. One of the most important hormones that play critical roles in breast cancer development is estrogen, and about 60% of breast cancer cases express estrogen receptor (Masood, 1992). Estrogen has an important role in regulating gene expression by binding to its intracellular receptor, which acts as a transcription factor by binding to response elements on the promoter region of target genes or by the activation of a variety of signal transduction pathways (e.g. ERK/MAPK, p38/MAPK, PI3K/AKT, PLC/PKC) that eventually regulate the expression of many different genes (Marino, Galluzzo, & Ascenzi, 2006). Another hormone that plays an important role in breast cancer development is Gonadotropin-releasing hormone (GnRH). Which stimulates pituitary gland secretion of Luteinizing and follicle stimulating hormones. GnRH exert its activity through binding to its receptor (GnRHR), expressed in pituitary tissues and other non-pituitary tissues like ovary, placenta, uterus, and breast (Cheng & Leung, 2005), and in many types of cancer including; prostate, ovary, and breast cancer cell lines (Harrison, Wierman, Nett, & Glode, 2004; Kakar, Grizzle, & Neill, 1994). Interestingly GnRH agonist was shown to have an anti- proliferative effect on breast cancer (Everest et al., 2001). Moreover, GnRH was found to decrease the invasiveness ability of breast cancer (von Alten et al., 2006).

GnRH receptor is a G protein-coupled receptor that activates phospholipase c (PLC) which in turn activates protein kinase c (PKC) and increases cytoplasmic level of calcium (Ca<sup>2+</sup>), which induces the synthesis and secretion of gonadotropin (Cheng & Leung, 2005).

In this project, the regulation of *TET* genes expression was studied in breast cancer at the transcriptional level in response to different hormones including Estrogen and GnRH on the expression of different *TET* genes and also *TET1* isoforms on the RNA level. The methylation pattern of the *TET1* gene promoter differs in different breast cancer cell line.

We also found that *TET1* has two isoforms that are differentially expressed in different cell types.

## **1.2.Hypothesis**

The expression of *TET1* is down regulated in breast cancer, thus we hypothesize this is due to promoter hyper methylation. In addition, breast cancer cell gene expression is highly affected by hormones. Consequently, we think that different hormones might also alter *TET* expression in various the breast cancer cell lines.

## **1.3. Objectives and specific aims**

Main Objective: Study at least two mechanisms responsible for altering *TET* expression in breast cancer cell lines.

Specific Targets:

- 1.3.1. Study the effect of estrogen and GnRH on the expression of *TET1*, *TET2* and *TET3*.
- 1.3.2. Use different signaling pathway effector inhibitors to elucidate how hormones alter *TET* gene expression.
- 1.3.3. Test the methylation status of *TET1* gene promoter in breast cancer cell lines that have low or normal expression levels of this .
- 1.3.4. Understand the expression pattern and regulation of *TET1* isoform.

## Chapter 2

### 2. Materials and methods

#### 2.1. Materials

**Table 2.1 list of materials.**

No	Material	Manufacture
1	MDEM/F12 media	Beit Haemek
2	RPMI (1640) media	Gibco Thermofisher
3	Charcoal striped Fetal bovine serum	Biological industries
4	Fetal bovine serum	Gibco Thermofisher
5	Hydrocortisone	Sigma
6	Insulin	Sigma
7	Epidermal growth factor (EGF)	Sigma
8	Cholera toxin	Sigma
9	Glutamine	Biological industries
10	Penicillin/streptomycin	Biological industries
11	DMSO	sigma
12	Sterile phosphate buffer saline PBS	Biological industries
13	Isopropanol biological gradient	Sigma
14	Ethanol biological gradient	Sigma
15	Chloroform biological gradient	Sigma
16	qScript™cDNA synthesis kit	Quanta Biosciences
17	SYBR® Green	Applied Biosystems
18	TRIZOL	Sigma
19	GnRH hormone	Biological industries
20	5-aza-2'-deoxycytidine	Sigma
21	H89	Calbiochem
22	Wartmannin	Sigma
23	Kn93	Calbiochem
24	NGIC-I	Calbiochem
25	EpiTect® Fast Bisulfite Conversion	QIAGEN
26	Blood/Cell DNA Mini Kit	Geneaid
27	Red master mix	larova
28	GoTaq® Green Master Mix	Promega
29	RNase A	



**Table 2.2 list of equipment's and tools.**

No	Equipment or tool	Company	Industrial country
1	Inverted microscope	Olympus ck40-SLP	Japan
2	Biological hood (HERA guard)	Heraeus	Germany
3	Biofuge Stratos Reconditioned	Heraeus 75005289R	Germany
4	Biofuge Fresco	Heraeus 75005521	Germany
5	Hera cell 150 CO2 Incubator	Heraeus	Germany
6	Labofuge 200 centrifuge	Heraeus	Germany
7	Autovortex SA6	Stuart Scientific	U.K
8	Water Bath Orbital Shaking	Grant OLS200	U.K
9	Water Bath	Grant LTD6G	U.K
10	SPIN-micropipette site	Nano Spinreact	china
11	Digital dry bath	Labnet	U.S.A
12	Elisa reader	BioTek EL-X800	U.S.A
13	Analytical Balance	METLER TOLEDO AB104	Switzerland
14	Autoclave	HIRAYAMA HV-110	U.S.A
15	RT-PCR	(Applied Biosystems 7500 FAST Real Time PCR	Singapore
16	PCR machine 96 well	Applied Biosystem #9902	Singapore

## **2.2.Methods**

### **2.2.1. Cell culture**

Breast cancer cell lines MCF7, MDA MB231, and T47D cells were grown in RPMI media (from Beit-Haemek), supplemented with 10% FBS, 1% glutamine, and 1% penicillin/streptomycin (all from Beit-Haemek). MCF10A cells were grown in DMEM/F12 media (from Beit-Haemek) supplemented with 5% horse serum, 1% glutamine, 1% Penicillin/Streptomycin (all from Beit-Haemek), 20 ng/mL EGF, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, and 100 ng/mL cholera toxin, (All from Sigma Aldrich). Cells were incubated in a humidity chamber at 37 °C with 5% CO<sub>2</sub>.

### **2.2.2. Cells freezing**

Freezing media was prepared to contain 10% DMSO (Sigma), 40% fetal bovine serum and 50% cell culture media. Cells were detached from the culture plate by adding 1ml trypsin. collected in freezing media and divided into aliquots in cryo-tube, and were stored in liquid nitrogen cell storage Tank.

### **2.2.3. Cell passage**

After removing the media, cells were washed with 1 ml X 1 PBS. Then, 1ml trypsin was added to cells and incubated in the humidity chamber at 37 °C until the cells were detached from the plate. Then cells were collected and divided into new culture plates depending on different experiment needs. Finally, cells were incubated in a CO<sub>2</sub> incubator at 37 °C.

#### **2.2.4. 5-aza-2'-deoxycytidine (5-aza-dc) treatment**

Cells were treated with 0.5-10  $\mu\text{M}$  5-aza-dc (Sigma) for 2 to 5 days. The medium containing 5-aza-dc was changed every 2 days.

#### **2.2.5. Hormone and inhibitor treatment**

Cells were cultured in RPMI media without phenol red supplemented with 10% charcoal-dextran-treated fetal bovine serum, 1% L-glutamine, and 1% penicillin streptomycin (All from Beit-Haemek) for 1-2 days before hormonal treatment. For experiments that included both hormone and kinase inhibitor treatments, inhibitors ;1  $\mu\text{M}$  Wortmannin from Sigma, 50  $\mu\text{M}$  Kn 93, 20  $\mu\text{M}$  H89, and 10  $\mu\text{M}$  NGIC-I from (CalBioChem) were added 30 min before hormonal treatment, then treated with  $10^{-9}$  M GnRH and Estradiol (both from Beit-Haemek).

#### **2.2.6. RNA extraction and reverse transcription-PCR and Real-Time PCR**

Total RNA was prepared using the TRI reagent (Sigma Aldrich) as described by the manufacturer. The concentration of RNA sample was measured using Nano-drop spectrophotometer, and the integrity of RNA was determined by running it on 0.8% agarose. Then one microgram of RNA was used for cDNA synthesis using qScript™ cDNA synthesis kit (Quanta Biosciences). Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and carried on Applied Biosystems® 7500 Real-Time PCR machine. Primers were designed at exon/exon boundaries to prevent amplification from genomic DNA. All measurements were performed in triplicate and standardized to the levels of the house keeping gene *hUBC*.

### 2.2.7. DNA extraction and bisulfite conversion

DNA was extracted using Genomic DNA mini kit (Geneaid). For bisulfite conversion, 1.5 to 3 µg of DNA were converted using EpiTect Fast Bisulfite kit (QIAGEN) according to the manufacturer's instructions.

### 2.2.8. Bioinformatics

*TET* Gene sequences were obtained from PubMed gene bank (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler, 2004). using the accession numbers NM\_030625.2 for *TET1*, NM\_001127208.2 for *TET2*, and NM\_001287491.1 for *TET3*. RT-PCR primers were designed using the primer3 website (Untergasser A et al., 2012). CpG islands prediction, methylation specific primers and bisulfite sequencing primers were designed using MethPrimer (Li LC and Dahiya R, 2002). Transcriptional Start sites were defined (recognized) using UCSC genome browser (Kent et al., 2002).

**Table 2.3 Methylation specific primer for TET1 two transcription starting sites.**

NO	target	Primer sequence
1	EXON1.20 0M	F.P 5'-ttttgggaatcgattttttatttc-3' R.P 5'-aaacctacaccaaccctcga-3'
2	EXON1.20 0UM	F.P 5'-ttttgggaattgattttttatttt-3' R.P 5'-caaacctacaccaaccctcaa-3'
3	EXON1.40 0M	F.P 5'-gttttgcgtttttggtttttc-3' R.P 5'-ccgaaaacattatttatctccga-3'
4	EXON1400 UM	F.P 5'-gtttgtgttttggtttttgt-3' R.P 5'-caaaaacattatttatctcaac-3'
5	EXON3 M	F.P 5'-ttgtttatttttggttaggttc-3' R.P 5'-aatcaactatcactaaacatctatccg-3'
6	EXON3 UM	F.P 5'- ttgtttatttttggttaggttg-3' R.P 5'-aatcaactatcactaaacatctatccac-3'

**Table 2.4 RT and conventional PCR primers.**

No.	Gene	Primer sequence
1.	<i>hUBC</i>	F.P. 5'-gtcgcagttcttgtttgtgg-3' F.P. 5'-gatggtgtcactgggctcaa-3'
2.	<i>TET1</i>	F.P. 5'-ccacagggacattcacaaca-3' R.P. 5'-catggagctgctcatcttga-3'
3.	<i>TET2</i>	F.P. 5'-ccgagacgctgaggaaatac-3' R.P. 5'-acatgctccatgaacaacca-3'
4.	<i>TET3</i>	F.P. 5'-cccacaaggaccagcataac-3' F.P. 5'-ccaagagtctgctggacac-3'
5.	<i>GnRHR</i>	F.P. 5'-gacccccacgaactacaact-3' R.P. 5'-ctgggtctgacaacctgtt-3'
6.	<i>E2R</i>	F.P. 5'-atcctgatgattggtctcgtct-3' R.P. 5'-ggatatggtccttctctccag-3'
7.	<i>TET1</i> Exon2_Exon4	F.P. 5'-gtgtaaccagcacagttcatg-3' R.P. 5'-tgtgtccacttctccacctc-3'
8.	<i>TET1</i> Exon1_Exon2	F.P. 5'-caagtcatgcagccctacct-3' R.P. 5'-cattttgttggtcccttg-3'
9.	<i>TET1</i> Exon3-Exon4	F.P. 5'-gaaaacaagaggccccagag-3' R.P. 5'-gcgttttatggttgcagtg-3'

**2.2.9. Methylation-specific PCR**

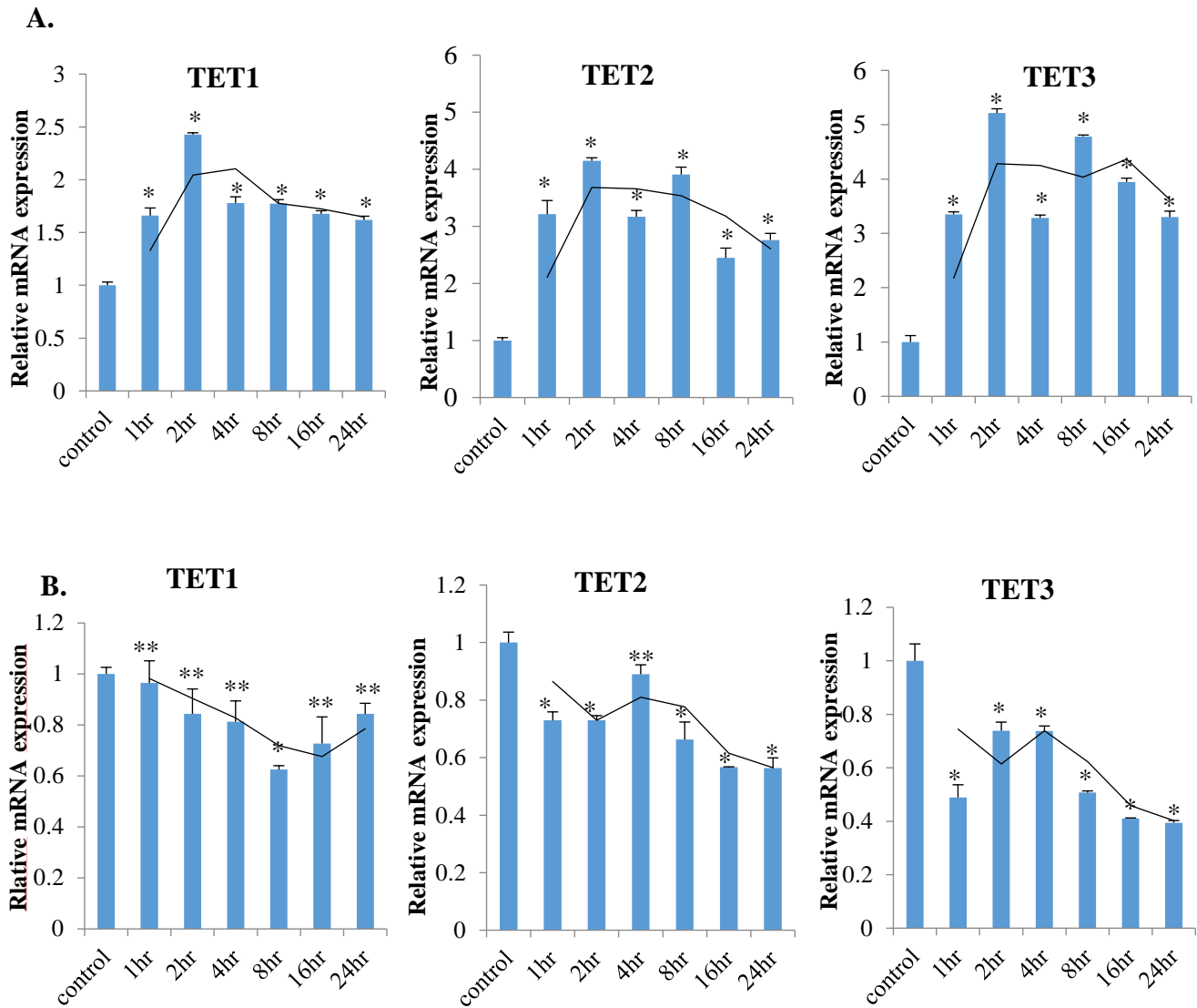
The primers for MSP were designed using MethPrimer as mentioned above. The primers were designed to cover two possible regulatory regions in *TET1* isoforms, which were identified using ENCODE Data on UCSC genome browser. The first region is in exon 1 and the second region is in the end of intron 2 before exon 3.

PCR conditions were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72°C for 30 s and a final extension at 72 °C for 5 min, PCR was performed using advantage PCR mix (cleantech).

### 3. Results

#### 3.1. Effect of GnRH on *TET* mRNA levels

In breast cancer, gene expression is highly regulated by different hormones (Bernhardt et al., 2016). GnRH hormone receptor was found to be expressed in breast cancer tissue (Eidne, Flanagan, Harris, & Millar, 1987). Moreover, GnRH was found to have an anti-proliferative effect on breast cancer cells. Thus, we wanted to study the effect of GnRH hormone on the transcription of *TET* mRNA in breast cancer cell lines that express GnRH receptor. To this end, MCF7 and T47D cells were incubated with GnRH for different time points. In MCF7, real-time PCR analyses showed that GnRH increased the expression of *TET1*, *TET2*, and *TET3* over all-time points tested, ranging from 2.5 to 5 folds with a peak at 2hrs of incubation (Fig.3.1.A). In comparison to MCF7, GnRH hormone has almost no effect at certain time points while lowering the expression of the different TET enzymes in others (Fig.3.1.B).



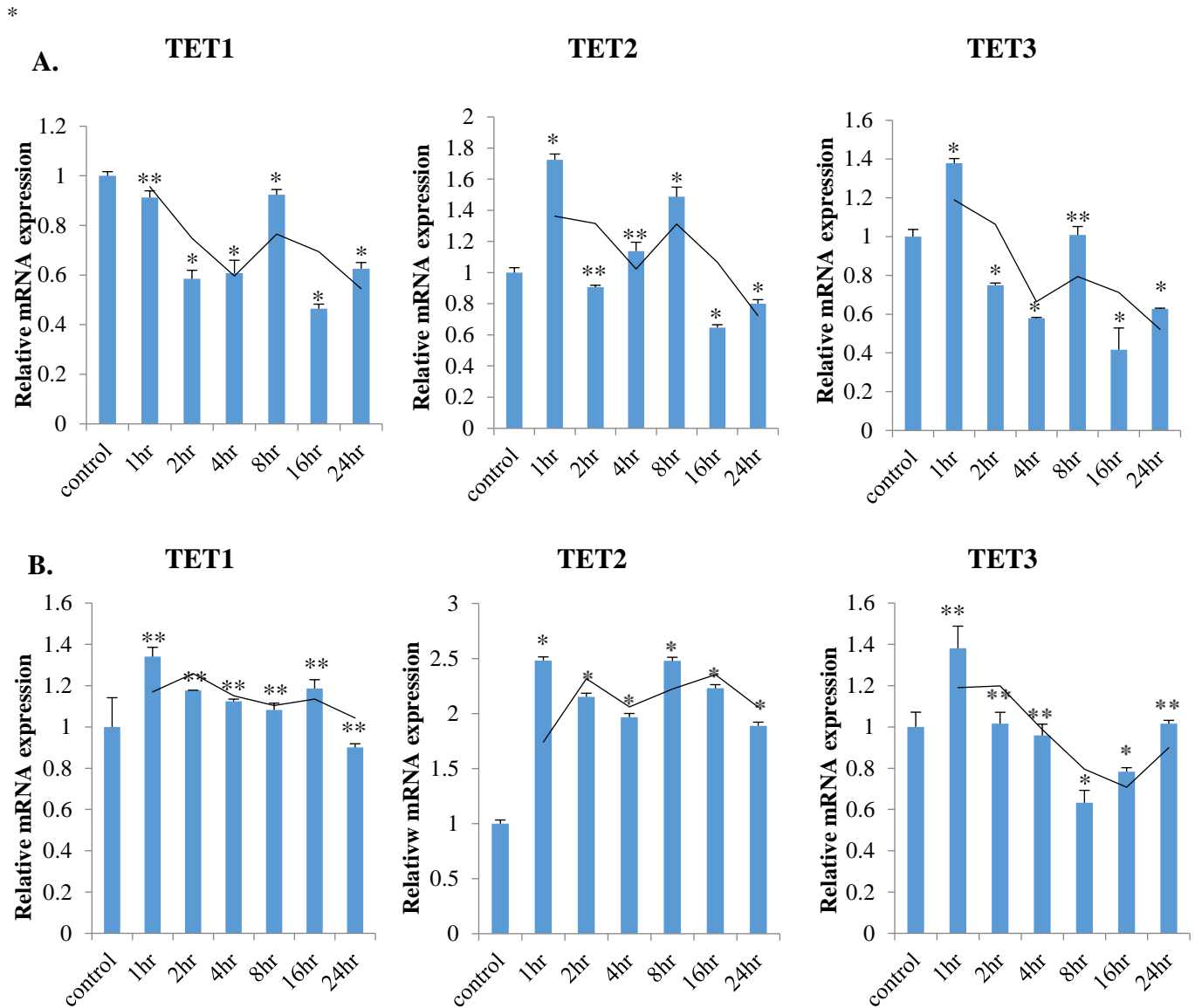
**Figure 3. 1. Effect of GnRH on *TET* gene expression in breast cancer cell lines.** (A). Real time PCR results showing the expression level of *TET1*, *TET2*, and *TET3* gene after treating MCF7 cells with  $10^{-9}$  GnRH hormone for the indicated time points. (B). Real time PCR results showing the expression level of *TET1*, *TET2*, and *TET3* gene after treating T47D cells with  $10^{-9}$  GnRH hormone for the indicated time points. The mRNA levels are shown after normalization to the level of the housekeeping gene *UBC* and relative to mRNA levels in control untreated cells. Bars represent SD of the mean. To test for the significance of difference between different time points and the control untreated cells, we did T-test to calculate p-value. (\*\* means that p-value > 0.05 and \* means that p-value < 0.05).

### 3.2. Estrogen decrease the expression of TET enzymes

Estrogen is a breast cell mitogen. It enhances cell proliferation which has mutagenic effect on breast cells that leads to breast cancer development (Yue, Yager, Wang, Jupe, & Santen, 2013). Estrogen produces its effect through its binding to estrogen alpha receptor, which in turn regulates gene expression by directly binding to DNA or indirectly by interacting with other proteins in the cell. (McDonnell & Norris, 2002). Because it affects gene expression, we tested the effect of estrogen on TET enzymes expression by treating MCF7 and T47D estrogen Alpha positive cells (appendix 2) with estrogen for different time points. Our qRT-PCR results in MCF7 cell line show that Estrogen lowers the expression of *TET1* and *TET3* most the time (Fig. 3.2.A) while increase or has almost no effect in the expression of *TET2* at certain time points. However in T47D cells qRT-PCR results were different *TET1* and *TET3* has no effect but *TET2* expression was increased most the time with peak of 2.5 fold (Fig. 3.2.B).

Estrogen has differential effect on different TET enzyme in different type of cells.





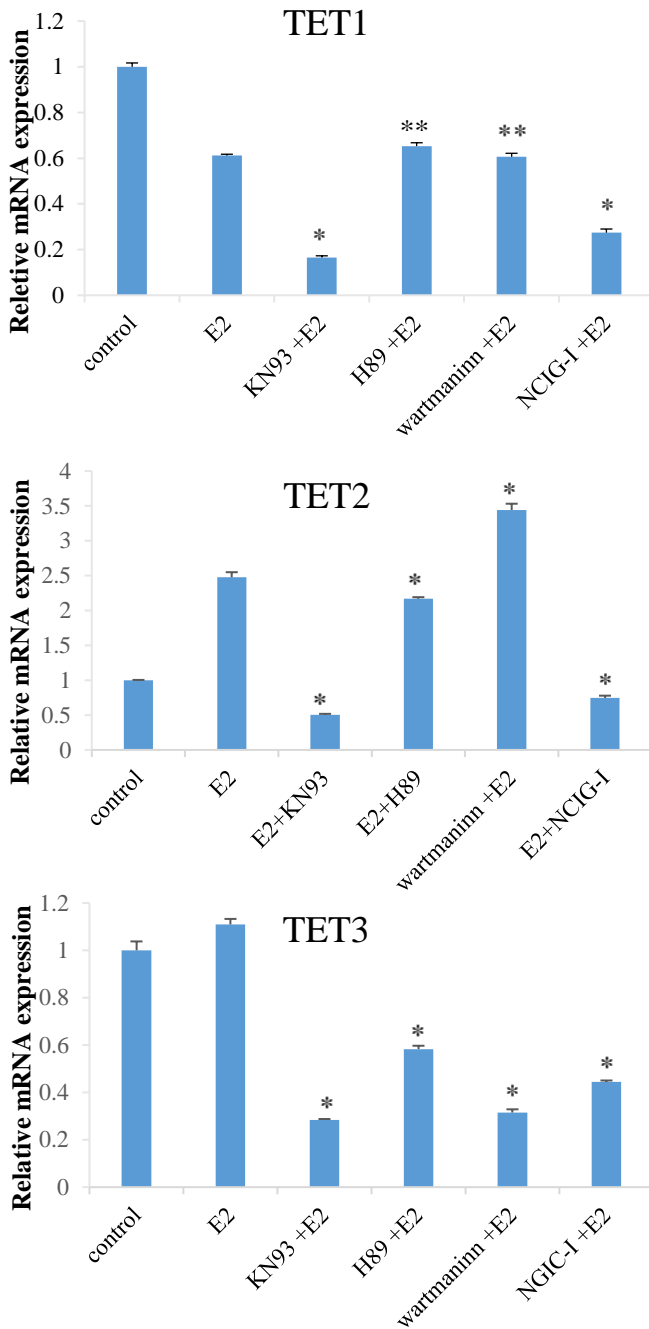
**Figure 3. 2. Effect of estrogen on *TET* gene expression in breast cancer cell lines.** (A). Real time PCR results showing the expression level of *TET1*, *TET2*, and *TET3* gene after treating MCF7 cells with  $10^{-9}$  Estrogen hormone for the indicated time points. (B). Real time PCR results showing the expression level of *TET1*, *TET2*, and *TET3* gene after treating T47D cells with  $10^{-9}$  Estrogen hormone for the indicated time points. The mRNA levels are shown after normalization to the level of the housekeeping gene *UBC* and relative to mRNA levels in control untreated cells. Bars represent SD of the mean. To test for the significance of between different time points and the control untreated cells, we did T-test to calculate p-value. (\*\* means that p-value > 0.05 and \* means that p-value < 0.05).

### 3.3. Kinase inhibitor effect on TET mRNA level

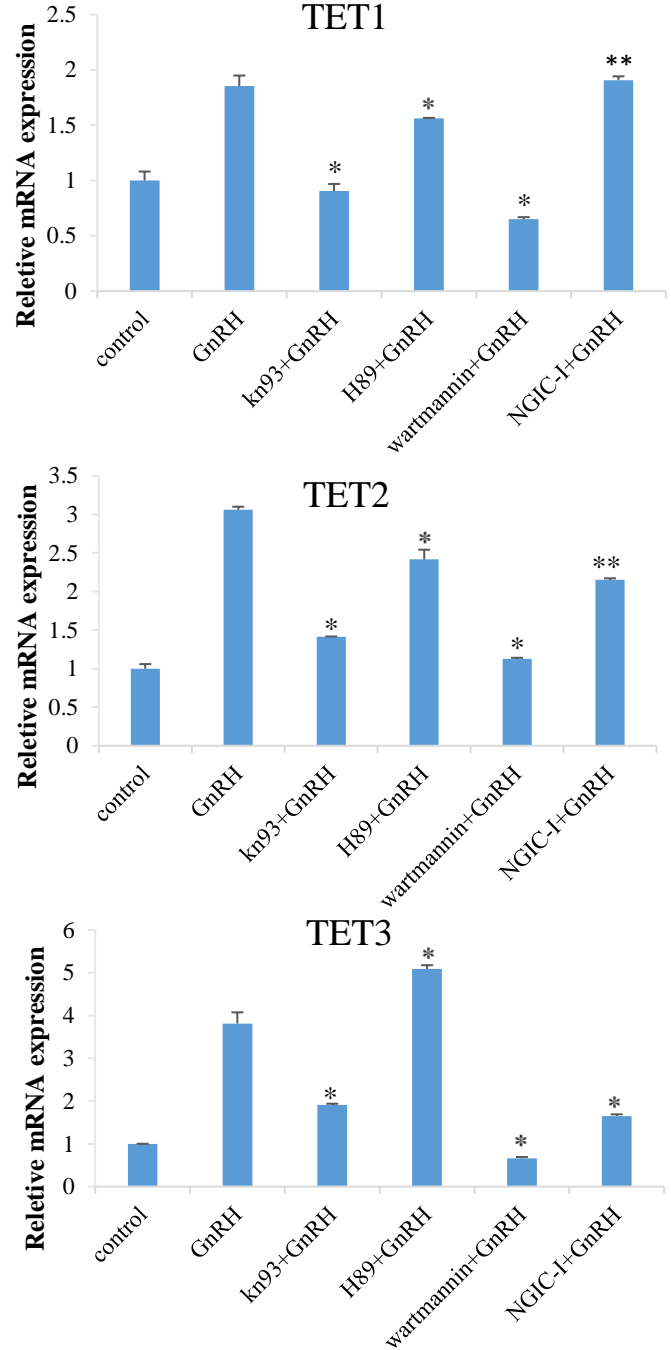
After screening for the effect of E2 and GnRH hormone effect on the expression of TET enzymes in breast cancer cell lines, we wanted to elucidate the intracellular signaling pathway(s) downstream of these hormone receptors that is responsible for regulating *TETs* gene expression. In order to do so, we co-treated both MCF7 and T47D cells with E2 and GnRH and different cellular kinase inhibitors including; PKA inhibitor (H89), CaMKs inhibitor (Kn93) or PI3K inhibitor (Wartmannin) and PKC inhibitor (NGIC-I). We can see the great effect of kn93 in reducing the expression of all TET enzyme with both estrogen and GnRH treatment (Fig. 3.3), its clearly demonstrated that Wartmannin has effect on *TET1* and *TET2* but not *TET3* in estrogen treated cells (Fig.3A), while affecting the expression of all TET enzyme treated with GnRH (fig.2.3.B). NGIC-I shows remarkable effect on all *TET* genes in estrogen treated cells (fig.3A), but has no effect in GnRH treated cells (Fig.3.3.B). H89 reduce the expression of *TET1* and *TET3* but not *TET2* in estrogen treated cells (Fig.3.3.A), while in GnRH treated cells it doesn't show the same effect on all *TET* genes (Fig. 3.3.B) .

All inhibitors have different effects on *TET* expression indicating these kinases are involved differentially in the cellular response to GnRH and Estrogen treatment GnRH treatment.

**A.**



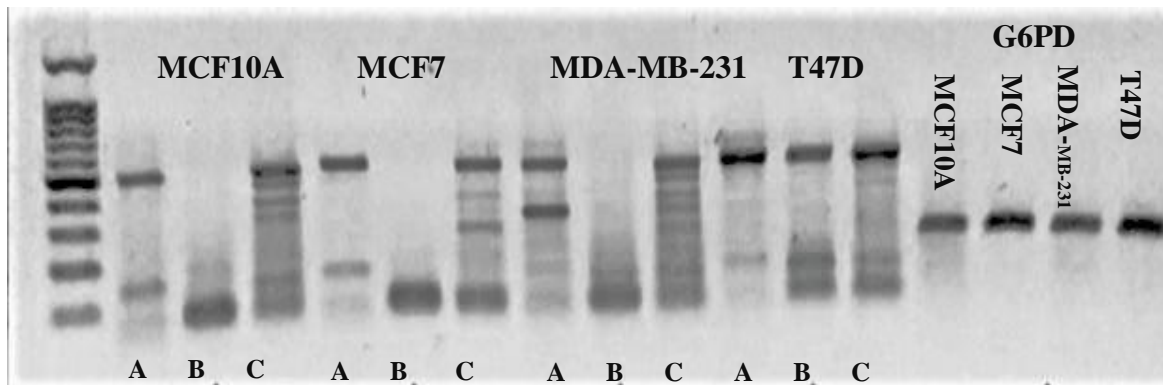
**B.**



**Figure 3. 3. Effect of kinases inhibitor with hormones on *TET* gene expression in breast cancer cell line.** Real time PCR results showing the expression level of *TET1*, *TET2*, and *TET3* mRNA after treating MCF7 cells with  $10^{-9}$  GnRH hormone for 2hrs after pre-treating the cells with H89 (PKA inhibitor), Kn93(CaMKs inhibitor) or Wartmanin (PI3K inhibitor). The mRNA levels are shown after normalization to the level of the housekeeping gene *UBC* and relative to mRNA levels in control MCF7 cells. Bars represent SD of the mean. To test for the significance of difference between different time points and the control cells, we did T-test to calculate p-value. (\*\* means that p-value>0.05 and \* means that p-value <0.05).

### 3.4. TET1 has two isoforms

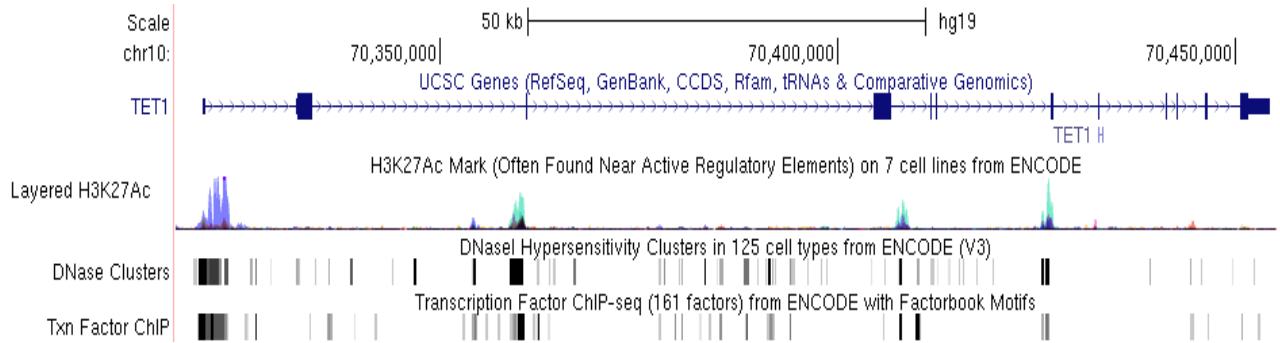
During real time and conventional PCR analyses of *TET1* RNA levels, using different sets of primers, we noticed the possible presence of different *TET1* isoforms, as shown in (Fig.3.4) that PCR results using primer targeting different exon of *TET1*, primers targeting exon 1 and 2 are expressed in T47D cells but not in other cell lines which gives hint these two exon might be lost and there is another *TET1* isoform that leak Exon1 and 2. while primer targeting the Exon 3 and 4 are expressed in all cell lines. Moreover, a recent work had just showed the presence of Tet1 isoform switch that regulates epigenetic memory erasure and mouse development (W. Zhang et al., 2016).



**Figure 3. 4. Expression pattern of *TET1* isoforms in breast cancer cell lines.** Conventional PCR results using primers that target different *TET1* exons in different breast cancer cell lines. A: 2-4 407bp, B: 1-2, and C: 3-4 PCR product 470, 466, and 464 respectively, we used G6PD as internal control. 100bp ladder.

Therefore, we performed some bioinformatics analysis on ENCODE data using the UCSC genome browser, to detect possible transcriptional start sites. Both DNaseI sensitivity cluster and localization of H3K27Ac3 clearly suggest that human *TET1* is expressed as two possible isoforms using two possible transcription initiation sites (TSS). To further confirm the presence of alternative TSS, we did analysis on published transcription factor chromatin immunoprecipitation data. This analysis showed that *TET1* has two possible TSS and one of those TSS leads to the transcription of a shorter isoform that lacks the first exon and possibly all or a part of exon 2 (Fig.3.5) which might lead to

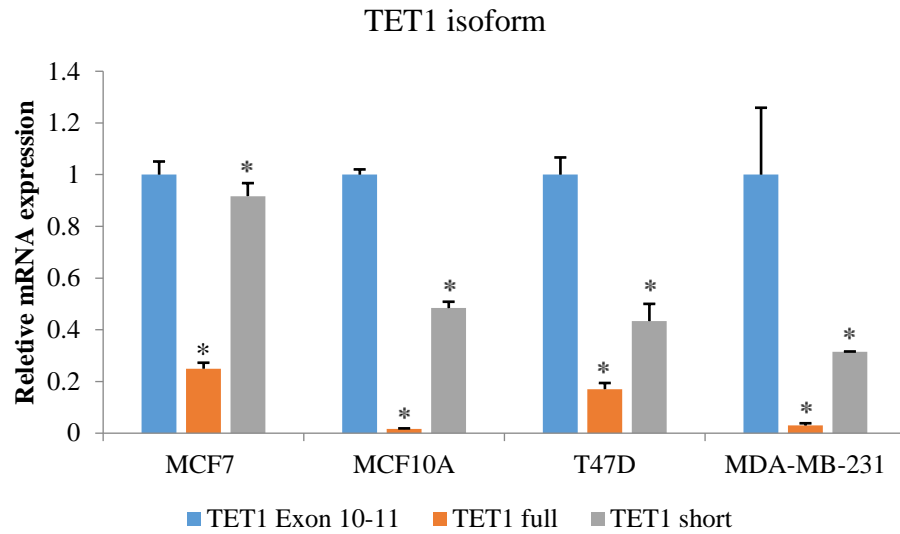
the translation of a shorter TET1 protein from an alternative translation start site present in exon 3, a finding that suggests the presence of a shorter TET1 protein.



**Figure 3. 5. *TET1* two possible promoter characterization using UCSC genome browser.** UCSC genome browser analysis of the 5' end of the human *TET1* gene showing the DNase clusters, H3K4me3 and Txn Factor chip localization to indicate possible transcription start-sites

To test this hypothesis and to confirm the bioinformatics data, we did real time PCR on RNA isolated from MCF7, T47D, MCF10A and MDA MB231 cells.

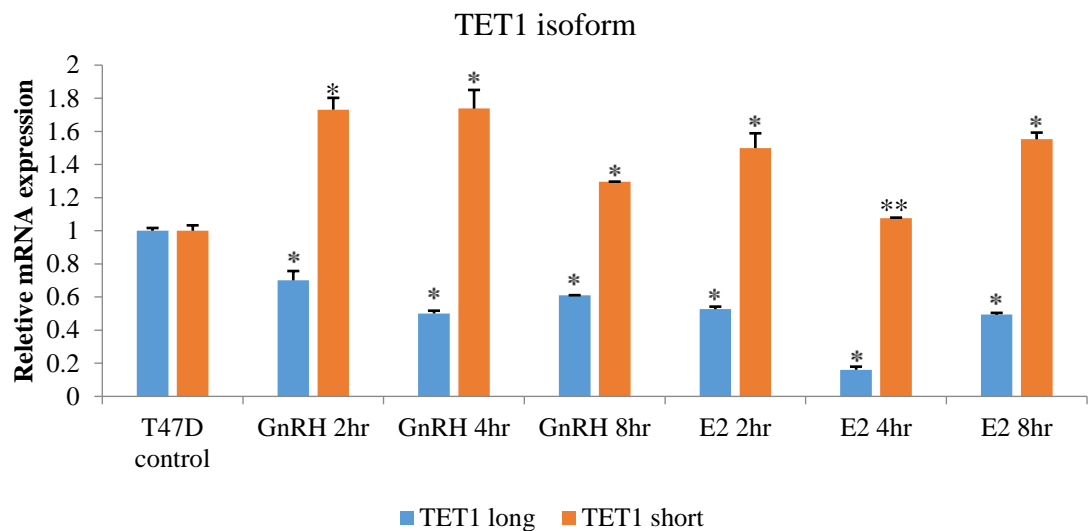
After treating RNA with DNase, we quantitated the expression of *TET1* using primers that target either the long or the short isoforms of *TET1*. The expression level of Exons 10 and 11 was used as our reference point. As shown in Fig.3.6, all cells express higher levels of the shorter *TET1* isoform regardless the cells being basal or luminal. Moreover, our results show that luminal cells express higher levels of the longer *TET1* isoform compared to very low expression levels of this isoform in basal cells.



**Figure 3. 6. Expression pattern of *TET1* isoforms in breast cancer cell lines.** qPCR results on different *TET1* isoforms. Exons 10-11 were used as our reference point. The mRNA levels are shown after normalization to the level of the housekeeping gene *UBC* and relative to mRNA levels in control untreated cells. Bars represent SD of the mean. To defined the significances in differences between *TET1full* and *TET1short* expression by T-test to calculate p-value. (\*\* means that p-value>0.05 and \* means that p-value <0.05), to a significant results the p-value <0.05.

### 3.5.TET1 isoform and hormone treatment

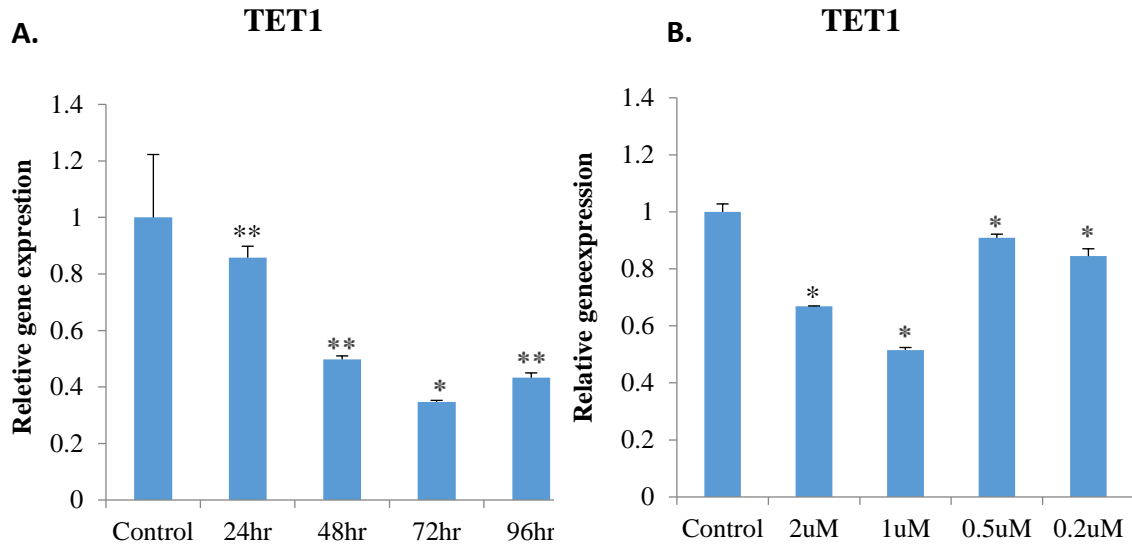
After examining the expression of *TET1* two isoforms in different breast cancer cell lines, we wanted to see the how do hormone treatment affects the expression of these two isoforms in T47D cells. Our qRT-PCR showed that both estrogen and GnRH increased the expression of *TET1* short isoform, while decreasing the expression of *TET1* long isoform (Fig.3.7).



**Figure 3. 7. Effect of hormones on the expression pattern of *TET1* isoforms in breast cancer cell lines.** qPCR results on different *TET1* isoforms. Exons 10-11 were used as our reference point. The mRNA levels are shown after normalization to the level of the housekeeping gene *UBC* and relative to mRNA levels in control untreated cells. Bars represent SD of the mean. To define the significance in difference between the expression of *TET1* long and short isoform in control untreated cells and different time point hormone treated cells by T-test to calculate p-value. (\*\* means that p-value>0.05 and \* means that p-value <0.05), to a significant results the p-value <0.05.

### 3.6.Methylation of *TET1* promoter; effect of 5-aza-2'-deoxycytidine on *TET1* mRNA level

Methylation of *TET1* promoter represent one mechanism behind its low expression in liver cancer (Thomson et al., 2016). Analogous to this, we wanted to know whether promoter methylation of *TET1* is the reason behind it's low expression in breast cancer cell lines. To this end, we tested *TET1* promoter methylation status in MDA MB 231 breast cancer cell line that expresses low levels of *TET1*. We treated the MDA MB 231 cell with the demethylation chemical, 5-aza-2'-deoxycytidine for different time points using different concentrations. Our qPCR analyses showed that 5-AZA decreases the expression of *TET1* in time dependent manner. And time (Fig.3. 8).



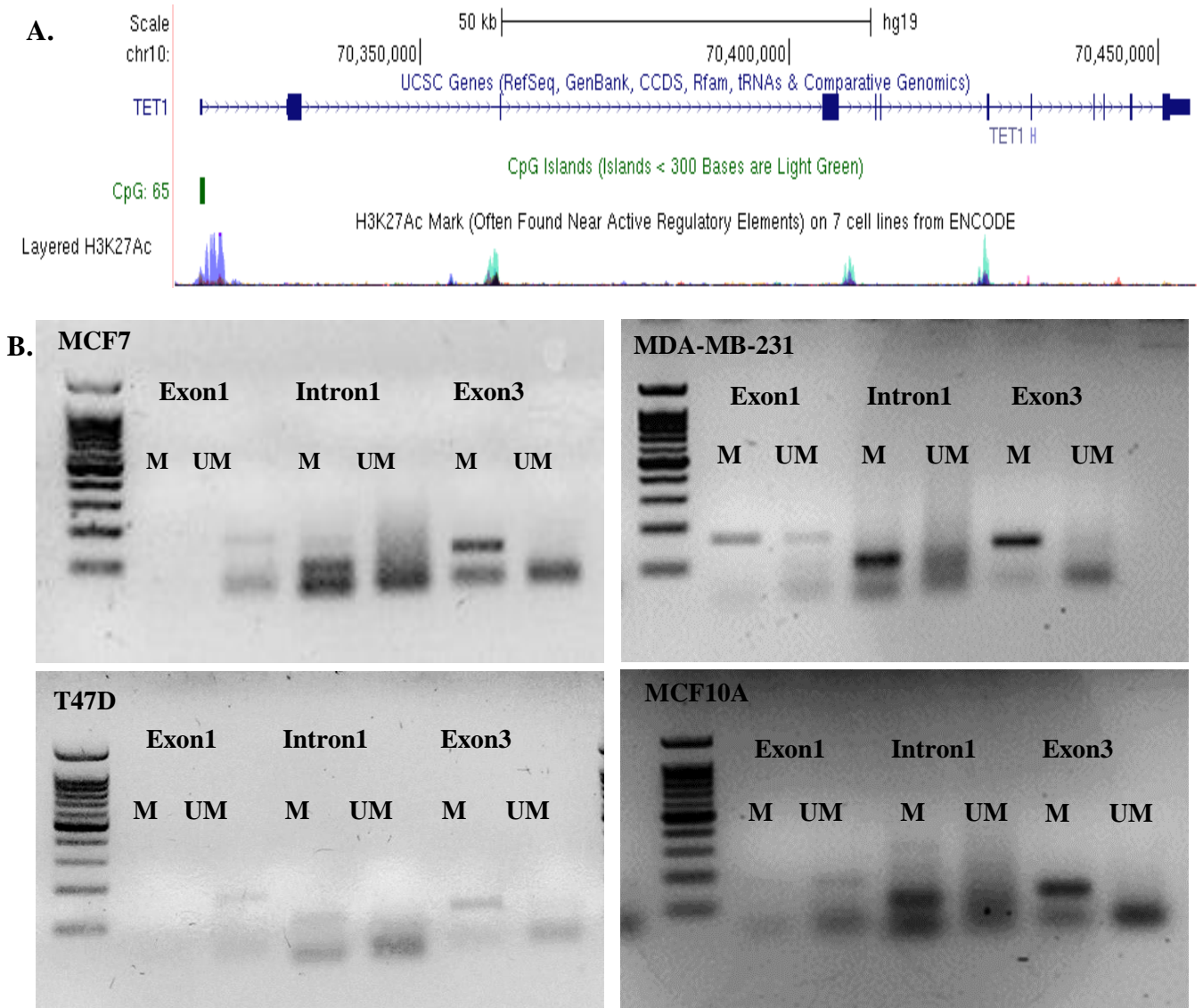
**Figure 3. 8. Effect of 5aza on the expression of *TET1* in breast cancer cell line.** (A):Real time PCR results showing the expression level of *TET1* mRNA after treating MDA-MB-231 cells with 2uM of 5-aza-2'-deoxycytidine for different time points. (B): Real time PCR results showing the expression level of *TET1* mRNA after treating MDA-MB-231 cells with different concentrations of 5-aza-2'-deoxycytidine for 48 hours. The mRNA levels are shown after normalization to the level of the housekeeping gene *UBC* and relative to mRNA levels in control MDA MB231cells. Bars represent SD of the mean. To define the significance of differences between the expressions of *TET1* long and short isoform in control untreated cells and 5-aza-2'-deoxycytidine treated cells by T-test to calculate p-value. (\*\* means that p-value>0.05 and \* means that p-value <0.05), to a significant results the p-value <0.05.

### 3.7.Methylation of *TET1* promoter by methylation specific PCR

Since treatment with 5-aza can reactivate transcription repressors that could repress the transcription of *TET1* and thus leads to reduced *TET1* expression instead of increasing it, we decided to study *TET1* promoter methylation pattern using methylation specific PCR. To do so, we first studied *TET1* promoter using UCSC genome browser to identify the localization of the CpG islands in the regulatory region of *TET1* (Fig.3. 9A). Based on our analysis, we then designed methylation specific primers that cover the regulatory region for *TET1* long isoform in EXON1 and *TET1* short isoform before EXON3, using MethPrimer free online software. Methylation-specific PCR (MSP) was performed on sodium bisulfite-treated DNA derived from MCF7, MDA-MB-231, T47D, and



MCF10A cell lines. MS-PCR result showed that the promoter of the long *TET1* isoform is un-methylated in MCF7 and T47D cell lines, while hypermethylated in MDA-MB-231 and in the transformed breast cell line MCF10A (Fig. 3.9B). The short isoform of *TET1* possible promoter before exon 3 is found to be methylated in all cell lines (Fig.3.9B).



**Figure 3. 9. Methylation of CpG Island in *TET1* promoter.** **A.** UCSC genome browser CpG island prediction in *TET1* regulatory region that start in EXON1 and end in the 3' of INTRON1. **B** methylation specific PCR for the regulatory region in EXON1, 3' of INTRON1 for the long *TET1* isoform, and before EXON3 the regulatory region of *TET1* Short isoform

### 4. Discussion

Proper gene expression regulation is very important for normal cell physiology and homeostasis. Cancer transformation and development is associated with aberrant gene expression. One of the aberrant gene expression regulation mechanisms in cancer is gene methylation. It has been shown that cancer has different genome methylation pattern compared to normal tissue. This methylation pattern of cancer genomes was thought to be related only to enzymes that are able of methylating CpG islands. However, it was recently discovered that there is a family of enzymes, TET family, that are able of demethylating genes and actively reverts methylation. These enzymes were found to be downregulated in different types of cancer, including breast cancer (L. Yang et al., 2015). In this research work different mechanisms that might be responsible for differential TET enzyme expression in breast cancer cell lines were investigated. Our data showed that different hormones affect the expression of TET enzymes in different manners. and at least *TET1* has two isoforms transcribed from two different transcription start sites.

Breast cancer is known as a hormone-dependent cancer. Different hormones can alter gene expression in breast cancer .through activation of different intracellular signaling cascades that influence gene transcription in a positive or a negative manner (Bernhardt et al., 2016). GnRH receptor was found to be expressed in different types of cancer including breast cancer (Cheng & Leung, 2005) . GnRH was found to have an anti-proliferative effect on breast cancer cells (Everest et al., 2001). Therefore GnRH was hypothesized to affect the expression of *TET genes* in different breast cancer cell lines. and that GnRH would increase the expression of tumor suppressor genes like these of the *TET* genes. In concordance with this hypothesis, GnRH increased the expression of all *TET* genes in MCF7 cell line, however in T47D cells it decrease *TET* genes expression. Although MCF7 and T47D cells are classified in the same subtype as luminal A breast cancer cells, there is difference in the expression pattern of *TETs* .A study was done on

comparing the proteomic analysis of these two cell lines found that T47D cells show, for example, down regulation of proteins involved in the growth regression compared to MCF7 and also T47D has over expression of anti-apoptotic protein compared to MCF7(Aka & Lin, 2012),and this might explain the difference in the *TET* expression as response to GnRH in these two cell lines. Regarding the regulatory effect of GnRH on the expression of *TET* genes, a study was conducted on pituitary cells reported that *TET1* expression was repressed in response to GnRH (Yosefzon et al., 2017). This opposite effect of GnRH on different cell lines might be explained by the fact that GnRHR is coupled to different G proteins in different cell lines. GnRH receptor in pituitary cells is coupled to Gq/G11, which upon ligand binding, stimulates phospholipase C, while in breast cancer GnRH receptor is coupled with Gi protein which interferes with Epidermal growth factor receptor(EGFR) (Aguilar-Rojas & Huerta-Reyes, 2009).

Another important hormone that plays an important role in breast carcinogenesis is estrogen. Estrogen was found to increase proliferation of breast cancer cells (Yue et al., 2013). Estrogen alpha receptor was found to be expressed in around 60% of breast cancer cells (Masood, 1992) . Eventually estrogen might play a role in the expression of TET enzymes in different breast cancer cell lines. And it may decrease the expression of tumor suppressor genes like those of the TET enzymes. Our data showed that estrogen decreased the expression of *TET1* and *TET3* but didn't affect *TET2* gene in MCF7 cell line. However, in T47D, cell line estrogen has no effect on *TET1* and *TET3* but increased the expression of *TET2*. Also in this context, T47D and MCF7 cell lines show a discrepancy in the estrogen effect on the expression of different enzyme in these two cell line. In fact, estrogen was found to have different effects on the expression of some genes in these two cell lines like *c-MYC*, *TGFBI*, and *THSBI* (Rangel, Villegas, & Rondon-Lagos, 2017).The differential effect of estrogen on the expression of TET enzymes in these two breast cancer cell lines could be a reflection of molecular heterogeneity of these breast cancer cell lines and also might be explained by the fact that estrogen might bind to different types of receptors other than estrogen alpha receptor like G-protein coupled estrogen receptor (GPER) that is expressed in T47D and MCF7 (Samartzis et al., 2014).

In order to show the downstream effectors of GnRH and estrogen that affects TET enzyme expression, we used different inhibitor of different GnRH and estrogen receptor downstream kinases including. Among the kinases that were tested to see their involvement in the hormone regulation of TET enzymes Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaMKs) was found to be involved in the GnRH and estrogen regulation of all *TET* genes. In addition, PKA was found to be involved in GnRH regulation of all *TET* genes, which is similar to results obtained in pituitary cells (Yosefzon et al., 2017). Furthermore, Phosphatidylinositol 3-kinase (PI3-k) was found to be involved in GnRH regulation of *TET1* and *TET2*. For estrogen treatment, PKA is involved in the estrogen receptor mediated regulation of *TET3* but not *TET1* and *TET2*.

Protein kinase C (PKC) which we found to be involved in the GnRH regulation *TET3* but not *TET1* and *TET2*.and PKC is involved in estrogen regulation of all *TET* genes, similar to previous studies that reported PKC to be rapidly activated in response to estrogen treatment (Boyan et al., 2003).

While trying to understand the expression pattern of TET enzymes, our data suggested that *TET1* might have two isoform. Conventional PCR results showed that not all exons of *TET1* gene were expressed in different cell lines. Exon1 and 2 were not expressed in MDA-MB-231 but are expressed in T47D cell line. Indeed a recent study in the mouse embryonic stem cells showed that there are two TET1 isoforms (W. Zhang et al., 2016). Thus, by employing some bioinformatics using the UCSC genome browsing website which offers data for ENCODE projects that can help in the identification of the regulatory regions of genes and can give an idea about the location of transcription starting site of a gene. Our analysis on published ChIP data for the histone modification H3K27Ac3 which is found near the active promoter and enhancers (Rivera & Ren, 2013), and we found a high-density of H3K27Ac3 localized in two regions, one at the 5' of the *TET1* gene in EXON 1 and the other region in the 3' of INTRON 2 before EXON3. In

addition, the DNase sensitivity clusters which represent the regulatory region depending on the fact that the regulatory region of gene show higher sensitivity do DNase activity since the chromatin structure in this region is not compact made them accessible for DNase activity (Tanaka, Zhao, Wu, & Hersh, 1998). Also, transcription factor binding from chromatin immunoprecipitation (ChIP) data shows a higher density of transcription factor in the two promoter regions mentioned earlier. These findings suggested the presence of a second transcriptional starting site that leads to the transcription of a *TET1* isoform that lacks Exon1 and Exon2 and the translation of truncated TET1 that lacks the CXXC domain since the coding sequence of CXXC domain is located in Exon2. Indeed, all these findings were confirmed using different sets of primers that target different *TET1* exons. We found that *TET1* short isoform is expressed in all breast cancer cell lines at different levels; MCF7 showed the highest expression level and MDA-MB-231 at the lowest level of *TET1* long isoform. This switch of TET1 isoform expression was found to have a role in mouse development. It was found that TET1 long isoform is expressed in embryonic stem cells while the short isoform is activated in somatic cells (W. Zhang et al., 2016). Similarly to our findings, a recent study showed that TET1 short isoform is activated in different types of cancer including breast cancer (Good et al., 2017).

The presence of the *TET1* long isoform in the low grade luminal breast cell lines its loss in the more aggressive cell line might indicate that the long isoform might have a tumor suppressor activity, and that the loss of this long *TET1* isoform contributes to breast tumorigenesis. The notion that, *TET1* short isoform differential expression pattern indicates that it has different roles and functions in breast tumorigenesis. Moreover TET1 short isoform lacks the CXXC domain that functions to identify the CpG islands which gives it the ability to de-methylate DNA in non CpG island regions. Of course this TET1 short isoform can still be active and bind to DNA sequences with the help of other proteins. Indeed, a new study found that TET1 interacts with methyl CpG binding domain protein Mbd1 that enhance the localization and binding of TET1 to the methylated CpG through its CXXC3 domain (P. Zhang et al., 2017). This shorter isoform that lacks CXXC domain can function Similar to the mechanism involved in TET2 demethylation activity

mediated in a CXXC independent manner by interacting with IDAX, which has the same structure as the CXXC domain (Ko et al., 2013). All this indicates that the short isoform might have different target genes which, can reflect on its function in cancer tumorigenesis. Not only *TET1* isoforms are differentially expressed in Breast cancer, but we also found that they are regulated differently by different hormones. The short isoform expression was increased with hormone treatment, while the long isoform expression was decreased upon hormone treatment. In order to understand the basis for this differential hormonal treatment effect on these isoforms the UCSC transcription factor chromatin immunoprecipitation data revealed that estrogen receptor 1(ESR1) has a binding site in the promoter region of *TET1* short isoform but not in the promoter of the longer isoform. These data affirms that activation of estrogen receptor1 by directly binds to the promoter of *TET1* short isoform and induces its transcription. In comparison, GnRH has the same effect on increasing the expression of the short isoform may be through the activation of estrogen response element since a study conducted in mouse pituitary cells showed that GnRH activates an estrogen element –luciferase reporter gene (Chen, An, Cheng, Hammond, & Leung, 2009).

In order to elucidate the functions of these two *TET1* isoforms in breast cancer tumorigenesis, knockout of these isoforms genes and their effect on cancer cells needs exploration using, for example, CRISPR-Cas9 technique.in addition, to overexpress each of these isoforms separately in different breast cancer cell lines, to test how would this affect cellular phenotypes. An investigation on their distribution in the cell, since this will give further a hint regarding the differential functions of these isoforms.

As discussed above, one of the key mechanisms involved in the regulation of gene expression at the transcription level is DNA methylation, where alterations in DNA methylation has been connected with the development of diseases including cancer (Siegfried & Simon, 2010) (Robertson, 2005). Low levels of TET1 enzyme in breast cancer may be due to methylation of its promoter, similar to previous studies on colorectal cancer and many another type of cancers which showed that down-regulation of *TET1*

mRNA is associated with promoter hyper methylation(Rawluszko-Wieczorek et al., 2015). To test this hypothesis in breast cancer cell lines, the low *TET1* expressing cell line MDA-MB-231 cells was treated with the demethylating chemical 5-aza-2'-deoxycytidine. Instead of detecting *TET1* increase the qRT-PCR results showed the expression of *TET1* mRNA was not restored, even upon using various 5-aza concentrations. The failure of 5-Aza to restore the expression of *TET1* may due to induction of the expression of a transcriptional repressor of *TET1*, or demethylation was not enough to reverse *TET1* silencing. A previous study found that the combination of 5-Aza and deacetylase inhibitor which was not used in the present study has synergistic effect on demethylation and reversing gene silencing (Cameron, Bachman, Myohanen, Herman, & Baylin, 1999). Since our experiments with 5-Aza were not conclusive, methylation specific PCR approach was tested through. Methylation specific PCR primers to test the methylation status of the CpG Islands in the promoters of *TET1* two isoforms. The results showed that in MDA MB231 cell line hypermethylation of the promoter of the long isoform in contrast to MCF7 and T47D cell lines where their long *TET1* isoform promoter was shown to be hypomethylated. This methylation pattern of the long isoform are in consistency with the long isoform expression pattern in MCF7, T47D and MDA MB231cells. MCF7 cell line is considered less tumorigenic than the MDA-MB-231 cell line which is derived from metastatic breast cancer. *TET1* long isoform promoter is hypermethylated and its expression is down-regulated in MDA-MB-231, a finding that is supported by a previous study that showed that the expression level of *TET1* is lost in metastatic cells compared to primary cells, which is associated with *TET1* promoter hypermethylation (Sang et al., 2015). Our data showed that CpG Island in the promoter of the *TET1* short isoform, was found to be methylated in all cell lines, which is inconsistent with the expression of *TET1* short isoform results which showed the *TET1* short isoform is expressed in all cell lines. An explanation could be while trying to identify the CpG Island in the second promoter using Meth Primer website, two additional CpG islands where detected and in fact we tested the methylation of one of these CpG islands, which is more upstream to the expected transcription start site. This of course ensures the necessity for testing the methylation status of the second CpG Island. In addition, one of the limitations of MD-PCR is that it does not tell us the exact methylated cytosines in

CpGs. Thus, it might be necessary to do bisulfate pyrosequencing, which shows exactly the pattern of methylation and how many of the cytosine in the CpG Island are methylated.



### 5. Conclusion and Recommendations

#### 5.1. Conclusions

TET enzyme in breast cancer is regulated by hormone, *TET1* has two isoforms the long isoforms that are differentially expressed in breast cancer. The *TET1* long isoform is down regulated in aggressive breast cancer due to hyper methylation in their promoter.

#### 5.2. Recommendations

Understanding the function of the TET enzyme can help to understand their regulation and role in breast cancer development. For this purpose, functional studies of these enzyme are required. Furthermore the regulation of the TET enzymes at the protein levels, their localization in the cytoplasm and nucleus will provide further insight to their contribution in understanding breast cancer biology.

## References

- Aguilar-Rojas, A., & Huerta-Reyes, M. (2009). Human gonadotropin-releasing hormone receptor-activated cellular functions and signaling pathways in extra-pituitary tissues and cancer cells (Review). *Oncol Rep*, *22*(5), 981-990.
- Aka, J. A., & Lin, S. X. (2012). Comparison of functional proteomic analyses of human breast cancer cell lines T47D and MCF7. *PLoS One*, *7*(2), e31532. doi:10.1371/journal.pone.0031532
- Barazeghi, E., Gill, A. J., Sidhu, S., Norlen, O., Dina, R., Palazzo, F. F., . . . Westin, G. (2017). A role for TET2 in parathyroid carcinoma. *Endocr Relat Cancer*, *24*(7), 329-338. doi:10.1530/ERC-17-0009
- Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Wheeler, D. L. (2004). GenBank: update. *Nucleic Acids Res*, *32*(Database issue), D23-26. doi:10.1093/nar/gkh045
- Bernhardt, S. M., Dasari, P., Walsh, D., Townsend, A. R., Price, T. J., & Ingman, W. V. (2016). Hormonal Modulation of Breast Cancer Gene Expression: Implications for Intrinsic Subtyping in Premenopausal Women. *Front Oncol*, *6*, 241. doi:10.3389/fonc.2016.00241
- Boyan, B. D., Sylvia, V. L., Frambach, T., Lohmann, C. H., Dietl, J., Dean, D. D., & Schwartz, Z. (2003). Estrogen-dependent rapid activation of protein kinase C in estrogen receptor-positive MCF-7 breast cancer cells and estrogen receptor-negative HCC38 cells is membrane-mediated and inhibited by tamoxifen. *Endocrinology*, *144*(5), 1812-1824. doi:10.1210/en.2002-221018
- Brisken, C., & O'Malley, B. (2010). Hormone action in the mammary gland. *Cold Spring Harb Perspect Biol*, *2*(12), a003178. doi:10.1101/cshperspect.a003178
- Bronowicka-Klys, D. E., Roszak, A., Pawlik, P., Sajdak, S., Sowinska, A., & Jagodzinski, P. P. (2017). Transcript levels of ten-eleven translocation type 1-3 in cervical cancer and non-cancerous cervical tissues. *Oncol Lett*, *13*(5), 3921-3927. doi:10.3892/ol.2017.5930
- Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., & Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet*, *21*(1), 103-107. doi:10.1038/5047
- Chen, J., An, B. S., Cheng, L., Hammond, G. L., & Leung, P. C. (2009). Gonadotropin-releasing hormone-mediated phosphorylation of estrogen receptor-alpha contributes to fosB expression in mouse gonadotrophs. *Endocrinology*, *150*(10), 4583-4593. doi:10.1210/en.2009-0455
- Cheng, C. K., & Leung, P. C. (2005). Molecular biology of gonadotropin-releasing hormone (GnRH)-I, GnRH-II, and their receptors in humans. *Endocr Rev*, *26*(2), 283-306. doi:10.1210/er.2003-0039
- Dawlaty, M. M., Breiling, A., Le, T., Barrasa, M. I., Raddatz, G., Gao, Q., . . . Jaenisch, R. (2014). Loss of Tet enzymes compromises proper differentiation of embryonic stem cells. *Dev Cell*, *29*(1), 102-111. doi:10.1016/j.devcel.2014.03.003
- Dawlaty, M. M., Ganz, K., Powell, B. E., Hu, Y. C., Markoulaki, S., Cheng, A. W., . . . Jaenisch, R. (2011). Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. *Cell Stem Cell*, *9*(2), 166-175. doi:10.1016/j.stem.2011.07.010

- Eidne, K. A., Flanagan, C. A., Harris, N. S., & Millar, R. P. (1987). Gonadotropin-releasing hormone (GnRH)-binding sites in human breast cancer cell lines and inhibitory effects of GnRH antagonists. *J Clin Endocrinol Metab*, *64*(3), 425-432. doi:10.1210/jcem-64-3-425
- Everest, H. M., Hislop, J. N., Harding, T., Uney, J. B., Flynn, A., Millar, R. P., & McArdle, C. A. (2001). Signaling and antiproliferative effects mediated by GnRH receptors after expression in breast cancer cells using recombinant adenovirus. *Endocrinology*, *142*(11), 4663-4672. doi:10.1210/endo.142.11.8503
- Global Burden of Disease Cancer, C., Fitzmaurice, C., Allen, C., Barber, R. M., Barregard, L., Bhutta, Z. A., . . . Naghavi, M. (2017). Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study. *JAMA Oncol*, *3*(4), 524-548. doi:10.1001/jamaoncol.2016.5688
- Good, C. R., Madzo, J., Patel, B., Maegawa, S., Engel, N., Jelinek, J., & Issa, J. J. (2017). A novel isoform of TET1 that lacks a CXXC domain is overexpressed in cancer. *Nucleic Acids Res*, *45*(14), 8269-8281. doi:10.1093/nar/gkx435
- Guiu, S., Michiels, S., Andre, F., Cortes, J., Denkert, C., Di Leo, A., . . . Reis-Filho, J. S. (2012). Molecular subclasses of breast cancer: how do we define them? The IMPAKT 2012 Working Group Statement. *Ann Oncol*, *23*(12), 2997-3006. doi:10.1093/annonc/mds586
- Guo, J. U., Su, Y., Zhong, C., Ming, G. L., & Song, H. (2011). Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell*, *145*(3), 423-434. doi:10.1016/j.cell.2011.03.022
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-674. doi:10.1016/j.cell.2011.02.013
- Harrison, G. S., Wierman, M. E., Nett, T. M., & Glode, L. M. (2004). Gonadotropin-releasing hormone and its receptor in normal and malignant cells. *Endocr Relat Cancer*, *11*(4), 725-748. doi:10.1677/erc.1.00777
- Hashimoto, H., Liu, Y., Upadhyay, A. K., Chang, Y., Howerton, S. B., Vertino, P. M., . . . Cheng, X. (2012). Recognition and potential mechanisms for replication and erasure of cytosine hydroxymethylation. *Nucleic Acids Res*, *40*(11), 4841-4849. doi:10.1093/nar/gks155
- Herman, J. G. (1999). Hypermethylation of tumor suppressor genes in cancer. *Semin Cancer Biol*, *9*(5), 359-367. doi:10.1006/scbi.1999.0138
- Jin, C., Lu, Y., Jelinek, J., Liang, S., Estecio, M. R., Barton, M. C., & Issa, J. P. (2014). TET1 is a maintenance DNA demethylase that prevents methylation spreading in differentiated cells. *Nucleic Acids Res*, *42*(11), 6956-6971. doi:10.1093/nar/gku372
- Jin, S. G., Zhang, Z. M., Dunwell, T. L., Harter, M. R., Wu, X., Johnson, J., . . . Pfeifer, G. P. (2016). Tet3 Reads 5-Carboxylcytosine through Its CXXC Domain and Is a Potential Guardian against Neurodegeneration. *Cell Rep*, *14*(3), 493-505. doi:10.1016/j.celrep.2015.12.044
- Kakar, S. S., Grizzle, W. E., & Neill, J. D. (1994). The nucleotide sequences of human GnRH receptors in breast and ovarian tumors are identical with that found in pituitary. *Mol Cell Endocrinol*, *106*(1-2), 145-149.
- Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., & Haussler, D. (2002). The human genome browser at UCSC. *Genome Res*, *12*(6), 996-1006. doi:10.1101/gr.229102
- Klose, R. J., & Bird, A. P. (2006). Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci*, *31*(2), 89-97. doi:10.1016/j.tibs.2005.12.008

- Ko, M., An, J., Bandukwala, H. S., Chavez, L., Aijo, T., Pastor, W. A., . . . Rao, A. (2013). Modulation of TET2 expression and 5-methylcytosine oxidation by the CXXC domain protein IDAX. *Nature*, *497*(7447), 122-126. doi:10.1038/nature12052
- Kohli, R. M., & Zhang, Y. (2013). TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*, *502*(7472), 472-479. doi:10.1038/nature12750
- LaMarca, H. L., & Rosen, J. M. (2007). Estrogen regulation of mammary gland development and breast cancer: amphiregulin takes center stage. *Breast Cancer Res*, *9*(4), 304. doi:10.1186/bcr1740
- Laukka, T., Mariani, C. J., Ihantola, T., Cao, J. Z., Hokkanen, J., Kaelin, W. G., Jr., . . . Koivunen, P. (2016). Fumarate and Succinate Regulate Expression of Hypoxia-inducible Genes via TET Enzymes. *J Biol Chem*, *291*(8), 4256-4265. doi:10.1074/jbc.M115.688762
- Lee, T. I., & Young, R. A. (2013). Transcriptional regulation and its misregulation in disease. *Cell*, *152*(6), 1237-1251. doi:10.1016/j.cell.2013.02.014
- Li, E., Bestor, T. H., & Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, *69*(6), 915-926.
- Li LC and Dahiya R. (2002). MethPrimer: designing primers for methylation PCRs. *Bioinformatics*. Retrieved from PMID: 12424112
- Li, Z., Cai, X., Cai, C. L., Wang, J., Zhang, W., Petersen, B. E., . . . Xu, M. (2011). Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood*, *118*(17), 4509-4518. doi:10.1182/blood-2010-12-325241
- Liao, J., Karnik, R., Gu, H., Ziller, M. J., Clement, K., Tsankov, A. M., . . . Meissner, A. (2015). Targeted disruption of DNMT1, DNMT3A and DNMT3B in human embryonic stem cells. *Nat Genet*, *47*(5), 469-478. doi:10.1038/ng.3258
- Marino, M., Galluzzo, P., & Ascenzi, P. (2006). Estrogen signaling multiple pathways to impact gene transcription. *Curr Genomics*, *7*(8), 497-508.
- Masood, S. (1992). Estrogen and progesterone receptors in cytology: a comprehensive review. *Diagn Cytopathol*, *8*(5), 475-491.
- McDonnell, D. P., & Norris, J. D. (2002). Connections and regulation of the human estrogen receptor. *Science*, *296*(5573), 1642-1644. doi:10.1126/science.1071884
- Mikeska, T., & Craig, J. M. (2014). DNA methylation biomarkers: cancer and beyond. *Genes (Basel)*, *5*(3), 821-864. doi:10.3390/genes5030821
- Rangel, N., Villegas, V. E., & Rondon-Lagos, M. (2017). Profiling of gene expression regulated by 17beta-estradiol and tamoxifen in estrogen receptor-positive and estrogen receptor-negative human breast cancer cell lines. *Breast Cancer (Dove Med Press)*, *9*, 537-550. doi:10.2147/BCTT.S146247
- Rasmussen, K. D., & Helin, K. (2016). Role of TET enzymes in DNA methylation, development, and cancer. *Genes Dev*, *30*(7), 733-750. doi:10.1101/gad.276568.115
- Rawluszko-Wieczorek, A. A., Siera, A., Horbacka, K., Horst, N., Krokowicz, P., & Jagodzinski, P. P. (2015). Clinical significance of DNA methylation mRNA levels of TET family members in colorectal cancer. *J Cancer Res Clin Oncol*, *141*(8), 1379-1392. doi:10.1007/s00432-014-1901-2
- Rivera, C. M., & Ren, B. (2013). Mapping human epigenomes. *Cell*, *155*(1), 39-55. doi:10.1016/j.cell.2013.09.011
- Robertson, K. D. (2005). DNA methylation and human disease. *Nat Rev Genet*, *6*(8), 597-610. doi:10.1038/nrg1655

- Samartzis, E. P., Noske, A., Meisel, A., Varga, Z., Fink, D., & Imesch, P. (2014). The G protein-coupled estrogen receptor (GPER) is expressed in two different subcellular localizations reflecting distinct tumor properties in breast cancer. *PLoS One*, *9*(1), e83296. doi:10.1371/journal.pone.0083296
- Sang, Y., Cheng, C., Tang, X. F., Zhang, M. F., & Lv, X. B. (2015). Hypermethylation of TET1 promoter is a new diagnostic marker for breast cancer metastasis. *Asian Pac J Cancer Prev*, *16*(3), 1197-1200.
- Scourzic, L., Mouly, E., & Bernard, O. A. (2015). TET proteins and the control of cytosine demethylation in cancer. *Genome Med*, *7*(1), 9. doi:10.1186/s13073-015-0134-6
- Siegel, R. L., Miller, K. D., & Jemal, A. (2017). Cancer Statistics, 2017. *CA Cancer J Clin*, *67*(1), 7-30. doi:10.3322/caac.21387
- Siegfried, Z., & Simon, I. (2010). DNA methylation and gene expression. *Wiley Interdiscip Rev Syst Biol Med*, *2*(3), 362-371. doi:10.1002/wsbm.64
- Tanaka, H., Zhao, Y., Wu, D., & Hersh, L. B. (1998). The use of DNase I hypersensitivity site mapping to identify regulatory regions of the human cholinergic gene locus. *J Neurochem*, *70*(5), 1799-1808.
- Thomson, J. P., Ottaviano, R., Unterberger, E. B., Lempiainen, H., Muller, A., Terranova, R., . . . Meehan, R. R. (2016). Loss of Tet1-Associated 5-Hydroxymethylcytosine Is Concomitant with Aberrant Promoter Hypermethylation in Liver Cancer. *Cancer Res*, *76*(10), 3097-3108. doi:10.1158/0008-5472.CAN-15-1910
- Tsai, K. W., Li, G. C., Chen, C. H., Yeh, M. H., Huang, J. S., Tseng, H. H., . . . Chang, H. T. (2015). Reduction of global 5-hydroxymethylcytosine is a poor prognostic factor in breast cancer patients, especially for an ER/PR-negative subtype. *Breast Cancer Res Treat*, *153*(1), 219-234. doi:10.1007/s10549-015-3525-x
- Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, & Rozen SG. (2012). Primer3 - new capabilities and interfaces. *Nucleic Acids Res*, *40*(15).
- von Alten, J., Fister, S., Schulz, H., Viereck, V., Frosch, K. H., Emons, G., & Grundker, C. (2006). GnRH analogs reduce invasiveness of human breast cancer cells. *Breast Cancer Res Treat*, *100*(1), 13-21. doi:10.1007/s10549-006-9222-z
- Wang, K. C., Kang, C. H., Tsai, C. Y., Chou, N. H., Tu, Y. T., Li, G. C., . . . Tsai, K. W. (2018). Ten-eleven translocation 1 dysfunction reduces 5-hydroxymethylcytosine expression levels in gastric cancer cells. *Oncol Lett*, *15*(1), 278-284. doi:10.3892/ol.2017.7264
- Wang, M., Yang, Q., Yuan, Q., Yi, X., Wang, Q., Tan, P., & Li, G. (2016). TET3 and BRCA1 co-repress EZH2 to inhibit the aggressive behavior of breast cancer cells. *Int J Clin Exp Med*, *9*(2), 1585-1593.
- Wu, M. Z., Chen, S. F., Nieh, S., Benner, C., Ger, L. P., Jan, C. I., . . . Izipisua Belmonte, J. C. (2015). Hypoxia Drives Breast Tumor Malignancy through a TET-TNFalpha-p38-MAPK Signaling Axis. *Cancer Res*, *75*(18), 3912-3924. doi:10.1158/0008-5472.CAN-14-3208
- Yang, H., Liu, Y., Bai, F., Zhang, J. Y., Ma, S. H., Liu, J., . . . Xiong, Y. (2013). Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation. *Oncogene*, *32*(5), 663-669. doi:10.1038/onc.2012.67
- Yang, L., Yu, S. J., Hong, Q., Yang, Y., & Shao, Z. M. (2015). Reduced Expression of TET1, TET2, TET3 and TDG mRNAs Are Associated with Poor Prognosis of Patients with Early Breast Cancer. *PLoS One*, *10*(7), e0133896. doi:10.1371/journal.pone.0133896

- Yin, R., Mao, S. Q., Zhao, B., Chong, Z., Yang, Y., Zhao, C., . . . Wang, H. (2013). Ascorbic acid enhances Tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. *J Am Chem Soc*, *135*(28), 10396-10403. doi:10.1021/ja4028346
- Yosefzon, Y., David, C., Tsukerman, A., Pnueli, L., Qiao, S., Boehm, U., & Melamed, P. (2017). An epigenetic switch repressing Tet1 in gonadotropes activates the reproductive axis. *Proc Natl Acad Sci U S A*, *114*(38), 10131-10136. doi:10.1073/pnas.1704393114
- Yue, W., Yager, J. D., Wang, J. P., Jupe, E. R., & Santen, R. J. (2013). Estrogen receptor-dependent and independent mechanisms of breast cancer carcinogenesis. *Steroids*, *78*(2), 161-170. doi:10.1016/j.steroids.2012.11.001
- Zhang, P., Rausch, C., Hastert, F. D., Boneva, B., Filatova, A., Patil, S. J., . . . Cardoso, M. C. (2017). Methyl-CpG binding domain protein 1 regulates localization and activity of Tet1 in a CXXC3 domain-dependent manner. *Nucleic Acids Res*, *45*(12), 7118-7136. doi:10.1093/nar/gkx281
- Zhang, W., Xia, W., Wang, Q., Towers, A. J., Chen, J., Gao, R., . . . Xie, W. (2016). Isoform Switch of TET1 Regulates DNA Demethylation and Mouse Development. *Mol Cell*, *64*(6), 1062-1073. doi:10.1016/j.molcel.2016.10.030
- Zhao, H., & Chen, T. (2013). Tet family of 5-methylcytosine dioxygenases in mammalian development. *J Hum Genet*, *58*(7), 421-427. doi:10.1038/jhg.2013.63

## تنظيم نسخ جينات TET في سرطان الثدي

إعداد: أبرار علي داود جاموس.

إشراف: الاستاذ الدكتور زيدون صلاح.

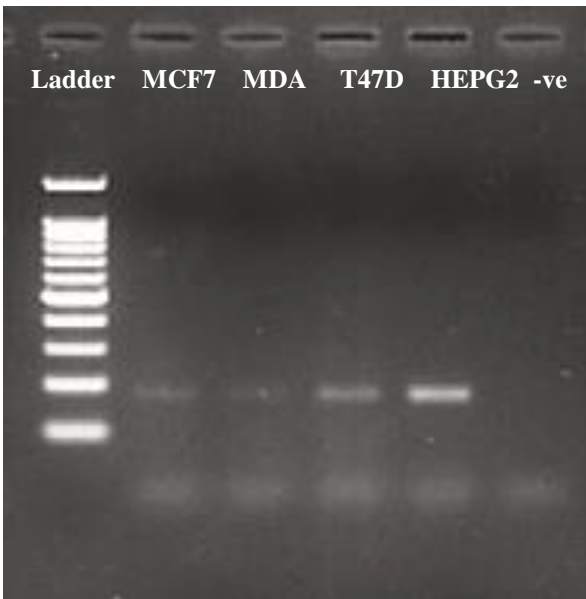
### الملخص

تحول الخلايا السرطانية وتطورها مرتبط باختلالات في التعبير الجيني والتي تحدث من خلال الطفرات الجينية أو من خلال التنظيم الفوق جيني. واحدة من آليات التنظيم الفوق جيني الرئيسية التي تختل في السرطان هي إضافة مجموعة الميثيل إلى الحمض النووي (DNA methylation) بينما أن الآليات المسؤولة عن إضافة مجموعة الميثيل إلى الحمض النووي واضحة ومدروسة جيدا، كان يعتقد أن عملية إزالة الميثيل عن الحمض النووي هي عملية سلبية (passive process) حتى تم اكتشاف عائلة (Ten-Eleven Translocation (TET والتي تقوم بالأزالة الفعالة لمجموعة الميثيل عن الحمض النووي عن طريق تفاعل hydroxylation لميثلي السيتوسين (mC) لتحويلها إلى مجموعة الهيدروكسيل السيتوسين (5hmC). يقل نشاط افراد عائلة TET في عدة أنواع من الأورام الخبيثة، مما يدل على أن وظيفة هذه البروتينات قد يساهم في تثبيط الأورام. وبالتالي، كانت فرضيتنا هي أن النشاط المنخفض لهذه الإنزيمات قد تكون مسؤولة عن زيادة نسبة مجموعة الميثيل المضافة على السيتوسين في الحمض النووي (hypermethylation) مما يؤدي إلى إختلالات في التعبير الجيني في سرطان الثدي. لذا قمنا بفحص بعض الآليات المنظمة التي تؤثر على تعبير TET أنزيمات في سرطان الثدي، بما في ذلك الاشارات الهرمونية و بعض مسارات نقل الاشارات المؤثرة عليها. وأيضا قمنا بفحص وجود مواقع بدأ النسخ الجيني المختلفة لجين *TET1*. ثم قمنا بفحص وجود مجموعة الميثيل على منطقة بدأ النسخ (promoter) ل *TET1* لإمكانية اعتبارها كآلية منظمة. نتائجنا أظهرت أن TET أنزيمات ينظمها العمل الهرموني و بعض الأنشطة الأنزيمية و بعض مسارات نقل الاشارات المؤثرة من النشاط الهرموني، مثل PKA و PKC و PI3k و CaMKs ، التي تشارك في التنظيم الهرموني ل TET أنزيمات. بالإضافة نتائجنا أظهرت أن TET1 لهو عدة نظائر (isoforms) مختلفة والتي لها مقاطع مختلفه من التعبير الجيني

في عدة أنواع من خلايا سرطان الثدي. ومن المثير للأهتمام أن هذه الهرمونات تقوم بالتنظيم بطرق مختلفة بواسطة النشاط الهرموني. وأيضاً كشفت أن تعبير النظير الاطول ل TET1 قد ثبت عن طريق وجود مجموعة الميثيل على منطقة بدأ النسخ (promoter). أجمالي نتائجنا تثبت أن تعبير TET أنزيم ينظم، على الاقل، في سرطان الثدي، على مستوى النسخ الجيني، وأن *TET1* لديه نظيران يختلفان في تعبيرهما الجيني وتنظيمهما في سرطان الثدي. مجتمعه نتائجنا تشير الى أن TET أنزيمات مهمة في نمو أورام الثدي و أن هناك حاجة إلى مزيد من البحوث لتوضيح المزيد من المسارات الجزيئية التفصيلية التي تشارك في تنظيم TET أنزيم في سياق تطور سرطان الثدي والتقدم.

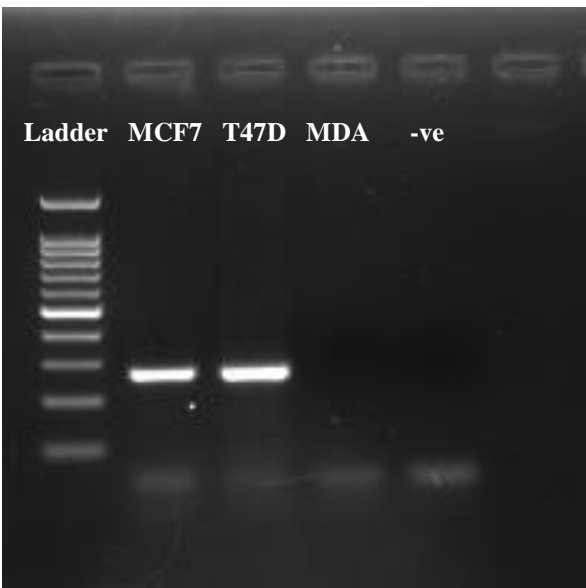


## Appendix

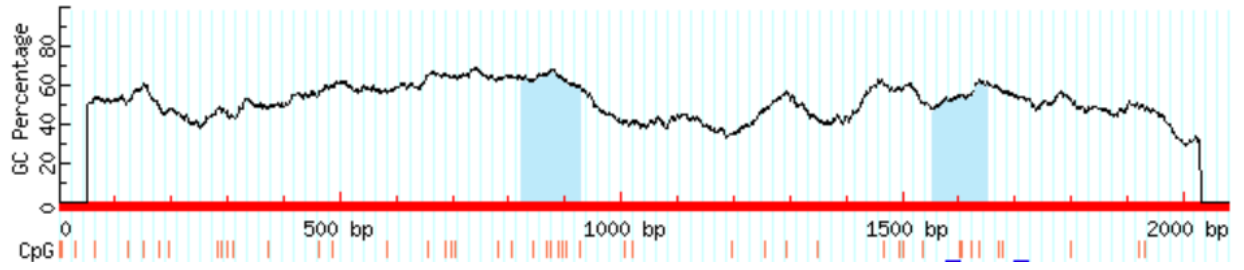


### Appendix 1: The expression of GnRH receptor in cancer cell lines.

Breast cancer cell lines (MCF7,MDA-MB-231,T47D) ,live cancer cell line (HEPG2) is positive control, and negative control.



### Appendix 2: The expression of Estrogen alpha receptor in breast cancer cell line MCF7,T47D express E2R ,and MDA-MB-231 as negative control



**Appendix 3: CpG Island in *TET1* short isoform promoter .CpG island prediction for the second promoter using MethPrimer website: show the presence of two CpG island**