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The Role and Expression Pattern of TET1 in Breast Cancer

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Dedication

Thank you, God my prayer and gratitude to you. You are the one who gave me the power to work hard and continue despite the obstacles until I reached this high and great status. True love that never changes or ends to those who continuously encouraged and helped me to achieve my goals, especially my dear sisters and brothers. I would also like to dedicate my work to my family, specially my wife, for her efforts and help to make this possible, thank you very much.

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With love and respect

Mahmoud Ali Mousa Al-Zahayqa

Declaration

I certify that this thesis was submitted for the degree of masters. It is the result of my own research, except where otherwise is acknowledged. I also certify that this thesis (or any part of the same) has not been submitted for any other university or institution.

Signed 

Mahmoud Ali Mousa Al-Zahayqa

Date: 18/12//2019

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I am glad to thank my family who supported and encouraged me during this period.

List of Abbreviations

%	Percent
°C	Celsius
Ab	Antibody
Amp	Ampicillin
Bp	Base pair
cDNA	Complementary DNA
CpG	Cytosine phosphate guanine
Cy3	Cyanine
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DW	Distilled water
EGF	Epidermal growth factor
FBS	Fetal bovine serum
Hrs	Hours
L	Litter
LB	Lysogeny broth
Min	Minutes
Ml	Milliliter
Ng	Nanogram
PBS	Phosphate buffer saline
RPM	Round per minute
RT	Room temperature
RT-PCR	Real time- polymerase chain reaction
SFM	Serum free media
Tri reagent	Trizol
Mg	Microgram
µL	Microliter
Mm	Micrometer
TSG	Tumor suppressor gene
IF	Immunofluorescent
IHC	Immunohistochemistry
E2	Estrogen
5mc	5-Methylcytosine
5hmc	5-hydroxymethylcytosine
DNMTs	DNA methyltransferase enzymes
TET	Ten Eleven Translocation enzymes
αKG	Alpha Ketoglutarate
DSBH	Double strand beta sheet
HEK	Human Embryonic Kidney cells
NP-40	Nonidet P-40
NaCl	Sodium chloride

TNBC	Triple negative breast cancer
Uc	Uncut
Nuc	Nuclear
Cyt	Cytoplasm
Tur	Terminus
Kb	kilo base pair
N	Normal
T	Tumor
WT	Wilde type
N-KO	Pre-cancerous tissue of KO mice
w-KO	*Wwox* knockout
p-KO	p53 knockout
D-KO	Double knockout for both p53 and Wwox
EV	Empty vector
Mut	Mutant
GnRH	Gonadotropin-releasing hormone
EV-M	MCF10A cells infected with empty vector
HRAS	MCF10A cells infected with *HRAS*
KO p53-EV	MCF10A cells *p53* KO infected with empty vector
KO p53-HRAS	MCF10A cells *p53* KO infected with *HRAS*
mut p53-EV	MCF10A cells mutated *p53* infected with empty vector
mut p53-HRAS	MCF10A cells mutated *p53* infected with *HRAS*
DAPI	4′,6-diamidino-2-phenylindole
PCR	Polymerase chain reaction

Abstract

Cancer is a genetic disease. Mutations and epigenetic alterations such as aberrant DNA methylation which results in altered gene expression is evident in all cancers studied, and is likely responsible for its major hallmarks. Methylation is maintained by DNA methyltransferases, however, methylation can be reversed by mechanisms that are poorly understood. Recently, functional demethylation was linked to hydroxylation of 5hmC by the TET family. TET1 is the most reduced member in a variety of human malignancies, suggesting a tumor suppressor function for this protein. In addition, published research showed controversial conclusions about TET1 function in breast cancer. Moreover, recent evidence showed that *TET1* has more than one isoform. Thus, our hypothesis proposes the different *TET1* isoforms may play different roles in breast cancer through differential expression pattern in different transformation contexts. In the present study, we tested the expression level and localization of TET1 enzyme in breast cancer samples using IHC, and the expression level using relative qRT-PCR in different breast cancer cell lines under different contexts. In addition, we tested the expression pattern of different *TET1* isoforms using in vitro and in vivo cell transformation models. We also tested the effect of TET1 overexpression in MDA MB231 cells using lentivirus vector containing *TET1* coding sequence on various cancer hallmarks. Our results demonstrate that TET1 has differential expression pattern in breast cancer embedded tissue samples compared to normal tissue. In addition, TET1 expression correlated with the differentiation level. From our hormone experiments, and in vitro as well as in vivo transformation studies, we clearly showed that different *TET1* isoforms are differentially expressed under different physiological and transformation contexts, and different *TET1* isoforms having different distribution pattern. Finally, we proved that TET1 full length is a tumor suppressor gene. In conclusion, our study demonstrates the role of TET1 in breast cancer is not straight forward one and this necessitates future studies to better characterize the TET1 function in breast cancer initiation and progression.

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1. Introduction

1.1. Cancer

Cancer is the second causing death worldwide after cardiovascular diseases (Mortality & Causes of Death, 2016). Cancer can cause death because it has the ability to invade adjacent tissues, and can spread to other body parts through circulation and lymphatic system (Tracey A. Martin, 2013). Normal cellular function is highly correlated with normal gene expression and an abnormal gene expression leads to different diseases including cancer. Cancer is characterized by specific abnormal gene expression pattern that leads to uncontrolled cell growth and leading to malignant transformation, tumor progression and invasion of distant body organs. Cancer cells acquire different properties that support their survival and progression. These phenotypes are called the hallmarks of cancer. Those hallmarks including sustained proliferative state, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, genome instability and mutations, inflammation, metabolic alterations and evading immune destruction (Hanahan & Weinberg, 2011). These characteristic phenotypes are associated with aberrant gene expression that disrupts the balance between oncogenes and tumor suppressor genes (TSGs) as well as other genes that maintain normal cell identify (Hanahan & Weinberg, 2011).

1.2. Breast cancer

According to the estimation by WHO in 2018, breast cancer is the most common type of cancers that affect women worldwide. It also results in the highest rate of cancer-related death in women. In 2018, more than 2 million cases among women were registered, and around 627,000 women died from breast cancer worldwide https://www.who.int/cancer/prevention/diagnosis-screening/breast-cancer/en/. In Palestine, according to the ministry of health, breast cancer is considered as the first cause of death in females. (The First Report On Oncology In Palestine - World Health). Breast cancer was classified based on histopathological

appearance of breast tissue under the microscope. The new classification of breast cancer is based on different molecular markers that reflects on cell origin and cell behavior or even response to different therapies. Currently, breast cancer is categorized into 4 types including, luminal A, luminal B, HER2/neu and basal like tumors. Although This molecular classification is successful in certain instances, it seems that this molecular based categorization of breast cancer is still immature and suffers from different problems including lack of reproducibility and poor definition of specific tumors such as basal-like tumors (Eliyatkin, Yalcin, Zengel, Aktas, & Vardar, 2015). These drawbacks and others in molecular classification of breast cancer may indicate that the current molecular markers are not enough for breast cancer classification. Consequently, this necessitates deeper understanding of the molecular events involved in breast tumorigenesis. The molecular changes involved in carcinogenesis can either be genetic changes that alter the DNA sequence or epigenetic changes that affect gene expression

1.3. Methylation

Genetic alterations and epigenetic reprogramming play an important role in tumor formation and progression (Brien, Valerio, & Armstrong, 2016). One of the most important epigenetic events that involves in gene regulation and has critical roles in normal tissue homeostasis as well as development of diseases including tumorigenesis is DNA methylation (Jones & Baylin, 2007).

DNA methylation is catalyzed and maintained by DNA methyltransferase enzymes (DNMTs) through the addition of a methyl group to cytosine at position 5 (Klose & Bird, 2006). DNMTs family includes three catalytic active enzymes, DNMT1, DNMT3A, and DNMT3B. DNMT1 enzyme maintains DNA methylation pattern by copying the methylation status mark onto newly synthesized DNA strands. On the other hand, DNMT 3A and 3B are responsible for denovo methylation synthesis (Liao et al., 2015). DNA methylation occurs at CpG islands, these islands are usually found at the centromeric tandem repeat units, and in the promoter area of many genes (Bansal & Pinney, 2017). DNA methylation of CpGs occurs frequently at gene regulatory regions, and is

associated with transcriptional silencing that involves binding of methylated cytosines (5mC) to DNA binding proteins. These proteins recruit histone deacetylases and other chromatin remodelers that collectively prevent the binding of transcriptional activators and thus transcription inhibition (Klose & Bird, 2006).

During normal development, DNA methylation is generated in a programmed manner. However, in cancer, alterations in DNA methylation are among the most common events that lead to loss of cell and tissue homeostasis, and characterized by hypo and hypermethylation patterns independent of each other. In addition, DNMT expression is deregulated. Altogether, these events lead to altered gene expression and genomic instability (Baba, Watanabe, & Baba, 2013; Robertson, 2005).

In cancer, DNA hypomethylation is seen mainly in oncogenes and genes associated with metastasis, while hypermethylation is a mechanism used by cancer cells to shut down the expression of TSGs (Daud Faran Asif, 2017). For example, in breast cancer, the oncogene protease urokinase involved in cell proliferation and migration, is hypomethylated and thus is overexpressed (Ehrlich, 2009). On the contrary, the TSG *CDKN2A* gene, which inhibits CDK, is hypermethylated and thus is silenced (Pfeifer, 2018).

Methylation status is a result of balance between methylation and demethylation processes. While the mechanisms responsible for methylation are well understood and well established, DNA demethylation was thought to likely occur passively by replacement of methyl group due to the reduction or absence in DNMTs activity (Klose & Bird, 2006; Wu & Zhang, 2014). However, recent evidence proved that this process is actively catalyzed by a family of enzymes that belong to the Ten Eleven Translocation (TET) hydroxymethylase family (Piccolo & Fisher, 2014; Wu & Zhang, 2014).

1.4. TET enzymes

TET family is named after a common translocation between chromosomes ten and eleven (Pastor, Aravind, & Rao, 2013). There are three known members of

TET proteins (TET1, TET2 and TET3). All these members have the same catalytic domain at the C terminus which consists of cytosine rich region, and double strand beta sheet (DSBH). This catalytic domain contains also binding sites for iron and alpha Ketoglutarate (αKG) cofactors, which are involved in the oxidation reactions of methylated cytosine (Rasmussen & Helin, 2016). In addition, TET1 and TET3 have CXXC zinc finger domain in their N-termini which is responsible for binding to DNA in unmethylated stretches of CpGs islands. The CXXC is missing in TET2, but TET2 still has the ability to bind DNA through IDAX protein which has the same structure similar to the CXXC domain (Iyer, Tahiliani, Rao, & Aravind, 2009; Ko et al., 2013; Wu & Zhang, 2011) Fig(1.1).

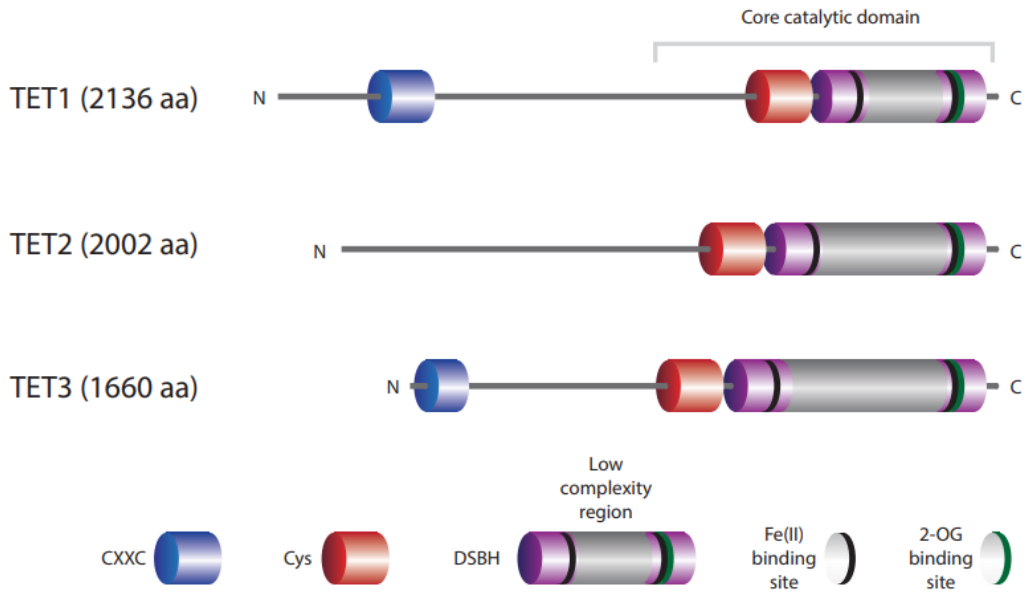

Figure 1.1 Schematic illustration for TET proteins structure. TET protein structure showing catalytic domain at C-terminal in all TET enzymes which consists of a cysteine-rich domain, DSBH domain with unknown function, and binding sites for iron and αKG cofactors. TET1 and TET3 have CXXC domain at N-terminal which through they can bind to DNA directly, and facilitate genomic target sites recruitment. (Xu et al., 2012; H. Zhang et al., 2010).

TET enzymes are hydroxymethylase enzymes. They catalyze DNA demethylation through the conversion of 5mc to 5-hydroxymethylcytosine (5hmc) which acts as a stable epigenetic marker. This modification prevents further DNA methylation by reducing the affinity of binding between DNMT and CpGs (Hashimoto et al., 2012). Moreover, TET enzymes can catalyze active demethylation through the oxidation of 5hmc to form 5-formylcytosine and 5-carboxylcytosine. Finally,

thymine DNA glycosylase enzyme can convert 5-carboxylcytosine to free cytosine through base excision repair system (Coey et al., 2016). During these oxidation reactions, TET enzymes convert αKG to succinate and CO2. It was found that the accumulation of oncometabolites like fumarate, 2-hydroxyglutarate, and succinate inhibits the activity of TET enzymes. These oncometabolites are αKG analogues. They compete with the substrate of TET enzymes to bind on their catalytic domains (Xiao et al., 2012). On the other hand, vitamin C (an antioxidant) was known to enhance the α-KG/Fe dependent dioxygenase activity. It directly interacts with TET enzyme catalytic domain, and induce its dioxygenase enzymatic activity to convert 5mc to its oxidation products (Yin et al., 2013).

1.4.1. Biological functions of TET enzymes

TET proteins have different common and distinct physiological functions in stem cell biology, embryonic development, cell differentiation, and neuronal cell biology (Rasmussen & Helin, 2016). In different physiological processes, there are different roles for these enzymes. For instance, Tet3 knockout causes neonatal sublethality in mice (Inoue, Shen, Matoba, & Zhang, 2015), while Tet2 knockout in mice increased the rate of myeloid malignancies (Li et al., 2011). In embryonic stem cells, which express high amount of Tet1, and as a result high amount of 5hmc, Tet1 knockdown in these cells showed a reduction in 5hmc without affecting the pluripotency properties of these cells (Dawlaty et al., 2011). Moreover, TET protein deregulation was shown to lead to different developmental defects and associated with pathological conditions including cancer (W. Sun, Guan, & Li, 2014; Yang et al., 2013).

1.4.2. TET enzymes and carcinogenesis

It has been found that the expression of different TET proteins is altered in both solid and liquid tumors (Rasmussen & Helin, 2016). In liquid cancers, loss of *Tet2* gene function plays a role in initiating aggressive myeloid cancer in a

mouse model (An et al., 2015). Moreover, mutations in *Tet2* were found to be the most common genetic alterations among hematological malignancies (Scourzic, Mouly, & Bernard, 2015). In solid tumors, different studies showed an association between low *TET* gene expression and loss of 5-hmC in different human tumors including: liver, lung, pancreatic, prostate and breast cancer (Yang et al., 2013).

1.4.3. TET family in breast cancer

Since their discovery, different studies demonstrated that TET family members are lost or downregulated in breast cancer. *TET* expression and 5hmc levels were significantly reduced and tightly linked to breast tumorigenesis (Yang et al., 2013), while activation of TET reduces cancer risk in mammary tissues (Romagnolo et al., 2016). In addition, downregulation of different *TET* gene expression through miR-22 is associated with poor prognosis of breast cancer (Song et al., 2013). Moreover, increasing *TET1* expression in breast cancer leads to up-regulation of the tumor suppressor miR-34a (Siyi Z, 2019). Also, TET enzymes were linked to breast cancer progression. For example, TET knockout repressed miR-200 and increased EMT genes (*ZEB1/2, BM1I* genes) (Song et al., 2013).

1.5. TET1

TET1 is located on chromosome 10 and consists of 12 exons. It encodes for about 6.4 kb mRNA, which is translated into 2136 amino acids (Abdel-Wahab et al., 2009). TET1 has different functions including; active promoter demethylation (Ito et al., 2010), histone deacetylation of both promoters of transcriptionally active (Almeida et al., 2017) as well as polycomb-repressed genes (Wu et al., 2011), and repressing the expression of specific genes through recruiting transcriptional repressors (Williams et al., 2011). *TET1* has at least two different isoforms, one of them lacks the first exon and possibly all or a part of exon 2 (Good et al., 2017). The expression of these two isoforms was shown to be differentially expressed in

different cellular contexts including stem cell differentiation (W. Zhang et al., 2016) and carcinogenesis (Good et al., 2017).

1.5.1. TET1 in carcinogenesis

In many studies about the correlation between TET1 expression and tumorigenesis, TET1 was identified as a tumor suppressor gene. Most studies showed that TET1 level is downregulated in most cancer types and that its low level is associated with poor prognosis. Studies performed on endometrial, gastric, hepatic, lung, and prostate cancers demonstrated that TET1 as well as 5hmC low levels are thought to be associated with tumor size, location, histological grade, invasion, metastasis, and cancer related death (Siyi, 2019). For instance, in colorectal cancer, TET1 loss was shown to correlate with late stages (Siyi, 2019), and in endometrial cancer, low *TET1* level is associated with high risk of metastasis to lymph nodes (Ciesielski et al., 2017). In discordance with its tumor suppressive functions, some studies demonstrated that TET1 has oncogenic activities. For example, TET1 upregulation due to hypoxic microenvironment decreased CpG methylation of hypoxia responsive elements, and thus more HIF-1α binding to its target genes that consequently lead to enhanced migration of colorectal cancer cells (Ma et al., 2019).

1.5.2.TET1 enzyme in breast carcinogenesis

The current available data about the role of TET1 in breast cancer is controversial. While several studies had shown that TET1 is a tumor suppressor gene that suppresses mammary gland tumorigenesis and metastasis, other studies showed that it is an oncogene that promotes cell proliferation and tumor progression. For example, in triple negative breast cancer patients (TNBC), EZH2 downregulation and thus TET1 upregulation is accompanied with p53 signalling activation, and growth promoting protein downregulation. On the other hand, low TET1 and high EZH2 expression levels is associated with

cancer promotion and poor outcomes (Yu et al., 2019). Moreover, in metastatic breast cancer cells and tumor tissues, *TET1* reduced expression level naturally due to promoter methylation or artificially because of TET1 siRNA enhanced cell migration, invasion and metastasis (Sang, Cheng, Tang, Zhang, & Lv, 2015). In contrast to these studies, different studies have shown that TET1 is a protumorigenic gene. For example, in a histopathological study, immunohistochemistry revealed that TET1 is overexpressed in breast cancer (Good et al., 2018). Moreover, in TNBC patients, high TET1 expression is associated with significantly worse overall survival. In addition, TET1 knockout in MDA MB231 TNBC cell line significantly reduces cellular migration and proliferation due to loss of phospho-4EBP1 (Good et al., 2018). Recently, it was found that at least two *TET1* isoforms are expressed in breast cancer cell lines, and it was demonstrated that the shorter isoform that lacks the CXXC domain is overexpressed in many breast cancer cell lines compared to untransformed and immortalized breast cells (Good et al., 2017).

1.6. Problem statement and study motivation

Cancer is characterized by aberrant DNA methylation that results in altered gene expression which is responsible for the development of major cancer hallmarks. TET1 has a vital role in gene expression regulation, and appears to protect genes from methylation (Jin et al., 2014). In breast cancer, reduced activity of TET1 is suggested to be responsible for hypermethylation and aberrant gene expression. The compelling evidence about the role of TET1 in breast tumorigenesis indicates that its role is controversial and that further investigation of the role of TET1 and its isoforms in mammary gland is highly needed.

1.7. Hypothesis

It was demonstrated that TET1 seems to have controversial functions in breast carcinogenesis, and it has at least two isoforms that are differentially expressed in breast cancer cell lines and tissue samples. In addition, no correlation was made

between the expression level of the different isoforms in different types of breast cancer. Also, the localization of TET1 in breast cancer tissue samples was not deeply studied. Thus, here we hypothesize that different TET1 isoforms have different distribution and expression levels and patterns in different breast cancer stages and types.

1.8. Objectives and specific aims

1.8.1. Main objective:

To investigate the expression pattern and level of TET1 enzyme isoforms in breast cancer clinical samples, different cell lines under different culture conditions, and in different breast cancer animal models. Moreover, we aimed to elucidate the phenotypic outcomes after TET1 enzyme overexpression.

1.8.2. Specific targets:

1.8.2.1. To determine the expression and localization pattern of TET1 protein in different stages of human breast tissues samples using immunohistochemistry.

1.8.2.2. To test the expression level of *TET1* isoform mRNA in breast cancer cell lines under different cell culture conditions using real time PCR.

1.8.2.3. To study the expression pattern of *TET1* isoforms in different breast cancer animal models.

1.8.2.4. To clone *TET1* gene in Lentiviral vector to evaluate the effect of *TET1* gene overexpressing on different breast cancer hallmarks.

2. Materials and Methods

Table 2-1. A: List of materials used in methodology.

No	Material	Manufacture
1	MDEM/F12 media	Biological industries
2	RPMI (1640) media	Gibco Thermofisher
3	Horse serum	Biological industry
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	Dimethyl sulfoxide	Sigma
12	PBS	Biological industries
13	Skim milk	Sigma
14	Anti-TET1 Ab 191698	Abcam
15	ECL	Thermofisher
16	GAG-pol plasmid	Addgene
17	VSV-G plasmid	Addgene
18	Mirus TransLTi	TransfectionExperts, MirusBio
19	Ampicillin	Sigma
20	Puromycin	Sigma
21	Maxi prep kit	Invitrogen
22	Mini prep kit	Macherey-Nagel
23	Paraformaldehyde	Electron Microscopy Siences
24	XTT kit	Biological industries
25	Anti-rabbit horse raddish peroxidase conjugated Ab	Bethyl
26	Isopropanol biological gradient	Sigma
27	Ethanol biological gradient	Sigma
28	Chloroform biological gradient	Sigma
29	qScript™cDNA synthesis kit	Quanta Biosciences
30	SYBR® Green	Applied Biosystems
31	TRIZOL	Sigma
32	Agarose	Hy-labs
33	100bp DNA ladder	Genedirex
34	Bradford Protein assay	BioRad

Table 2-2. B: List of materials used in methodology.

No	Material	Manufacture
35	DNase1 kit	Biolabs
36	Polyacrylamide	Biological industries
37	GoTaq® Green Master Mix	Promega
38	Triton X-100	Sigma
39	Goat serum	Biological industries
40	Anti-TET1 N-terminus GTX125888 Ab	Gene TEX
41	Cy3	Bethyl
42	DAPI	Sigma
43	Xylene	LOBA CHEMIE
44	Ethanol	Biolabs
45	Citrate buffer	Sigma
46	Hydrogen peroxidase	Sigma
47	CAS	Invetrogen
48	Tween	Sigma
49	Mayer's hematoxylin	BioGnost
50	FH-TET1-pEF	Addgene
51	PSF-LENTI-CMV	Sigma
52	Kpn I	Biolabs
53	Xba I	Biolabs
54	BstUI	Biolabs
55	Gel/PCR extraction	Hy-Lab
56	1 Kp DNA ladder	Thermo-fisher
57	T4 DNA ligase	Biolabs
58	DH5α	Agilent Technologies
59	BamHI	Biolabs
60	0.22 μm filters	JET BIOFIL
61	Methanol	Sigma
62	Coomassie blue	BioRad
63	Matrigel	Invitrogen
64	Chamber slide	Lab-Tek
65	DNA extraction	Geneaid
66	Protease inhibitors	Sigma
67	HEPES	Biological industries
68	KCl	Biological industries
69	EDTA	Sigma
70	DTT	Thermo Scientific
71	Nonidet P-40	Sigma
72	Tris base	Sigma
73	NaCl	pubChem
74	Glycerol	Sigma

Table 2-3: List of Equipment and tools used in methodology

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	Ultracentrifuge	BECKMAN COULTER optima LE80H	U.S.A
11	SPIN-micropipette site	Nano Spinreact	china
12	Digital dry bath	Labnet	U.S.A
13	Elisa reader	BioTek EL-X800	U.S.A
14	Analytical Balance	METLER TOLEDO AB104	Switzerland
15	Autoclave	HIRAYAMA HV-110	U.S.A
16	RT-PCR machine	(Applied Bio-systems 7500 FAST Real Time PCR	Singafora
17	PCR machine	Applied Biosystem #9902	Singapore
18	SD semi-dry transfer cell	BioRad	U.S.A
19	G:BOX Chemi XX6 Gel Imaging System	Geneflow	UK

2.1. Cell culture

Breast cancer cell lines including MCF7, MDA MB231, T47D, HCC70, Sum 149, BT 549 cells, and Human Embryonic Kidney (HEK 293T) cells were grown in RPMI media (Gibco-Thermofisher), supplemented with 10% FBS (Gibco-Thermofisher), 1% glutamine, and 1% penicillin/streptomycin (Biological Industries). MCF10A cells were grown in DMEM/F12 media (Biological industries) supplemented with 5% horse serum (Biological industries), 1% glutamine, 1% Penicillin/Streptomycin (Biological industries), 20 ng/mL EGF, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, and 100 ng/mL cholera toxin (Sigma Aldrich). All Cells were incubated in humidity chamber on 37 °C with 5% CO2.

2.2. Cell thawing

To restore frozen cells from liquid nitrogen, cryotubes containing cells were immediately transferred from the liquid nitrogen to a 37 °C water bath. After thawing, cells were transferred to a new conical tube containing 5 ml fresh medium. Afterwards, cells were resuspended in fresh media and cultured on the tissue culture plates.

2.3. Cell passage

To pass cells, old media was aspirated, 1.0 ml trypsin-EDTA was added to each plate and then part of trypsin was aspirated. Afterwards, cells were incubated in CO2 incubator at 37 °C until the cells completely detached. Finally, cells were mixed with the fresh media and certain amount of cells was passed, and incubated in CO2 incubator at 37 °C.

2.4. Cell Freezing

Freezing media was prepared to contain 70% growth media, 20% fetal bovine serum (Gibco-Thermofisher), and 10% DMSO (Sigma).

To freeze cells, they were washed and trypsinized as mentioned above. When ready, cells were collected in freezing media and transferred to cyrotubes, stored at -80 °C, and then finally transferred and stored in liquid nitrogen.

2.5. Bioinformatics

TET1 mRNA sequence was retrieved from gene bank https://www.ncbi.nlm.nih.gov/ website using the accession number NM_030625.2. All primers used in this work were designed using primer 3 software http://primer3.ut.ee/. For virtual plasmid digestion and cloning NEB cutter software http://nc2.neb.com/NEBcutter2/ was used.

2.6. RNA extraction, cDNA synthesis and qRT-PCR

Tri reagent (Sigma Aldrich) was used for RNA extraction following manufacturer's protocol. DNA contamination was avoided by using DNase I treatment kit (biolabs). cDNA was synthesized using Q-Script cDNA synthesis kit according to manufacturer instructions (Quanta Biosciences). Relative quantitative real-time PCR (qRT-PCR) was performed using Applied Biosystems® 7500 Real-Time PCR machine using power SYBR Green Mix (Applied Biosystems). The sequence of primers used to detect the presence of *TET1* mRNA is F.P_5'-ccacagggacattcacaaca-3' (forward primer), R.P_5'-catggagctgctcatcttga-3' (reverse primer). To measure the expression of ectopic *TET1* in cell clones we used HA-F.P_5'-GAGGATACCCCTACGACGTG-3' (forward primer that targets HA tag) and *TET1*-R.P_5'-TCCCTTGGTTGTCTTTCGTAG-3' (reverse primer that targets *TET1*). To test the effect of *TET1* over-expression on the expression of cancer hallmark related genes, we used the primers listed in table 1 in appendix1. In order to check for the presence of different TET1 isoforms, we used exon specific primers designed to target specific TET1 exons according to published primer sequences (Good et al., 2017). For exon specific expression analysis, the relative TET1 isoform expression was in relation to TET1 exon 10.

2.7. PCR and sequencing

DNA extraction was done by Geneaid (GB-100) kit. PCR was perfomed using PCR machine (Applied Biosystem ,9902) using GoTaq® Green Master Mix (Promega) protocol. PCR products were run on standard agarose gel (Hy-labs), using 100bp DNA ladder (Genedirex, DM001-R500) as a marker. PCR products were cleaned using Gel/PCR extraction (Hy-Lab, EX-GP 200) kit. Sanger sequencing service was purchesd from a commercial company. Sequencing primers were *TET1* F.P_5'-gtgtaaccagcacagttcatg-3' and *TET1* R.P_5'-

tgtgtccacttctccacctc-3’. Sequencing results were analyzed using chromas® software.

2.8. Protein subcellular fractionation

Nuclear and cytoplasmic extracts were prepared as follows. First, cells were scraped in PBS, and after centrifugation, the cell pellet was reconstituted in a hypotonic lysis buffer (10 mmol/liter HEPES (pH 7.9), 10 mmol/liter KCl, 0.1 mmol/liter EDTA) supplemented with 1 mmol/liter DTT and a broad-spectrum mixture of protease inhibitors (Sigma-Aldrich). The cells were allowed to swell on ice for 15 min, and then Nonidet P-40 was added, and cells were lysed by vortex. After centrifugation, the cytoplasmic fraction was collected. Afterward, nuclear extracts were obtained by incubating nuclei in a hypertonic nuclear extraction buffer (20 mmol/liter HEPES (pH 7.9), 0.42 mol/liter KCl, 1 mmol/liter EDTA) supplemented with 1 mmol/liter DTT for 15 min at 4 °C. The nuclear fraction was collected after centrifugation.

2.9. Western Blot analysis

Cells were lysed by using Nonidet P-40 lysis buffer containing 50 mM Tris (pH 7.5),150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors. Protein concentration was quantified using Bradford protein assay (BioRad.) Samples were run on polyacrylamide gel (Biological industries), transferred to Nitrocellulose membrane by SD semi-dry transfer cell (BioRad). Membranes were blocked with skim milk (Sigma) and immunoblotted with primary antibody (anti-TET1 191698, Abcam). Afterwards membranes were washed and blotted with secondary anti-rabbit horse raddish peroxidase conjugated antibody (Bethyl, A120-101P) according to the manufacturer’s instructions. Signal was visualized using Western Protein ECL substrate (Thermofisher), and G: BOX Chemi XX6 Gel Imaging System (Geneflow).

2.10. Immunofluorescence (IF)

Cells were seeded on round slide coverslips in 12-well plates. Twenty-four hours later cells were fixed with 4% paraformaldehyde (Electron Microscopy Siences), permeabilized with 0.25% Triton X-100 (Sigma). Then cells were blocked using 10% goat serum (Biological Industries). Afterwards, Cells were incubated 1 hr at room temperature (RT) with primary antibodies (TET1 C-terminus 191698 from Abcam, TET1 N-terminus GTX125888 from Gene TEX). After washing excess primary antibody, using TTBSX1, cells were incubated in dark with either anti-mouse or anti-rabbit fluorescent secondary antibody (Cy3, Bethyl, A90-516C3 and A120-201C3). DAPI was used as a counter stain for nuclei (Sigma). Photos were taken using fluorescent microscope (Olympus Ck-40).

2.11. Immunohistochemistry (IHC)

5 µm tumor sections were deparaffinized and rehydrated with Xylene (LOBA CHEMIE) 3 times for 5 min, then 100 % ethanol (BioLab) 3 times for 2 min, 95 % ethanol 2 times for 2 min, 80 % ethanol for 3 min, and then transferred into distilled water (DW) for 5 min. After that, tissues were denatured for 4 min in pressure cooker in citrate buffer (Sodium citrate dehydrate and Citric acid, both Sigma-Aldrich) 0.01 mol/L, pH 6.0. Then slides were cooled to RT in a cold water bath, and then washed and incubated with DW for 5 min. Afterwards slides were blocked by 3% Hydrogen peroxidase (Sigma-Aldrich), washed and incubated with DW for 5 min. After that, slides were blocked again with CAS® block (Invetrogen,00-8120) reagent for 15 min. Then the sections were incubated with anti-TET1 Abs (TET1 191698, Abcam) overnight at 4 °C according to manufacturer's recommendations. Next, slides were washed 3 times for 5 min each with X1 TTBS (100 ml TBS X10, 900 mL DW, and 0.5 mL Tween, Sigma-Aldrich). Then, slides were incubated with secondery antibody conjugated to HRP (Bethyl, A120-101P). To visualize the stained tissue, slides were incubated with HRP chromogen substrate (Impress HRP reagent kit, vector laboratories). Finally, slides were counterstained with Mayer's hematoxylin (BioGnost).

2.12. *TET1* gene cloning

2.12.1. Digestion of FH-TET1-pEF and PSF-LENTI – LENTIVIRUS

FH-TET1-pEF (Addgene, Plasmid #49792) was used as a template. The viral vector was PSF-LENTI - LENTIVIRUS PUROMYCIN CMV PLASMID was our destination vector (Sigma-Aldrich, OGS269). 20 µg of both FH-TET1-pEF and PSF-LENTI were digested with Kpn I (BioLab, R0142S) and Xba I (BioLab, R0145S). BstUI (BioLab, R0518S) was used to digest the FH-TET1-pEF vector backbone (Appendix2). Digestion volumes and conditions are shown in table 1. Then, digestion products were run on 0.7 % agarose (hy-labs). Specific bands were purified from the gel using Gel/PCR extraction (Hy-Lab, EX-GP 200) kit.

Table 2-4*:* Digestion volumes and conditions

	FH-TET1-pEF (µL)	**PSF- Lenti (µL)**	
20 µg template	38	30	**First digestion Incubated at 37 °C For 3 hrs**
KpnI enzyme	3	3	
Buffer 1.1	5	5	
Ultra-Pure H_2O	24	32	
XbaI enzyme	3	3	**Second digestion Incubated at 37 °C For 3 hrs**
Cut smart buffer	5	5	
BstUI enzyme	3	--	**Third digestion BstUI Incubated at 60 °C For 3 hrs**
Cut smart buffer	5	--	

2.12.2. Ligation

In order to clone *TET1* gene with the viral vector PSF-Lenti, different ligation molar ratios (1:3, 1:5, and 1:10) were used. Molar ratios were calculated using

the online calculator http://www.insilico.uni-duesseldorf.de. Ligation was done according to T4 DNA ligase (BioLabs, M0202S) protocol.

2.12.3. Transformation

Chemically competent DH5α bacteria (Agilent Technologies Cat # 210518) tube was taken out from - 80 °C, and directly transferred to and incubated on ice for 30 min. 50 µl of DH5α was mixed with 5 µL of ligation products. After 30 min incubation on ice, bacterial tubes were quickly transferred and incubated at 42 °C for 1 min. Then tubes were returned directly to ice for 5 min, and then transferred and incubated at RT for 5 min. Transformed DH5α cells were grown in 1mL LB liquid with shaking for 2 hrs at 30 °C, then centrifuged at 8000 rpm for 1 min. Then, most of supernatant was removed and cells were resuspended in the residual media left. Finally, transformed DH5α cells were grown on Ampicillin (Sigma-Aldrich) LB agar plates and incubated at 30 °C for 24 hrs.

2.12.4. Digestion of meni-prep products

To test which colony has TET1-PSF-Lenti, digestion was done using BamHI enzyme (Biolabs, R01363) which cuts one time in both PSF-Lenti and TET1 mRNA sequence (appendix 3).

2.12.5. Plasmid preparation (Maxi-perp)

After growing the bacteria in 0.5 L LB media containing antibiotic selection marker at 30 °C overnight with shaking, cells were collected and Maxi-preb was done using HiPure Plasmid Maxiprep kit (Invitrogen, K210006) according to manufacturer instructions.

2.13. Lentivirus preparation

Lentivirus particles were generated by three plasmid expression system, in which HEK 293T cells were co-transfected with the following three vectors: packaging GAG- pol (Addgene), envelope pCMV-VSV-G (Addgene), and TET1-PSF Lenti or PSF lenti vector. One day before the transfection, HEK-293T cells were plated to be 60% confluent. On the next day, cells were fed with fresh medium and transfected using MIRUS (TransfectionExperts, MIR2300) transfection reagent according to manufacturer instructions. Briefly, 2.2 µg packaging GAG-Pol, 1.2 µg Envelop VSVG, and 5 µg of each TET1-PSF-lenti and PSF-lenti vector were transferred to tubes containing 21 µl MIRUS mixed with 2ml serum free medium. After 15 min incubation at RT the mixture was dropped on cell culture media. 24 hrs after transfection, cell culture media was changed with a fresh media. 2 and 3 days after transfection, cell culture media containing the viral particles was collected and centrifuged for 10 min at 53000 rpm to get rid cellular debris. Finally, the collected virus containing media were centrifuged at 40000 rpm for 2 hrs using BECKMAN COULTER (OptimaTM LE-80K Ultracentrifuge). After centrifugation, most of the media was removed and the viral particles were then resuspended and filtered using 0.45 µm filters (JET BIOFIL).

2.14. Infection and selection

0.5 million MDA MB231 cells were infected with 0.75 mL of viral particles suspension. Cells were incubated with viral particles for two days in CO2 incubator at 37 °C. Next, media containing viruses was removed, and replaced with refresh one for one day. We repeated the infection procedure twice. To select for clones, cells were grown in media containing 1 µg/mL of puromycin (Sigma) until control un infected cells completely died.

2.15. Cell count

$3x10^4$ cells were seeded in 6 well plate in triplicates and cells were counted over 3 days as follows. Cells were first trpsynized and collected into 15 ml conical

tubes and centrifuged at 1600 RPM for 10 min. Then the supernatant was removed and cells were re-suspended in 1 mL media. Next, 10 µl of the homogenous supernatant was counted using counting chamber slides.

2.16. XTT test

2 $X10^3$ cells were seeded in triplicate in 96 well plate and cell proliferation was assessed over 3 days using XTT kit (Biological industries) according to the manufacturer's instructions. Results were read using ELISA reader, (BioTek EL-X800).

2.17. Wound healing

2.5 $x10^5$ cells were seeded in triplicates in 12 well plates. Upon reaching 100% confluency, cell monolayer was scratched using the 10 µl plastic pipette tip. After removing floating cells, the same area in plate was photographed over the needed period of time using a camera attached to Inverted Microscope (Olympus, CK-40)

2.18. Survival assay

200 cells were seeded in triplicates in 6 well plate. Each 3-4 days, the media was changed until cell colonies were visible by naked eye. Then the media was removed and wells were washed with PBSX1 (Biological industries). Then PBS was aspirated and wells were left to dry. After drying, cells were fixed with absolute methanol (Sigma) for about 15 min at RT. Afterwards, wells were left to dry and then stained using Coomassie blue (Bio-Rad) for about 15 min. Finally, the stain was removed and wells were washed using tap water.

2.19. 3D culture assay

To coat cell culture wells, reduced growth factor Matrigel (Invitrogen) was thawed on ice overnight to liquefy it. Then 40µl of the liquid matrigel was used to coat each well of chamber slide (Lab-Tek). After coating, the chamber slides were incubated at 37 °C in order to dry. After preparing the lower layer, 200 µl of cell suspension containing $2.5x10^4$ cells/mL was mixed with 200 µl of 4% Matrigel containing cell culture media and added to the lower layer. Cells were then incubated in CO2 incubator at 37 °C, observed, and photographed over about 2 weeks.

3. Results and discussion

3.1.TET1 enzyme expression in breast cancer tissue samples.

To determine the prevalence of altered TET1 level in breast cancer in Palestinian patients, we examined it's expression in different types of human breast cancer samples by IHC using antisera against TET1 enzyme. Distinct TET1 expression levels and localization were seen in normal and tumor tissues, or when comparing tumors from different patients. For example, TET1 expression was lacking in most of the tumors tested compared to normally appearing tissue obtained from the same patient (Fig 3.1). In addition, we noticed that the expression level of TET1 correlates with the degree of differentiation of the tumor. Positive brown staining is evident in well-differentiated tumors (Fig 3.2 arrows), which is lost when the same tumor progresses towards a poorly differentiated one (Fig 3.2 arrow heads). Moreover, the expression pattern of TET1 enzyme varied between tumors obtained from different patients (Fig 3.3). These results indicate that TET1 expression is very heterogeneous in breast cancer samples and its expression correlates at least with breast cancer cell differentiation. Our results are in agreement with different studies that have shown TET1 expression is variable between different subtypes of breast cancer or in the same subtype of cancer that have specific molecular markers (Collignon et al., 2018; Good et al., 2018). For example, the expression of TET1 was shown to negatively correlate with miR-29a and this negative correlation was stronger in estrogen receptor (ER) negative breast cancer samples compared to ER+ ones (Pei, Lei, & Liu, 2016). In another research that studied the role of TET1 in TNBC, TET1 expression was shown to inversely correlate with the expression of EZH2 (Yu et al., 2019). In these two studies, TET1 was proved to be a TSG. On the other hand, TET1 expression pattern in tissue samples was shown to correlate with poor prognosis in breast cancer patients (Wu et al., 2015). In this study, in comparison to the two above discussed studies, TET1 was shown to be an oncogene and that its expression is elevated and correlates with the hypoxic level in breast cancer samples (Wu et al., 2015). In conclusion, TET1 staining pattern seems to be controversial and extended investigations are needed to better understand the expression pattern of TET1 in different breast cancer subtypes. For instance, in

different studies that looked at TET1 expression in tissue samples, using IHC, the presence of different TET1 isoforms was not taken into consideration, especially in light that those different TET1 isoforms might have different functions, expression patterns and cellular localization. Moreover, in most of studies, the anti-TET1 antibodies used to detect TET1 expression are raised against TET1 C-terminus (Tsai et al., 2015; Wang et al., 2018), which can affect the detection of full length TET1 enzyme which is thought to be the TSG version of TET1. Thus, in order to have a better picture about the true TET1 expression pattern in breast cancer tissue, it might be important to use anti-TET1 antibodies that can detect TET1 N-terminus.

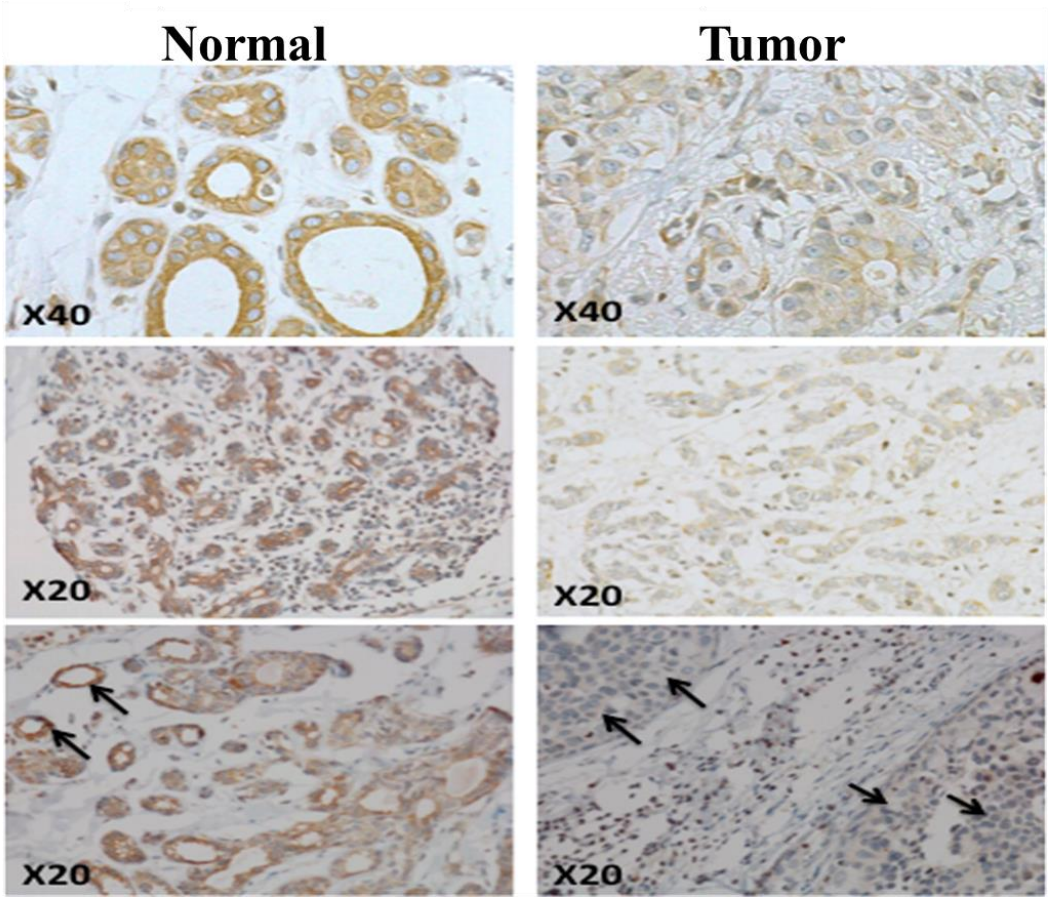

Figure 0.1 TET1 expression in breast cancer samples. Representative images of TET1 IHC staining showing loss or weak staining of TET1 in malignant cells (right panel, arrow heads) compared to benign or normally appearing cells of mammary tissue alveoli or ducts (left panel, arrows). Brown color indicates positive staining for TET1.

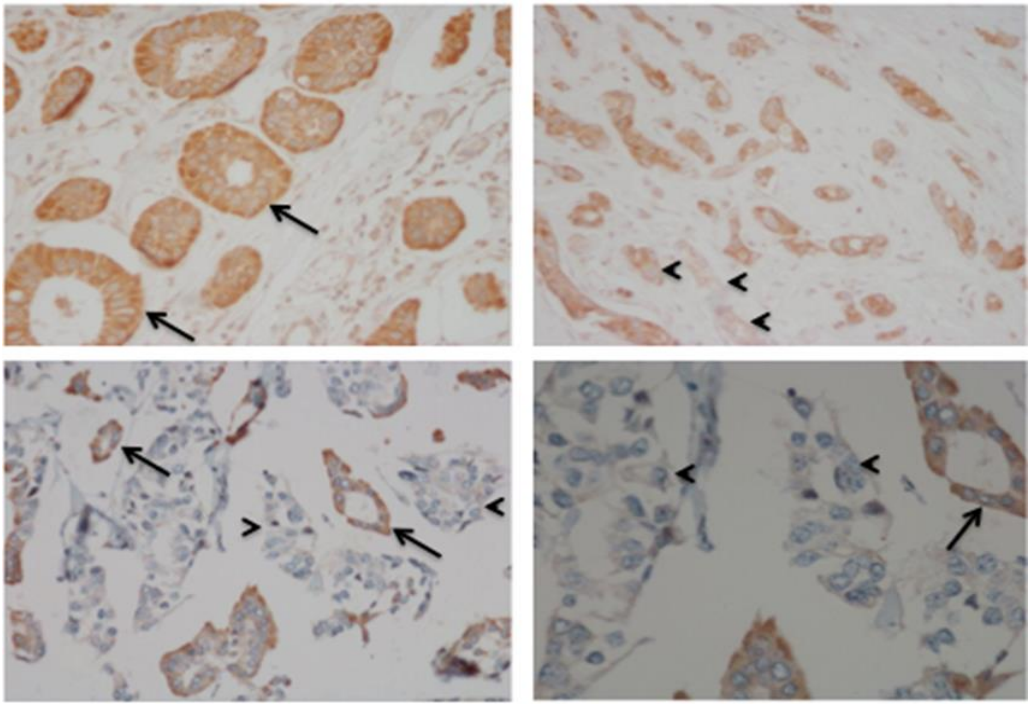

Figure 0.2 Correlation between TET1 expression and differentiation level in breast cancer samples. Representative images of TET1 IHC staining showing loss or decreased levels of TET1 expression in poorly differentiated (arrow heads) compared to well differentiated tissue (arrows). Brown color indicates positive staining for TET1.

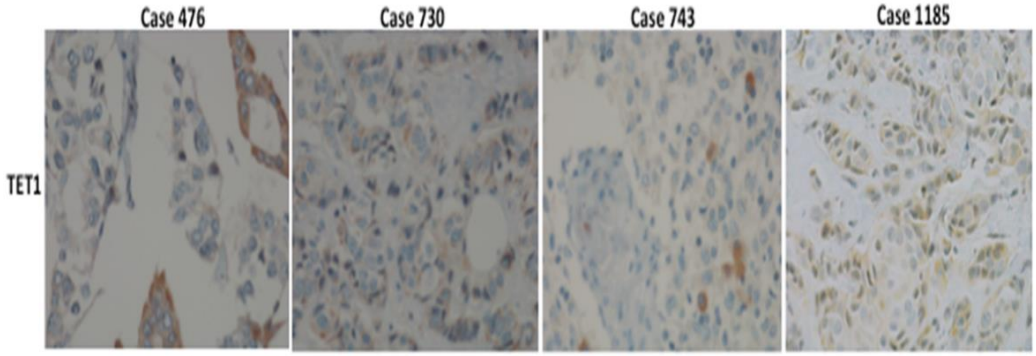

Figure 0.3 IHC staining pattern of TET1 enzyme in breast cancer samples. Representative images of TET1 IHC staining showing different expression level ranging from heterogeneous (case 476), weak (case 730), negative (case 743), and mixed cytoplasmic and nuclear (case 1185) staining. Brown color indicates positive staining for TET1.

3.2. Effect of Estrogen and GnRH on *TET1* isoforms mRNA.

Previous data showed that *TET1* has at least three different isoforms (Good et al., 2017). In addition, unpublished results from our lab (Jamoos et al, unpublished data) demonstrated that the expression of these different isoforms is affected by

hormonal treatment. In order to test the effect of different hormonal treatments on the expression of the specific different isoforms in different breast cancer cell lines, we treated hormone receptor positive cells (MCF7 and T47D cells) with GnRH and Estrogen (E2) for 4hrs. Our qRT-PCR results demonstrated that different cells showed different *TET1* isoform expression pattern after hormonal treatment. For example, in MCF7 cells, GnRH almost has no effect except that it slightly decreases the shorter isoform B11, while E2 treatment significantly decreases mRNA level of the different *TET1* isoforms (Fig 3.4.A). In T47D cells, GnRH treatment slightly increases different *TET1* isoforms except the shorter isoform B15, while E2 significantly decreases the shorter isoform B15 with almost no effects on other isoforms (Fig 3.4.B).

Our results here demonstrate that not only there is a difference in the expression pattern of *TET1* various isoforms between different breast cancer cell lines, but also in the presence of differential effect of different hormones on their expression in different cells. This can lead to the conclusion that *TET1* isoforms can have variable behavior in different cell lines under different cell contexts, which indicates that those different *TET1* isoforms can have different functions.

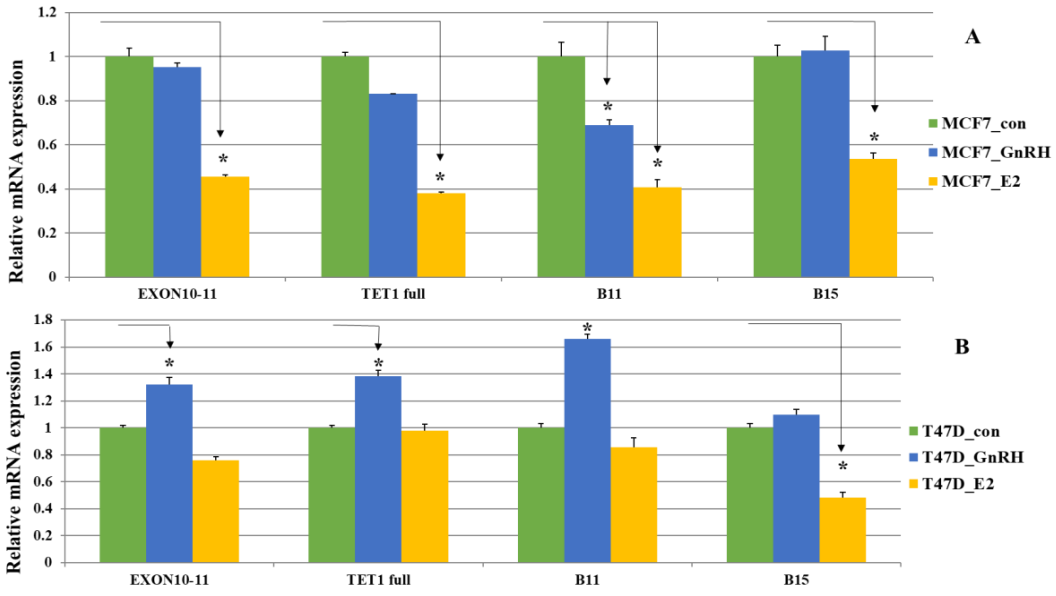

Figure 0.4 Effect of Estrogen and GnRH on *TET1* isoform mRNA levels in MCF7 and T47D cells. Relative qRT-PCR results showing the expression level of different *TET1* isoforms in MCF7 (A) and T47D (B) treated with E2 and GnRH. The mRNA levels are shown after normalization to the level of the housekeeping gene *hUBC* and relative to mRNA levels in control untreated cells. Bars represent SEM. * indicates that p-value is <0.05.

3.3. The expression pattern of different TET1 isoforms in different transformation background in MCF10A cells.

After showing that different cell contexts have differential effect on *TET1* isoforms, we aimed to test the effect of cell transformation on the expression level of different *TET1* isoforms. We wanted to test the effect of different cell transformation models on *TET1* isoforms expression. To do so, we transformed MCF10A cells harboring different *p53* variants with *HRAS G12V* oncogene. Our results show that, cells that overexpress HRAS on either p53 knockout or R175H p53 backgrounds, all isoforms of *TET1* mRNA global level increases. This is seen from the increased expression of exons 10-11 that supposed to be expressed in all *TET1* isoforms (Fig. 3.5). The same results were obtained using primers that target the full length of *TET1* (Fig. 3.5). Upon testing the expression level of the short *TET1* isoforms (B11 and B15), we noticed that overexpression of HRAS G12V or p53 R175H or p53 knockout reduced the expression of both B11 and B15 *TET1* isoforms. Moreover, in p53 R175H and p53 knockout cells HRAS G12V rescued the reduction in *TET1* B11 and B15 isoforms.

Our findings from this experiment provide another evidence that the expression of different *TET1* isoforms is subject to changes per changing cellular context. This indicates that the conclusion about the expression pattern of different *TET1* isoforms in cancer versus normal tissues needs further and deeper investigation in order to assign each isoform with it’s function in breast cancer initiation and progression.

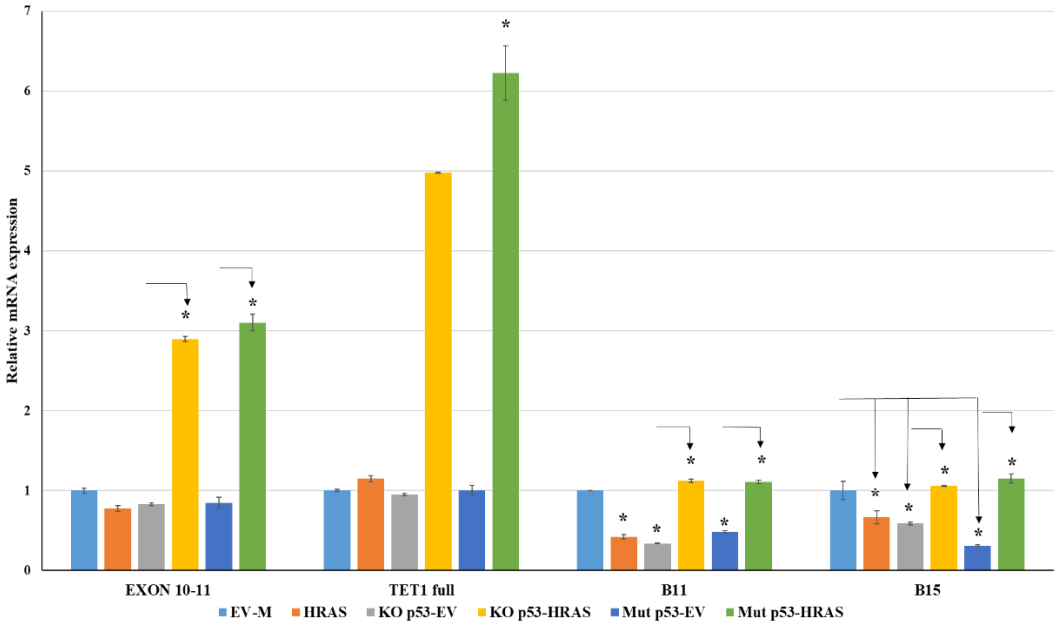

Figure 0.5 The expression pattern of different *TET1* isoforms in different transformation background in MCF10A cells. Relative qRT-PCR results showing the expression level of different *TET1* isoforms in the indicated cell lines. mRNA levels are shown after normalization to the level of the housekeeping gene *hUBC* and relative to mRNA levels in MCF10A cells infected with EV. Bars represent SEM. * indicates that p-value is <0.05. EV-M: MCF10A cells infected with empty vector, HRAS: MCF10A cells infected with *HRAS*, KO p53-EV: *p53* KO MCF10A cells infected with empty vector, KO p53-HRAS: *p53* KO MCF10A cells infected with *HRAS*, mut p53-EV: MCF10A cells mutated *p53* infected with empty vector, mut p53-HRAS: MCF10A cells mutated *p53* infected with *HRAS*.

3.4. The expression pattern of the short *Tet1* isoform in different animal models.

To elucidate whether the presence of a specific *TET1* isoform is relevant to breast tumorigenesis, we tested the expression of the N-terminus truncated (short) and canonical (full length) *Tet1* in normal and tumor mammary tissues obtained from either *p53* and *WWOX* single and double knockout mice, which give rise to basal triple negative mammary gland tumors (from Prof. Rami Aqeilan, Hebrew University) or from an MMTV-PyMT transgene mouse model that give rise to luminal tumor type (RNA provided by Dr. Itay Ben-Borath, Hebrew university). In the first model (basal tumors), we noticed a drastic reduction in the expression level of *Tet1* in all tumors tested compared to normal mammary gland tissue without preferential expression of any of *Tet1* exones (Fig. 3.6 A&B). In the MMTV-py-MT model (luminal tumors), qRT-PCR readily detected low levels of

exon 1 in tumor tissue while a twofold expression level of the shorter *Tet1* gene in tumor tissue suggesting the possible relevance of this isoform in breast tumorigenicity (Fig 3.6 C&D)

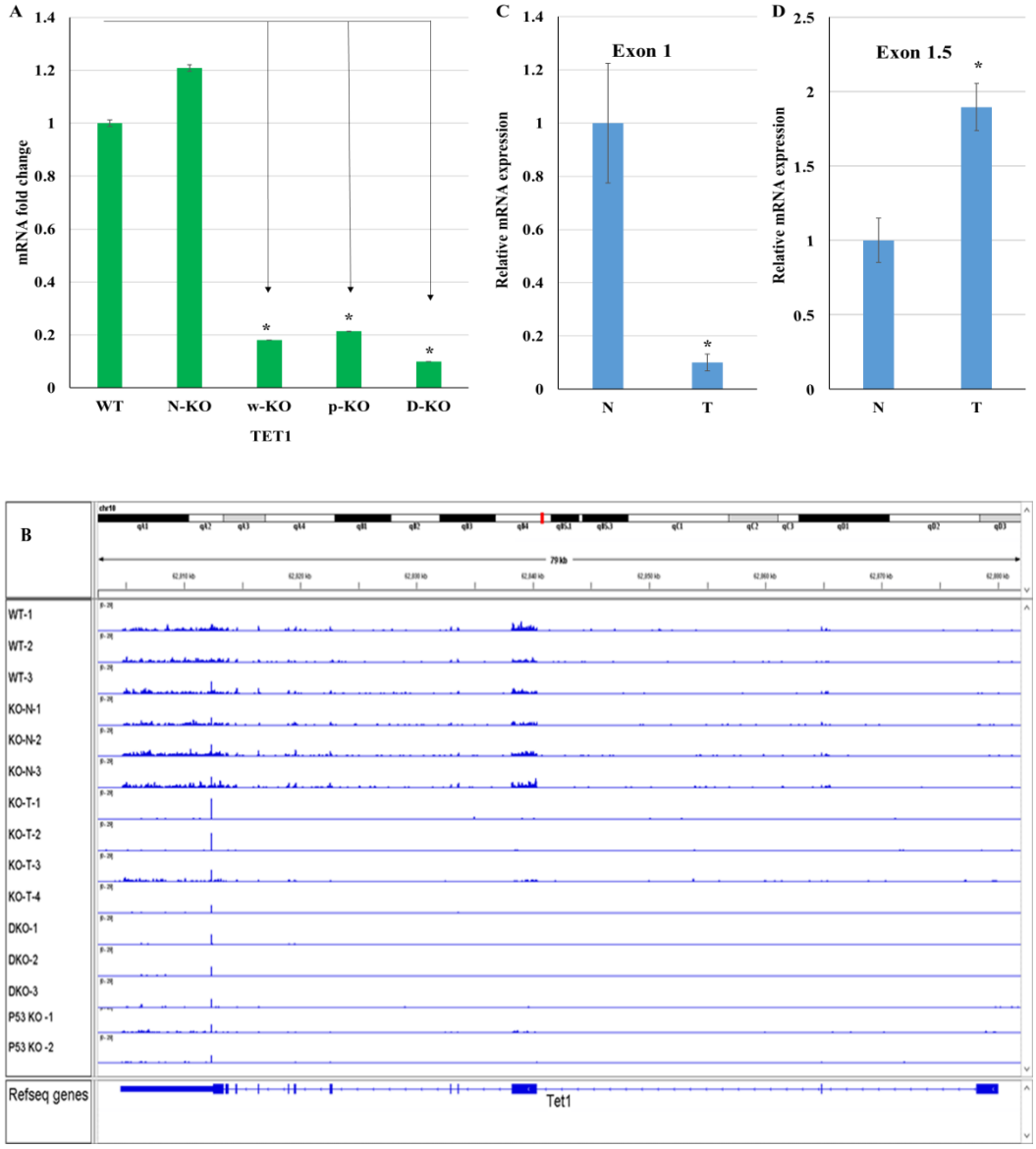

Figure 0.6 Expression pattern of different *Tet1* isoforms in different breast cancer animal models. A&B. RNA seq data for RNA extracted from conditional mammary gland *p53* and *Wwox* single and double knockout (w-KO: *Wwox* knockout, p-KO: *p53* knockout, D-KO: double knockout for both *p53* and *Wwox*) mice. RNA obtained from normal mammary gland tissue isolated from wild type mice (WT) or from pre-cancerous tissue of D-KO mice (N-KO) were used as control samples. **C&D.** qRT-PCR on RNA extracted from mouse mammary tumors (T) and normal tissue (N), after DNase treatment, for Exons 1 (represents the longer *Tet1* isoform) and 1.5 of *Tet1* (represents the shorter isoform). The mRNA levels are shown after normalization to levels of *Rpl0*. Bars represent SEM, n=6. * indicates that p-value is <0.05.

While our results from the luminal breast cancer model are in concordance with a recent publication that found *TET1* short isoform is overexpressed in breast cancer tissues and cell lines, and that this short isoform might behave as an oncogene (Good et al., 2017), our results from the basal breast cancer model indicate that TET1 is a tumor suppressor gene that gets deleted in specific types of breast cancer, which is in agreement with studies which showed *TET1* expression level is attenuated in breast cancer and that TET1 behaves as a tumor suppressor gene (Yang, Yu, Hong, Yang, & Shao, 2015). In fact, our findings here, in addition to the controversy about the function of *TET1* gene, indicates that *TET1* gene and its isoforms differential expression pattern might not be a straight forward pattern and necessitates further studies that would elucidate better the expression pattern and TET1 role in breast tumorigenesis.

3.5. TET1 different isoforms have different cellular distribution

After our results from the animal models, and to understand the expression pattern of different *TET1* isoforms, and to test whether these isoforms have different cell distribution. We tested the expression pattern of *TET1* in different cell lines. We first did qRT-PCR. As shown in Fig. 3.7A, *TET1* expression is highest in MCF7 compared to other cell lines. Then we tested TET1 expression by IF. Our results show that TET1 is expressed more in the nucleus and specifically in the nucleolus (Fig. 3.7B). Moreover, while our qRT-PCR shows highest expression in MCF7, IF results show differences in the expression but not to the extent observed in qRT-PCR. Taking into consideration our results from animal models showed different isoforms of *Tet1* in mouse tissue, we tried to explain this discrepancy between the protein and mRNA levels by hypothesizing that our PCR primers and the Ab used in IF recognize specific parts of the protein and not all isoforms. To test our hypothesis, we re-did the IF experiment using two different antibodies (Abs), one raised against TET1 N-terminus and the other against the C-terminus. In this experiment, only the anti-C-terminus Ab gave a signal (Fig. 3.7C) indicating that TET1 might be modified at its N-terminus in a way or another. Following this observation, we thought that these potential different isoforms may be distributed differentially in the cell. To test this, we did

subcellular fractionation of cellular proteins and ran the nuclear and cytoplasmic fractions in western blot analysis. As shown in Fig. 3.7D, both fractions express different isoforms to different extents, where the TET1 shorter isoform is expressed in the nucleus, the longer isoform is expressed in the cytoplasm. Our findings here indicate that the distribution of different TET1 isoforms is different in different cell compartments.

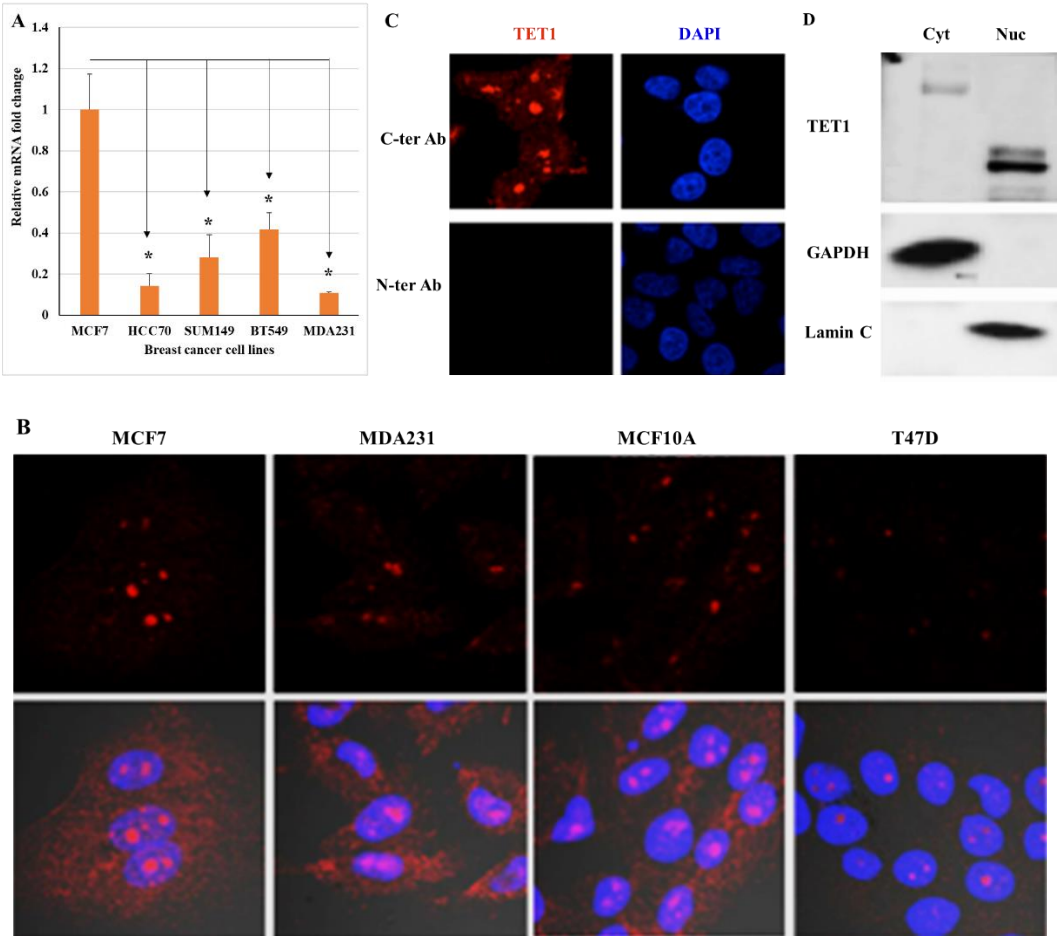

Figure 0.7 Different *TET1* isoforms have different cellular distribution. A. Relative qRT-PCR results showing the relative *TET1* mRNA expression level in the indicated cell lines. The mRNA levels are shown after normalization to the level of the housekeeping gene *hUBC* and relative to mRNA levels in MCF7 control cells. Bars represent SEM. * indicates that p-value is <0.05. **B.** IF staining of TET1 in different cell lines. Orange signal represents positive signal while blue signal represents DAPI DNA counter stain. **C.** MCF7 breast cancer cell line stained with Anti-TET1 Abs raised against either the C-terminus (C-ter) or the N-terminus (N-ter) of the protein. No staining was observed with the Ab raised against the N-terminus of the protein. Orange signal represents positive signal while blue signal represents DAPI DNA counter stain. **D.** Subcellualr fractionation of MCF7 breast cancer cell line lysates into cytoplasmic (Cyt) and nuclear (Nuc) fractions and western blot analysis shows the presence of different TET1 isoforms in the nucleus and cytoplasm.

3.6. *TET1* gene cloning in Lentiviral vector

3.6.1. Isolation of *TET1* fragment and digestion of the lentiviral destination vector

In order to study the function of TET1 in breast cancer, we first cloned *TET1* into a lentiviral vector. To do so, we excised out *TET1* mRNA from FH-TET1-pEF plasmid by using KpnI and XbaI enzymes. However, the size of the plasmid backbone and that of *TET1* mRNA were very close and didn't allow good separation in gel electrophoresis (*TET1* mRNA is 6494 and its back bone is 6104 bp) (Fig 3.8.A). In order to overcome this obstacle, we further digested the plasmid with BstUI enzyme that digests the back bone only but not *TET1* mRNA. Because BstUI enzyme digests 28 times in the plasmid backbone and that the largest fragment size didn't exceed 700 bp, we easily retrieved *TET1* mRNA fragment from the gel (Fig 3.8.B) Lane5.

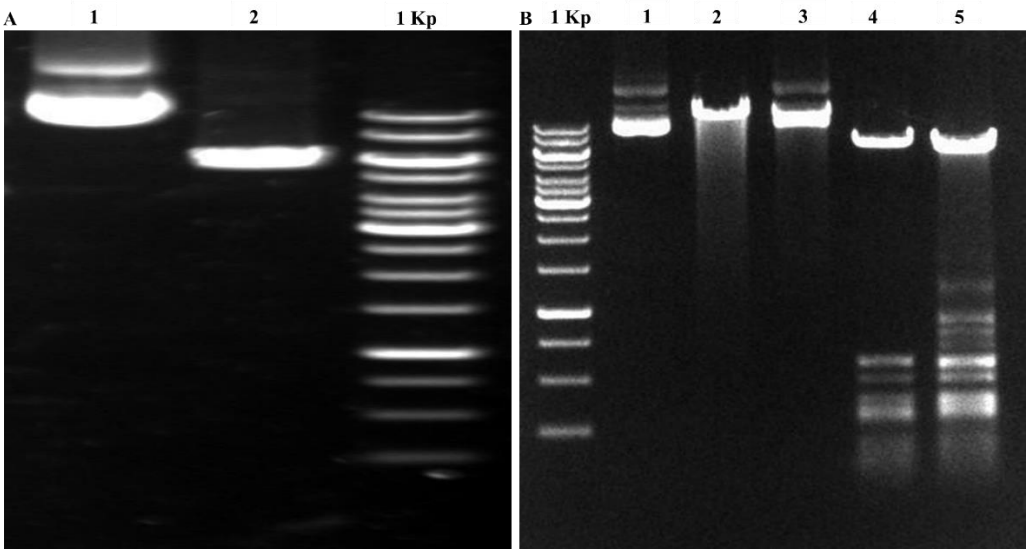

Figure 0.8 Digestion of FH-TET1-pEF and PSF-lentiviral vectors. A. Agarose gel photograph showing FH-TET1-pEF digestion with KpnI and XbaI. Lane 1: FH-TET1-pEF uncut, lane 2: FH-TET1-pEF cut with KpnI and XbaI. **B.** Agarose gel photograph showing FH-TET1-pEF digestion with KpnI, XbaI, and BstUI enzymes. Lane1: FH-TET1-pEF uncut, lane2: FH-TET1-pEF cut with KpnI, lane3: FH-TET1-pEF cut with XbaI, lane4: FH-TET1-pEF cut with BstUI, lane5: FH-TET1-pEF triple cut with KpnI, XbaI, and BstUI. 1Kb: 1Kb DNA ladder.

3.6.2. Digestion of meni-prep products to validate successful *TET1* cloning into PSF-Lentiviral vector.

After cutting *TET1* mRNA using KpnI and XbaI enzymes, the same restriction enzymes were used to digest our destination lentiviral vector. Following that, the products were ligated as described in the materials and methods section. To confirm successful *TET1* mRNA cloning in lentiviral vector, we prepared minipreps from several bacterial colonies transformed with the ligation product. Then, the minipreps were digested using BamH1 that cuts one time in both PSF-Lenti and *TET1* mRNA sequence (appendix 3). Gel electrophoresis analysis of the digested products revealed that 2 colonies out of the 14 colonies that we analyzed were positive and carried TET1-PSF lentiviral vector (Fig 3.9) lanes 1&5. These results confirm the successful cloning of *TET1* cDNA in PSF-lentiviral vector.

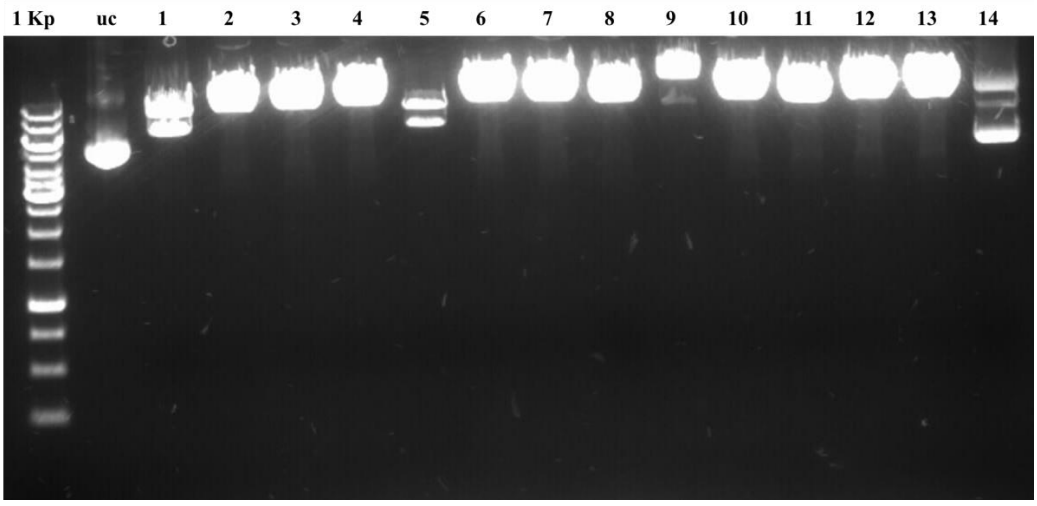

Figure 0.9 Testing for successful *TET1* cloning by analysing cloning menipreps. Agarose gel photograph showing menipreps digestion with BamHI enzyme (Lanes 1-14), Lane uc: TET1-PSF-lenti uncut meniprep control. 1Kb: 1Kb DNA ladder.

3.6.3. PCR, Digestion, and sequencing for TET1-PSF-Lenti vector maxi-prep

To obtain large amount of TET1-PSF-Lenti to be further used for viral particle preparation and cell infection, we did maxi-prep plasmid isolation. In order to further confirm cloning success, we did conventional PCR on the viral vector using *TET1* specific primers. As shown in Fig 3.10.A, a positive PCR product was obtained from both our positive control sample as well as from our cloned

vector; lanes 1&2 respectively. Of note, no PCR product was revealed in both, the negative control plasmid or from the no template PCR reaction; lanes 3&4 respectively. To further confirm cloning success, we digested TET1-PSF-lenti vector with BamHI enzyme and got the expected DNA fragments as shown in Fig. 3.10 B&C. Finally, to show that the cloning process didn't result in mutations in *TET1* cDNA, we sequenced the plasmid using *TET1* specific primers. The sequencing results showed that indeed *TET1* is cloned into the vector and that no mutations are found in the cloned *TET1* cDNA (Appendix 4). Altogether, these results confirm that *TET1* was successfully cloned into our viral vector and that the cloned *TET* mRNA is mutation free.

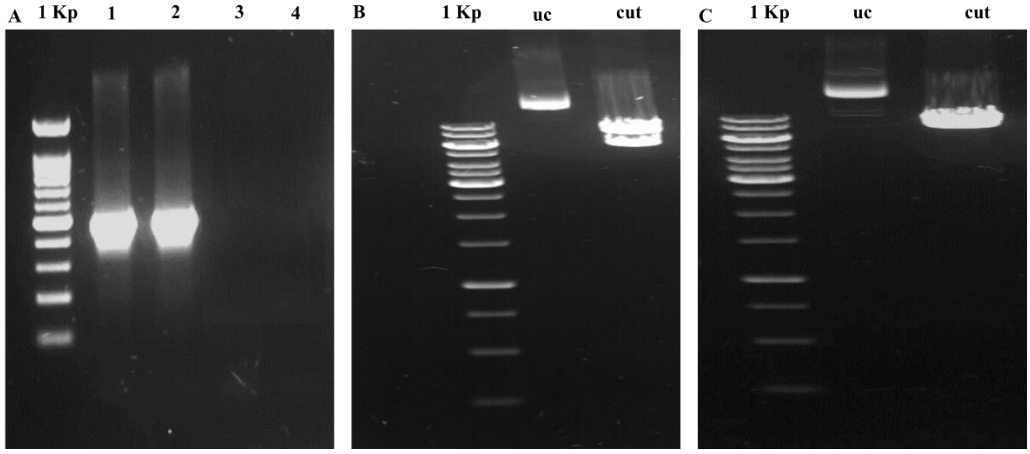

Figure 0.10 PCR on and digestion of maxi-prep product A. Agarose gel photograph showing conventional PCR results using primers that target *TET1* gene using different templates: Lane1: FH-TET1-pEF positive control, lane2: cloned TET1-PSF-Lenti plasmid, lane3: PSF-Lenti plasmid as negative control, and lane4: no template DNA as negative control. Agarose gel photograph showing the digestion of TET1-PSF-Lenti with BamHI. uc: uncut TET1-PSF-Lenti and cut: TET1-PSF-Lenti (**B**) and digestion of PSF-lenti empty vector with BamHI (**C**). Uc: uncut PSF-Lenti. Cut: PSF-Lenti empty vector. 100 bp: 100bp DNA ladder. 1Kb: 1Kb DNA ladder.

3.7. Generation of TET1 overexpressing MDA MB231 cell

In order to study the function of TET1 enzyme in breast cancer, we infected breast cancer cell line MDA MB231 with either viral particles expressing HA-tagged full length *TET1* (TET1-PSF-Lenti) or empty viral vector control (PSF-

Lenti). Two days later, we added puromycin to select for TET1 positive cells. The same was done for control cell selection. As shown in Fig. 3.11 A, after one week of selection, only control non infected cells died, while the control empty vector and TET1 overexpressing cells were resistant to puromycin selection. To insure TET1 overexpression in our clones, we did qRT-PCR on RNA extracted from both the control and TET1 overexpressing cells. First we did qRT-PCR using TET1 specific primers and indeed, our results from this experiment proved that we have about 10 fold *TET1* overexpression in our clones compared to control clones (Fig. 3.11B). To further verify TET1 overexpression and to ensure that the overexpression that we got with *TET1* specific primers is not transient and didn't result from the cloning and selection process, we used a primer in which the forward primer targets the HA tag while the reverse one targets TET1. The results confirmed overexpression of TET1 in our clones (Fig. 3.11C).

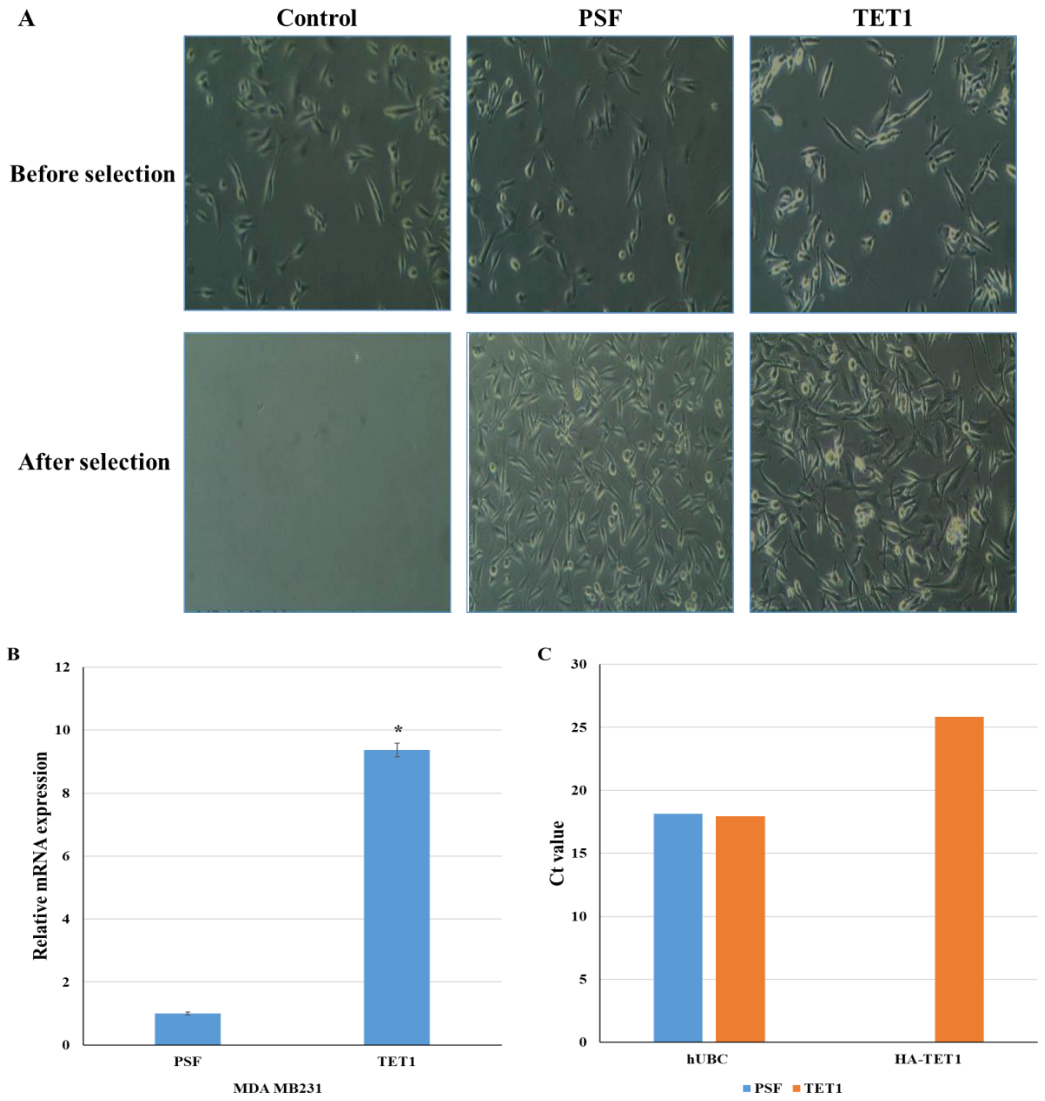

Figure 0.11 Generation and validation of TET1 clones. A. Representative pictures of MDA MB231 before and after selection with puromycin. **B&C.** qRT-PCR on mRNA extracted from either MDA MB231 infected with *TET1* (TET1-PSF-lenti) or MDA MB231 infected with empty vector (PSF-lenti) using either *TET1* specific primers (**B**) or primer pair where the forward primer targets HA-tag while the reverse primer targets *TET1* (**C**). Results are shown after normalization to the level of the housekeeping gene *hUBC* and relative to mRNA levels in MDA MB231 control cells infected with empty vector. Bars represent SEM. * indicates that p-value is <0.05.

3.8. Effect of TET1 overexpression on MDA MB231 cell phenotypes

Throughout their development and progression, cancer cells acquire common phenotypic characteristics named cancer hallmarks (Hanahan & Weinberg, 2011). In order to learn about how TET1 affects these hallmarks, we tested the effect of TET1 overexpression on different hallmarks including cell proliferation,

migration, survival and growth in 3D culture. To test TET1 overexpression effect on cell proliferation, we compared the growth rate of TET1 overexpressing cells to the growth rate of control cells using both cell count and xtt assays. In both assays, TET1 overexpression inhibited cell proliferation by about 35% compared to control cells (Fig. 3.12 A&B). Another cancer cell hallmark that we tested is cell migration. To evaluate TET1 overexpression on cell migration, we performed wound healing assay. As it appears in Fig. 3.12 C, TET1 overexpression inhibited cell migration capacity by about 20 % in comparison to control cells.

Cell autonomy and survival independence on cell-cell communication is a hallmark that characterizes cancer cell growth. To elucidate the effect of TET1 manipulation on cell survival, we did cell survival assay by culturing a few number of cells over a big surface area. In comparison to control cells, TET1 manipulated cells showed a lower cell survival index. The survival index was lowered by approximately 50% (Fig. 3.12 D).

Disruption of cellular morphogenesis and organization is a phenotype that is related to transformation and carcinogenesis. To evaluate whether TET1 has an effect on this phenotype, we performed 3D mammosphere formation assay. We cultured both TET1-overexpressing and control cells in Matrigel 3D culture setup. In this assay, TET1 overexpression appeared to have no effect on oncogenic disrupted 3D growth mode of MDA MB231 (Fig 3.12 E). Altogether, phenotypic characterization of TET1-overexpressing cells revealed that at least the full length of TET1 is a proliferation, survival and migration inhibitor in the breast cancer cell line MDA MB231. While our results here are in concordance with previous findings which revealed TET1 is a tumor suppressor gene (Wu et al., 2019; Yang et al., 2015), these results are in contrast with other published data that defined TET1 as an oncogene (Good et al., 2018; Huang et al., 2013). TET1 was shown to inhibit breast cancer cell migration (Hsu et al., 2012), while in the contrary TET1 expression was shown to correlate with more aggressive breast cancer and bad prognosis (Good et al., 2018).

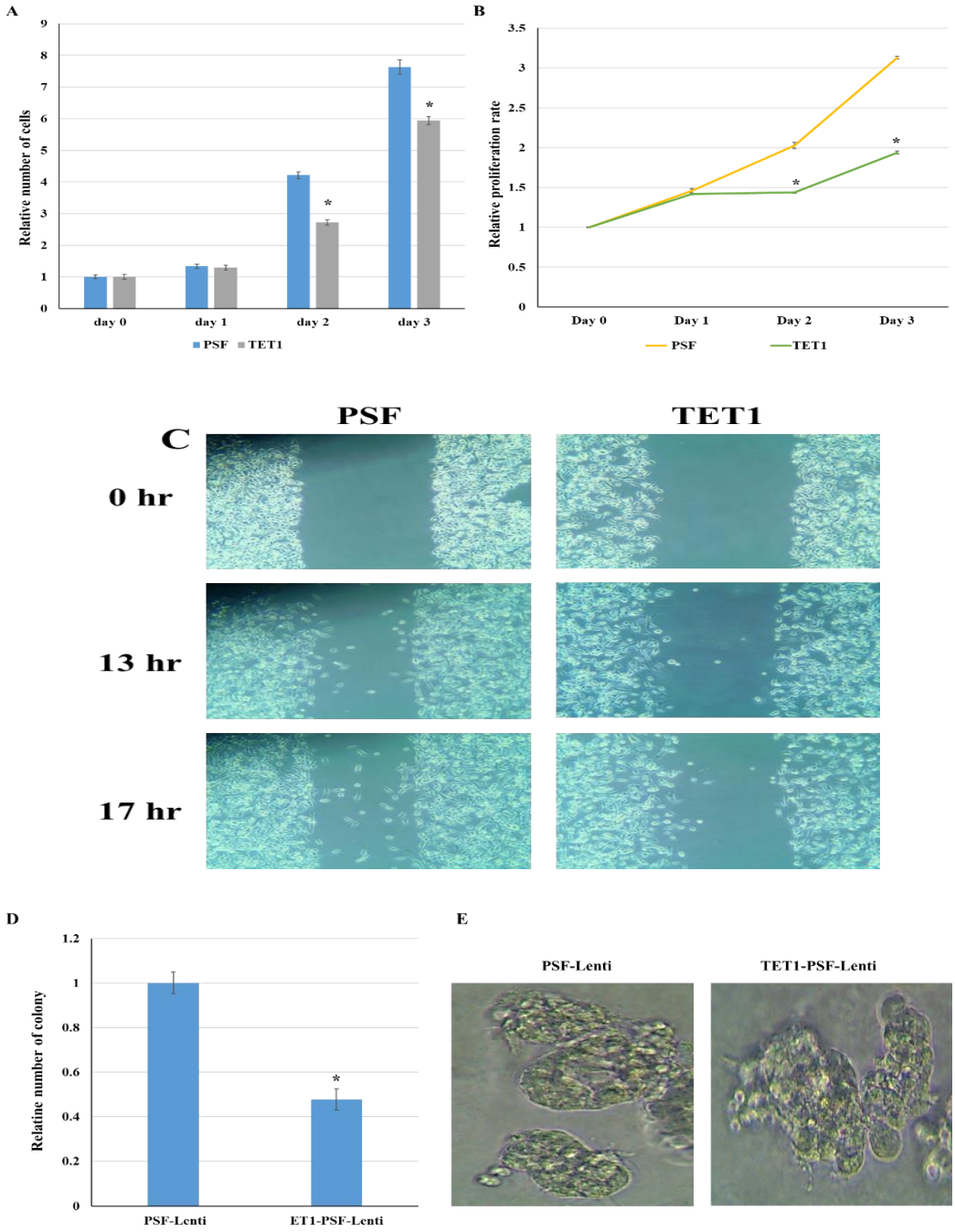

Figure 0.12 Effect of TET1 overexpression on MDA MB231 cell phenotypes. Representative graph showing the relative proliferation rate of TET1 overexpressing compared to control cells using cell count assay (**A**) and XTT assay (**B**). Cell growth was monitored over three days. **C.** Representative images showing the migration capability of TET1 overexpressing cells in comparison to control cells using wound healing assay. Cell growth was monitored after 13&17 hrs. **D.** Representative statistical analysis of the survival rate of TET1 overexpressing cells in comparison to control cells using cell survival assay. **E.** Representative images showing 3D colony organization on day 8 of TET1 overexpressing cells in comparison to control cells using 3D culture assay. The mammosphere formation was monitored for over about 2 weeks. All experiments were done in triplicates. Bars represent SEM. * indicates that p-value is <0.05. PSF: PSF-Lentiviral, TET1: TET1-PSF-Lentiviral

3.9. Effect of *TET1* overexpression on other genes like Oncogenes and TSG

In order to try to explain the phenotypes related to TET1 overexpression at the molecular level, we tested the effect of TET1 manipulation on the expression level of different genes that are linked to the tested cancer hallmarks. We tested the expression of both oncogenes and tumor suppressor genes in MDA MB231 cells infected with either empty vector or TET1 lentiviral vector. The list of the tested genes included *AKT 1, Cyclin B1, IDH1, Nanog, PCDH7, SLIT2, Snail1,* and *Wnt-5A*. As expected, some genes didn't show any significant change in gene expression, while the expression of others was either induced or reduced upon TET1 overexpression. Interestingly, genes behaved as expected in correlation with TET1 suppressive function, ie; tumor suppressor genes either didn't change (*PCDH7*) or were induced (*SLIT2*) (Fig.3.11), while oncogenes were repressed (*IDH1*, *Cyclin B1*, *Nanog*, *AKT1*) (Fig. 3.11). SLIT2 is a SLIT protein family member that was shown to act as a tumor suppressor gene in different types of cancers (Dallol et al., 2002; Dinesh K. Ahirwar, 2016; Mohamed et al., 2019; Yuan et al., 2016). In breast cancer SLIT2 was shown to suppress breast carcinogenesis and progression by attenuating β- catenin signaling pathway (Prasad, et al, 2008). On the other hand, *IDH1*, *Cyclin B1*, *Nanog*, *AKT1* are genes that are known to be involved in the pathogenesis of different types of cancers in general and breast cancer in specific (Bergaggio & Piva, 2019; Dai et al., 2017; Jeter, et al, 2015; X. Sun et al., 2017; G. Zhang, et al, 2016). For example, IDH1 activating mutations were shown to increase serum level of the oncometabolite 2-hydroxygluterate in breast cancer patients (Fathi et al., 2014). Cyclin B1 is a cell cycle regulator that is overexpressed in many types of cancer (Dong, et al, 2002; Yasuda et al., 2002). It was shown to be associated with poor breast cancer prognosis (Aaltonen et al., 2009). Nanog is a pluripotency factor that was found also to be involved in breast carcinogenesis. It is overexpressed in breast cancer samples (Nagata et al., 2014) and promotes breast cancer tumorigenesis, invasion and metastasis (Lu, et al, 2014). AKT also acts as an oncogene in breast cancer. While activating mutations are detected in breast cancer samples (Dunlap et al., 2010), wt AKT increased activity was shown in different studies to correlate with breast cancer development and progression (Choi et al., 2019; Hinz & Jucker, 2019). Taken together, the gene expression

profile after FL-TET1 overexpression in MDA MB231 breast cancer cells prove that FL-TET1 acts a tumor suppressor gene.

Overall, in the current research, we proved that at least FL-TET1 is a tumor suppressor gene, a finding which is in agreement with different previous studies that demonstrated that TET1 inhibits breast cancer cell growth and metastasis (Sang et al., 2015; Yu et al., 2019). However, our results are in disconcordance with other studies that proved that TET1 is an oncogene (Good et al., 2017; Good et al., 2018). One explanation to this discrepancy could stem from the fact that *TET1* has different isoforms, and that each isoform can behave in a different manner. For example, in the studies that showed TET1 is an oncogene, the short TET1 isoform was the studied isoform. The dual TET1 function in cancer that stems from the presence of different isoforms is not unique only to TET1. Different known cancer genes were also found to behave in a manner similar to *TET1* gene. For example, *p63* different isoforms behave sometimes in even an antagonistic manner. While, for example, the full length TAp63 acts usually as a tumor suppressor gene in specific tumor types (Mitani et al., 2011), the N-terminus truncated shorter isoform of p63 (ΔNp63) acts as an oncogene in the same types of cancer (Mitani et al., 2011). Although this simple explanation can solve the issue of functional discrepancy related to *TET1* gene in breast cancer, this explanation can be oversimplifying things. For instance, our study showed that the expression pattern of *TET1* in different breast cancer samples is different, not only on the quantity level, but also on the distribution level. Moreover, our results demonstrate that the expression pattern of the different isoforms is different between different cell lines. Moreover, while our results here show that all *TET1* isoforms are totally deleted in one breast cancer animal model (basal breast cancer model), they show that we have a switch in *TET1* isoform in another breast cancer mouse model (polioma virus luminal model). These findings and others may indicate that *TET1* gene regulation, pattern of expression as well as its role in breast tumorigenesis are more complicated and need further and deeper analysis.

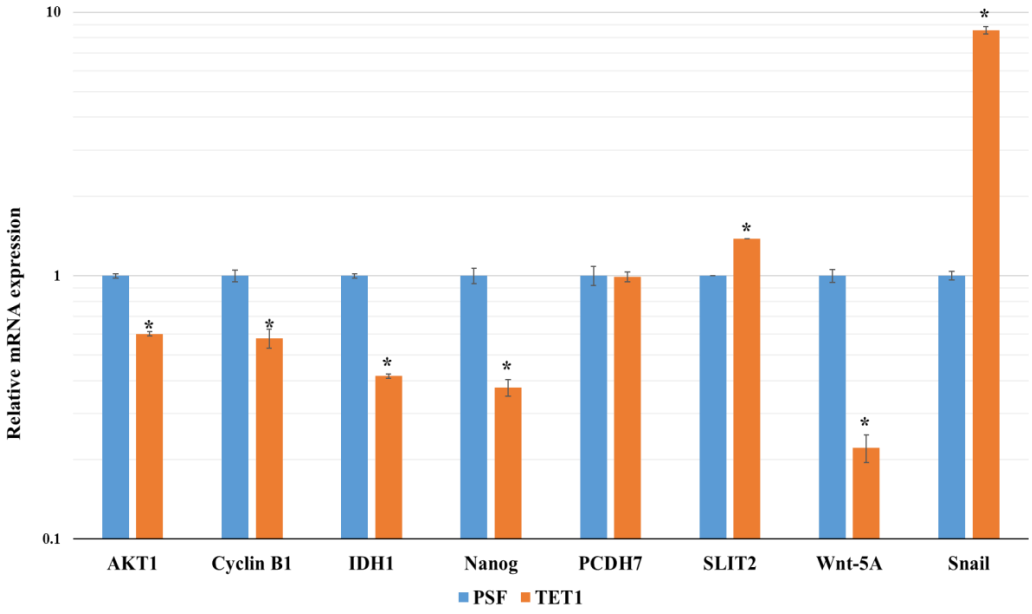

Figure 0.13 Effect of *TET1* overexpression on other genes like Oncogenes and TSGs. Relative qRT-PCR results showing the expression level of different TET1 target genes in MDA MB231 cell line. mRNA levels are shown after normalization to the level of the housekeeping gene *hUBC* and relative to mRNA level of each gene in MDA MB231 infected with EV. Bars represent SEM. * indicates that p-value is <0.05. PSF: PSF-Lentiviral, TET1: TET1-PSF-Lentiviral.

4. Conclusions and Future Directions

Previous studies have shown different conclusions about TET1 role in breast cancer. In these studies, researchers did not study the function of different TET1 isoforms which might have led to controversial conclusions about TET1 functions and breast tumorigenesis. In this study, we clearly demonstrated that elucidating TET1 function in breast cancer is complicated by the fact that TET1 has different isoforms that have different distribution and expression pattern in different cell contexts. This fact insures the need for future research that dissects the different functions of different TET1 isoforms. This can be achieved by either the overexpression of distinct and specific isoform in immortalized non transformed mammary gland cells or by knocking out the specific TET1 isoforms using for example the CRISPR-Cas technology.

5. References

1. Aaltonen, K., Amini, R. M., Heikkila, P., Aittomaki, K., Tamminen, A., Nevanlinna, H., & Blomqvist, C. (2009). High cyclin B1 expression is associated with poor survival in breast cancer. Br J Cancer, 100(7), 1055-1060. doi:10.1038/sj.bjc.6604874

2. Abdel-Wahab, O., Mullally, A., Hedvat, C., Garcia-Manero, G., Patel, J., Wadleigh, M., Levine, R. L. (2009). Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood, 114(1), 144-147. doi:10.1182/blood-2009-03-210039

3. Almeida, L. O., Neto, M. P. C., Sousa, L. O., Tannous, M. A., Curti, C., & Leopoldino, A. M. (2017). SET oncoprotein accumulation regulates transcription through DNA demethylation and histone hypoacetylation. Oncotarget, 8(16), 26802-26818. doi:10.18632/oncotarget.15818

4. An, J., Gonzalez-Avalos, E., Chawla, A., Jeong, M., Lopez-Moyado, I. F., Li, W., Rao, A. (2015). Acute loss of TET function results in aggressive myeloid cancer in mice. Nat Commun, 6, 10071. doi:10.1038/ncomms10071

5. Baba, Y., Watanabe, M., & Baba, H. (2013). Review of the alterations in DNA methylation in esophageal squamous cell carcinoma. Surg Today, 43(12), 1355-1364. doi:10.1007/s00595-012-0451-y

6. Bansal, A., & Pinney, S. E. (2017). DNA methylation and its role in the pathogenesis of diabetes. Pediatr Diabetes, 18(3), 167-177. doi:10.1111/pedi.12521

7. Bergaggio, E., & Piva, R. (2019). Wild-Type IDH Enzymes as Actionable Targets for Cancer Therapy. Cancers (Basel), 11(4). doi:10.3390/cancers11040563

8. Brien, G. L., Valerio, D. G., & Armstrong, S. A. (2016). Exploiting the Epigenome to Control Cancer-Promoting Gene-Expression Programs. Cancer Cell, 29(4), 464-476. doi:10.1016/j.ccell.2016.03.007

9. Choi, E., Kim, E., Kim, J. H., Yoon, K., Kim, S., Lee, J., & Cho, J. Y. (2019). AKT1-targeted proapoptotic activity of compound K in human breast cancer cells. J Ginseng Res, 43(4), 692-698. doi:10.1016/j.jgr.2019.07.001

10. Ciesielski, P., Jozwiak, P., Wojcik-Krowiranda, K., Forma, E., Cwonda, L., Szczepaniec, S., Krzeslak, A. (2017). Differential expression of ten-eleven translocation genes in endometrial cancers. Tumour Biol, 39(3), 1010428317695017. doi:10.1177/1010428317695017

11. Coey, C. T., Malik, S. S., Pidugu, L. S., Varney, K. M., Pozharski, E., & Drohat, A. C. (2016). Structural basis of damage recognition by thymine DNA glycosylase: Key roles for N-terminal residues. Nucleic Acids Res, 44(21), 10248-10258. doi:10.1093/nar/gkw768

12. Collignon, E., Canale, A., Al Wardi, C., Bizet, M., Calonne, E., Dedeurwaerder, S., Fuks, F. (2018). Immunity drives TET1 regulation in cancer through NF-kappaB. Sci Adv, 4(6), eaap7309. doi:10.1126/sciadv.aap7309

13. Dai, F., Yu, W., Song, J., Li, Q., Wang, C., & Xie, S. (2017). Extracellular polyamines-induced proliferation and migration of cancer cells by ODC, SSAT, and Akt1-mediated pathway. Anticancer Drugs, 28(4), 457-464. doi:10.1097/CAD.0000000000000465

14. Dallol, A., Da Silva, N. F., Viacava, P., Minna, J. D., Bieche, I., Maher, E. R., & Latif, F. (2002). SLIT2, a human homologue of the Drosophila Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers. Cancer Res, 62(20), 5874-5880.

15. Daud Faran Asif, N. M. a. U. R. (2017). DNA Methylation in Cancer Tissues. Cell Science & Therapy, 8(3), 2157-7013.

16. 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

17. Dinesh K. Ahirwar, M. W. N., Mohamad Elbaz, Kontestine Shilo and Ramesh Ganju. (2016). Slit2 inhibits breast cancer growth and metastasis by modulating tumor microenvironment. In American Association for Cancer Research (Vol. 76, pp. 1538-7445).

18. Dong, Y., Sui, L., Watanabe, Y., Sugimoto, K., & Tokuda, M. (2002). Clinical relevance of cyclin B1 overexpression in laryngeal squamous cell carcinoma. Cancer Lett, 177(1), 13-19. doi:10.1016/s0304-3835(01)00770-4

19. Dunlap, J., Le, C., Shukla, A., Patterson, J., Presnell, A., Heinrich, M. C., Troxell, M. L. (2010). Phosphatidylinositol-3-kinase and AKT1 mutations occur early in breast carcinoma. Breast Cancer Res Treat, 120(2), 409-418. doi:10.1007/s10549-009-0406-1

20. Ehrlich, M. (2009). DNA hypomethylation in cancer cells. Epigenomics, 1(2), 239-259. doi:10.2217/epi.09.33

21. Eliyatkin, N., Yalcin, E., Zengel, B., Aktas, S., & Vardar, E. (2015). Molecular Classification of Breast Carcinoma: From Traditional, Old-Fashioned Way to A New Age, and A New Way. J Breast Health, 11(2), 59-66. doi:10.5152/tjbh.2015.1669

22. Fathi, A. T., Sadrzadeh, H., Comander, A. H., Higgins, M. J., Bardia, A., Perry, A., Borger, D. R. (2014). Isocitrate dehydrogenase 1 (IDH1) mutation in breast adenocarcinoma is associated with elevated levels of serum and urine 2-hydroxyglutarate. Oncologist, 19(6), 602-607. doi:10.1634/theoncologist.2013-0417

23. 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

24. Good, C. R., Panjarian, S., Kelly, A. D., Madzo, J., Patel, B., Jelinek, J., & Issa, J. J. (2018). TET1-Mediated Hypomethylation Activates Oncogenic Signaling in Triple-Negative Breast Cancer. Cancer Res, 78(15), 4126-4137. doi:10.1158/0008-5472.CAN-17-2082

25. 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

26. 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

27. Hinz, N., & Jucker, M. (2019). Distinct functions of AKT isoforms in breast cancer: a comprehensive review. Cell Commun Signal, 17(1), 154. doi:10.1186/s12964-019-0450-3

28. Hsu, C. H., Peng, K. L., Kang, M. L., Chen, Y. R., Yang, Y. C., Tsai, C. H., Juan, L. J. (2012). TET1 suppresses cancer invasion by activating the tissue inhibitors of metalloproteinases. Cell Rep, 2(3), 568-579. doi:10.1016/j.celrep.2012.08.030

29. Huang, H., Jiang, X., Li, Z., Li, Y., Song, C. X., He, C., Chen, J. (2013). TET1 plays an essential oncogenic role in MLL-rearranged leukemia. Proc Natl Acad Sci U S A, 110(29), 11994-11999. doi:10.1073/pnas.1310656110

30. Inoue, A., Shen, L., Matoba, S., & Zhang, Y. (2015). Haploinsufficiency, but not defective paternal 5mC oxidation, accounts for the developmental defects of maternal Tet3 knockouts. Cell Rep, 10(4), 463-470. doi:10.1016/j.celrep.2014.12.049

31. Ito, S., D'Alessio, A. C., Taranova, O. V., Hong, K., Sowers, L. C., & Zhang, Y. (2010). Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature, 466(7310), 1129-1133. doi:10.1038/nature09303

32. Iyer, L. M., Tahiliani, M., Rao, A., & Aravind, L. (2009). Prediction of novel families of enzymes involved in oxidative and other complex modifications of bases in nucleic acids. Cell Cycle, 8(11), 1698-1710. doi:10.4161/cc.8.11.8580

33. Jeter, C. R., Yang, T., Wang, J., Chao, H. P., & Tang, D. G. (2015). Concise Review: NANOG in Cancer Stem Cells and Tumor Development: An Update and Outstanding Questions. Stem Cells, 33(8), 2381-2390. doi:10.1002/stem.2007

34. 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

35. Jones, P. A., & Baylin, S. B. (2007). The epigenomics of cancer. Cell, 128(4), 683-692. doi:10.1016/j.cell.2007.01.029

36. 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

37. 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

38. 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

39. 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

40. Lu, X., Mazur, S. J., Lin, T., Appella, E., & Xu, Y. (2014). The pluripotency factor nanog promotes breast cancer tumorigenesis and metastasis. Oncogene, 33(20), 2655-2664. doi:10.1038/onc.2013.209

41. Ma, L., Qi, T., Wang, S., Hao, M., Sakhawat, A., Liang, T., Huang, Y. (2019). Tet methylcytosine dioxygenase 1 promotes hypoxic gene induction and cell migration in colon cancer. J Cell Physiol, 234(5), 6286-6297. doi:10.1002/jcp.27359

42. Mitani, Y., Li, J., Weber, R. S., Lippman, S. L., Flores, E. R., Caulin, C., & El-Naggar, A. K. (2011). Expression and regulation of the DeltaN and TAp63 isoforms in salivary gland tumorigenesis clinical and experimental findings. Am J Pathol, 179(1), 391-399. doi:10.1016/j.ajpath.2011.03.037

43. Mohamed, G., Talima, S., Li, L., Wei, W., Rudzki, Z., Allam, R. M., Murray, P. G. (2019). Low Expression and Promoter Hypermethylation of the Tumour Suppressor SLIT2, are Associated with Adverse Patient Outcomes in Diffuse Large B Cell Lymphoma. Pathol Oncol Res, 25(3), 1223-1231. doi:10.1007/s12253-019-00600-9

44. Mortality, G. B. D., & Causes of Death, C. (2016). Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet, 388(10053), 1459-1544. doi:10.1016/S0140-6736(16)31012-1

45. Nagata, T., Shimada, Y., Sekine, S., Hori, R., Matsui, K., Okumura, T., Tsukada, K. (2014). Prognostic significance of NANOG and KLF4 for breast cancer. Breast Cancer, 21(1), 96-101. doi:10.1007/s12282-012-0357-y

46. Pastor, W. A., Aravind, L., & Rao, A. (2013). TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. Nat Rev Mol Cell Biol, 14(6), 341-356. doi:10.1038/nrm3589

47. Pei, Y. F., Lei, Y., & Liu, X. Q. (2016). MiR-29a promotes cell proliferation and EMT in breast cancer by targeting ten eleven translocation 1. Biochim Biophys Acta, 1862(11), 2177-2185. doi:10.1016/j.bbadis.2016.08.014

48. Pfeifer, G. P. (2018). Defining Driver DNA Methylation Changes in Human Cancer. Int J Mol Sci, 19(4). doi:10.3390/ijms19041166

49. Piccolo, F. M., & Fisher, A. G. (2014). Getting rid of DNA methylation. Trends Cell Biol, 24(2), 136-143. doi:10.1016/j.tcb.2013.09.001

50. Prasad, A., Paruchuri, V., Preet, A., Latif, F., & Ganju, R. K. (2008). Slit-2 induces a tumor-suppressive effect by regulating beta-catenin in breast cancer cells. J Biol Chem, 283(39), 26624-26633. doi:10.1074/jbc.M800679200

51. 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

52. Robertson, K. D. (2005). DNA methylation and human disease. Nat Rev Genet, 6(8), 597-610. doi:10.1038/nrg1655

53. Romagnolo, D. F., Daniels, K. D., Grunwald, J. T., Ramos, S. A., Propper, C. R., & Selmin, O. I. (2016). Epigenetics of breast cancer: Modifying role of environmental and bioactive food compounds. Mol Nutr Food Res, 60(6), 1310-1329. doi:10.1002/mnfr.201501063

54. Sang, Y., Cheng, C., Tang, X. F., Zhang, M. F., & Lv, X. B. (2015). Hypermethylation of TET1 promoter is a new diagnosic marker for breast cancer metastasis. Asian Pac J Cancer Prev, 16(3), 1197-1200. doi:10.7314/apjcp.2015.16.3.1197

55. 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

56. Siyi Z, X. Y. a. W. W. (2019). Regulation Activity of Ten-Eleven Translocation Family in Solid Cancers. Journal of Molecular and Genetic Medicine, 13(1): 405. doi:10.4172

57. Song, S. J., Poliseno, L., Song, M. S., Ala, U., Webster, K., Ng, C., Pandolfi, P. P. (2013). MicroRNA-antagonism regulates breast cancer stemness and

metastasis via TET-family-dependent chromatin remodeling. Cell, 154(2), 311-324. doi:10.1016/j.cell.2013.06.026

58. Sun, W., Guan, M., & Li, X. (2014). 5-hydroxymethylcytosine-mediated DNA demethylation in stem cells and development. Stem Cells Dev, 23(9), 923-930. doi:10.1089/scd.2013.0428

59. Sun, X., Zhangyuan, G., Shi, L., Wang, Y., Sun, B., & Ding, Q. (2017). Prognostic and clinicopathological significance of cyclin B expression in patients with breast cancer: A meta-analysis. Medicine (Baltimore), 96(19), e6860. doi:10.1097/MD.0000000000006860

60. Tracey A. Martin, L. Y., Andrew J. Sanders, Jane Lane, and Wen G. Jiang. (2013). Cancer Invasion and Metastasis: Molecular and Cellular Perspective. Landes Bioscience.

61. 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

62. 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

63. Williams, K., Christensen, J., Pedersen, M. T., Johansen, J. V., Cloos, P. A., Rappsilber, J., & Helin, K. (2011). TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature, 473(7347), 343-348. doi:10.1038/nature10066

64. Wu, H., D'Alessio, A. C., Ito, S., Xia, K., Wang, Z., Cui, K., Zhang, Y. (2011). Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. Nature, 473(7347), 389-393. doi:10.1038/nature09934

65. Wu, H., & Zhang, Y. (2011). Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. Genes Dev, 25(23), 2436-2452. doi:10.1101/gad.179184.111

66. Wu, H., & Zhang, Y. (2014). Reversing DNA methylation: mechanisms, genomics, and biological functions. Cell, 156(1-2), 45-68. doi:10.1016/j.cell.2013.12.019

67. Wu, J., Li, H., Shi, M., Zhu, Y., Ma, Y., Zhong, Y., Peng, C. (2019). TET1-mediated DNA hydroxymethylation activates inhibitors of the Wnt/beta-catenin signaling pathway to suppress EMT in pancreatic tumor cells. J Exp Clin Cancer Res, 38(1), 348. doi:10.1186/s13046-019-1334-5

68. Wu, M. Z., Chen, S. F., Nieh, S., Benner, C., Ger, L. P., Jan, C. I., Izpisua 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

69. Xiao, M., Yang, H., Xu, W., Ma, S., Lin, H., Zhu, H., Guan, K. L. (2012). Inhibition of alpha-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. Genes Dev, 26(12), 1326-1338. doi:10.1101/gad.191056.112

70. Xu, Y., Xu, C., Kato, A., Tempel, W., Abreu, J. G., Bian, C., Shi, Y. G. (2012). Tet3 CXXC domain and dioxygenase activity cooperatively regulate key genes for Xenopus eye and neural development. Cell, 151(6), 1200-1213. doi:10.1016/j.cell.2012.11.014

71. 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

72. 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

73. Yasuda, M., Takesue, F., Inutsuka, S., Honda, M., Nozoe, T., & Korenaga, D. (2002). Overexpression of cyclin B1 in gastric cancer and its clinicopathological significance: an immunohistological study. J Cancer Res Clin Oncol, 128(8), 412-416. doi:10.1007/s00432-002-0359-9

74. 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

75. Yu, Y., Qi, J., Xiong, J., Jiang, L., Cui, D., He, J., Tao, R. (2019). Epigenetic Co-Deregulation of EZH2/TET1 is a Senescence-Countering, Actionable Vulnerability in Triple-Negative Breast Cancer. Theranostics, 9(3), 761-777. doi:10.7150/thno.29520

76. Yuan, M., Guo, H., Li, J., Sui, C., Qin, Y., Wang, J.,Ye, J. (2016). Slit2 and Robo1 induce opposing effects on metastasis of hepatocellular carcinoma Sk-hep-1 cells. Int J Oncol, 49(1), 305-315. doi:10.3892/ijo.2016.3506

77. Zhang, G., Liu, Z., Xu, H., & Yang, Q. (2016). miR-409-3p suppresses breast cancer cell growth and invasion by targeting Akt1. Biochem Biophys Res Commun, 469(2), 189-195. doi:10.1016/j.bbrc.2015.11.099

78. Zhang, H., Zhang, X., Clark, E., Mulcahey, M., Huang, S., & Shi, Y. G. (2010). TET1 is a DNA-binding protein that modulates DNA methylation and gene transcription via hydroxylation of 5-methylcytosine. Cell Res, 20(12), 1390-1393. doi:10.1038/cr.2010.156

79. 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

6. Appendices

Appendix 1.

Table 6-1: List of the designed primers for target genes

No	Gene	Primers	Reference
1	*AKT1*	F.P_5'- cacaaacgaggggagtacat -3' R.P-5'- tgcgccacagagaagttg-3'	NM_005163.2
2	*Cyclin B1*	F.P_5'- gtcaccaggaactcgaaaat -3' R.P-5'-ttaccaatgtccccaagagc-3'	NM_031966.3
3	*IDH 1*	F.P_5'- ctacatagctatgatttaggc -3' R.P-5'- ctcaaccctcttctcatcagg-3'	NM_001282386.1
4	*Nanog*	F.P_5'- acggagactgtctctcctct-3' R.P-5'- tttgcgacactcttctctgc-3'	NM_024865.3
5	*PCDH7*	F.P_5'-atggaaaatgattcaaggcctc-3' R.P_5'-aggctggctcttcttcctct-3'	NM_001173523.1
6	*SLIT2*	F.P_5'-gctatacaggcttgatctcagtg-3' R.P_5'-ctgaatgccccatcttcaat-3'	NM_004787.4
7	*Snail1*	F.P_5'- acactggcgagaagcccttt -3' R.P-5'- gcctggcactggtacttctt -3'	NM_005985.3
8	*Wnt5A*	F.P_5'- atgaagaagtccattggaat -3' R.P-5'- ctgggcgaaggagaaaaata -3'	NM_003392.4
9	*hUBC*	F.P 5'- gtcgcagttcttgtttgtgg-3' R.P 5'-gatggtgtcactgggctcaa-3'	NM_021009.6

Appendix 2

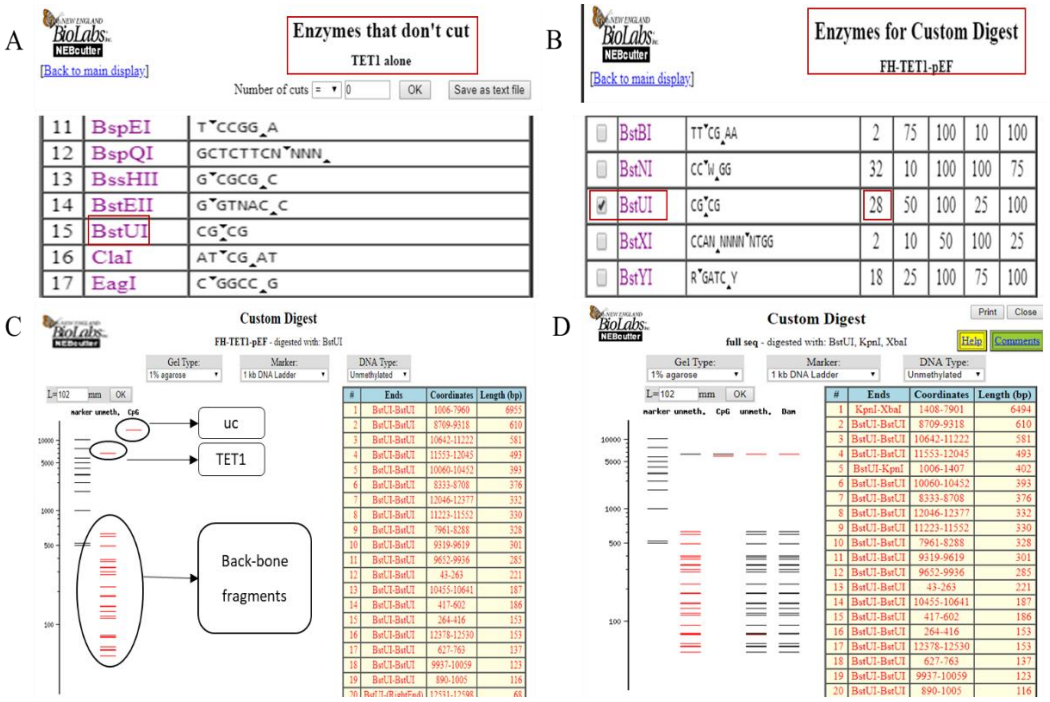

Figure 6.1 Bioinformatics and design of FH-TET1-pEF. A. Bioinformatics result from NEB cutter for HA-*TET1* mRNA coding sequence digested with BstUI enzyme. **B**. Bioinformatic result from NEB cutter for FH-TET1-pEF full sequence digested with BstUI enzyme. **C**. Bioinformatic gel picture for FH-TET1-pEF full sequence digested with BstUI enzyme. **D**. Bioinformatics gel picture for FH-TET1-pEF full sequence digested with triple enzymes KpnI, XbaI, and BstUI.

Appendix 3

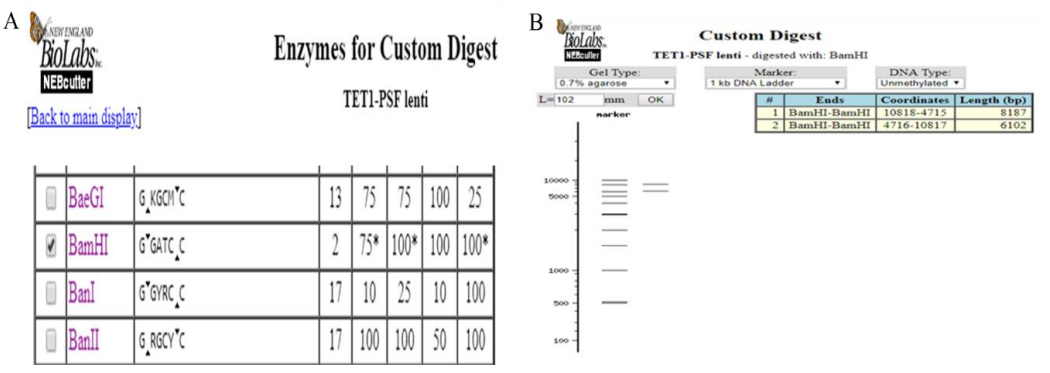

Figure 6.2 Digestion of TET1-PSF-Lenti with BamHI. A. Bioinformatic result from NEB cutter for TET1-PSF-Lenti digested with BamHI enzyme. **B**. Bioinformatic gel picture for TET1-PSF-Lenti digested with BamHI enzyme.

Appendix 4

Figure 6.3 sequencing of *TET1* cloned in PSF-Lenti viral plasmid. Sequence alignment of cloned *TET1* against reference sequence using Blast web-available software.

دور TET1 وأنماط انتاجه في سرطان الثدي

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اشراف : الدكتور زيدون صلاح

الملخص:

السرطان من الامراض التي يمكن ان تنتج عن اعتلال التعابيرالجينية (ترجمة الجينات الى بروتينات). حيث ان الطفرات في الجينات اوالتغيرات الفوق جينية مثل اضافة مجموعة الميثيل الى تسلسل القواعد النتيروجينية في المادة الوراثية والتي تؤدي إلى تغيير التعابير الجينية, وهي مثبته في جميع الدراسات التي تخص مرض السرطان ، ومن الممكن ان تكون مسؤولة عن السمات المشتركة لمرض السرطان. ان ارتباط مجموعة الميثيل في القواعد النيتروجينية للمادة الوراثية يحافظ عليها مجموعة انزيمات تعرف ب DNMTs, كما ان عملية ربط مجموعة الميثيل على المادة الوراثية هي عملية عكسية, لكن هذه الالية لم تكن واضحة حتى تم اكتشاف مجموعة من الانزيمات تعرف بعائلة TET. اظهرت بعض الدراسات ان الانزيم TET1 هو الاكثر انخفاضا في حالة الاصابة بمرض السرطان, وعليه يمكن ان تكون وظيفة هذا الانزيم تثبيط المرض, الا ان دراسات اخرى اظهرت ان هذا الانزيم يمكن ان ينشط مرض السرطان. الدراسات الحديثة اظهرت ان لهذا الانزيم اكثر من شكل وبالتالي يمكن ان ينتج اكثر من بروتين. واستنادا على هذا, فاننا نفترض ان وجود عدة اشكال من هذا الانزيم من الممكن ان يكون له عدة وظائف في مرض السرطان من خلال اختلاف تعابيره الجينية في الخلايا. من اجل اختبار هذه الفرضية, قمنا بفحص كمية ومواقع وجود هذا الانزيم في عينات سرطان الثدي باستخدام الصبغ المناعي, وكذلك فحص نسبة تواجدة في بعض انواع من خلايا سرطان الثدي المختلفة باستخدام تقنية qRT-PCR. علاوة على ذلك, تم فحص اثر اشكال هذا الانزيم باستخدام نماذج من خلايا سرطانية في المختبر وكذلك داخل جسم الفئران. كما تم ادخال هذا الانزيم على خلايا ثدي سرطانية من نوع MDA MB231 من اجل دراسة تاثيره على بعض السمات لمرض السرطان. اظهرت النتائج ان كمية انتاج هذا الانزيم تختلف بين الخلايا الطبيعية والسرطانية, وان لهذا الانزيم علاقة في انقسام وتمايز الخلايا. كما ان اشكال هذا الانزيم تختلف كمياتها باختلاف الظروف الفسيولوجية والمرضية, وان لها توزيع مختلف داخل الخلايا. وقد برهنت تجاربنا على ان التسلسل الكامل لهذا الانزيم يثبط من تكون وانتشار مرض السرطان اذا تم افرازة في خلايا

الثدي السرطانية من نوع MD MB231. وفي المحصلة, اظهرت نتائجنا ان وظيفة هذا الانزيم لا تنحصر باتجاه واحد, ولهذا هناك حاجة لدراسات تفصيلية لتساعد في فهم اعمق لوظيفة هذا الانزيم في تكون وتطور مرض سرطان الثدي.