ROLE OF TET2 IN LUMINAL DIFFERENTIATION AND HORMONE THERAPY RESPONSE IN BREAST CANCER

by

MI RAN KIM

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THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Chun Ju Chang, Co-Chair
Roswell Park Cancer Institute

Dr. Kari Ekenstedt, Co-Chair
Department of Basic Medical Sciences

Dr. Sophie A. Lelièvre
Department of Basic Medical Sciences

Dr. Sulma Mohammed
Department of Comparative Pathobiology

Approved by:

Dr. Laurie A. Jaeger

Head of the Department of Basic Medical Sciences

I would like to dedicate my dissertation to Dr. Chun-Ju Chang, who gave me the opportunity to follow my academic passion and help me to endure all the hard times. I could not have completed this without her support and encouragement. I dedicate this to my parents, Sung Tae Kim and In Suk Sim, and my sister, Kyoung Ae Kim who always trust me and support my decision with endless love. Finally, I dedicate this to my family, my husband, Song Kang, and my kids, Andrew, Brandon, and Catherine who understand why mom could not spend more time to play with them and support me to overcome the difficulties throughout this journey over the years.

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ABSTRACT

Epigenetic mechanisms, including DNA methylation, play an important role in regulation of stem cell fate and tumorigenesis. The Ten-Eleven-Translocation 2 (TET2) is a core enzyme for DNA demethylation by catalyzing the conversion of 5-methylcytosine (5mC) to 5-hydromethylcytosine (5hmC). It has been shown that TET2 is the main regulator of hematopoietic stem cell homeostasis and loss of TET2 is highly associated with hematopoietic malignancies. Our previous work has also shown that loss of TET2 expression is linked to promotion of an epithelial-mesenchymal-transition phenotype and expansion of a breast cancer stem cell-like population with skewed asymmetric cell division in vitro (1); however, the in vivo role that TET2 plays in regulation of mammary stem cell (MaSC) fate and development of mammary pathology has yet to be determined. Here, using our newly established mammary-specific Tet2-knockout mouse model, the data reveals for the first time that TET2 plays a pivotal role in mammary gland development via directing MaSC to luminal lineage commitment in vivo. Furthermore, we find that TET2 coordinates with FOXP1 to target and demethylate FOXA1, GATA3, and ESR1, key transcription factors that orchestrate mammary luminal lineage specification and endocrine response and are often silenced by DNA methylation in aggressive human breast cancers. Finally, loss of TET2 expression leads to promotion of mammary tumor development with defective luminal cell differentiation and tamoxifen resistance in a PyMT;Tet2 deletion breast cancer mouse model. As a result, this study provides a previously unidentified role for TET2 in governing luminal lineage specification and endocrine response that underlies resistance to anti-estrogen treatments.

CHAPTER 1. **INTRODUCTION**

1.1 Motivation

Epigenetic control plays a key role in regulation of stem cell fate and tumorigenesis. My research project is focused on determining the physiological and pathological roles of a critical epigenetic regulator, TET2, in regulation of mammary stem cell fate, mammary gland development, and tumor formation. Our recent work has shown that knock-out of *Tet2* in mouse mammary glands leads to disrupted mammary epithelial morphology and aberrant mammary stem cell (MaSC) fate determination with defective luminal differentiation, potentially contributing to an expansion of the MaSC population and development of mammary lesions with a basal-like phenotype (2). Since aberrant MaSC fate is often linked to tumorigenesis, our studies will not only reveal novel mechanisms by which stem cell fate is regulated but also provide new strategies for use in breast cancer treatment.

1.2 Background

The mammary gland is a remarkably dynamic organ that undergoes developmental and morphological changes after birth and through puberty, pregnancy, lactation, and involution. The mammary gland epithelium is a ductal system composed of two types of epithelial cells: the luminal epithelial cells lining mammary ducts and the basal/myoepithelial cells surrounding the luminal cells and separating mammary epithelium from extracellular matrix. MaSCs can self-renew and produce multipotent progenitors, which further differentiate into distinct cell lineages to generate the functional mammary epithelium. Luminal progenitor cells can differentiate into

ductal and alveolar cells, and basal/myoepithelial progenitor cells give rise to the myoepithelial cells during mammary gland development (3–5). The significant expansion of the mammary epithelium during puberty and pregnancy particularly reflects the regenerative capability of the MaSCs/progenitor cells. One of the important regulators to control stem cell fate (self-renewal vs differentiation) is the epigenetic mechanism, such as histone modification, DNA methylation, chromatin remodeling, and non-coding RNAs (6,7). DNA methylation is especially known to play an important role in regulation of cellular processes in stem cells, such as cell proliferation and differentiation by suppressing gene expression (6,8). Moreover, it has been reported that DNA methylation is the main regulator for maintaining cancer stem cell (CSC) properties in various cancer types (8–11). TET is the main enzyme for DNA demethylation by catalyzing the conversion of 5-methylcytosine (5mC) to 5-hydromethylcytosine (5hmC) and play a pivotal role in regulation of embryonic and adult stem cell homeostasis (12,13) TET family, TET1, TET2 and TET3 (Figure 1-1) (12) work in concert to control pluripotency and differentiation of embryonic stem cells and each of them can partially compensate the function of other TET protein (14–16). Particularly, TET2 is mainly expressed in the mammary tissue among TET proteins (The Human Protein Atlas), and its expression is often reduced by microRNAs in breast cancer, prostate cancer, and acute myeloid leukemia (12,17–19). In addition to post-transcriptional repression of TET2, genetic alteration of *TET2* is commonly observed in hematopoietic malignancies (20–22) and *TET2* loss of function variant is often detected in breast cancer (23). Previous studies have uncovered that TET2 is critical for maintenance of hematopoietic stem cell self-renewal, proliferation, and differentiation (24–26). It has been proposed that inactivation of TET2 is one of the first genetic alterations in the onset of tumor development, followed by oncogenic events that trigger hematological malignancies (12,27). It has been shown that TET2 expression can hinder breast

tumor progression by upregulating tumor suppressor genes that include miR-200c (17) and Caspase-4 (28). We have also demonstrated that TET2 is an essential epigenetic regulator that governs polarity of stem cell division and stem cell fate decision using in vitro and in vivo models (2).

1.3 Thesis goal and approach

The goal of this thesis is to elucidate a new role for the epigenetic modifier TET2 in the regulation of stem cell fate determination, mammary tumor development, and therapeutic response to breast cancer treatment. To achieve this goal, I have investigated the role of TET2 mediated epigenetic mechanisms involved with sensitivity to a cytodifferentiation agent, all-trans retinoic acid (ATRA), using breast cancer cell lines (1).

To further understand the role of TET2 in the regulation of mammary gland development and tumorigenesis in vivo, I have generated mammary specific *Tet2* knock-out mice and observed the morphological difference between knock-out mice and wild type mice. Knock-out mice were generated by site-specific recombinase technology, Cre-Lox recombination. Bacteriophage P1 Cre recombinase recognizes 34 base pair repeats called LoxP and catalyzes site-specific DNA recombination between two LoxP sites (Figure 1-2 A). This DNA recombination allows DNA modifications, such as insertion, deletion, translocation, and inversion, at the specific DNA sites. Also, it can be targeted in specific cell types by using cell type-specific promoters to drive the expression of the Cre recombinase. For example, MMTV (mouse mammary tumor virus) promoter has been routinely used to express Cre recombinase specifically in the mammary gland epithelial cells (Figure 1-2 B). Therefore, we bred $Tet2^{fl/fl}$ mice (the Jackson lab) with MMTV-Cre transgenic

mice (the Jackson lab) to generate mammary gland specific *Tet2* knock out mice. Then we observed the effects of TET2 ablation on whole mammary gland and mammary epithelial cells using whole mount staining and immunofluorescence staining with specific lineage markers: luminal cells (CK8, MUC1) and basal/myoepithelial cells (CK14, SMA). Realtime RT-PCR and Western blot analyses were used to determine expression of the genes and proteins involved in luminal cell differentiation and endocrine response.

The data has shown that TET2 indeed is an important epigenetic regulator that governs MaSC fate, particularly in luminal cell differentiation, by inducing demethylation of luminal differentiation-associated genes, such as *FOXA1*, *GATA3*, and *ESR1*, which encodes estrogen receptor (ER) α protein. ERα is an essential transcription factor (TF) for the mammary gland development (29). FOXA1 and GATA3 are well-known pioneer TFs that directly bind to condensed chromatin and initiate transcriptional events by opening chromatin to facilitate DNA binding of ERα (30). Moreover, the function of FOXA1 and GATA3 is not only limited in pioneer role to recruit ERα to promoter regions of the ERα target genes but also is critically related to determination of the ERα activity by interplaying with ERα and altering gene expression patterns in the mammary gland (31–35). Therefore, FOXA1, GATA3, and ESR1 are essential TFs for estrogen-related luminal differentiation and mammary development directing to mammary morphogenesis, estrogen-responsive growth, as well as luminal type breast cancer formation. Since it has known that these genes are often silenced by DNA methylation in basal-like breast cancers that are highly resistant to anti-estrogen treatments (36,37), we further investigated the function of TET2 in mammary tumor development and a therapeutic response to ERα targeting endocrine therapy using *Tet2* deletion breast cancer mouse models, which is generated by breeding

Tet2 deletion mouse with an established breast cancer Polyoma Middle T Oncoprotein (PyMT) mouse model (Figure 1-3). Together, this describes an important epigenetic mechanism involved in regulation of mammary stem cell homeostasis with significant implications in breast tumorigenesis and hormone therapy resistance.

1.4 Thesis layout

In Chapter 2, I summarize two articles, “Retinoic acid directs breast cancer cell state changes through regulation of TET2-PKCζ pathway”, published in Oncogene (2017) 36, 3193–3206 (1), and “Determine the roles of epigenetic regulators in mammary stem cell fate decision using genetically modified mouse models”, my Master Thesis deposited in 2017 (2). I have extended the investigation of TET2 in breast tumor development in vivo during my Ph.D. program. In Chapter 3, I present data demonstrating that genetic deletion of TET2 accelerates mammary tumor development and that loss of TET2 expression confers resistance in breast cancer cells to hormone therapy.

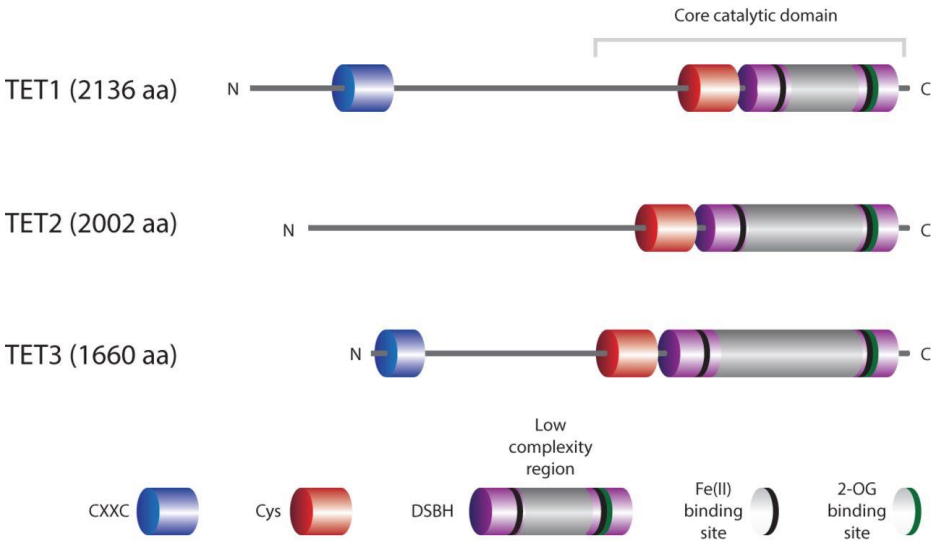


Figure 1-1. Protein structure of TET family proteins

The catalytic domain in C-terminal core is shared by all TET enzymes. however, TET2 does not have an N-terminal CXXC DNA binding domain.

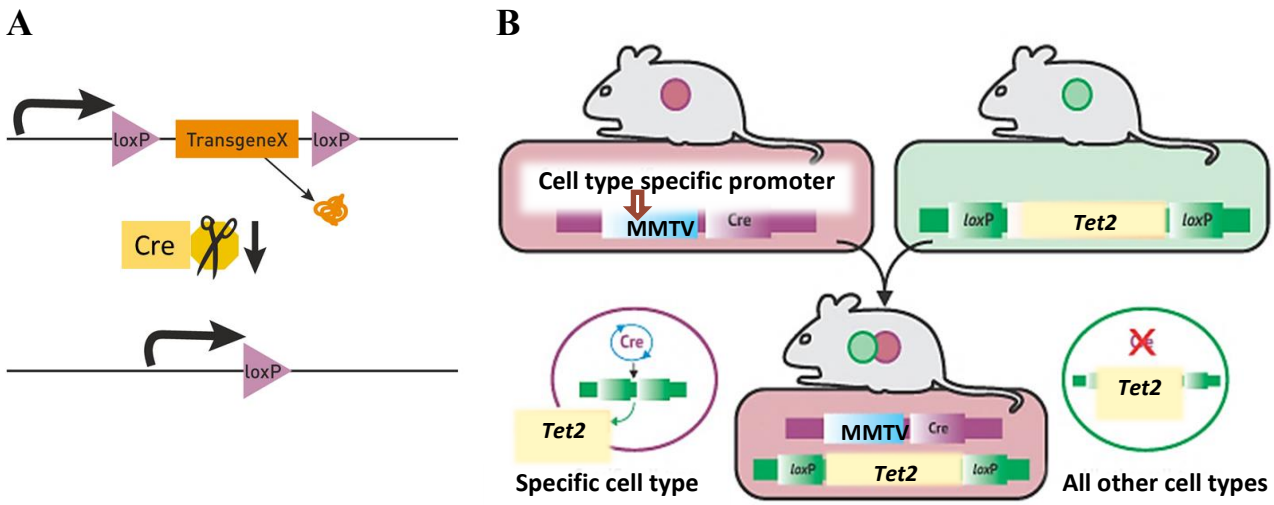


Figure 1-2. Generation of conditional *Tet2* knock-out mice using Cre-Lox recombination

(A) A schematic model of Cre function. (B) Use of Cre/Lox system to generate tissue-specific *Tet2* gene knock-out mice.

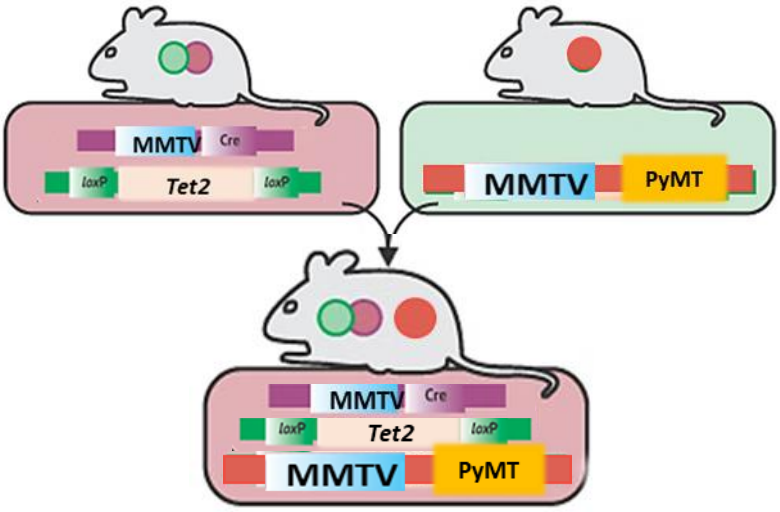


Figure 1-3. Generation of conditional *Tet2* deletion breast cancer mouse model

Tet2 deletion PyMT breast cancer mouse was generated by breeding the MMTV-PyMT breast cancer mouse with the MMTV-*Tet2* deletion mouse model.

CHAPTER 2. **RECENTRLY ESTABLISHED ROLE OF TET2 IN BREAST CANCER STEMNESS *IN VITRO***

2.1 Retinoic acid directs breast cancer cell state changes through regulation of TET2-PKCζ pathway

We have described the TET2 signaling pathway in controlling breast cancer stem cell fate determination responding to All-Trans Retinoic Acid (ATRA) treatment, which is one of the cancer therapeutic strategies to target cancer stem cells (38–40). The cancer stem cell is a small subset of cancer cells and has been thought to be responsible for cancer progression, relapse, and resistance to the treatment (41–43). ATRA is a cytodifferentiation agent and induces cancer stem cells into differentiation, allowing conventional cancer drugs to defeat the cancer cells (38). Even though ATRA treatment results in 90% complete remission in acute promyelocytic leukemia patients (44), many aggressive/relapsed breast cancer patients failed to gain a significant benefit from ATRA treatment (45). To reveal the mechanisms associated with ATRA, first, we found that ATRA induced interaction between TET2 and the retinoic acid receptor β (RARβ) and also facilitated nuclear localization of RARβ- TET2 complex. Genome-wide chromatin immunoprecipitation sequencing data showed that this complex binds to the promoter regions of the genes associated with the cell differentiation and non-coding RNAs, such as miR-200c, a tumor-suppressive microRNA. Therefore, recruitment of TET2 by RARβ promoted miR-200c activation along with the increased level of 5hmc on the miR-200c promoter. In response to ATRA treatment, activated miR-200c targeted and suppressed PKCζ, which is pivotal for mediating stem cell asymmetric division. Through the suppression of PKCζ, ATRA directed the stem cell fate to

the differentiation fate and decreased the cancer stem cell-like population in breast cancer cell lines (Figure 2-1).

We also determined that the RARβ-TET2-miR200c-PKCζ signaling pathway linked to the clinical phenotypes of breast cancer patients. Expressions of TET2 and miR-200c were overexpressed in normal, benign lesions and well-differentiated low-grade breast tumor, whereas PKCζ was overexpressed in aggressive high-grade breast tumor specimens. Together, the study reveals the important role of TET2 in directing breast cancer stem cell state in vitro (1).

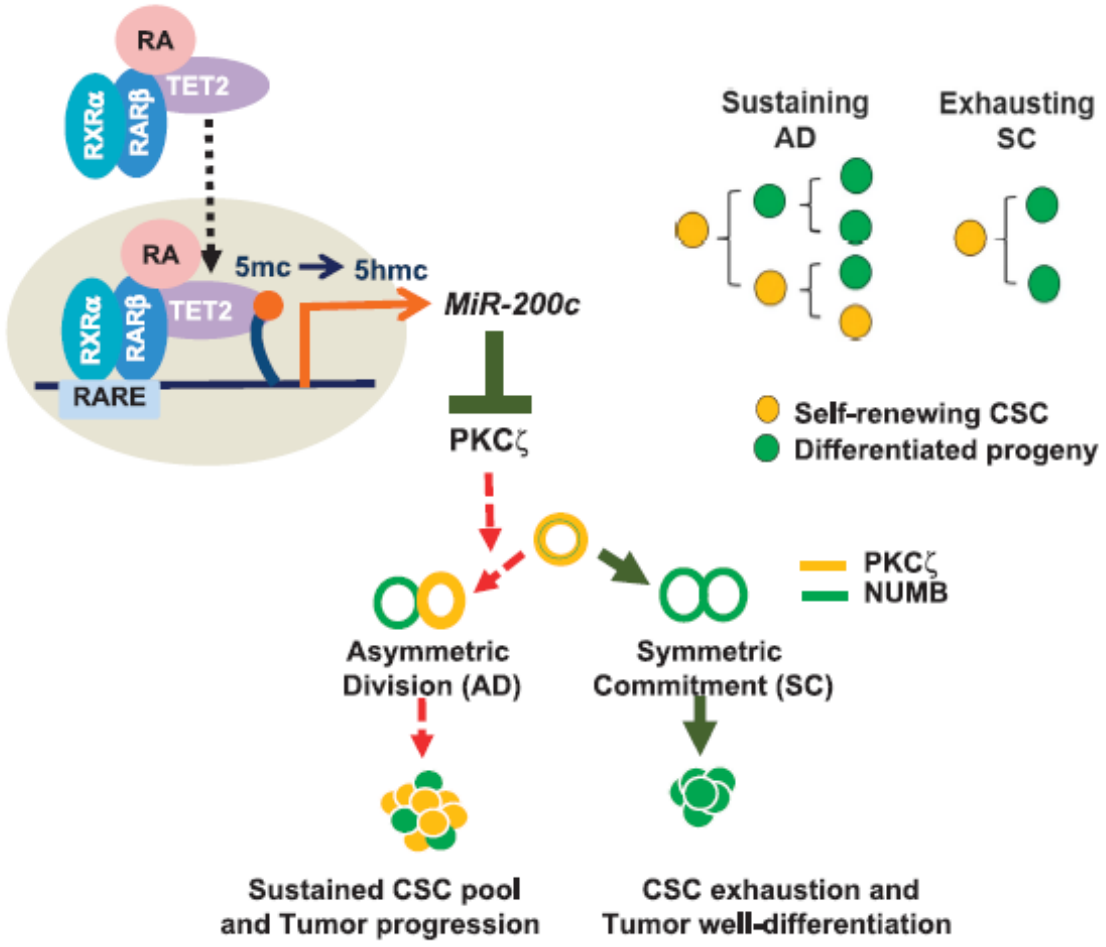


Figure 2-1 Retinoic acid directs breast cancer cell state changes through regulation of TET2-PKCζ pathway.

A proposed model illustrating how ATRA directs breast cancer stem cell state changes. Oncogene (2017) 36, 3193–3206.

2.2 Roles of TET2 in mammary stem cell fate using genetically modified mouse models

To elucidate the function of TET2 in mammary gland development, we generated *Tet2* knock out mice by genetically deleting *Tet2* in the mouse mammary gland (2). The *Tet2*$^{fl/fl}$ mouse, which has two insertions of LoxP sites flanking exon 3 of *Tet2* gene mated with MMTV-Cre transgenic mice, which expressed Cre recombinase mainly in mammary glands. We observed morphological differences between the mammary glands from Tet2 wild type (WT: *Tet2*$^{+/+}$; MMTV-Cre), and Tet2 knock out (KO: *Tet2*$^{fl/fl}$; MMTV-Cre) mice in three different developmental stages: puberty, pregnancy, and lactation, using these stains: whole mount stain, H&E, and trichrome stain. The *Tet2* KO mammary glands in puberty (7weeks) showed elongated ductal branches, enlarged terminal end buds and extensive side branching, as well as significant fibrosis near the ductal area (Figure 2-2 A). *Tet2* KO mammary glands during pregnancy and lactation display severe defects in lobuloalveolar development with decreased milk production compared to the wild type mammary gland (Figure 2-2 A). In addition to microscopic morphologic changes, *Tet2* deletion increases mammary stem cell populations (MaSC, $CD24^{+}CD29^{hi}$) but decreases luminal cell populations (Lum, $CD24^{+}CD29^{lo}$), especially the mature luminal cells (ML, $CD24^{+}CD29^{lo}CD61^{-}$) (Figure 2-2 B). Along with the changes in mammary epithelial cell subsets, loss of *Tet2* dysregulates the commitment of basal/myoepithelial cells (CK14 positive) and luminal cells (CK8 positive) in the mammary gland (Figure 2-2 C). Besides the cellular alterations, on the molecular level, the *Tet2* KO mammary gland down-regulates expression of luminal cell marker proteins (Figure 2-2 D). These results imply that TET2 plays a critical role in mammary gland development, especially in luminal cell differentiation.

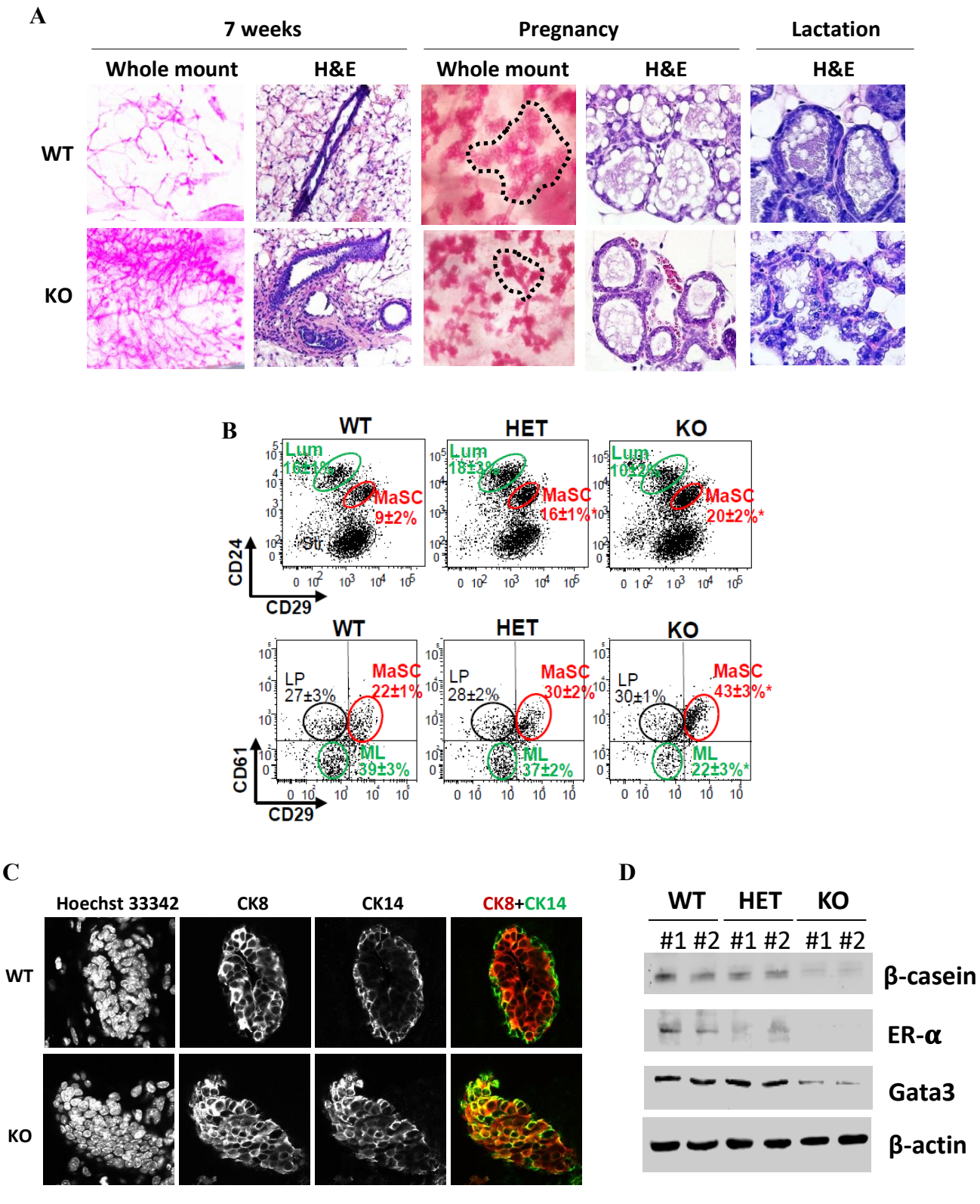


Figure 2-2. Role of TET2 in mammary gland development.

(A) Whole mount and H&E staining of mammary glands from WT and KO 7-week-old virgin, late pregnancy, and lactating female mice (Dotted area represents terminal alveoli). (B) Flow cytometry analysis showing the percentage of basal cell/mammary stem cell (MaSC, $Lin^-CD24^+CD29^{hi}$) and luminal cell (Lum, $Lin^-CD24^+CD29^{lo}$) populations (top panel) and the percentage of MaSC ($Lin^-CD24^+CD29^{hi}CD61^{hi}$), luminal progenitor cell (LP, $Lin^-CD24^+CD29^{lo}CD61^{hi}$), and mature luminal cell (ML, $Lin^-CD24^+CD29^{lo}CD61^{lo}$) populations (bottom panel). Str stands for stroma cells. (C) Immunofluorescence images of mammary glands from WT and KO- 7-week-old female mammary glands stained with CK8 and CK14. (D) The protein expression levels of β-casein, ERα, and GATA3 in mammary cells isolated from 7-week-old WT, HET and KO female mice.

CHAPTER 3. ROLE OF TET2 IN TUMOR DEVELOPMNET AND HORMONE THERAPY RESPONSE IN BREAST CANCER

3.1 Introduction

According to the American Cancer Society, "breast cancer is one of the most common cancers among women and the second leading cause of cancer death in the United States" (46). About 80% of breast cancers are estrogen receptor (ER) positive breast cancers according to Breastcancer.org (47). These cancers respond to hormonal therapies including selective ER modulators, selective ER down regulators, aromatase inhibitors, and luteinizing hormone-releasing hormone. Tamoxifen is one of the most widely used selective ER modulators, that competes with estradiol, most predominant estrogen, to bind to ERα (48). Tamoxifen and ER complex blocks expression of estrogen-regulated genes and inhibits estrogenic effects in breast cancer cells (48–50). Five years of adjuvant tamoxifen therapy for ER-positive breast cancer treatment reduces the annual breast cancer death rate by 31% and the absolute reduction in mortality of 9.2% at 15 years (51,52). Despite the benefit of endocrine therapy, 50% of ERα positive breast cancers eventually exhibit either *de novo* or acquired to hormonal therapy (53). Endocrine resistance causes adverse progression and tumor recurrence and it remains a significant therapeutic challenge (53). Although the mechanisms of endocrine resistance are unclear, it has been reported that epigenetic mechanisms play an important role in resistance to hormonal therapy (36). Therefore, it is essential to elucidate the mechanisms of how cancer cells gain resistance to hormonal therapeutic reagents to develop beneficial strategies as well as to discover a therapeutic target for breast cancer treatment.

We previously demonstrated that the loss of TET2 leads to down-regulation of ERα level in the mouse mammary gland (2). Since ERα is a therapeutic target for luminal type breast cancer, I hypothesized that loss of TET2 in breast cancer cells desensitizes them to selective ER antagonists, such as Tamoxifen, leading to treatment effects of hormonal therapy being diminished. To this end, we generated a *Tet2* KO breast cancer mouse model by breeding *Tet2* KO mice with PyMT breast cancer mice. The PyMT mouse model possesses morphological biomarker similarities to human breast cancer progression and metastasis (54,55). The PyMT mouse carries the Polyoma middle T oncoprotein that activates multiple oncogenic pathways associated with the Src family, Ras, and PI3 kinase (56,57). These pathways are also dysregulated in human breast cancer, in cases where rapid transformation of epithelial cells to malignant cells occurs (58,59). More than half of PyMT mice develop mammary lesions as early as 4 weeks of age (hyperplasia, adenoma), followed by early carcinoma at 8-12 weeks of age, and advanced carcinoma at 14 weeks of age (54). Unlike other breast cancer mouse models, premalignant and early stage mammary tumors in PyMT mouse express ER, recapitulating human luminal breast cancer (54). Therefore, by using a *Tet2* deletion PyMT breast cancer mouse model we can study an important epigenetic mechanism involved in regulation of breast tumorigenesis and hormone therapy resistance.

3.2 Material and Methods

3.2.1 Animals

The *Tet2* $^{fl/fl}$ mouse line (*Tet2*tm1.1Iaai, 017375), which possess a loxP site flanking exon 3 in the *Tet2* gene (26), the MMTV-Cre transgenic mouse line (Tg(MMTV-cre)4Mam/J, 003553), which expresses Cre recombinase under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter, and the MMTV-PyMT transgenic mouse line (FVB/N-

Tg(MMTV-PyVT)634Mul/J) were purchased from the Jackson Laboratory. *Tet2* $^{fl/fl}$ mice were bred with MMTV-Cre mice. Three lines of mammary gland specific *Tet2* knock-out mice were created: 1) *Tet2*$^{+/+}$; MMTV-Cre (Tet2-WT), 2) *Tet2*$^{fl/+}$; MMTV-Cre (Tet2-HET), and 3) *Tet2*$^{fl/fl}$; MMTV-Cre (Tet2-KO) mice were generated. To produce the *Tet2* knock-out breast cancer mouse model, MMTV-PyMT mice were mated with *Tet2* knock out mice and two mouse phenotypes were generated: 1) *Tet2*; PyMT (WT-PyMT), and 2) *Tet2*$^{fl/+}$; MMTV-Cre; PyMT (MUT-PyMT). Either Tamoxifen (25mg/kg) or corn oil (mock control) were administrated to WT-PyMT and MUT-PyMT mice as intraperitoneal injections 5 days per week for 4 weeks (n=6). Tumor growth was determined by using the formula Volume = (Width^2 × Length)/2 via caliper measurement. After euthanizing each mouse, mammary glands were collected for further evaluation. All animal experiments were conducted with the approval of the Purdue Animal Care and Use Committee.

3.2.2 Mammary gland histology

Mammary glands were harvested from the mice, fixed in 10% buffered formalin, and processed for paraffin-embedded blocks in the Histology Research Laboratory at Purdue University. Hematoxylin and eosin stained slides and unstained slides (5μm-thick) of formalin-fixed paraffin-embedded mammary gland sections for immunostaining were provided from the Histology Research Laboratory at Purdue University.

3.2.3 Immunohistochemistry staining

Unstained tissue slides of formalin-fixed paraffin-embedded mouse mammary glands and tissue microarray slides (BRC1501, Pantomics) were deparaffinized and rehydrated. After heat-induced antigen retrieval, sections were incubated with 5% BSA and then incubated with primary antibodies, anti-Cre (#15036, Cell signaling), anti-Ki67 (Invitrogen, PIPA519462), anti-TET2

(ABE364, Millipore Sigma), and anti-ERα (#ab32063, Abcam) overnight at 4°C. Next, the appropriate secondary antibodies (#115-035-003 and #111-035-003, Jackson Immunoresearch) were applied to the section and visualized with the DAB chromogen kit (BioCare medical). Slides were counterstained with hematoxylin. Images were taken with an Olympus BX53 upright microscope. For human breast cancer tissue microarray, the histological grading and pathological annotation (tumor grade and subtype) were provided by Pantomics. The correlation between the expression levels of the proteins and with the tumor grade was analyzed using Chi-Square test.

3.2.4 Immunofluorescence staining

The fresh mouse mammary gland was sliced and fixed in 4% paraformaldehyde for one hour. The tissue slices were incubated with primary antibodies, anti-CK8 (#ab59400, Abcam, 1:250), and anti-CK14 (#ab7800, Abcam, 1:250), in PBS containing 0.5% Triton X-100 and 5% BSA overnight at 4°C. The specimens were washed with PBS containing 0.1% tween 20 three times and incubated with fluorochrome-conjugated secondary antibodies: Rhodamine Red – conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (Jackson immunoresearch, 1:400), overnight at 4°C. After washing, the samples were incubated with Trihydrochloride (Hoechst 33342, #H3570, Life technology, 1:1000) 10min at room temperature for nucleus staining. Tissue samples were placed on glass slides and mounted with mounting media. Images were taken by an Olympus FV10i-LIV laser scanning microscope.

3.2.5 Generation of single-cell suspensions from mouse mammary glands

Mammary glands from the mice were minced and digested in EpiCult-B medium: EpiCult-B mouse medium (Stemcell Technologies), 5% fetal bovine serum (FBS) and 50 ug/mL gentamycin (Stemcell Technologies), supplemented with Gentle Collagenase/Hyaluronidase, for 16 hours at

37°C. Cells were collected by centrifuging at 350 g for 5 minutes and resuspended in one part Hank's balanced salt solution (HBSS) with 2% FBS and 4 parts Ammonium Chloride Solution (Stemcell Technologies) to lysis red blood cells. The resultant pellet was dissociated with prewarmed 0.25% trypsin-EDTA, followed by prewarmed 5 mg/mL dispase II and 0.1 mg/mL DNase I. After then single-cell suspensions were obtained by filtration through a 40 μm cell strainer.

3.2.6 Flow Cytometry analysis

Cells at a concentration of 1×10^6 per 100 μl of Stain Buffer (BD Biosciences) were incubated with primary antibodies: FITC-conjugated anti-CD29 (#561796, BD Biosciences), APC-conjugated anti-CD24 (#562349, BD Biosciences), PE-conjugated anti-CD61 (#561910, BD Biosciences), and PerCP-Cy™5.5 Mouse Lineage Antibody Cocktail (#561317, BD Biosciences). Stained cells were subjected to BD Canto II flow cytometry system. Data (Mean%±SD) was evaluated by FCS Express 6 (Denovo Software) from three independent experiments with gating boundaries determined by using antibody isotype controls.

3.2.7 Targeted bisulfite sequencing

Isolated cells from mammary glands of Tet2 WT and Tet2 KO mice were stained with Zombie Violet™ Fixable Viability Kit (#423113, Biolegend). After washing, cells were stained with FITC-conjugated anti-CD29 (#561796, BD Biosciences), APC-conjugated anti-CD24 (#562349, BD Biosciences), and PerCP-Cy™5.5 Mouse Lineage Antibody Cocktail (#561317, BD Biosciences). Stained cells were sorted by BD FACS Aria Fusion into two cell populations: MaSC ($Lin^-CD24^+CD29^{hi}$) and Lum ($Lin^-CD24^+CD29^{lo}$). Genomic DNA was extracted from each cell population using Blood & Cell Culture DNA Mini Kit (#13323, Qiagen) according to the

manufacturer's instructions, and quality and quantity of DNA were assessed using a NanoDrop spectrophotometer. Three CpG regions of ERα (Mus musculus strain C57BL/6J chromosome 10, GRCm38.p4 C57BL/6J, 1) Sequence ID: NC_000076.6 Range 1: 4609905 to 4610205, 2) Sequence ID: NC_000068.7 Range 1: 9879791 to 9879988, 3) Sequence ID: NC_000078.6 Range 1: 57546217 to 57546401) analyzed by targeted bisulfite sequencing from Zymo Research (Irvine, CA). Briefly, assays were designed targeting CpG sites in the specified regions of interest (ROI) using primers created with DNA-specific primer design tool, Rosefinch (Zymo Research). Samples were bisulfite converted using the EZ DNA Methylation-LightningTM Kit (Zymo Research) according to the manufacturer's instructions. Multiplex amplification of all samples using ROI specific primer pairs. The resulting amplicons were pooled for harvesting and subsequent barcoding according to the Fluidigm instrument's guidelines. After barcoding, samples were purified and then prepared for massively parallel sequencing using a MiSeq V2 300bp Reagent Kit and paired-end sequencing protocol according to the manufacturer's guidelines. Sequence reads of each sample (Stem WT, Stem KO, Lum WT, and Lum KO) were identified using standard Illumina base-calling software. A boxplot was then generated to display the distribution of the methylation levels measured for each site within a region. The boxplot displays the median DNA methylation levels as well as the distribution of methylation levels within a sample. The boxplots show the 2nd quartile (median) as a yellow line, and 1st quartile and 3rd quartile as the bottom and upper bounds of the box, respectively. The upper whisker extends from the upper edge of the box to the largest value no further than 1.5 * IQR (or Interquartile Range) from the edge. The lower whisker extends from the lower edge of the box to the smallest value at most 1.5 * IQR from the edge.

3.2.8 Western blot and immunoprecipitation assay.

Western blotting and immunoprecipitation were performed according to standard protocols with the following antibodies: anti-TET2 (#61389, Active Motif, 1:1000), anti-β actin (#A5316, Sigma, 1:5000), anti-ERα (#ab32063, Abcam, 1:1000), anti-GATA3 (#PA520892, Thermo Fisher Scientific, 1:1000), anti-FOXA1 (sc101058, Santa Cruz, 1:1000), anti-FOXP1 (#4402T, Cell Signaling Technologies, 1:1000) and anti-TET2 (#36449, Cell Signaling Technology) antibodies.

3.2.9 Chromatin Immunoprecipitation (ChIP) assay and real-time qPCR

The sequences of the *Esr1* and *Gata3* promoters were obtained from UCSC Genome Database. Analysis of putative TF binding sites on *Esr1*, *Gata3* and *Foxa1* promoter was done by TRED (Cold Spring Harbor Laboratory) and MatInspector (Genomatix). The chromatin immunoprecipitation (ChIP) experiment was modified from the EZ-CHIP (Upstate) protocol using anti-FOXP1 (#4402T, Cell Signaling Technologies, 1:1000) and anti-TET2 (#36449, Cell Signaling Technology) antibodies. Sequential ChIP was performed as described previously (60). Primers used for the sequences of the *Esr1* and *Gata3* promoters were: Esr1-F(5'- CTGGGCAGGTGCCTAGTAGT), Esr1-R(5'- CAGTGAATTTCTGGTCCTCCTCA), Gata3-F(5'- ACCCCTGCCCAGGATTAGTA), Gata3-R(5'- AACAACCCGAACCTGACTCC), Foxa1-F(5'- AAACCAGACGGTCGCCTCTAGC), Foxa1-R(5'- TCAGTCCACTCCACTCCCCTT). For real-time qPCR, total RNA was extracted from cells by using Direct-zol™ RNA MiniPrep Plus (Zymo Research). RNA was reverse-transcribed by using Superscript II kit (Invitrogen). The results were analyzed by the Light Cycler 96 (Roche), and the quantification of RNA levels was normalized to GAPDH as CT (difference of cycling threshold) = CT (target) – CT (control).

3.2.10 Cell culture and stable cell line generation

The immortal normal mammary epithelial cell line, MCF12A, and the breast cancer cell line, MCF7, were purchased from American Type Culture Collection. MCF12A cells were grown in DMEM-F12 medium supplemented with 5% horse serum, epidermal growth factor (20ng/ml), insulin (10ng/ml), cholera toxin (100ng/ml), hydrocortisone (500ng/ml), penicillin (50 U/ml), and streptomycin (50 U/ml). MCF7 cells were cultured with DMEM medium supplemented with 10% FBS, penicillin (50 U/ml), and streptomycin (50 U/ml). For the stable cell line, shRNA against FOXP1 either pLV[CRISPR]-hCas9:T2A: Puro-U6>h*TET2*[gRNA#7970] were co-transfected with lentiviral packaging plasmids (pPAX2 and pMD2.G) into 293T cells. After 48 hours incubation, Lentiviruses were harvested and used to transduce to the target cells. Stable cell lines were selected with puromycin (2 mg/mL) treatment within 7 days.

3.2.11 Glucosylation of genomic 5hmC followed by methylation-sensitive qPCR.

Genomic DNA from the mouse mammary glands was treated with T4 Phage β-glucosyltransferase (T4-BGT, New England Biolabs) and UDP-Glucose (UDP-Glc) according to the manufacturer's instruction (EpiMark® 5-hmC and 5-mC Analysis Kit, New England Biolabs). Glucosylated genomic DNA (100 ng) was digested with 10 U of HpaII, MspI or no enzyme (control group) at 37°C overnight, followed by inactivation for 20 min at 80°C. The HpaII- or MspI-resistant fraction was quantified by qPCR using primers designed around at least one HpaII/MspI site, and normalizing to the mock digestion control. The calculation of quantitation of 5-hydroxymethylcytosine at a specific CCGG Site follows manufacturer's instruction (EpiMark® 5-hmC and 5-mC Analysis Kit, New England Biolabs). Primers used for the sequence of Esr1 and Gata3 promoters are: Esr1-F(5'-CTGGGCAGGTGCCTAGTAGT), Esr1-R(5'-

CAGTGAATTTCTGGTCCTCCTCA), Gata3-F(5'- ACCCCTGCCCAGGATTAGTA), Gata3-R(5'- AACAACCCGAACCTGACTCC).

3.2.12 Quantification and Statistical analysis

Each experiment was conducted independently for at least three times. The animal cohorts for experiments are with the same strain, sex, size, and allocated to different experimental groups by randomization. The sample size was chosen based on power analysis (desired power=80% and significance $P<0.05$). Differences between individual groups were analyzed by Student's t test or by one-way ANOVA test for multiple group analysis. Experimental models were randomly distributed over treatment groups. The dose-response curves were evaluated using GraphPad Prism software to determine whether the curves were statistically different with respect to the fitted midpoints (log EC50) using the sum-of-squares F test. If not otherwise noted, no methods were used to determine whether the data met assumptions of the statistical approach; no inclusion/exclusion criteria/cases were applied. All analyses were carried out using Microsoft Excel or GraphPad Prism 7 and presented as means± the standard deviation of the mean (SD). P value of 0.05 or lower was considered statistically significant for all experiments. The statistical parameters can be found in the figure legends. All data were presented as means± the standard deviation of the mean (SD). Statistical calculations were performed with Microsoft Excel analysis tools and GraphPad Prism. Differences between individual groups were analyzed by two-tailed paired t test. P values of <0.05 were considered statistically significant.

3.3 Results

3.3.1 TET2 is required for expression of luminal differentiation-associated genes

Previously we observed lower protein expression levels of ERα and GATA3 in *Tet2* knock out mouse models (2). Therefore, we hypothesized that TET2 governs luminal lineage commitment by controlling the expression of luminal differentiation-associated genes by catalyzing 5mc to 5hmc. First, we evaluated the mRNA levels of genes associated with luminal cell differentiation (*Esr1, Gata3, Foxa1*) as well as their DNA demethylation levels on the promoter regions of these 3 genes in mammary cells from Tet2 WT and Tet2 KO mice. We showed that deletion of *Tet2* reduced mRNA levels (Figure 3-1 A) and 5hmc levels (Figure 3-1 B) of all three luminal differentiation-associated genes.

Furthermore, to determine the effect of TET2 in DNA demethylation on the promoter region of *Esr1* in stem cell vs. luminal cell populations, we conducted bisulfite sequencing in stem cells (MaSC) and luminal cells (Lum) sorted from Tet2-WT and -KO mammary glands. Bisulfate sequencing results showed that methylation of *Esr1* gene was significantly lowered in the luminal cells compared with mammary stem cells. However, deletion of *Tet2 c*ould increase methylation level of *Esr1* in the luminal cell population to a level similar to that in the stem cell population (Figure 3-1 C). These data suggest that TET2 plays a role in maintaining luminal differentiation gene expression via DNA demethylation.

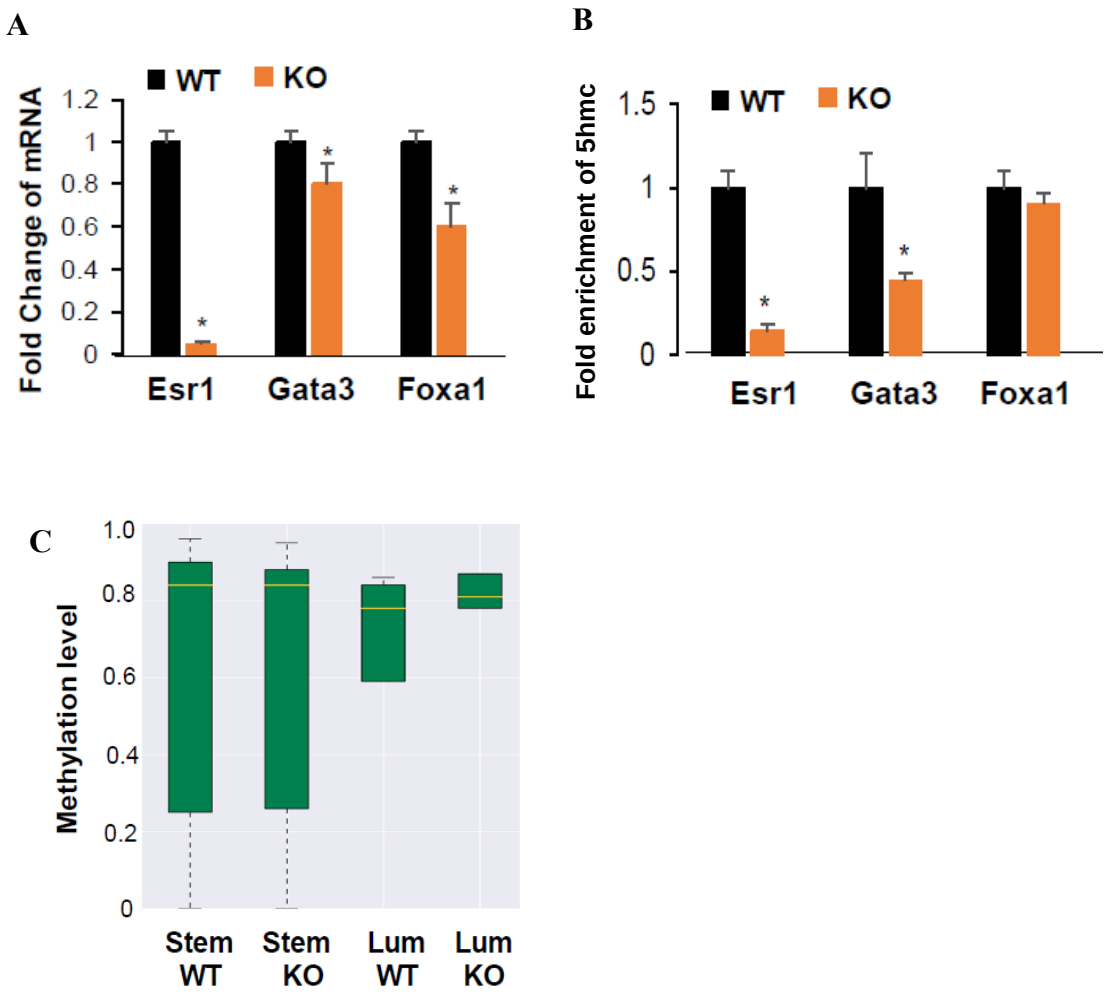


Figure 3-1. TET2 is required for expression of luminal differentiation-associated genes

(A) The mRNA expression levels and (B) fold enrichment of 5hmc of the luminal differentiation-associated genes (*Esr1*, *Gata3*, and *Foxa1*) and (C) ratio of methylation level of the *Esr1* gene in mammary cells isolated from 7-week-old WT and KO female mice. Asterisks indicate $P < 0.05$ and error bars denote SD)

3.3.2 TET2 interacts with FOXP1 to regulate luminal differentiation-associated gene expression

To determine the mechanism by which TET2 modulates luminal cell differentiation, we used Genomatix software (www.genomatix.de) (62) to identify the putative TFs that have consensus binding sites within the promoter regions of luminal differentiation-associated genes, including *FOXA1, GATA3,* and *ESR1*. TET2 requires DNA binding partners such as TFs, in order to be recruited to a target gene regulatory element (12) because TET2 does not have a DNA-binding domain called the CXXC domain unlike other TET proteins, TET1 and TET3 (Figure 1-1) (61). We found that Forkhead box protein P1 (FOXP1) had a high matrix similarity score of greater than 0.95 for targeting these genes. It has been reported that FOXP1 expression is positively correlated with hormone receptor status in human breast cancer (63–65). Recently Fu NY *et al.* revealed that FOXP1 is a crucial TF that orchestrates mouse MaSC differentiation and mammary gland development (66). To validate the promoter analysis result, we next performed a co-immunoprecipitation assay to reveal the direct physical interaction between TET2 and FOXP1. We showed that endogenous TET2 is reciprocally immunoprecipitated with FOXP1 in mammary epithelial cells from the Tet2 WT mouse (Figure 3-2 A, upper panel). The interaction between TET2 and FOXP1 can also be observed in the human mammary epithelial cell line, MCF12A (Figure 3-2 A, lower panel). To confirm whether FOXP1 indeed regulates ERα expression, we knocked-down FOXP1 by shRNA in mouse mammary epithelial cells and in human breast cancer MCF7 cells, and found that FOXP1 knockdown cells showed significantly decreased expression of ERα (Figure 3-2 B). Next, using Sequential Chromatin Immunoprecipitation (ChIP) assay, we showed that TET2-FOXP1 complex co-occupied in regulatory elements of *Gata3*, *Foxa1*, *and Esr1* genes (Figure 3-2 C). We further revealed that FOXP1 could bind to *Esr1* genes via putative FOXP1 binding motifs in the promoter region (binding sites at 198bp through 996bp upstream

TSS) and also in the enhancer region (binding sites at 3160bp through 3653bp upstream TSS), where FOXP1-TET2 mainly co-occupied in the GC-rich enhancer region by sequential ChIP analysis (Figure 3-2 D). This result agreed with Rasmussen KD *et al.* suggesting that TET2 binding to enhancers plays a pivotal role for chromatin accessibility and recruitment of TFs (67). Concordantly, we showed that deletion of Tet2 abolished FOXP1 recruitment to the *Esr1, Gata3 and Foxa1* genes (Figure 3-2 E). Together, these data suggest that FOXP1 directs TET2 binding to specific genes (*Esr1, Gata3, and Foxa1*), where TET2 is required for FOXP1 chromatin recruitment to coordinately mediate expression of the genes involved in luminal cell differentiation.

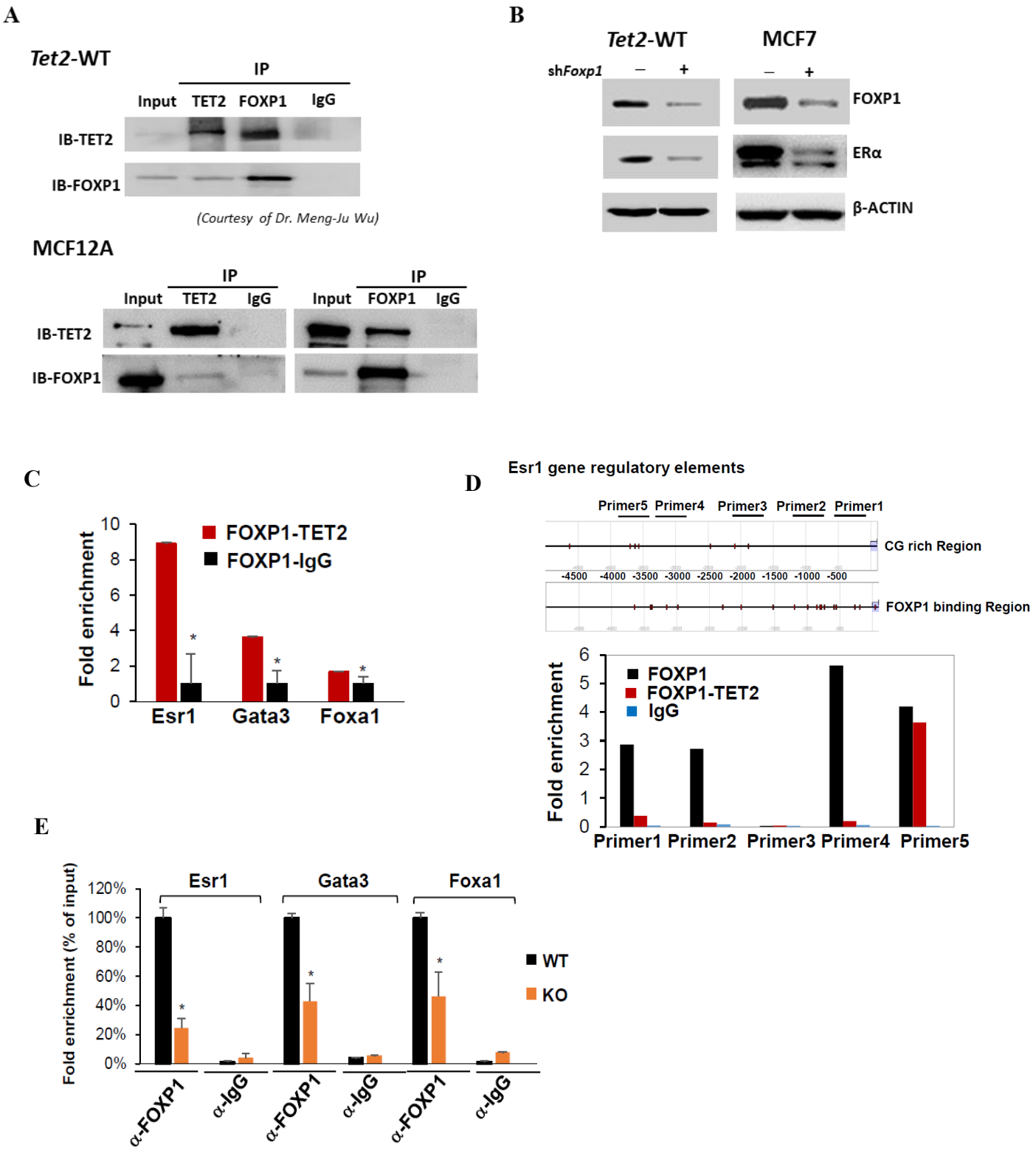


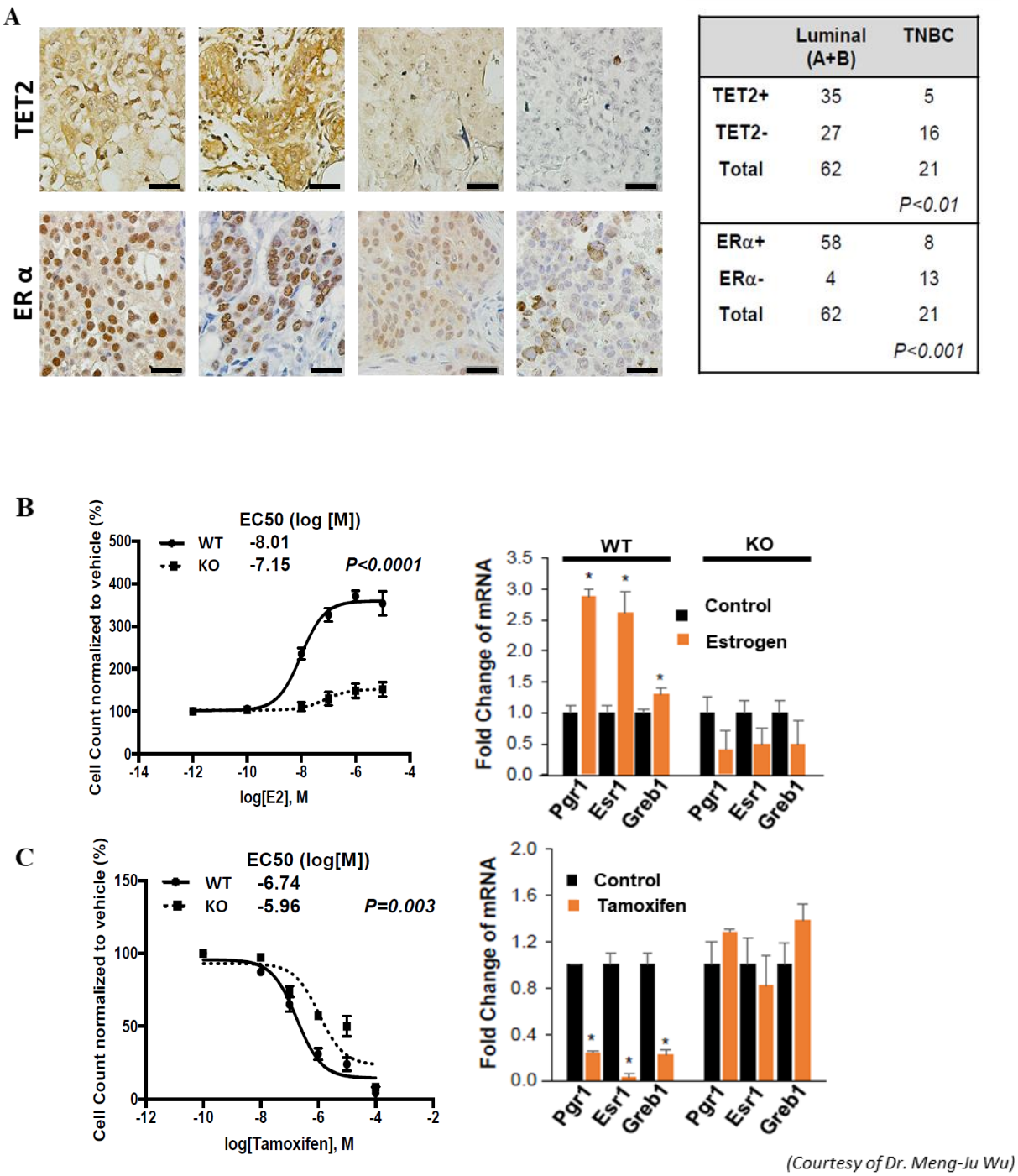
Figure 3-2. TET2-FOXP1 transcription complex mediates gene expression associated with luminal cell differentiation

(A) Reciprocal co-immunoprecipitation showing endogenous FOXP1 and TET2 interaction in mammary cells isolated from 7-week-old WT female mice (upper panel) and human normal mammary epithelial cells, MCF12A (lower panel). (B)The ERα expression level in mammary cells stably expressed shFOXP1 or the control vector. Mammary cells were isolated from 7-week-old WT female mice (left) and luminal breast cancer cell line, MCF7 cells (right). (C) Fold enrichment of FOXP1-TET2 complex bound to regulatory elements of *Esr1*, *Gata3* and *Foxa1* using Sequential-ChIP-qPCR analysis. (D) Diagram showing putative GC-rich region and FOXP1 binding motif within the regulatory elements of *Esr1* gene (promoter and enhancer regions) (upper panel). Fold enrichment of FOXP1 and FOX-TET2 complex bound to each putative binding motif (low panel). (E) Fold enrichment of FOXP1 in the binding regions of *Esr1*, *Gata3* and *Foxa1* promoters in the mammary cells of 7-week-old WT and KO female mice. n=3 independent experiments, asterisks indicate $P<0.05$. Error bars denote ±SD.

3.3.3 Loss of TET2 confers endocrine resistance

To investigate the clinical relevance of TET2 and ERα interaction in human breast cancer, we analyzed the staining profiles of TET2 and ERα expression in tissue microarrays with 83 tumor samples from breast cancer patients. TET2 expression was correlated with ERα expression in luminal breast cancer while triple-negative breast cancer tissues lost expression of both TET2 and ERα expression (Figure 3-3 A, $P<0.001$). Since TET2 deletion leads decreased expression of ERα, We next asked whether loss of TET2 expression conferred endocrine resistance in mammary epithelial cells and breast cancer cells. To determine the role of TET2 in endocrine resistance, cells from Tet2-WT and Tet2-KO mouse mammary glands were treated with various concentrations of estradiol (E2) and Tamoxifen, which is the most commonly used selective ER modulators for breast cancer hormone therapy (68). We found that loss of TET2 abrogated estrogen dependent cell growth and also reduced differentiation-associated mRNA expression levels of ER target genes compared to cells from Tet2-WT mouse mammary glands (Figure 3-3 B). Similarly, cells from Tet2-WT mice were sensitive to Tamoxifen, which blocked 50% of the cell growth with a half-maximal effective concentration (EC50) at $10^{-6.74}$M, accompanied by decreased expression levels of ER target genes, *Pgr*, *Esr1*, and *Greb1* (Figure 3-3 C). However, loss of TET2 significantly diminished the sensitivity to Tamoxifen as indicated by the elevated EC50 (to $10^{-5.96}$M) and resistance to Tamoxifen-mediated repression of ER target genes (Figure 3-3 C). Together, these data suggest that loss of TET2 is associated with ER-negative breast cancer and confers Tamoxifen resistance in tumor cells. Deficient TET2 expression may contribute to the development of hormone resistant in basal-like human breast cancers.

To further verify the role of TET2 in the regulation of ERα expression and tamoxifen resistance in human breast cancer cells, we generated stable TET2-KO cells using the CRISPR-Cas9 system in a human luminal breast cancer cell line, MCF7. First, we showed that loss of TET2 led to down-regulation of ERα protein levels (Figure 3-4 A) as well as ERα target gene expression (Figure 3-4 B). Next, we treated the TET2-WT-MCF7 cells and TET2-KO-MCF7 cells with estrogen and Tamoxifen. Consistent with the results from mouse mammary epithelial cells, we found that compared to TET2-WT-MCF7 cells, TET2-KO-MCF7 cells had a decreased sensitivity to estrogen-induced cell proliferation (Figure 3-4 C). Loss of TET2 developed resistance to Tamoxifen-mediated cell growth inhibition (Figure 3-4 D) as well as colony formation inhibition (Figure 3-4 E). Together these data suggest that loss of TET2 may contribute to loss of ERα expression and defective ERα signaling to confer endocrine resistance.



	Luminal (A+B)	TNBC
TET2+	35	5
TET2-	27	16
Total	62	21
		P<0.01
ERα+	58	8
ERα-	4	13
Total	62	21
		P<0.001

(Courtesy of Dr. Meng-Ju Wu)

Figure 3-3. Loss of TET2 confers endocrine resistance.

(A) Representative images (left) and chi-square analysis (right) of TET2 and ERα expression in 83 human breast tumor specimens of luminal breast cancer and triple-negative breast cancer. (-): negative/low staining, (+): positive/high staining (scale bar: 20μm). (B-C) Dose-response curve showing normalized percentage of surviving cells with EC50 and mRNA expression of mammary cells isolated from WT and KO 7-week-old female mice treated with (B) estrogen (estradiol E2, 10^{-7}M, upper panel) and (C) tamoxifen (10^{-7}M) or control vehicle (lower panel) for 72 hours.

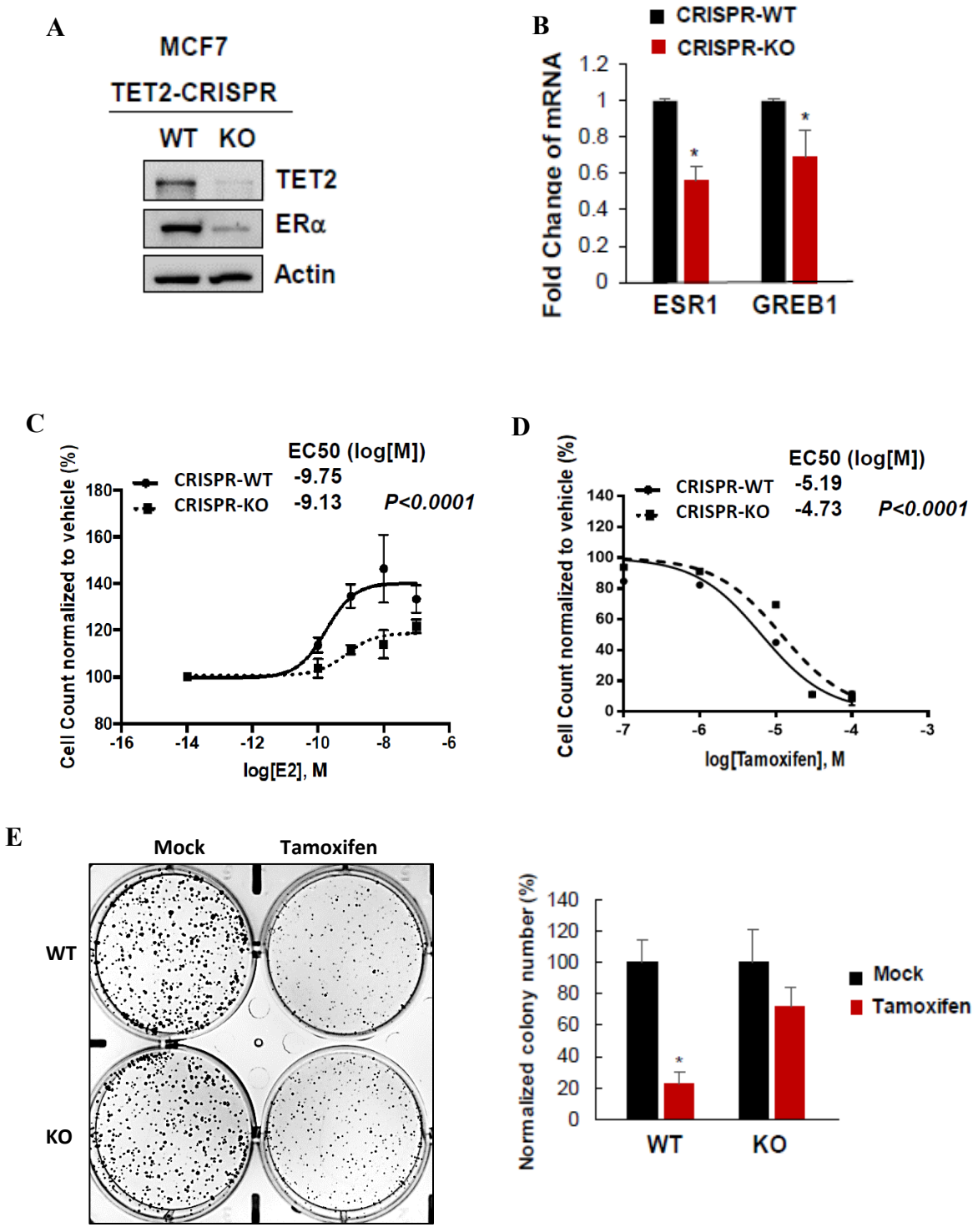


Figure 3-4. Loss of TET2 confers Tamoxifen resistance in breast cancer cells in vitro.

(A) ERα expression, and (B) *ESR1* and *GREB1* gene expression levels of CRISPR-TET2 expressing (CRISPR-KO) and a vector expressing (CRISPR-WT) MCF7 cells. (C, D) Dose-response curve showing normalized percentage of surviving cells with EC50 of CRISPR-WT- and CRISPR-KO- MCF7 cells treated with (C) estradiol (E2) and (D) tamoxifen or control vehicle in phenol red free charcoal stripped FBS media for 72 hours. (E) Representative images of colony formation (left) and the normalized percentage of colony formation (right) of CRISPR-WT- and CRISPR-KO- MCF7 cells treated with tamoxifen ($5*10^{-6}$M) or the control vehicle. n=3 independent experiments, asterisks indicate $P<0.05$. Error bars denote ±SD.

3.3.4 Loss of TET2 promotes mammary tumor development

To further determine the role of TET2 in breast tumorigenesis, we generated a *Tet2*-deletion breast cancer mouse model by breeding the *Tet2*-deletion mouse model with an established breast cancer mouse model, MMTV-PyMT (PyMT). This PYMT mouse model expresses polyoma middle T oncogenic protein in mouse mammary epithelium and develops spontaneous luminal-like mammary tumors at 4-6 weeks of age, progressing to mammary carcinoma around 8-12 weeks of age (54,69). We collected the mammary glands from WT-PyMT (*Tet2*;PyMT) and MUT-PyMT (MMTV-Cre;$Tet2^{fl/+}$;PyMT) at 5 weeks of age to verify the effects of TET2 deletion in the early onset of mammary tumor development. Compared with WT-PyMT, TET2 deletion led to enhanced tumor cell proliferation in MUT-PyMT mammary glands (Figure 3-5 A). Loss of TET2 also increased the MaSC population (Figure 3-5 B) and resulted in aberrant lineage commitment from a predominantly luminal phenotype to a mixed lineage phenotype with CK8/CK14 double-positive staining in the mammary lesions (Figure 3-5 C). Moreover, compared with WT-PyMT mice, MUT-PyMT mice generated more tumor lesions (6.3±2.5 vs 3.7±2.5) with significantly larger tumor size (0.479±0.66 cm^2 vs 0.103±0.06 cm^2, Figure 3-5 D). Next, we aimed to determine the role of TET2 in Tamoxifen resistant in the PyMT mammary tumors. We treated 7 weeks old WT-PyMT and MUT-PyMT mice with Tamoxifen (TAM, 25mg/Kg) or corn oil (control vehicle) intraperitoneally for 5 consecutive days per week for 4 weeks. Compared with the control group, we found that Tamoxifen significantly reduced WT-PyMT tumor growth (Figure 3-5 E) and converted an aggressive tumor phenotype to a phenotype similar to normal ductal architecture (with single-layered epithelium), indicating a tumor involution phenotype induced by Tamoxifen (Figure 3-5 F). However, MUT-PyMT mice failed to respond to Tamoxifen treatment (Figure 3-5

E, F). Together these data suggest that loss of TET2 accelerates mammary tumor development and confers Tamoxifen resistance in breast cancer.

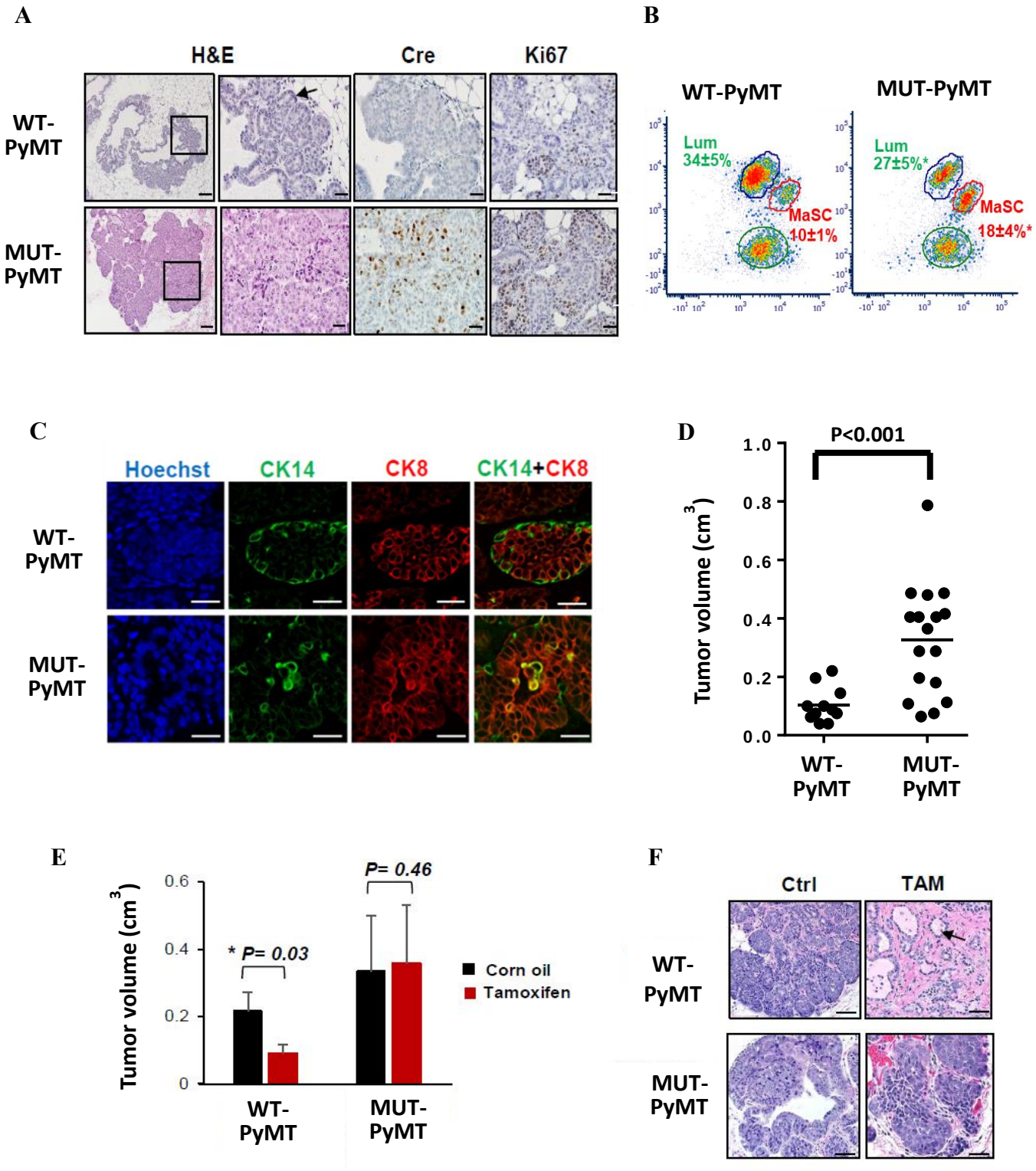


Figure 3-5. Loss of TET2 promotes mammary tumorigenesis and contributes to tamoxifen resistance in vivo.

(A) H&E and immunostaining of Cre and Ki67 in mammary gland sections isolated from 5-week-old female *Tet2*$^{fl/+}$;PyMT and MMTV-Cre;*Tet2*$^{fl/+}$; PyMT mice (arrow indicates premalignant tumor cell proliferation, scale bar: 50 μm). (B) Flow cytometry analysis showing the percentage of basal cell/mammary stem cell (MaSC, Lin$^-$CD24$^+$CD29^{hi}) and luminal cell (Lum, Lin$^-$ CD24$^+$CD29^{lo}) populations isolated from 6-week-old female *Tet2*$^{fl/+}$;PyMT and MMTV-Cre;*Tet2*$^{fl/+}$;PyMT mice. (C) immunofluorescence images showing co-staining of CK8 (red) and CK14 (green) in mammary glands of 7-week-old female *Tet2*$^{f/+}$; PyMT and MMTV-Cre;*Tet2*$^{fl/+}$;PyMT mice (scale bar: 50 μm). (D) Scatter plot showing the tumor volume (Width^2×Length/2) of tumors collected from 13 (±2days) week-old female *Tet2*$^{f/+}$;PyMT and MMTV-Cre;*Tet2*$^{fl/+}$;PyMT mice. (E) Tumor volume, and (F) tumor phenotype of 7-week-old female *Tet2*$^{f/+}$; PyMT and MMTV-Cre;*Tet2*$^{fl/+}$;PyMT mice treated with corn oil or tamoxifen (i.p. 25 mg/kg) for 5 consecutive days per week for 4 weeks (arrow indicates single layered epithelium, scale bar: 100 μm).

3.4 Discussion

DNA hypermethylation in cancer is associated with silencing of differentiation genes as well as tumor suppressor genes (70,71). It has been reported that loss of function mutations of TET2 in hematopoietic systems is associated with hematological malignancies (16,21,25). TET2 is mostly considered as a tumor suppressor in breast cancer *in vitro* (1,28), however, *in vivo* role of TET2 in breast cancer has not yet been established. Therefore, we have established a new mammary-specific *Tet2* knock-out mice model and a *Tet2* knock-out-PyMT breast cancer mouse model. These mouse models have been used in this study and successfully demonstrated the role of TET2 in mammary gland development, mammary tumorigenesis, and hormone therapy resistance *in vivo*. We have identified that TET2 and FOXP1 form a novel chromatin complex to specifically target and transactivate genes associated with luminal cell differentiation by DNA demethylation. Furthermore, we have shown that TET2 deprivation leads to aberrant luminal lineage commitment and promotes mammary tumor development with therapeutic resistance to Tamoxifen. These findings not only reveal novel epigenetic mechanisms involved in stem cell fate determination, but also important implications for therapeutic strategies and prediction biomarkers of hormone resistance in breast cancer.

DNA demethylation patterns are determined by TET2 enzyme activity and its recruitment to the DNA. The activity of TET2 can be regulated by metabolites, such as 2-oxoglutarate- (2-OG) and the cofactor Fe(II) (72) and by post-translational modification, phosphorylation, and acetylation of TET2 (73,74). Since TET2 does not have DNA binding site unlike other TET family members, TET2 interacts with tissue specific TFs, such as WT1 (75), RUNX1 (76), early B-cell factor 1 (EBF1) (77), and RAR β (1), to activate transcription of respective target genes. TET2

also can be recruited to enhancers by TFs such as C/EBPa, Klf4, and Tfcp2l1 and then facilitate the activity of enhancers during reprogramming in B cells (78). Recently Wang *et al*. have reported that TET2 occupies in the enhancer region with MLL3 to facilitate recruitment of TFs such as ERα (79). In our current study, we have shown that TET2 is associated with transcriptional activation of *ERα* as well as other luminal differentiation-associated genes, such as *GATA3* and *FOXA1*. Furthermore, our data has revealed that TET2-FOXP1 plays a critical role in transactivation of luminal differentiation-associated genes. FOXP1 is known as an estrogen-induced TF and enhances ER target transcription in MCF7 cells (63,64). FOXP1 expression is also positively correlated with breast cancer cell sensitivity to endocrine therapy, including Tamoxifen (64). This evidence provides important clinical relevance of the TET2-FOXP1 axis to ER signaling regulation and hormone therapy sensitivity in luminal breast cancer (Figure 3-6).

ER-positive breast cancers comprise about 80% of all breast cancers (47) and are responded well to hormone therapies. However, almost half of ER-positive breast cancers eventually develop intrinsic or acquired resistance towards hormone therapy (53). Tamoxifen is one of the most widely used hormone/endocrine therapies in breast cancer. Despite the benefit of Tamoxifen in ERα positive breast cancer, almost half of the patients treated with adjuvant Tamoxifen treatment relapse with adverse progression at the end (80). Our in vitro and in vivo findings in this study consistently demonstrate that deletion of *TET2* may contribute to development of therapeutic resistance to Tamoxifen, and functional TET2 status may be used as a potential biomarker to predict Tamoxifen sensitivity in breast cancer. It has been reported that high concentration treatment of vitamin C in leukemic cells can block aberrant self-renewal and induce DNA demethylation by functionally restoring TET2 (81). Sant *et al.* demonstrated that high

concentration of vitamin C treatment suppresses triple-negative breast cell survival by increasing 5hmC levels in vitro (82), and Lee *et al.* have also reported that high concentration vitamin C treatment can synergize with Tamoxifen in MCF7 cell growth inhibition (83). These results support our findings that TET2 serves as an important target for breast cancer prevention and treatment, and our in vivo *Tet2* deficient breast cancer mouse model will be a valuable tool for the development of new therapeutics.

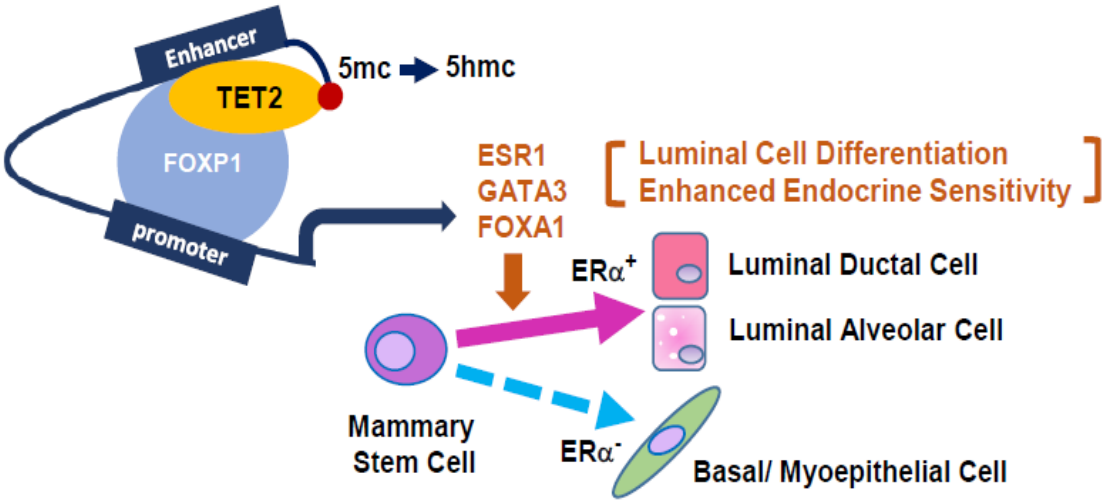


Figure 3-6. A proposed model of the role of TET2 in regulation of luminal cell differentiation and endocrine response

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