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Date: 17-04-2016

**Characterizing Novel Molecular Mechanisms
Involved in Breast Cancer
Tumourigenesis/Progression and Tamoxifen
Resistance**

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BEng. (Hons.) HK; M. Phil. HK

A thesis submitted in partial fulfilment of the requirements for
The Joint Degree of Doctor of Philosophy
at Imperial College London (ICL) and the University of Hong Kong
(HKU)

April 2016

Abstract of thesis entitled

**“Characterizing novel molecular mechanisms involved in breast cancer
tumorigenesis/progression and tamoxifen resistance”**

Submitted by

GONG Chun

For the degree of The Joint Degree of Doctor of Philosophy
at Imperial College London (ICL) and the University of Hong Kong (HKU)
in April 2016

Breast cancer is the most common cancer among women. Breast cancer tumorigenesis could be due to loss/mutation of BRCA1, yet the mechanisms underlying BRCA1-associated tumorigenesis are not well understood. Tamoxifen stands in the first-line for treatment of oestrogen receptor (ER) positive breast cancers but drug resistance significantly compromises its clinical efficacy in preventing cancer progression and improving survival. The mechanism of tamoxifen resistance is not fully understood and there is no clinical in-use biomarker that can reliably predict tamoxifen response.

My PhD study aims at characterizing novel molecular pathways that are involved in breast cancer tumorigenesis/progression and tamoxifen resistance, focusing on two gaps in breast cancer research: 1) the role of BRCA1-mediated epigenetic regulation of gene expression; 2) identifying biomarker that can be used for prediction of tamoxifen resistance.

In chapter 3, regulation of tumour suppressor FOXO3a by BRCA1 through EZH2-mediated epigenetic mechanism is studied. Knockdown or overexpression of BRCA1 suppresses or induces FOXO3a expression at both mRNA and protein levels, respectively. In addition, FOXO3a gene expression is negatively regulated by EZH2 in basal cell lines where BRCA1 is suppressed or mutated but not in BRCA1-wild-type MCF-7 cells, as chemical inhibition or siRNA-mediated depletion of EZH2 enhanced FOXO3a expression in basal cell lines. Moreover, ChIP assays showed that DNMT1, DNMT3a and DNMT3b and H3K27me3 are recruited to FOXO3a promoter in basal cell lines but not in MCF-7, and depletion of BRCA1 in MCF-7 induces the deposition of DNMTs and H3K27me3 to FOXO3a promoter. It is also found BRCA1 suppressed cancer cell proliferation at least partially through FOXO3a. Consistent with the *in vitro* data, FOXO3a promoter methylation scores are significantly higher in BRCA1-mutated tumours comparing with non-BRCA1 mutated tumours. These data collectively suggest that BRCA1 can suppress promoter methylation and enhance transcription of FOXO3a indirectly through binding to EZH2 and restraining its methyltransferase activity. The findings could contribute to better understanding of BRCA1-associated tumorigenesis and the regulation of FOXO3a in breast cancer.

In chapter 4, the role of BQ323636.1, a splice variant of NCOR2 in tamoxifen resistance is studied and its efficacy in predicting tamoxifen resistance in breast cancer patients is also evaluated. It was found that overexpression of BQ323636.1 conferred resistance to tamoxifen in both breast cancer cell lines and orthotopic mouse model. Mechanistic studies showed that BQ323636.1 overexpression compromised the suppressive role of NCOR2 in regulating

oestrogen-response element activity shown by luciferase assay and rescues the transcriptional suppression of tamoxifen on ER-target genes. Co-immunoprecipitation also showed that BQ323636.1 could bind to NCOR2 and inhibit the formation of co-repressor complex, which could explain how BQ323636.1 interferes with functions of NCOR2. Unlike NCOR2 that is localized in the nucleus, BQ323636.1 is found to be expressed in both nucleus and cytoplasm while nuclear BQ323636.1 level is significantly higher in tamoxifen resistant cells. More importantly, in human breast cancer tissue microarray, nuclear BQ323636.1 overexpression is significantly associated with tamoxifen resistance as well as patients' survival. The findings lead to the proposal of using BQ323636.1 as a biomarker for prediction of tamoxifen resistance.

(Total 499 words)

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April 2016

Declarations

I declare that the thesis and the research work represent my own work, except where due acknowledge is made, and that is has not been previously included in a thesis dissertation, or report submitted to this University or other institution for a degree, diploma or other qualifications.

Signature  _____

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Abbreviations

5'-aza-dC	5'-azacitidine-decitabine
aa	Amino acid
AF1	Activation domain 1
AKT	Protein kinase B
AML	Acute myeloid leukaemia
ATCC	American type culture collection
BARD	BRCA1-associated RING domain protein 1
BRCA1	Breast cancer 1
ChIP	Chromatin immunoprecipitation
CO ₂	Carbon dioxide
Co-IP	Co-immunoprecipitation
DCIS	Ductal carcinoma <i>in situ</i>
DBD	DNA-binding domain
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNMTs	DNA methyltransferases
E2	Estradiol
EMT	Epithelial-to-mesenchymal transition

ER	Oestrogen receptor
ER+	Oestrogen receptor positive
ER-	Oestrogen receptor negative
ERE	Oestrogen response element
EtOH	Ethanol
EV	Empty vector
EZH2	Enhancer of zeste homolog 2
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	The US Food and Drug Administration
FISH	Fluorescence <i>in situ</i> hybridization
FOXO3a	Forkhead box O3a
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCLC	Glutamate-cysteine ligase catalytic subunit
GEO	Gene expression omnibus
GFP	Green fluorescent protein
GR	Glucocorticoids receptor
GSTs	Glutathione S-transferases
H&E	Hematoxylin and Eosin
H3K27	Histone 3 lysine 27
H3K27me3	Trimethylation of H3K27

HATs	Histone acetyl transferases
HDACs	Histone deacetylases
HKE293FT	Human embryonic kidney 293 fast transform
HER2	erb-b2 receptor tyrosine kinase 2
HMT	Histone methyltransferase
HO-1	Heme oxygenase-1
IDC	Invasive ductal carcinoma
IGFR1	Insulin-like growth factor-1 receptor
IgG	Immunoglobulin G
IgG (M)	Moue immunoglobulin G
IgG (R)	Rabbit immunoglobulin G
IHC	Immunohistochemistry
IKK	IkappaB kinase
ILC	Invasive lobular carcinoma
IMEM	Improved minimum essential medium
IP	Immunoprecipitation
KConFab	The Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer
LH	Luteinsing hormone
MAPK	Mitogen-activated protein kinase
MeDIP	Methylated DNA immunoprecipitation

MEFs	Mouse embryonic fibroblasts
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
mut	Mutant
NCOR2	Nuclear co-repressor 2
NLS	Nuclear localisation signal
NP-40	Nonidet P40 substitute
NQO1	NAD(P)H: quinone oxidoreductase 1
NRF2	NF-E2–related factor 2
ns	No significance
NSC	Non-silencing control
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween 20
PI3K	Phosphoinositide 3-kinase
PMRT	Post-mastectomy radiotherapy
PMSF	Phenylmethanesulfonyl fluoride
PR	Progesterone receptor
PRC2	Polycomb repressive complex 2
P/S	Penicillin/streptomycin
PVDF	Polyvinylidene difluoride
RANKL	Receptor activator of NfκB ligand
RD	Repression domain

ROS	Reactive oxygen species
RR	Risk ratio
RT	Room temperature
RT-qPCR	Real-time quantitative PCR
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERM	Selective estrogen receptor modulator
SIRT1	NAD ⁺ -dependent deacetylase sirtuin-1
SRB	Sulforhodamine B
Tam	Tamoxifen
TBST	Tris-buffered saline tween-20
TMA	Tissue microarray
WHO	World Health Organization
WT	Wild-type
VEGF	Vascular endothelial growth factor

Symbols

$^{\circ}\text{C}$	Degree Celsius
bp	Base pair
Ct	Cycle threshold
h	Hour
L	Litter
min	Minute
ml	Millilitre
mM	Millimolar
s	Second
μg	Microgram
μl	Microliter
V	Voltage
x g	Times gravity

Chapter 1 Introduction

1.1 Breast cancer

Breast cancer refers to the disease in which malignant cells develop from epithelial cells in breast tissues, namely the milk ducts and lobes, which are present in a background of fat and connective tissue. The majority of breast cancers originate from either ducts (ductal carcinoma) or lobes (lobular carcinoma).

1.1.1 Breast cancer epidemiology

Breast cancer is the most common type of invasive cancer in women worldwide. According to World Health Organization (WHO), an estimated 1.67 million new cases were diagnosed in 2012, accounting for 25% of all cancers. As shown in Figure 1.1, the incidence rates vary significantly across the regions, being highest in the more developed regions including Western Europe (96 per 100,000) and Northern America (94 per 100,000) and lowest in middle Africa (27 per 100,000). The high incidence rate in more developed regions may be associated with the lifestyle, use of oestrogen for delaying menopause, and breast feeding status, which will be discussed in more details in the next section. The mortality rate varies less drastically across world regions. Breast cancer ranks as the second most common cause of cancer death (after lung cancer) in women in more

developed countries while it remains as the most frequent cause of death from cancer in less developed regions. Breast cancer can also occur in men, but at much lower rate compared to women.

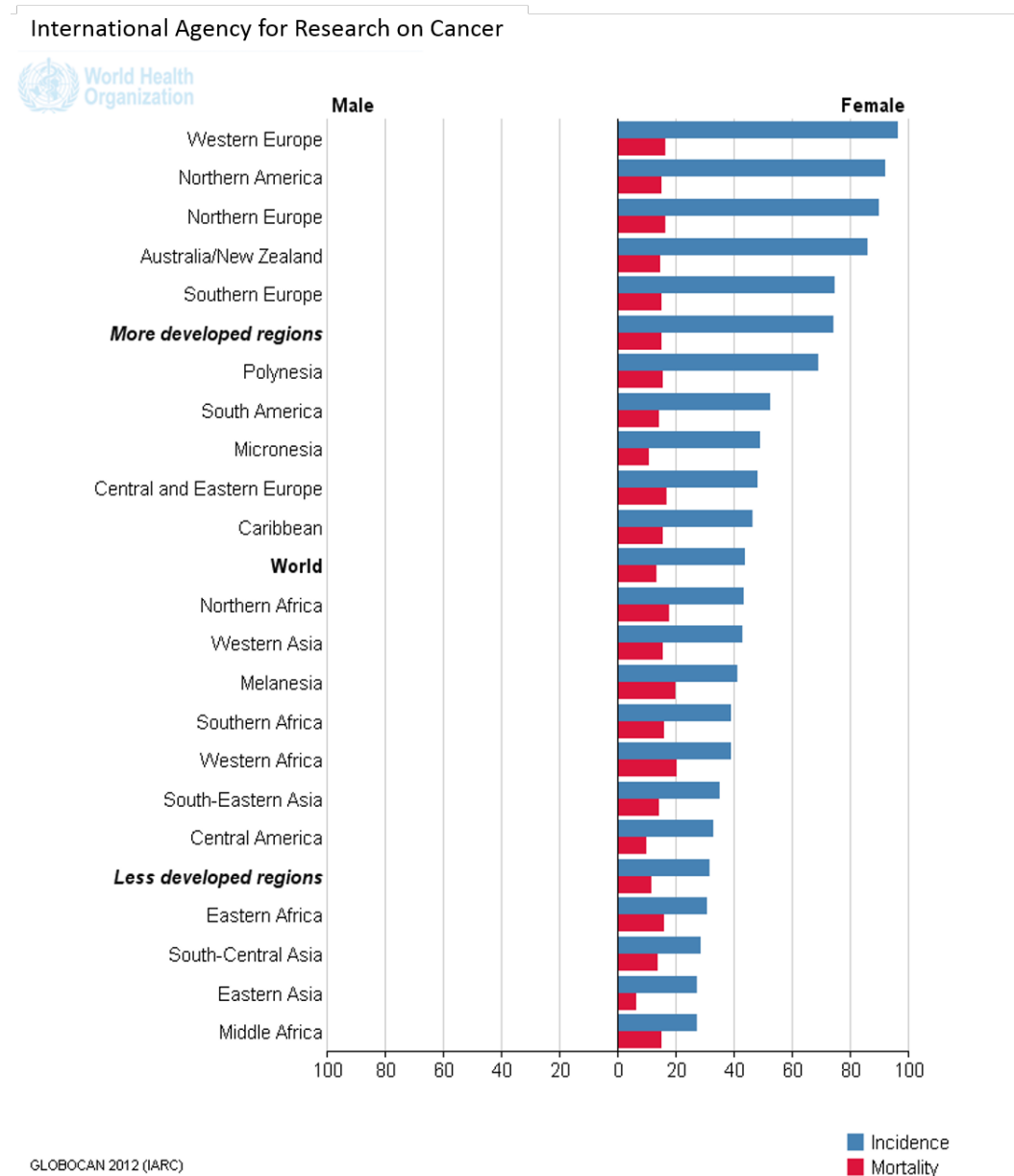


Figure 1.1 Age-standardised rates (per 100,000) of breast cancer incidence and mortality worldwide according to the estimation by GLOBOCAN in 2012 (GLOBOCAN 2012).

1.1.2 Breast cancer risk factors

The risk of developing breast cancer varies from individual to individual. There are some well recognized factors that are associated with higher risk of breast cancer.

1.1.2.1 Age and gender

Women has about 100 times higher risk of developing breast cancer than men (Hill, Khamis et al. 2005), which could be because women have higher levels of oestrogen and progesterone that will promote initial breast cancer growth. Risk of breast cancer also increases with the ageing process. According to American Cancer Society, about 1/8 invasive breast cancers were diagnosed in women younger than 45 while about 2/3 are found in women who are 55 or older. However, it is noted that the disease occurred in young women is generally more aggressive than that in older women and the prognosis is often poorer which could be due to negative oestrogen receptor status or higher grade at diagnosis (Peppercorn 2009).

1.1.2.2 Lifestyle

Lifestyle can affect a person's risk to breast cancer. Examples of such risk factors include: age of first pregnancy, obesity, use of hormonal contraceptives, hormone replacement therapy and alcohol consumption (Hulka and Moorman 2008). Experimental data have provided strong support on the notion that oestrogen promotes the growth and development of breast cancer cells (Clemons and Goss 2001). Therefore, accumulative high oestrogen exposure from personal lifestyle

is closely related to breast cancer risk. For instance, women with late first full-term pregnancy and late menopause, as well as women who use hormonal contraceptives or oestrogen-replacement therapy after menopause are reported to be correlated with higher risk of developing breast cancer (Hulka and Moorman 2008). The role of oestrogen in breast cancer tumourigenesis and progression will be discussed in detail in section 1.2 and 1.3.

1.1.2.3 Genetic factor

Genetic factors predispose women to breast cancer risk and are closely related to hereditary breast cancer. Hereditary breast cancer accounts for approximately 5-10% of all the cases (Honrado, Benitez et al. 2005). The best recognised genetic factors are breast cancer susceptibility genes (BRCA1 and BRCA2), germline mutations of which accounts for the majority of familial cases of breast and ovarian cancers. Although much more rarely reported, mutations in other genes such as p53 (Merino and Malkin 2014), PTEN (Fackenthal, Marsh et al. 2001), Phosphoinositide 3-kinase (PI3K) (Zardavas, Phillips et al. 2014), ATM (Mao, Chung et al. 2012), E-cadherin (Xie, Li et al. 2011), are also associated with inherited breast cancer. Besides occurring in hereditary breast cancer, reduced BRCA1 expression or mutation has also been frequently reported in sporadic breast cancer (Wilson, Ramos et al. 1999, Yang, Sakurai et al. 2001, Janatova, Zikan et al. 2005), highlighting the importance of BRCA1 in breast cancer tumourigenesis and progression.

1.1.3 Breast cancer classification

Breast cancer can be classified according to its histo-pathological characteristics, receptor status, and more recently, molecular features.

1.1.3.1 Histopathology

According to its histo-pathological characteristics, breast cancer can be classified broadly into the non-invasive carcinoma ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS), invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). IDC is the most common type of invasive breast cancer, accounting for about 80% of all the cases. Breast cancer can also be classified according its grade or stage. Breast cancer can be low grade (grade 1) when cancer cells are well-differentiated, intermediate grade (grade 2) when cells are moderately differentiated, or high grade (grade 3) if the cells are poorly differentiated. Staging is another way to classify breast cancer according to the size of tumour, the involvement of axillary lymph node and if there is metastasis present (TNM system). Prognosis of patients who are at late stage (stage III or IV) is usually much poorer than those who are at early stage.

1.1.3.2 Receptor status

Breast cancer classification can be based on the expression of three receptors including oestrogen receptor (ER), progesterone receptor (PR) and erb-b2 receptor tyrosine kinase 2 (also called human epidermal growth factor receptor/HER2). The expression levels of these receptors are assessed by immunohistochemistry (IHC), and HER2 overexpression can be further

confirmed by fluorescence *in situ* hybridization (FISH), which will provide valuable information for selection of appropriate therapies. ER positive (ER+) patients can be treated with endocrine therapy while HER positive (HER+) individuals can be treated with targeted therapy utilizing HER2-specific monoclonal antibody. On the contrary, triple negative patients who express none of the receptors have a comparatively poor prognosis due to lack of treatment options (Dent, Trudeau et al. 2007).

1.1.3.3 Molecular subtyping

Breast cancer is a highly heterogeneous disease and traditional ways of classification cannot fully capture the breadth of the disease and do not help in selecting treatment regimens. A more recent approach to classify breast cancer is according to its molecular features as determined by gene expression profiling (Andre and Pusztai 2006, Viale 2012). The five intrinsic subtypes of breast cancer according to molecular features are: luminal A, luminal B, HER2-enriched, basal-like and normal-like (Perou, Sorlie et al. 2000) and the molecular features of the five subtypes in the gene expression array is shown in figure 1.2. Luminal A and luminal B subtypes both express similar level of ER while luminal A tumours have lower level of proliferation/cell cycle related genes or proteins and higher level of luminal genes or proteins including PR and FOXA1 (Prat, Pineda et al. 2015). HER2-enriched subtype is distinguished by its high expression of HER2-related genes and low expression of basal-related genes such as keratin 5 and FOXC1. The basal-like subtype is the clinically most aggressive one and is characterized by high expression of proliferation-related genes and low expression of luminal-related genes, such as ER and FOXA1. There is evidence

suggesting that basal-like tumours belong to a distinct type of disease that is far different from other subtypes of breast cancer and, according to its molecular features, it is more similar to squamous cell lung cancer than to luminal A or B breast cancer (Prat, Adamo et al. 2013, Hoadley, Yau et al. 2014). Notably, BRCA1 mutation is associated with basal-like phenotypes (Foulkes, Stefansson et al. 2003, Prat, Cruz et al. 2014) and the role of BRCA1 in breast cancer tumorigenesis will be discussed in the next section.

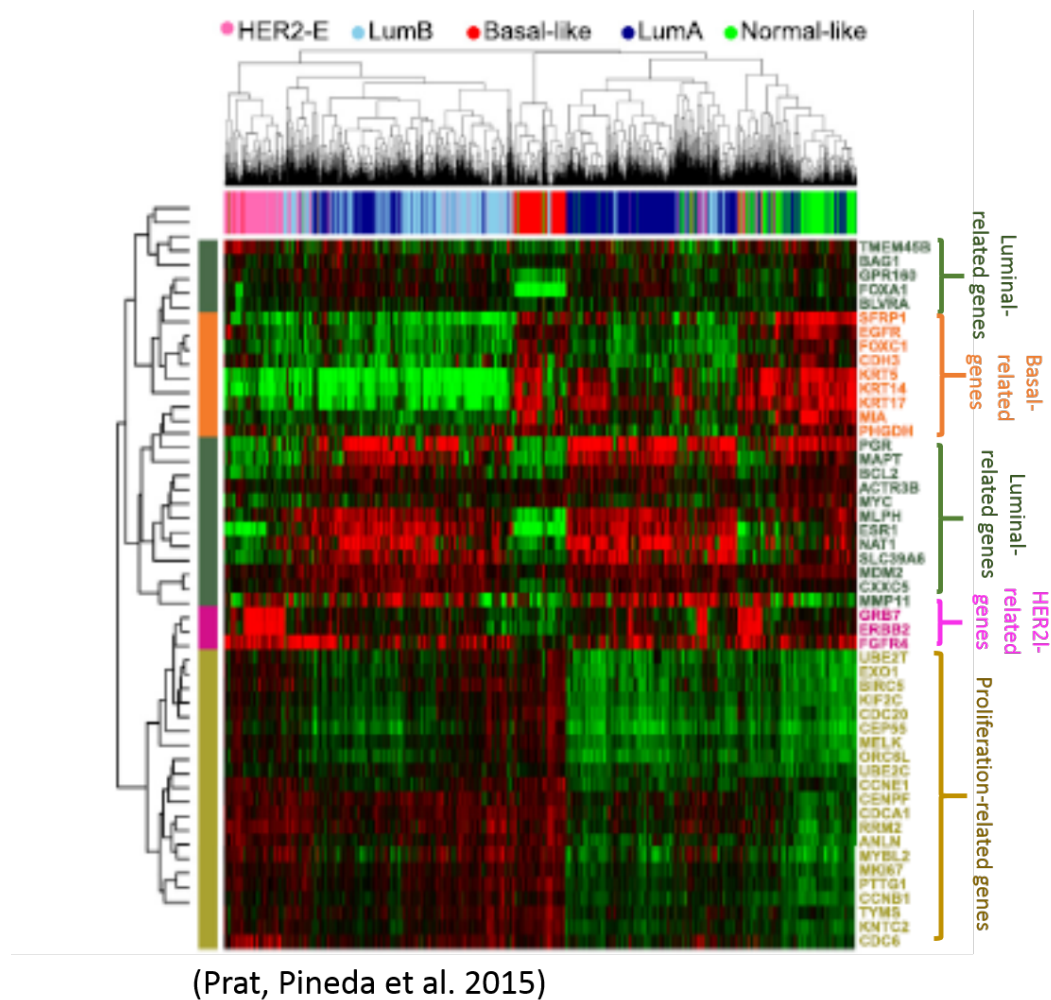


Figure 1.2 Molecular signatures of the five intrinsic subtypes of breast cancer (Prat, Pineda et al. 2015).

1.2 Breast cancer tumourigenesis

Breast tissue mainly contains two cell types: outer basal cells and inner luminal cells. The basal cells are consisted mainly of the differentiated myoepithelial cells and some mammary stem cells (Shackleton, Vaillant et al. 2006, Sleeman, Kendrick et al. 2006). The luminal cells include differentiated milk-secreting cells, hormone receptor-expressing cells and progenitors for hormone receptor-expressing cells and secretory cells (Booth and Smith 2006, Oakes, Naylor et al. 2008) . Breast cancer is thought to arise from either basal mammary stem cells or luminal progenitor cells, as these cells have long lifespan thus are prone to the accumulation of mutations/alterations required for tumour formation (Smalley and Ashworth 2003). There are several key factors that can substantially contribute to the tumourigenesis in breast.

1.2.1 BRCA1 in breast cancer tumourigenesis

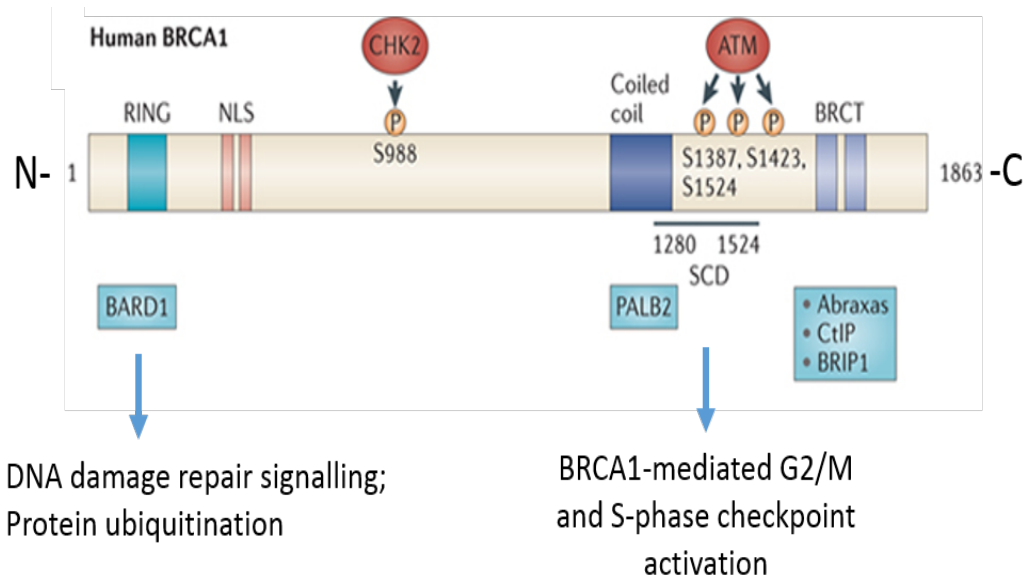
As mentioned previously, mutation or loss of BRCA1 is closely associated with both hereditary and sporadic breast cancer. BRCA1 is a master regulator of genome integrity by regulating multiple processes such as DNA replication and repair, transcriptional regulation and chromatin remodelling, thus a powerful suppressor of tumour formation (Jackson and Bartek 2009, Huen, Sy et al. 2010). The majority of breast cancers with BRCA1 germline mutations develop basal-like phenotype, however, the origin of the BRCA1-associated basal-like tumour is still under debate (Lim, Vaillant et al. 2009, Molyneux, Geyer et al. 2010, Prat, Pineda et al. 2015).

BRCA1 is a protein of 1,836 amino acids (aa) that harbours a highly conserved N-terminal RING domain and tandem BRCT domains at the C-terminal (Huen, Sy et al. 2010)) (Figure 1.3). The RING domain is a motif that is normally present in E3 ubiquitin ligases and it is important for the stable interaction of BRCA1 with BRCA1-associated RING domain protein 1 (BARD1) (Meza, Brzovic et al. 1999). Although its functions are not yet fully understood, the BRCA1-BARD1 interaction has been implicated in DNA damage signalling and DNA repair to maintain genome stability. The RING domain also interacts with E2 ubiquitin-conjugating enzymes to modulate mono- or poly-ubiquitination of multiple proteins and in turn affects their degradation, subcellular localization and/or activity (Starita and Parvin 2006, Eakin, Maccoss et al. 2007). There is a CHK2 phosphorylation site in the central region of BRCA1 protein that regulates DNA double-strand break repair functions of BRCA1 (Zhang, Willers et al. 2004). The SQ/TQ cluster domain contains approximately ten ATM phosphorylation sites which are important for BRCA1-mediated G2/M and S-phase checkpoint activation (Cortez, Wang et al. 1999, Xu, O'Donnell et al. 2002). The C-terminal BRCT domains are responsible for interactions of BRCA1 with numerous cell cycle checkpoint proteins, chromatin remodelling factors and components of transcriptional machinery (Anderson, Schlegel et al. 1998, Yarden and Brody 1999, Bochar, Wang et al. 2000).

Besides its traditional roles in DNA damage repair and maintaining genome integrity, recent experimental evidence also suggests that BRCA1 is able to suppress breast cancer tumourigenesis through regulation of other cellular processes. For instance, BRCA1 binds to promoter of TWIST and transcriptionally suppresses its expression, which in turn inhibits epithelial-to-

mesenchymal transition (EMT), mammary luminal stem cell dedifferentiation and expansion of basal-like tumours (Bai, Chan et al. 2014). Another study found that BRCA1 silencing in cancer-associated fibroblasts promotes the production of ketone bodies, a high-energy mitochondrial fuels, to metabolically accelerate cancer cell growth (Salem, Howell et al. 2012). BRCA1 can also down-regulate intracellular levels of reactive oxygen species (ROS) by binding to NRF2 (NF-E2-related factor 2) and activate NRF2-driven antioxidant signalling pathway (Gorrini, Baniasadi et al. 2013). As a result, loss of BRCA1 would result in high levels of ROS accumulation, which contribute to the perturbation of homeostasis, DNA damage, and ultimately, the initiation and progression of cancer (Waris and Ahsan 2006).

Although extensively studied, it is still not well understood why BRCA1 loss or mutation could give rise to basal-like tumours. Another recent finding highlights the role of BRCA1 in epigenetic regulation of gene expressions (Wang, Zeng et al. 2013), which might provide important clues for better understanding of BRCA1-associated tumourigenesis. In accordance with that, our previous study suggests that loss/mutation of BRCA1 contributes to suppression of FOXA1, one of the luminal markers, through an epigenetic mechanism that involves EZH2 (Gong, Fujino et al. 2014). However, the role of BRCA1 in modulating gene expression through epigenetic mechanisms and its association with breast cancer tumourigenesis is not well understood. Epigenetic alterations that cause suppression of tumour suppressors or overexpression of oncogenes have considerable impact on breast cancer tumourigenesis, which will be introduced in the next section.



(adapted from Roy, Chun et al. 2012)

Figure 1.3 Functional structure of BRCA1 (Roy, Chun et al. 2012). There are several important features in BRCA1 protein: 1) the RING domain which is responsible for interaction with BARD1; 2) the phosphorylation site by CHK2; 3) the SCD domain phosphorylated by ATM; and 4) the BRCT domain important in interaction of BRCA1 with DNA damage repair proteins, chromatin remodelling factors etc.

1.2.2 Epigenetics and epigenetic alternations in breast cancer tumourigenesis

Epigenetic alterations refer to the changes that could switch genes on and off and affect how genes are read and transcribed without affecting DNA sequences. The main epigenetic modifications include histone modification, DNA methylation and RNA mediated gene silencing (Karsli-Ceppioglu, Dagdemir et al. 2014). This section will focus on discussing histone modification and DNA methylation and their roles in breast tumourigenesis.

1.2.2.1 Epigenetics

The chromosome is a highly packed structure consisting of DNA and histones, as illustrated in figure 1.4. In the nucleus, double-strand DNA wraps around the histone core consisting of two copies of four types of histone proteins (H2A, H2B, H3 and H4) to form structural subunits of chromosome known as nucleosomes. The tails of histones allow them to be covalently modified at the post-translational level (histone modification). The vast number of nucleosomes then pack tightly into a coil that twist into another larger coil, and so on so forth, to produce highly condensed supercoiled chromatin fibres, which fold further to form loops in the chromosome.

Histone modification, including acetylation, methylation, phosphorylation, sumoylation, poly (ADP)-ribosylation and ubiquitination, are the main epigenetic mechanisms that regulates chromatin structure hence DNA accessibility and gene expression (Karsli-Ceppioglu, Dagdemir et al. 2014). Histone acetylation is catalysed by histone acetyl transferases (HATs), and causes the decondensation of chromatin and activation of gene transcription. On the contrary, histone

deacetylation is catalysed by histone deacetylases (HDACs) and generally causes chromatin condensation and gene silencing. Another type of histone modification is methylation of arginine or lysine residues, which is catalysed by histone methyltransferases (HMTs) and is carried out within the tails of histone H3 and H4 either in a mono-, di- or trimethylation state (Albert and Helin 2010). H3K4me2/3 (histone 3 lysine 4 di-/trimethylation) and H3K79me3 (histone 3 lysine 79 trimethylation) are associated with transcription activation while H3K27me2/3 (histone 3 lysine 27 di-/trimethylation) and H4K20me3 (histone 4 lysine 40 trimethylation) are involved in gene silencing (Martin and Zhang 2005, Hublitz, Albert et al. 2009).

Another important epigenetic mechanism is DNA methylation, which refers to a process of covalently addition of methyl group at cytosine residues of DNA template catalysed by DNA methyltransferases (DNMTs). Methylation or demethylation occurs at CpG islands in gene promoters, causing suppression or activation of transcription. There are eight subtypes of DNMTs that have been discovered, only three of which have catalytic methyltransferase activity: DNMT1, DNMT3a and DNMT3b (Veeck and Esteller 2010). Among the three, DNMT1 is the most abundant one with highest methyltransferase activity in mammalian cells while DNMT3a and DNMT3b are mainly expressed during embryo development and considered as *de novo* methyltransferase (Okano, Bell et al. 1999).

Traditionally, histone modification and DNA methylation were studied as two separate pathways in epigenetic regulation of gene expression. However, there has been evidence showing that the two mechanisms cooperate to silence gene

expression. It is found that EZH2 (Enhancer of Zeste homolog 2), a H3K27 methyltransferase, interacts with DNMTs and is associated with DNMTs activity *in vivo* and, therefore, it was suggested that EZH2 serves as a recruiting platform for DNMTs in gene silencing (Vire, Brenner et al. 2006). Epigenetic mechanisms provide efficient and rapid regulations of gene expression by activation/suppression of transcription and they are often altered during breast cancer tumourigenesis, which will be discussed in the next section.



Figure 1.4 Chromosome is a highly packed structure consisting of DNAs and histones (David Sadava from Scitable by nature EDUCATION).

1.2.2.2 Epigenetic alternations in breast cancer tumourigenesis

Epigenetic mechanisms often get disturbed during breast cancer tumourigenesis. Breast cancer frequently displays hyper-methylation at DNA promoters of specific genes. Examples of these genes include ER, PR, retinoic acid receptor- β (RAR β), and BRCA1 (Widschwendter and Jones 2002, Yamashita, Tokunaga et al. 2015). Silencing of RAR β leads to cell depolarization and dedifferentiation and transformation of mammary epithelial cells (Bistulfi, Pozzi et al. 2006). BRCA1 silencing by hypermethylation can lead to impaired DNA damage repair and continuous accumulation of mutations which could ultimately lead to the onset of breast cancer, as introduced previously. Investigation into the roles of histone modifications during breast cancer tumourigenesis are relatively new compared to studies on DNA methylation and there are only limited studies about histone modification patterns in breast cancers (Karsli-Ceppioglu, Dagdemir et al. 2014). Changes in histone modification status during tumourigenesis could result in closing of chromatin that was previously actively transcribed in normal cells or opening of chromatin that was condensed in normal cells (Lo and Sukumar 2008), producing overall effects that are similar to DNA methylation—the altered gene transcription.

The epigenetic alterations often arise from aberrant expression or activity of the catalytic enzymes such as DNMTs, HADCs and HMTs. For example, DNMT1 is found to be expressed at higher levels in breast tumours and is essential for mammary stem cell maintenance and tumourigenesis (Pathania, Ramachandran et al. 2015). In a cohort of 130 sporadic breast cancer patients, DNMT3b is found to be overexpressed in 30% of the patients (Girault, Tozlu et al. 2003). Protein

expression of HDAC1 is increased in hormone receptor-positive breast tumours while HDAC2 overexpression is associated with higher-grade tumours, and HDAC3 overexpression is correlated with both hormone receptor-negative tumours and higher-grade tumours (Muller, Jana et al. 2013). Elevated expression of EZH2, a catalytic subunit of polycomb repressive complex 2 (PRC2) that is responsible for trimethylation of H3K27 as a methyltransferase, is associated with aggressiveness of breast cancer (Kleer, Cao et al. 2003). Another study shows that BRCA1 activity is regulated by EZH2 in benign breast cells and EZH2 overexpression in the non-transformed mammary epithelial cell line MCF10A resulted in aberrant mitoses and genomic instability (Gonzalez, DuPrie et al. 2011). More interestingly, BRCA1 can interact with EZH2 and inhibit its catalytic activity in inducing trimethylation of H3K27 in epigenetic silencing (Wang, Zeng et al. 2013). Although there is emerging evidence to suggest interactions between BRCA1 and epigenetic mechanisms, the roles of such interaction in breast tumourigenesis and aetiology are still not well understood.

As there are massive epigenetic alterations during breast cancer tumourigenesis, targeting epigenetic mechanisms is considered as attractive therapeutic approach. For example, 5'-azacitidine-decitabine (5'-aza-dC), a DNMTs inhibitor, has been shown to be able to inhibit DNA methylation, breast cancer cell growth and re-express silenced genes such as ER and E-cadherin (Ferguson, Lapidus et al. 1995, Graff, Herman et al. 1995). An inhibitor of EZH2, 3-Deazaneplanocin A, can deplete EZH2 levels and induce apoptosis in breast cancer cells without affecting normal cells (Tan, Yang et al. 2007). GSK126 is another selective EZH2 inhibitor which can restrict tumour growth and increase survival in a mouse model of diffuse large B-cell lymphoma (McCabe, Ott et al. 2012).

1.2.3 Hormones in breast cancer carcinogenesis

As introduced previously, accumulative exposure to hormones is closely associated with higher breast cancer risk. Two hormones, namely oestrogen and progesterone, are of great importance to breast development as well as breast cancer carcinogenesis. During breast development, oestrogen is required for pubertal ductal elongation (Mallepell, Krust et al. 2006), while progesterone is essential for side branching of ducts and alveologenesis in mammary epithelium (Briskin, Park et al. 1998).

Progesterone is an endogenous steroid hormone that is mainly produced in the ovary by the corpus luteum starting from the onset of puberty. In the breast, it binds to progesterone receptor (PR) and regulates a range of signalling pathways that are important in cell proliferation, stem cell activation and tumour-promoting (Briskin, Hess et al. 2015). PR can promote cell proliferation through stabilizing the mRNA of receptor activator of NfκB ligand (RANKL) and enhancing its expression (Tanos, Sflomos et al. 2013), as RANKL acts as a mitogen that promotes tumour formation (Gonzalez-Suarez, Jacob et al. 2010). PR also regulates the expression of Wnt4, which acts as a central activator of mammary epithelium stem cells and of their niches and is exclusively transcribed in PR positive cells (Briskin, Hess et al. 2015, Rajaram, Buric et al. 2015). In addition, PR can physically interact with cyclinD1 to form transcription complexes that cooperatively induces cell-cycle dependent transcriptional programmes in breast cancer cells (Dressing, Knutson et al. 2014).

Early studies have already shown that repeatedly activation of PR signalling pathway, such as the use of combined hormone therapy can promote breast

cancer tumorigenesis, as chemically (7,12-Dimethylbenzanthracene) induced carcinogenesis can be enhanced by progesterone administration (Jabara, Marks et al. 1979, Aldaz, Liao et al. 1996). The tumour-promoting effects of PR-signalling can be achieved through: 1) its interaction with cyclinD1 to enhance cell cycle progression, 2) regulating the expression of RNAKL to promote early pathogenesis of mammary carcinoma (Gonzalez-Suarez, Jacob et al. 2010) and/or 3) regulating of Wnt4 expression as well as Wnt singling pathway, as illustrated in figure 1.5. Collectively, these different modes of regulation would result in enhanced cell mitosis/proliferation, activation of stem cells and ultimately increased risk for breast cancer.

PR is also an oestrogen-target gene which is often co-expressed with oestrogen receptor (ER), with some exceptions where PR is found to be expressed independent of ER (Hilton, Doan et al. 2014, Hilton, Santucci et al. 2014). In breast, oestrogen binds to ER and activates the downstream signalling pathways. It is generally accepted that ER plays a more significant role in breast cancer than PR as it can regulate expression of PR and other key pathways during tumourigenesis and disease progression. ER is also an important factor for the selection of appropriate treatments and for predicting prognosis. The roles of oestrogen, ER and ER-signalling pathways will be introduced in details in the next section.

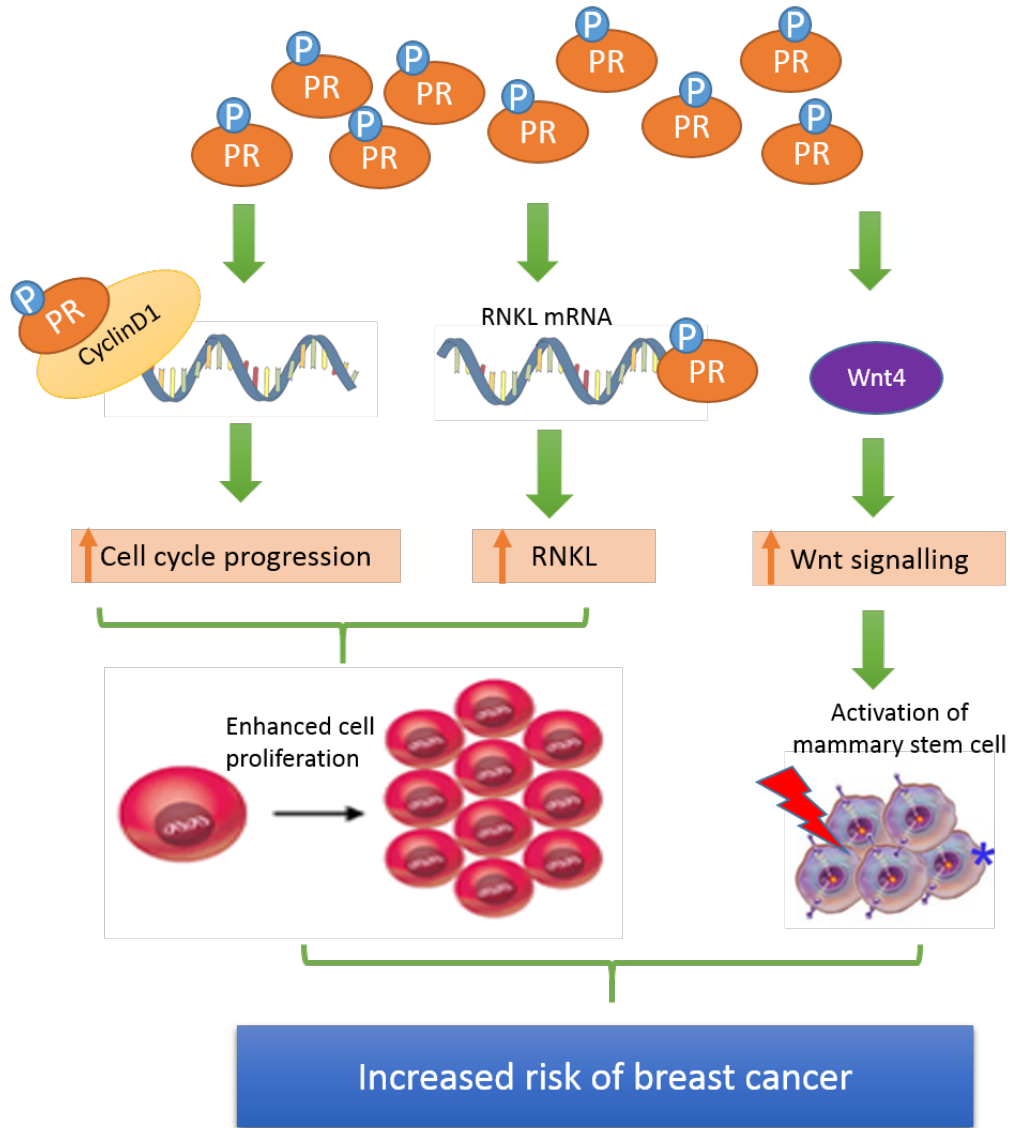


Figure 1.5 The proposed mechanisms explaining tumour-promoting effects of PR-signalling. Activation of PR signalling pathway can promote the cyclinD-dependent cell cycle progression and the expression of RNKL protein, which collectively contribute to enhanced cell proliferation. PR can also regulate expression Wnt4, which would subsequently activates Wnt signalling and mammary stem cell. P: progesterone. PR: progesterone receptor.

1.3 Estrogen and estrogen receptor signaling

1.3.1 Oestrogen

Oestrogen is the primary sex hormone in female that is important in regulating development of the reproductive system and secondary sex characteristics. There are three forms of oestrogens naturally occurring in women: estrone (E1), estradiol (E2), and estriol (E3). E2 is the predominant oestrogen with the highest serum level and estrogenic activity throughout reproductive years and, therefore, is used in biomedical research to study the oestrogen-signalling pathway. Circulating oestrogen level can be affected by menstrual cycle, pregnancy, lactation, menopause and body mass index (Ruggiero and Likis 2002).

The physiological functions of oestrogen are organ-dependent. In breast, it stimulates proliferation of glandular and ductal tissues as well as alveolar growth, contributing to breast tissue development in puberty (Warner, Nilsson et al. 1999). Oestrogen exerts its effects on cells that expression oestrogen receptors by binding to the receptors and activating the downstream signalling pathways.

1.3.2 Oestrogen receptor

Oestrogen receptors (ER) are the receptors that are generally located in the nucleus of cells and bind with oestrogen. They are ligand-inducible transcription factors consisting of two subtypes: ER α and ER β , and they regulate transcription of genes involved in cell proliferation and/or differentiation (Leclercq 2002).

ER α and ER β share structural similarity consisting of five functional domains encoded by eight exons (Fig. 1.6). The five domains from N-terminal to C-terminal are: 1) activation domain 1 (AF1) that is able to activate gene transcription without ligand (Tora, White et al. 1989); 2) DNA-binding domain (DBD) that determines the specific recognition of the oestrogen response element (ERE) in DNA (Kumar, Green et al. 1987); 3) D domain that contains a nuclear localisation signal (NLS) and is responsible for signalling nuclear localization of ER (Zilli, Grassadonia et al. 2009); 4) ligand-binding domain (LBD) that contains the activation function 2 (AF2) and binds to ligand; 5) the C-terminal F domain whose functions are not yet fully understood but are suggested to be modulation of ligand binding and transcription activation (Skafar and Zhao 2008).

The distribution and functions of ER α and ER β are different. ER β is found to be the predominant form in normal mammary gland and benign breast disease while dominant ER α expression seems to be the characteristics of breast cancer (Roger, Sahla et al. 2001, Shaw, Udokang et al. 2002). Studies showed that ER β is likely to function as a tumour suppressor in breast cancer, as overexpression of ER β induces G2 cell cycle arrest and inhibits oestrogen-induced proliferation of ER α -positive breast cancer cells (Paruthiyil, Parmar et al. 2004, Strom, Hartman et al. 2004) and is associated with better prognosis (Lazennec 2006). On the contrary,

ER α activation causes enhanced cell proliferation and is associated with breast cancer tumourigenesis. Therefore, inhibiting estrogenic activity of ER α has been employed as an anti-breast cancer therapeutic stratagem over the last two decades. Moreover, due to its dominant expression in breast cancer, clinical evaluation of ER expression by immunohistochemistry is only on ER α expression. This thesis will focus on ER α and ER α will be referred to as “ER” in short throughout the thesis if not otherwise specified. The functions of ER-signalling and its roles in breast cancer tumourigenesis will be introduced in the following section.

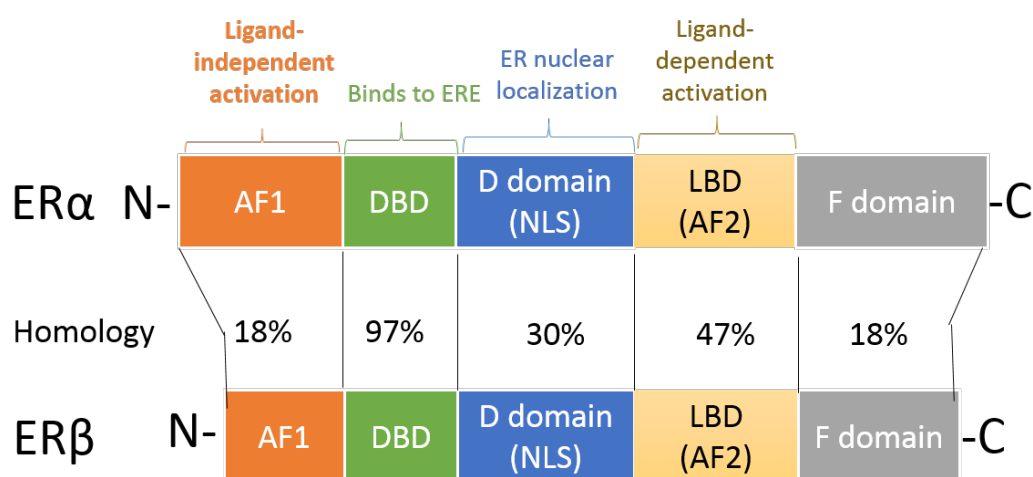


Figure 1.6 Schematic representation of ER α and ER β protein structures. Homology of each functional domain is shown as percentage.

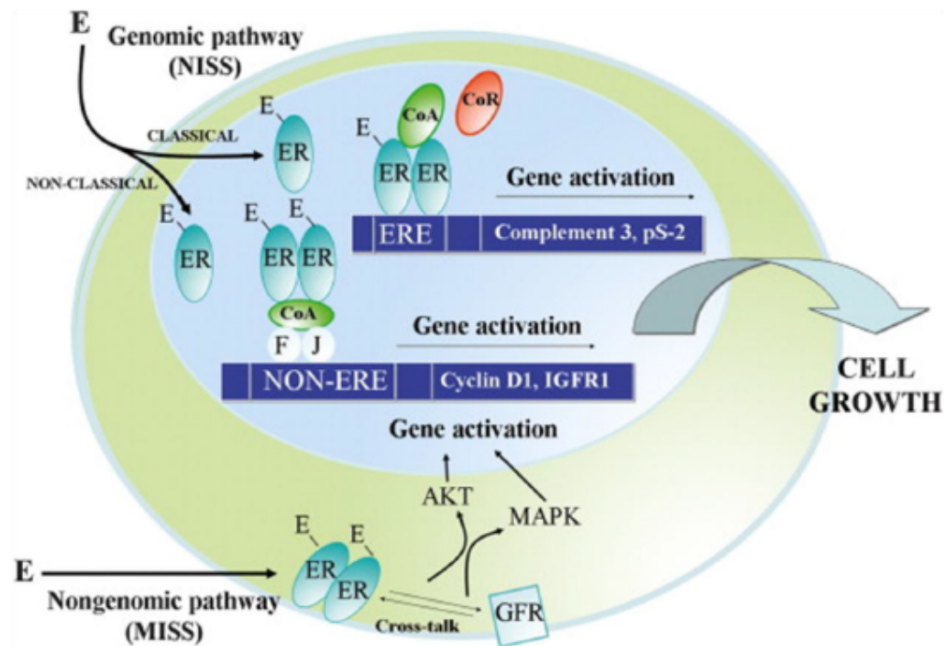
1.3.3. Oestrogen receptor signalling and its role in breast cancer tumourigenesis and progression

ER is activated by binding of oestrogen to its LBD, which induces a series of modifications including conformational changes, phosphorylation, dissociation from the chaperon proteins and homo, or hetero-dimerization (Osborne, Schiff et al. 2001). The activated ER dimers then dynamically and sequentially recruit various regulatory protein complexes (co-activators or co-repressors) to act through two pathway ways: the genomic pathway and non-genomic pathway (Zilli, Grassadonia et al. 2009), as illustrated in figure 1.7.

In the genomic pathway, the oestrogen-ER complex can then regulate gene expression through the “classical pathway” by direct binding to ERE in the promoter regions and promoting the transcription of oestrogen-targeted genes. ER signalling can also modulate transcription of genes lacking ERE in their promoters through “non-classical pathway” in which ER binds to other transcription factors that recognize alternative regulatory DNA sequences in the promoters, e.g. cyclinD1 and insulin-like growth factor-1 receptor (IGFR1) (Eeckhoute, Carroll et al. 2006, Maor, Mayer et al. 2006).

In the non-genomic pathway, ER signalling promotes gene expression indirectly through binding to the p85 subunit of phosphoinositide-3-kinase (PI3K) and adaptor proteins and activating PI3K as well as the mitogen-activated protein kinase (MAPK) pathway, which would further activates downstream protein kinase B (AKT) to promote cell proliferation and survival (Simoncini, Hafezi-Moghadam et al. 2000, Castoria, Migliaccio et al. 2001). The crosstalk between

genomic and non-genomic ER signalling pathways can further amplify its effects on cell survival and proliferation (Kelly and Levin 2001).



(Adapted from Zilli, et al., 2009)

Figure 1.7 The genomic and non-genomic pathway of ER-signalling (Zilli, Grassadonia et al. 2009).

The tumorigenic role of ER signalling largely relies on its role in transcriptional activation of various genes that are important in cell cycle progression, proliferation and apoptosis. For instance, binding of oestrogen to ER could stimulate the expression of cyclinD1, one of the master regulators of cell cycle progression (Eeckhoute, Carroll et al. 2006, Yamaga, Ikeda et al. 2013). ER signalling also transcriptionally regulates the expression of the anti-apoptotic

Bcl2, which contributes to apoptosis resistance and cell survival in various types of cancer (Romano, Adriaens et al. 2010). ER signalling can also suppress the expression of cyclin-dependent kinase (CDK) inhibitor p21^{Waf1/Cip1} (Musgrove, Caldon et al. 2011). In addition, the roles of ER signalling in mediating EMT and breast cancer metastasis have been frequently reported. ER can bind to the promoter and regulate expression of metastasis tumour antigen 3 (MTA3), a gene implicated in EMT (Fujita, Jaye et al. 2003). ER also transcriptionally regulates the epithelial adhesion molecule E-cadherin, which is implicated in the early stages of metastatic dissemination (Oesterreich, Deng et al. 2003, Onder, Gupta et al. 2008). There is also emerging evidence showing that non-genomic ER signalling pathway could promote tumourigenesis through regulating antioxidant pathway. Activation of PI3K-AKT pathway by ER signalling can potentiate NF-E2-related factor 2 (NRF2) in BRCA1-deficient cells to induce expression of antioxidant genes, protecting cells from reactive oxygen species-induced cell death and sustaining the BRCA1-deficient tumour (Gorrini, Gang et al. 2014).

As a result, targeting oestrogen signalling pathway to inhibit breast cancer progression, known as endocrine therapy, has been a well-established therapeutic approach for women who are ER positive, which will be introduced in greater details in the next section.

1.4 Breast cancer treatment regimes

There are mainly five treatment options available for breast cancer patients including surgery, chemotherapy, radiotherapy, endocrine therapy and targeted therapy, which can be employed alone or in combination. The treatment regimen is usually determined according to the stage of the disease, the size of the tumour, the receptor status (ER, PR and HER2) of the tumour and the health condition of the patient.

1.4.1 Surgery

Surgery is the most common breast cancer therapy, in which either the entire breast (mastectomy), only the area of cancer (lumpectomy or wide local excision) or a quarter of the breast tissue (quadrantectomy) is removed by operation. The latter two types of surgeries are referred to as breast conserving surgery. The type of the surgery is selected according to the size of the cancer and of patient's breast, if the cancer has metastasized and patient's personal wishes. Axillary lymph nodes may also be removed during the surgery if there is suspected metastasis.

The removed tumours, surrounding breast tissues as well as the lymph nodes (if any) are sent to the pathological laboratory for determination of the histological type, stage, grade, receptor status as well as lymph node metastasis, which provide valuable information for designing treatment regimen to control the disease progression or prevent relapse/metastasis.

1.4.2 Radiotherapy

Radiotherapy, also commonly referred to as radiation therapy, is the therapy that uses high-energy radiation (photons or charged particles) to cause direct or indirect ionization of atoms in DNAs of the exposed tissues and, ultimately, cell death. Radiotherapy is usually well-tolerated, with side effects such as skin redness, itching and soreness occurring only at the radiated areas.

Radiotherapy is often applied to patients who undergo breast conserving surgery to remove the remaining cancer cells as much as possible. However, for patients who receive mastectomy, the benefits of post-mastectomy radiotherapy (PMRT) are under debate (Oliai and Hurvitz 2015). Although the benefits of reducing locoregional and overall recurrence with PMT were observed, there has not been proven benefits in promoting overall survival of the patients (Ebcctg, McGale et al. 2014, Chapman and Jagsi 2015). Moreover, the meta-analysis performed does not stratify the patients by biological subtypes, which is known to closely correlate with risk of locoregional recurrence (Lowery, Kell et al. 2012).

Comparing with radiotherapy, systemic therapies including chemotherapy, endocrine therapy and targeted therapy have well-recognised benefits in preventing disease recurrence and improving patients' survival, and are more commonly prescribed to patients.

1.4.3 Chemotherapy

Chemotherapy is a systemic therapy that utilises cytotoxic agents to either stop cancer cell proliferation or kill the cells. It can be applied before surgery (neoadjuvant) to reduce the size of the tumour or after surgery (adjuvant) to prevent disease recurrence. It is often the sole option for patients with advanced-stage metastatic disease in order to destroy cancer cells as much as possible.

There are four types of common chemotherapeutic agents including taxanes, alkylating agents, antimetabolites and anthracyclines. Functions of these agents and examples are given in table 1.1. The chemotherapeutic drugs can be administered alone or, more frequently, in combination to achieve better treatment outcomes. Examples of the common FDA-approved combinational chemotherapeutic agents include: FEC (Fluorouracil, Epirubicin Hydrochloride, Cyclophosphamid), CMF (Cyclophosphamid, Methotrexate, Fluorouracil) and TAC (Docetaxel, Doxorubicin hydrochloride, Cyclophosphamid).

The benefits of chemotherapy, including to prolong survival months, delay the progression of breast cancer and improve quality of life are well-documented whilst the side-effects cannot be overlooked. Because the chemotherapeutic agents are not cancer-specific, they cause considerable amount of damages to normal cells, resulting in common side effects, such as hair loss, nausea, vomiting, loss of appetite, and fatigue (Bacic, Druzijanic et al. 2010). Chemotherapy-induced peripheral neuropathy also frequently occurs in early-stage breast cancer and is strongly associated with docetaxel-based regimen (Pereira, Fontes et al. 2015). Moreover, resistance to chemotherapy is a common problem seen in the clinic, which could arise from alterations in drug

transportation, activation of anti-apoptotic and cell-survival pathways, presence of cancer stem cells or induced EMT (Chuthapisith, Eremin et al. 2010, Pinto, Widodo et al. 2013).

Besides chemotherapy, endocrine therapy is another treatment option for patients who are oestrogen receptor positive, and will be introduced in the next section.

Table 1.1 Functions of chemotherapeutic agents and examples.

Category	Functions	Examples
Taxane	Disrupt microtubule functions during mitosis and inhibit cell division	Paclitaxel, docetaxel
Alkylating agents	Crosslink DNA strands and cause abnormal base pairing	Cisplatin, cyclophosphamide
Antimetabolites	Incorporate into DNA/RNA and interfere with cell division	Methotrexate, 5-fluorouracil
Anthracyclines	Intercalate between DNA/RNA strands to form free radicals to break the strands, inhibit topoisomerase enzyme and impair DNA replication/transcription	Doxorubin, epirubicin

1.4.4 Endocrine therapy

Endocrine therapy aims to block the pro-proliferative ER-signalling pathway in ER-positive patients to suppress cancer cell growth. This can be achieved through either stopping oestrogen synthesis or blocking oestrogen binding to the receptors. Since ovary is the primary organ that synthesizes oestrogen in premenopausal women, ovary ablation either by surgical removal of ovary (oophorectomy) or using chemical inhibitors can stop oestrogen synthesis. Luteinising hormone (LH) blocker (e.g. Zoladex) is used in pre-menopausal women to block the hormone produced in the pituitary gland that would stimulate release of oestrogen from ovary. Aromatase inhibitor (e.g. anastrozole, letrozole) is used in post-menopausal women to inhibit the conversion of androgen to oestrogen in the adipose tissue. Binding of oestrogen to its receptors can be blocked by selective oestrogen receptor modulators (SERMs, e.g. tamoxifen and raloxifene) or pure oestrogen antagonists (e.g. fulvestrant). The functions of SERMs are tissue-dependent. For example, tamoxifen acts as oestrogen antagonists in breast but as agonists in uterus and bone. In comparison, fulvestrant is a pure anti-oestrogen agent which not only blocks binding of oestrogen to its receptors but also degrades ER.

Endocrine therapy can be employed before surgery (neoadjuvant) to shrink the tumours or after surgery (adjuvant). In adjuvant setting, endocrine therapy is often applied for a consecutive of 5 years after finishing chemotherapy to prevent the cancer recurrence. Recent clinical trials also suggest additional endocrine therapies such as sequential intake of tamoxifen and aromatase inhibitor may provide greater benefits to patients than taking a single drug for 5 years (Regan,

Neven et al. 2011). However, these strategies are not approved by FDA yet. Endocrine therapy can also be prescribed for prevention of breast cancer in the high-risk population. The Breast Cancer Prevention Trial has found that five-year intake of tamoxifen can reduce the risk of developing invasive breast cancer by 50% in high-risk postmenopausal women (Vogel, Costantino et al. 2006). However, although not as severe as chemotherapy, endocrine therapy usually generates side effects that vary from drug to drug, as summarized in table 1.2. Moreover, endocrine resistance is commonly reported, which causes uncontrolled disease progression and ultimately cancer relapse or metastasis. Tamoxifen, the first-line endocrine therapeutic agent, and the mechanisms associated with tamoxifen resistance will be introduced in details in the section 1.5.

Table 1.2 FDA-approved endocrine therapeutic drugs and their adverse effects.

Drug	FDA-approved use	Side effects
Tamoxifen	Cancer prevention in both pre- and post-menopausal women; treatment of metastatic breast cancer; adjuvant treatment of early-stage breast cancer in both pre- and post-menopausal women.	Risk of blood clots, stroke, increased risk of endometrial and uterine cancers; mood swing and depression
Raloxifene	Cancer prevention in post-menopausal women.	Risk of blood clots and stroke in certain patients
Fulvestrant	Treatment of metastatic breast cancer in post-menopausal women.	Gastrointestinal symptoms, loss of strength
Aromatase inhibitors	Adjuvant treatment of early-stage breast cancer in post-menopausal women; Treatment of metastatic breast cancer in post-menopausal women if tamoxifen fails.	Risk of heart attack and heart failure, bone loss, joint pain, mood swing and depression

1.4.5 Targeted therapy

In addition to systemic chemotherapy and endocrine therapy, targeted therapy is a relative new approach that aims at specifically targeting cancer cells that usually overexpress pro-proliferative cell surface marker. HER2 is the only FDA-approved therapeutic target for treatment of breast cancer, which is overexpressed in around 20% of patients. A number of drugs have been developed that target HER2, including trastuzumab, pertuzumab, and lapatinib. Trastuzumab is usually used for treatment of both early and late stage HER2-overexpressed breast cancer while pertuzumab is given to patients in combination with trastuzumab and chemotherapy for neoadjuvant therapy or the treatment of late stage breast cancer. Lapatinib is for treatment of breast cancers at advance stages when the trastuzumab no longer works. A more recent and promising approach is to conjugate the anti-HER2 drug with cytotoxic agent to enable specific recognition of cancer cells and subsequent complete destruction to achieve better treatment outcome. Ado-trastuzumab is a FDA-approved conjugated drug for the treatment of HER2-positive metastatic breast cancer patients who have previously failed trastuzumab and taxane (Lambert and Chari 2014). Because targeted therapy is more cancer-specific, it generates much milder adverse effects including weakness, diarrhoea, headaches and rashes. Heart damage is also reported but the effects are usually transient and disappear after the treatment is stopped. Although specific and effective, the number of molecular targets identified is very limited. As a result, one of the major focuses of cancer research nowadays is to identify novel molecular cancer markers with potential of being used as cancer-specific therapeutic targets.

1.5 Tamoxifen and tamoxifen resistance

1.5.1 Tamoxifen

As introduced previously, tamoxifen is the first-line endocrine therapy with the widest spectrum of FDA-approved applications. It has been a great success in reducing breast cancer recurrence as well as in lowering incidence of contralateral breast tumour (Lewis and Jordan 2005). Evidence also shows that five-year tamoxifen treatment reduces risk of developing invasive breast cancer by 55% and the protection can last for at least five years after the treatment stops (Lewis and Jordan 2005). In addition, tamoxifen treatment has been shown to be able to preserve bone density in post-menopausal breast cancer patients (Love, Mazess et al. 1992).

Tamoxifen is the SERM with tissue-dependent oestrogen agonistic or antagonist activity (McDonnell 1999). In breast, tamoxifen acts as an antagonist by competing with oestrogen for binding to AF2 of ER, inhibiting the interaction between ER and co-activators and inducing recruitment of co-repressors to suppress the transcription of oestrogen-response genes (Privalsky 2004, Nettles and Greene 2005). The outcome is suppressed oestrogen-stimulated breast cancer cell growth. In other tissues such as cardiovascular cells and bone cells, tamoxifen acts as oestrogen agonistic that could stimulate ER-signalling pathway.

1.5.2 *Tamoxifen resistance*

Tamoxifen are primarily prescribed to patients who express ER. Although tamoxifen has been a great success in preventing and treating breast cancer over the past 20 years, resistance to this drug stands out as a prominent issue. Among the 70% of ER-positive patients who are treated with tamoxifen, up to half of the patients with metastatic breast cancer fail to respond to the drug (Ring and Dowsett 2004) and one-third of the treated patients experience disease relapse (Thomas and Gustafsson 2011). A tremendous amount of the research efforts have been devoted to the understanding of tamoxifen resistance over the past decade, but yet it is not fully understood. More importantly, there has not been any clinically available biomarker that could predict tamoxifen resistance.

Tamoxifen resistance can be either “*de novo*” where ER-positive tumours initially fail to respond to the treatment or “acquired” where initial sensitive tumours stop to respond (Lewis and Jordan 2005). In the acquired resistance, a minority of the patients lose ER expression through the treatment course while the majority of them retain ER expression that is no longer responding to inhibition by tamoxifen (Johnston, Sacconi-Jotti et al. 1995, Dowsett and Haynes 2003).

The proposed mechanisms for tamoxifen resistance are summarized in table 1.3. The resistance could come from: 1) abnormality in tamoxifen metabolism; 2) aberrantly activated cellular kinase or signal transduction pathways in favour of cancer cell survival and proliferation; 3) altered expression levels or functions of ER co-regulators; 4) up- or down-regulation of microRNAs; 5) activation of breast cancer stem cells. Regardless of the number of mechanisms reported, only

one of them has been translated into a patent biomarker for predicting tamoxifen resistance.

In 2006, the FDA recommended to include information on genotyping of *CYP2D6*, a metabolic enzyme that catalyses 4-hydroxylation of tamoxifen (Dehal and Kupfer 1997, Coller, Krebsfaenger et al. 2002) to predict patient outcomes for tamoxifen treatment. However, subsequent studies have shown inconsistent and conflicting results. Some studies showed that *CYP2D6* genotype is associated with shorter relapse free survival and disease-free survival while others could not find such associations (Dezentje, Guchelaar et al. 2009, Hoskins, Carey et al. 2009). In addition, a number of studies did not find statistically significant relationships with disease recurrence. Therefore, there are as yet not enough solid data to support *CYP2D6* genotyping as a robust biomarker for predicting tamoxifen resistance.

Table 1.3 The proposed mechanisms for tamoxifen resistance.

Mechanism	Target	Reference
Abnormal drug metabolism	Mutations in CYP2D6	(Dieudonne, Lambrechts et al. 2009)
	Mutations in ABCC2	(Kiyotani, Mushiroda et al. 2010)
Aberrantly activated cellular kinase or signal transduction pathways	Overexpression of HER2	(Cui, Germer et al. 2012)
	Nuclear factor- κ B	(Yde, Emdal et al. 2012)
	Overexpression of tyrosine-protein kinase Src	(Larsen, Laenkholm et al. 2015)
	Activation of PI3K/AKT pathway	(Campbell, Bhat-Nakshatri et al. 2001, Clark, West et al. 2002)
Altered expression or functions of co-regulators	Down-regulation of NCOR1	(Cottone, Orso et al. 2001)
	Overexpression of AIB1	(Osborne, Bardou et al. 2003)
	Alternative splicing of NCOR2	(Zhang, Gong et al. 2013)
Change in expression levels of microRNAs	Overexpression of miR-221/222	(Zhao, Lin et al. 2008)
	Over-expression of miR-301	(Shi, Gerster et al. 2011)
	Down-regulation of miR-342	(Cittelly, Das et al. 2010)
	Down-regulation of miR-574-3p	(Ujihira, Ikeda et al. 2015)
Breast cancer stem cell activation	JAG1-NOTCH4-dependent breast cancer stem cell activation	(Simoes, O'Brien et al. 2015)
	Over-expression of Nanog, Oct3/4 and Sox2	(Arif, Hussain et al. 2015)

1.6 Research initiative

Breast cancer is the most common cancer among women. Breast cancer tumourigenesis could be due to loss/mutation of BRCA1, altered epigenetic regulations or accumulated exposure to hormones that subsequently activates multiple pro-proliferative pathways. BRCA1 is one of genes that are the most intensively studied in the context of breast tumourigenesis and breast cancer aetiology, yet the mechanisms are not well understood.

Tamoxifen has been the first-line treatment option for breast cancer for over 20 years but the drug resistance significantly compromises its clinical efficacy in preventing cancer progression and improving patients' survival. Although there has been huge amount of research work trying to address the mechanism underlying tamoxifen resistance, it is still not fully understood. More importantly, there is no clinical in-use biomarker that can reliably predict tamoxifen response. Therefore, patients who are *de novo* resistant to tamoxifen cannot be distinguished, resulting in sub-optimised treatment regimen, which costs time, money and, in the worst cases, patients' lives.

The aim of my PhD projects is to characterize novel molecular pathways that are involved in breast cancer tumourigenesis/progression and tamoxifen resistance. The thesis will focus on two research gaps identified from the literature review: 1) the role of BRCA1-mediated epigenetic regulation of gene expression and its potential role in the breast cancer tumourigenesis/progression; 2) identifying biomarker that can be used for prediction of tamoxifen resistance.

In chapter 3, regulation of tumour suppressor FOXO3a by BRCA1 through EZH2-mediated epigenetic mechanism is studied. The findings unveil a novel regulatory mechanism of FOXO3a expression and could contribute to the better understanding of the tumour suppressor role of BRCA1 in breast cancer.

In chapter 4, the role of a splice variant of NCOR2 in conferring tamoxifen resistance is studied both *in vitro* and *in vivo* and the mechanisms are also explored. Its efficacy in predicting tamoxifen resistance in breast cancer patients is also evaluated. The results suggest that this splice variant of NCOR2 is a very promising biomarker for prediction of tamoxifen resistance after surgery and prior to when tamoxifen treatment starts.

Chapter 2 Materials and Methods

2.1 Cell culture

Human breast cancer cell lines MCF7 and ZR-75-1 were purchased from American Type Culture Collection (ATCC) and were re-authenticated by short tandem repeat profiling (Zhang, Gong et al. 2013). MCF7 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) and ZR-75-1 was cultured in Improved Minimum Essential Medium (IMEM) supplemented with 5% FBS and 1% P/S. LCC2 and AK-47 are the two tamoxifen resistant cell lines derived from MCF7 and ZR-75-1, respectively and were kindly provided by Dr. Robert Clarke (Georgetown University Medical School, Washington, D.C.) (Wong, Dai et al. 2006) and have been used in our previous study (Zhang, Gong et al. 2013). LCC2 and AK-47 were both cultured in IMEM supplemented with 5% charcoal-stripped FBS and 1% P/S. The human breast carcinoma cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468 and HCC70 with either low wildtype BRCA1 expression or mutated BRCA1 expression (Lehmann, Bauer et al. 2011) were originated from the American Type Culture Collection ((LGC standards, Middlesex, UK) and were authenticated by Cancer Research UK (London, UK). MCF7, MDA-MB-231 and MDA-MB-468 cell lines were maintained in DMEM (Sigma-Aldrich, Poole, UK) supplemented with 10% fetal calf serum (FCS) (First Link Ltd, Birmingham, UK), 4 mM glutamine and 100 U/ml P/S (Sigma-Aldrich, UK). MDA-MB-436 cells were maintained in

Leibovitz's L-15 medium (Gibco) supplemented with 10% horse serum (Invitrogen), while HCC70 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FCS, 4 mM glutamine and 100 U/ml P/S (Sigma-Aldrich, UK). HEK 293FT was cultured in DMEM supplemented with 10% FBS and 1% P/S. All the cells were cultured in 37 °C incubator supplied with 5% CO₂. The cell lines that were used in this thesis and their characteristics are summarized in Table 2.1.

Table 2.1 Cell lines used in the thesis and their characteristics.

Cell line	Characteristics
MCF-7	Human breast cancer cell line; Luminal-type, BRCA1-wild type, ER-positive and tamoxifen sensitive
ZR-75-1	Human breast cancer cell line, ER-positive and tamoxifen sensitive
LCC2	Human breast cancer cell line derived from MCF-7, ER-positive, tamoxifen resistant
AK-47	Human breast cancer cell line derived from ZR-75-1, ER-negative, tamoxifen resistant
HCC70	Human breast cancer cell line; basal-type, low BRCA1 wild-type, ER-negative
MDA-MB-231	Human breast cancer cell line; basal-type, low BRCA1 wild-type, triple-negative
MDA-MB-436	Human breast cancer cell line; basal-type, mutated BRCA1, ER-negative
MDA-MB-468	Human breast cancer cell line; basal-type, mutated BRCA1, ER-negative
HEK 293FT	Human embryonic kidney cells; ER-negative, used for lentiviral transfection

2.2 Chemicals and Plasmids

Tamoxifen and 17- β -estradiol (E2) were purchased from Sigma and were dissolved in ethanol for *in vitro* assay. For *in vivo* assay, tamoxifen was dissolved in ethanol and diluted in peanut oil (Sigma). MG132 was purchased from Sigma and was dissolved in dimethyl sulfoxide (DMSO). EZH2 inhibitor GSK126 was purchased from Millipore and was dissolved in DMSO. 5'-aza-dC was purchased from Sigma and was dissolved in DMSO.

Lentiviral vector pCDH-CMV-MCS-EF1-Puro-GFP-6xHis-BQ323636.1 was cloned and constructed by Dr. Tori Chan, the previous lab manager in Department of Pathology, the University of Hong Kong. PCMV6-Entry-NCOR2 plasmid as well as the pCMV6-Entry empty vector were purchased from OriGene. BRCA1-HA and BRCA1-mutant plasmid was a kind gift from Dr. Suhwan Chang (Department of Biomedical Sciences, Asan Medical Center, 388-1 Pung-nap2 Dong Songpa Gu, Seoul, Korea). BRCA1-mutant plasmid bears a cancer-predisposing point mutation C61G in the RING finger domain of BRCA1 which impairs the interaction between BRCA1 and BRAD1, and is generated by site directed mutagenesis. The luciferase reporter ERE-TK-Luc was a gift from Dr. Carolyn Smith (Baylor College of Medicine, Houston, TX). The maps for the vectors are attached in Appendix II. The plasmids were expanded in *E. coli* and purified using either Mediprep kit (QIAGEN) or Maxiprep Kit (Invitrogen) according to manufacturers' protocol

2.3 Total RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA was extracted from the cells using either TRIzol reagent (Invitrogen) or RNeasy Kit (QIAGEN). For the TRIzol protocol, cells seeded in 6-well plate were lysed in 1ml TRIzol at room temperature for 5 min. Two hundred microliter of chloroform was added and the samples were centrifuged at 13,000xg for 15 min at 4⁰C. The upper phase was saved, added with 500μl of isopropanol and allowed for precipitation at -20⁰C for overnight. On the second day after centrifugation, the RNA pellets were washed twice with ice-cold 70% ethanol, air-dried and dissolved in diethylpyrocarbonate (DEPC)-treated water. RNA concentration was measured by NanoDrop (Invitrogen). For the RNeasy Kit protocol, total RNA was extracted according to manufacturer's instruction. RNA was eluted with DEPC-water and concentration was measured by NanoDrop (Invitrogen).

After RNA extraction, the concentration of RNAs were measured using Nanodrop. Up to 2 μg of total RNAs were reverse transcribed into cDNA by SuperScript III reverse transcriptase (Invitrogen) following manufacturer's protocol. In brief, RNA was mixed with 1μl of dNTPs and 1μl of Oligo dT or random hexamer (Invitrogen) and DEPC-treated H₂O was added to bring the final volume to be 13μl. After incubation at 65°C for 5 minutes, 1μl of DTT, 1μl of RNaseOUT recombinant RNase inhibitor, 4μl of 5x first Strand buffer and 1μl of SuperScript III RT enzyme were added into the mixture and further incubated as

following: 25°C for 5 minutes, 42°C for 90 minutes, 75°C for 15 minutes and hold at 15°C. After cDNA synthesis, cDNAs were diluted 10-fold using H₂O.

Real-time quantitative PCR (RT-qPCR) reaction was carried out with the ABI 7900HT Fast Real-time PCR system (Applied Biosystems). Each reaction consisted of 10µl of 2x Power SYBR Green PCR Master Mix (Applied Biosystems), 2µl of cDNA template, 0.5µl of 10µM forward and reverse primer and H₂O. The final volume per reaction was 20µl. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or ribosomal protein L19 was used as housekeeping genes for normalization and the data were calculated either by $\Delta\Delta C_t$ method or absolute quantification using standard curve. The primer sequences used were presented in Table 2.2.

Table 2.2 Sequences of the primers used in this thesis

Gene	Sequence of the primer
BCL2	Forward: 5'-AAGATTGATGGGATCGTTGCC-3' Reverse: 5'-TATTCCAATTCCTTTTCGGATCTT-3'
BQ323636.1	Forward: 5'- AAGGTGGAGCGCATCGAGAAC-3' Reverse: 5'-GCATCTGCTTCTCCAGGTTCTCTG-3'
BRCA1	Forward: 5'-GCGGACTCCCAGCACAGA-3' Reverse: 5'-GCTTCTTCCATTCTTTTCTCTCACACA -3'
Cyclin D1	Forward 5'-TCCTCTCCAAAATGCC AGAG-3' Reverse 5'-GGCGGATTGGAAATGAACTT-3'
ER α	Forward: 5'-TGATCAGGTCCACCTTCTAGAATG-3' Reverse: 5'-CGCCAGACGAGACCAATCAT-3'
EZH2	Forward: 5'-ATGCGACTGAGACAGCTCAA -3'

	Reverse: 5'-CGCTGTTTCCATTCTTGGTT-3'
FOXO3a	Forward: 5'-TCTACGAGTGGATGGTGC GTT-3'
	Reverse: 5'-CGACTATGCAGTGACAGGTTGTG-3'
FOX M1	Forward: 5'-TGCAGCTAGGGATGTGAATCTTC-3'
	Reverse: 5'-GGAGCCCAGTCCATCAGAACT-3'
GATA3	Forward 5'-CCGAGGGGCTGGTTTCCTTGA-3'
	Reverse 5'-GACTCTGCAATTCTGCGAGC-3'
GAPDH	Forward: 5'-GAAGGCTGGGGCTCATTT-3'
	Reverse: 5'-CAGGAGGCATTGCTGATGAT-3'
L19	Forward: 5'-GCGGAAGGGTACAGCCAAT-3'
	Reverse: 5'-GCAGCCGGCGCAAA-3'
NCOR2	Forward: 5'-ACGAGGTGTCAGAGATCATCGA-3'
	Reverse: 5'-TGATGAACTTGATGCGCTGCT-3'
pS2	Forward: 5'-GAGAACAAGGTGATCTGCGCCC-3'
	Reverse: 5'-ACGAACGGTGTCGTCGAAACAG-3'
ChIP primers	
Primers 1	Forward: 5'-AGCCGAAGACAGCACAGACT-3'
	Reverse: 5'-TGCGTGCGTTTGTTTATGTT-3'
Primers 2	Forward: 5'-TTTGTACGCGTCGAACTAGC-3'
	Reverse: 5'-TCAACGACCCCATACACAAA-3'
Primers 3	Forward: 5'-ACTTGAAGCCGAGTTGTGGT-3'
	Reverse: 5'-GGGGCTCTGACTGCTTACTG-3'
Primers 4	Forward: 5'-GAATGCTGGCATTTCCTCTC-3'
	Reverse: 5'-CACAAATTGCCTCGACCTTT-3'

2.4 Western blot analyses

Cells were lysed in 1x Cell Signalling buffer (Cell Signalling) supplemented with protease inhibitor (Roche) and phenylmethanesulfonyl fluoride (PMSF) by incubating on ice for 10 min. Supernatant was collected after centrifuging at the 13,000xg for 10 min and protein concentration was measured using either DC protein assay or BCA protein assay protocol. The absorbance of samples was measured by microplate reader (Tecan) and concentrations were calculated according to the standard curve obtained from serial dilution of BSA. Samples were diluted to the equal concentration using the cell lysis buffer, added with sodium dodecyl sulphate (SDS) loading dye, denatured at 95°C for 5mins and stored at -20°C for western blot analyses.

In western blot analysis, SDS-polyacrylamide gels were made according to the recipe. The percentage of gel was selected according to the molecular weight of the proteins to be analysed. The gels were loaded with 10 to 20 µg of proteins per well and ran at 80V for stacking and 120V for separation of proteins according to their molecular weights. After gel electrophoresis, the proteins in the gel were transferred onto polyvinylidene difluoride (PVDF) or nitrocellulose membrane (Millipore) at a constant voltage of 100V for 90 minutes at 4°C in transfer buffer containing 20% methanol or 10% ethanol. After transfer, the membranes were stained with Ponceau S (Sigma) to check the quality of the transfer, de-stained by washing three times in 1x Tris-buffered saline Tween-20 (TBST), and blocked for one hour with either 5% non-fat milk or 5% bovine serum albumin (BSA) at room temperature. After blocking, the proteins were probed with antibody against primary antibodies diluted in 5% non-fat milk or 5% BSA for overnight at 4°C.

Primary antibodies used include: BQ3236363.1 (1:1000, homemade, US Provisional licence patent no. UHK.198P), NCOR2 (1:3000, Abcam), ERa (1:1000, Santa Cruz), 6xHis-tag (1:1000, Wako), FOXO3a (1:3000, Millipore), GATA3 (1:1000, Santa Cruz), EZH2 (1:1000, Diagenode), BRCA1 (1:1000, Millipore), HDAC3 (1:3000, Abcam), TBLR1 (1:3000, Abcam), β -tubulin (1:1000, Santa Cruz), lamin B (1:3000, Santa Cruz) and α -actin (1:10000, Sigma). After primary antibody incubation, the membranes were washed three times with 1xTBST, and incubated with corresponding secondary antibodies for one hour at room temperature. Secondary antibodies were: horseradish peroxidase-conjugated anti-rabbit antibody (1:10000, Dako), horseradish peroxidase-conjugated affiniPure anti-mouse antibody (1:30000, Jackson Immuno Research Laboratories or Dako) and horseradish peroxidase-conjugated anti-goat antibody (1:3000, DAKO). The membrane was washed in three times 1x TBST, applied with ECL Plus Western Blotting Detection Reagents (Amersham, GE Healthcare or PerkinElmer) and exposed to Hyperperformance Chemiluminescence films (Amersham, GE Healthcare). The films were developed in an automatic x-ray film processor.

2.5 Cell viability/proliferation assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assays were employed to measure the cell viability after tamoxifen treatment. Cells were seeded at a density of 6×10^3 cells/well. On the day of conducting MTT assay, cells were incubated with culture medium containing 10% MTT (USB, Affymetrix) at 37 °C for 4 hours. After incubation, the medium was discarded and replaced with 100 mL isopropanol with 4 mmol/L HCl and 0.1% (v/v) NP-40 to dissolve the purple precipitates. Extinction of the samples was measured by the Infinite 200 microtiter plate reader (Tecan) at 570 nm with a reference wavelength at 750 nm. Background MTT readings were subtracted from the samples. Data were presented as the percentage of cell viability relative to the untreated control.

Sulforhodamine B (SRB) assay was used to quantify the cell proliferation. Cells were seeded in 96-wells prior to the assay. On the day of harvesting, the cells were firstly fixed in 40% trichloroacetic acid at 4°C for 1 hour, washed three times with tap water and stained with SRB solution at room temperature for 1 hour. After staining, the cells were washed with 1% acetic acid and dried overnight. The protein-bound dye was dissolved in 10mM Tris base solution and the absorbance was measured at 492nm using Tecan Microplate reader. Data were presented as the percentage of cell proliferation relative to the reading on day 0.

2.6 Lentiviral transfection for generation of stable cell line

The BQ323636.1-overexpression stable cell lines were generated using lentiviral system. All the procedures were performed in class II Biosafety cabinet and extra precautions were taken when handling the virus.

Lentiviruses were produced using human embryonic kidney 293 fast transform cells (HEK293FT, 293FT in short). Early passage 293FT cells were seeded at 5×10^5 cells/well in 6-well plates 48 hours before transfection. On the day of transfection, the cells were ideally 80-90% confluent. 293FT cells were co-transfected with the lentivirus expression plasmid (pCDH-CMV-MCS-EF1-Puro-GFP-6xHis-BQ323636.1 or the empty vector as control) and packaging plasmids using Lipofectamine 2000 (Invitrogen). Lipofectamine 2000 (10 μ l) reagent was diluted in 250 μ l of plain medium and the mixture was incubated at room temperature for 5 minutes. The mixture was then added with lentivirus expression plasmid (1 μ g) and 2.6 μ l pPACK Packaging Plasmid Mix (System Biosciences) and incubated further at room temperature for 15 minutes. Meanwhile, the 293FT cells to be transfected were replaced with fresh complete medium. When the incubation finished, the DNA-Lipofectamine complexes were added to the cells and the cells were incubated overnight in 37 $^{\circ}$ C incubator with 5% CO $_2$. On the next day, the medium containing lipofectamine and plasmids were discarded and the cells were further incubated in 1ml of fresh medium containing 30% (v/v) FBS.

The viruses were collected 48 hours post transfection. The viruses-containing

medium was harvested and centrifuged at 2000xg for 5 minutes at room temperature to remove cell debris. The viral supernatant was stored at -80 °C until use.

Target cells (MCF7 and ZR75-1) were plated at density of 3 to 5×10^5 per well in 6-well plates 24 to 48 hours before viral infection. On the day of transduction, the old culture medium was removed from the culture plates and replaced with 1ml of fresh complete medium supplemented with Polybrene (Sigma) at a final concentration of 12 μ g/ml. The target cells were infected by adding 400 μ l of prepared viral supernatant. After incubating for 24 hours, the polybrene-containing medium was removed and replaced with 1ml of complete medium without Polybrene. The cells were allowed to recover by culturing in complete medium for 3 to 7 days post-transfection. The infected cells expressed green fluorescent protein (GFP), which can be visualized under fluorescent microscope three to four days after transfection. The successfully infected cells were selected by gradual increase of puromycin (Sigma) concentration from 0.5 μ g/ml until a pure population in which all the cells expressed GFP was obtained.

2.7 Transient transfection

Transient transfection was performed using either lipofectamine 2000 (Invitrogen) or FuGENE 6 (Promega) following manufacturers' protocol. Cells were seeded into six well plates or culture dishes to achieve approximately 70% confluency on the day of transfection.

For the transfection using lipofectamine 2000, the desired amount of plasmid DNA and lipofectamine reagent was diluted in 250 μ l of plain DMEM and incubated at room temperature for 5 min separately. They were then mixed together and incubated for additional 30 min at room temperature. After incubation, the mixture was added into the cells to be transfected, and the final volume was brought up to 2ml for six well plates with plain DMEM medium. Six hours after transfection, the transfection mixture was removed from the cells and complete medium was added to the cells.

For the transfection using FuGENE, desired amount of plasmid DNA was diluted in plain Opti-MEM medium (Invitrogen), incubated at room temperature for 5 min, and mixed with FuGENE at a ratio of 3:1. The mixture was further incubated at room temperature for 25 min and then added to the cells cultured in complete growth medium. Cells were harvested 24 hours after transfection.

2.8 Knockdown using siRNA

All siRNAs for the work were ON-TARGET*plus* SMARTpool siRNA purchased from Dharmacon Thermo Scientific. The SMARTpool siRNAs used in this study were: siBRCA1 (L-003461-00), siEZH2 (L-004218-00) and the ON-TARGET*plus* Non-Targeting Pool (D-001810-10). All siRNA pools were resuspended to 20 μ M in 1x siRNA buffer (Thermo Scientific) prior to use.

SiRNAs or non-silencing controls (NSC) were delivered into the cells seeded in 6 well plates or 150mm culture dishes using Oligofectamine (Invitrogen) following manufacturer's instruction. In brief, siRNA or NSC was diluted in plain Opti-MEM medium and mixed with Oligofectamine at a ratio of 1.5:1. The mixture was incubated at room temperature for 25 minutes. The volume was brought up to 500 μ l for 6-well plate and 5ml for 150mm culture dish using plain Opti-MEM, and added back to the cells that had been washed twice with 1xPBS. Four hours later, the complete growth medium was added to the cells and cells were harvested 72 h after transfection.

2.9 Luciferase assay

The luciferase assay was performed using Dual-Luciferase[®] Reporter assay system (Promega). The ERE-E1b-Luc firefly reporter and pRL-SV40 were co-transfected with NCOR2 or its empty vector into MCF7 or ZR75-1 cells with or without BQ323636.1 overexpression using Lipofectamine 2000 transfection reagent (Invitrogen). The procedures for transfection are previously described in section 2.7. The ratio between firefly reporter and pRL-SV40 was 50:1. The total amount of plasmid DNA used was 1.5µg. Twenty-four hours after transfection, cells were treated with E2 or tamoxifen or vehicle control (ethanol). After another 24 hours of treatment, cells were harvested using Passive Lysis buffer provided by the manufacturer and the activities of firefly and renilla luciferases were measured sequentially according to manufacturer's protocol. The intensity of firefly and renilla luminescence was recorded by Infinite F200 (Tecan). Background readings were subtracted from those of the samples and the firefly luminescence were normalized by dividing with the corresponding renilla luminescence readings. Data were presented as ratios relative to the vector control.

2.10 Co-immunoprecipitation (Co-IP)

Cells were lysed in IP lysis buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol, pH 7.4) by incubating on ice for 10 min with occasional vortexing. The lysate was centrifuged at maximum speed at 4 °C for 10 minutes and the supernatant was transferred to a clean Eppendorf tubes. The lysate was pre-cleared with 30µl of pre-washed Dynabeads Protein A/G (A beads for rabbit antibodies and G beads for mouse antibodies, Invitrogen, Life Technologies, UK) by rotating at 4 °C for 4 hours. After pre-clearing, protein concentration was measured and the lysate was split into equal amount of proteins per tube and incubated with the specific primary antibodies including NCOR2 (Abcam), HDAC3 (Abcam), ER (Santa Cruz) or Immunoglobulin (IgG) negative control (DAKO), all diluted at the ratio of 1:200 at 4 °C for O/N with gentle rotating. On the second day, 40µl of pre-washed beads were added to the antibody-lysate mixture and incubated at 4 °C for another 4 h. After incubation, the beads were washed five times with cold PBS supplemented with protease inhibitor and boiled at 100 °C for 5 min to elute the proteins. The supernatant was transferred to clean tube using magnetic stand (Invitrogen). Proteins were separated by SDS-PAGE gel electrophoresis, transferred to nitrocellulose membrane and probed with the antibodies same as Western blotting.

2.11 Immunofluorescent staining

Immunofluorescent staining was done with the help of Ms Ellen Man, our lab manager (Rm323, Department of Pathology, University Pathology Building, Queen Mary Hospital, HKSAR). Cells were seeded on round glass slides in 12-well plate and cultured in 37⁰C incubator. When harvesting, cells were fixed with mixture of methanol and acetone (Millipore, Germany/ Merck, UK) at the ratio of 50:50 for 10 min at -20 ⁰C. The slides were then incubated overnight at 4 ⁰C with the homemade primary mouse anti-BQ323636.1 (1:50, US Provisional licence patent no. UHK.198P). On the second day, after washing with 1x PBS, the slides were incubated with secondary goat anti-mouse IgG-FITC (1:2000, Life Technologies, US) for an hour at 37 ⁰C. After washing with 1xPBS, the glass slides were mounted with mounting medium with DAPI (Life Technologies, US) and left to dry at room temperature for overnight. The slides were examined by Carl Zeiss LSM 710 confocal laser scanning microscope (HKU Faculty Core Facility). Images were captured under the same settings (laser power, gain, offset) and analysed using the software ZEN 2011 version 5.5 SP1. The images were adjusted for brightness or contrast using linear transformation and the parameters of all the images were kept the same. For each slide, pictures of at least three independent fields were taken. The intensities of fluorescence were quantified using ImageJ software (developed by National Institutes of Health, USA) (Collins 2007).

2.12 Subcellular fractionation

Subcellular fractionation was performed using the NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) following manufacturers' protocol. In brief, 1×10^6 cells were harvested by centrifuging at 500 xg for 5 minutes. The cells were washed twice with PBS and 100 μ l of ice-cold CER I buffer was added into the cell pellet followed by vigorous vortex for 15 seconds to fully suspend the cells. After 10 min incubation on ice, 5.5 μ l of ice-cold CER II buffer was added and the tube was vortexed vigorously for 5 s and further incubated on ice for 1min. The lysate was then centrifuged at maximum speed for 5 minutes at 4 ⁰C and the supernatant which contained cytoplasmic proteins was immediately transferred to a clean pre-chilled tube. The insoluble pellet which contained nuclei was washed twice with ice-cold PBS to remove the residue cytoplasmic proteins and suspended in 50 μ l of ice-cold NER. The mixture was vortexed on highest setting for 15 seconds followed by incubation on ice for a total of 40 min with vortexing every 10 minutes. The tube was centrifuged at maximum speed for 10 min and the supernatant which contains nuclear extract was transferred to a clean pre-chilled tube. The cytoplasmic and nuclear extracts were stored in -80⁰C until further use.

2.13 Chromatin immunoprecipitation (ChIP)

The bindings of proteins to DNA were studied by ChIP. The recipes of all the buffers used in ChIP are summarized in table 2.3

On the first day, the magnetic beads were incubated with antibodies. Dynabeads Protein A/G (40µl per antibody per sample) was washed with 200µl of TSE I buffer for three times and diluted with 40µl of TSE I buffer. Four microgram of antibodies including Anti-BRCA1 (Millipore, 07-434), Anti-EZH2 (Diagenode; C15410039), DNMT1 (Abcam; ab87656), DNMT3a (Abcam; ab2850), DNMT3b (Abcam; ab13604), H3K27me3 (Abcam; ab6002), rabbit/mouse IgG negative control (DAKO) were first separately diluted in Buffer D at a ratio of 1:9, mixed with diluted Dynabeads and then rotated overnight at 4⁰C. All the buffers were supplemented with protease inhibitor and were kept on ice prior to use.

On the second day, cells cultured in 150mm dish with 90% confluency were crosslinked with 10ml of 1% formaldehyde for 10 min, rinsed with ice-cold 1x PBS, incubated with 0.25M glycine diluted in 1xPBS for 5 min to stop the crosslinking reactions. The cells were harvested with 2ml of scrapping buffer by scrapping. After a sequential wash with PBS, Buffer I and Buffer II, cell pellet was resuspended in 300µl of Lysis buffer, incubated on ice for 10 min and sonicated for 20 min with the setting of 30 seconds on and 30 seconds off in cold room. The samples were then centrifuged at maximum speed for 10 min and supernatant was then diluted in 300µl of Buffer D from which 100µl was taken as INPUT. The remaining cell lysate was split into equal amount for each antibody and mixed with Dynabeads prepared on Day 1 by rotating overnight at 4⁰C. All

the buffers were supplemented with protease inhibitor and were kept on ice prior to use.

On the third day, after sequential wash with ice-cold TSE I, TSE II and TE buffer supplemented with protease inhibitor, 100µl of elution buffer was added to the Dynabeads and the mixture was rotated at RT for 1 h. Eluted samples were collected in clean eppendorfs and the Dynabeads was re-eluted with another 100µl of elution buffer for 1 hour. The 200µl samples were pooled together and crosslinking was reversed by incubating at 65°C for overnight.

On the next day, DNA in the lysate was purified using QIAquick PCR Purification Kit (QIAGEN) following manufacturer's instructions. In brief, 1ml of Buffer PB was added to the DNA mixture and the liquid was applied to QIAquick column and centrifuged at 13,000xg for 1 min. The flow-through was discarded the columns were washed twice with Buffer PE. DNA was eluted with 30-50 µl of RNase-free water. The genes of interest were quantified by RT-qPCR using primers listed in Table 2.2. Results were calculated by $\Delta\Delta C_t$ method and expressed as fold-change relative to the IgG control.

Table 2.3 Recipes of the buffers used in ChIP.

Buffer Name	Recipes
Buffer I	0.25% Triton X-100; 100mM EDTA; 0.5mM EGTA; 10mM HEPES pH6.5; H ₂ O
Buffer II	200mM NaCl; 10mM EDTA; 0.5mM EGTA; 10mM HEPES pH6.5; H ₂ O
Lysis Buffer	1% SDS. 10mM EDTA, 500mM Tris-HCL pH8.1; H ₂ O
Buffer D	1% Triton X-100; 2mM EDTA; 20mM Tris-HCL pH8.1; 150mM NaCl; H ₂ O
TSE I	0.1% SDS, 1% Triton X-100, 2mM EDTA; 20mM Tris-HCL pH8.1; 150mM NaCl; H ₂ O
TSE II	0.1% SDS, 1% Triton X-100; 2mM EDTA; 20mM Tris-HCL pH8.1; 500mM NaCl; H ₂ O
TE Buffer	100mM Tris-HCL pH8.0; 1mM EDTA; H ₂ O
Scraping Buffer	100mM Tris-HCL pH9.4; 0.1% SDS; H ₂ O
Elution Buffer	0.1mM NaHCO ₃ ; 1%SDS; H ₂ O

2.14 Orthotopic mouse model

Female nude mice at the aged 6 to 7 weeks were used for this study. On the day of inoculation, mice were anesthetized by intra-peritoneal injection of ketamine+xylazine. One million of ZR75 cells or five millions of MCF7 cells were mixed with Matrigel (BD Bioscience) at a ratio of 1:1 and the 100 μ l cell mixture were injected into abdominal mammary fat pad of the mice. When the tumours were palpable, mice were randomized into treatment and control groups where treatment group received tamoxifen dissolved in peanut oil (Sigma) by subcutaneous injection for 6 consecutive days per week and control group received solvent only. The tumour sizes were measured regularly using calliper and the tumour volume was calculated as longest diameter x (shortest diameter)²/2. At the endpoint of the experiments, the mice were euthanized by intra-peritoneal injection of pentobarbital. Livers and lungs were dissected out, washed three times with 1xPBS and put under PE IVIS Spectrum in vivo imaging system (HKU Faculty core facility) for visualization of GFP signals. License for conducting experiments on animals has been obtained from Department of Health, Hong Kong. All the procedures have been reviewed and approved by HKU Committee on the Use of Live Animals in Teaching and Research (CULATR No.:3259-14).

2.15 Pulse-chase assay

The pulse-chase assay was performed in a controlled room dedicated to radioactive substances. Before pulse labelling, the cells to be labelled were washed twice with 1X PBS and incubated in plain DMEM without methionine and cysteine (DMEM –met/cys, Gibco) for one hour. While waiting, ^{35}S -methionine stock (43.3 mCi/ml, Perkin-Elmer) was diluted in DMEM –met/cys supplemented with 10% FBS (working concentration was 0.2 mCi per 100mm culture dish (hot medium)). After cell starvation, hot medium was added into the cells and cells were labelled with ^{35}S for 1 hour. After the pulse period, the hot medium was removed, and cells were washed twice with 1X PBS to remove the residue radioactive substance. After washing, one dish of cells was harvested and labelled as 0h. The remaining dishes of cells were cultured in non-hot medium, which was the normal cell culture medium for a period of 2h, 4h and 6h (chase period). Cell pellets were collected and lysed for immunoprecipitation (IP) using anti-NCOR2 antibody (Abcam) or anti-BQ323636.1 antibody (homemade, US Provisional licence patent no. UHK.198P). The procedures for immunoprecipitation have been described in the previous section. After IP, all the samples were loaded onto polyacrylamide gel for protein separation. The gels were then dried in vacuum drier for 2h and the dried gels were fixed onto filter paper and exposed to X-ray film in cassette for 24 hours and 48 hours. The X-ray films were developed using automatic film processor.

2.16 Hematoxylin and Eosin (H&E) staining

The H&E staining of mouse xenografts was done with the help of Ms Ellen Man. The slides were soaked in xylene three times, 5 minutes each, then were rehydrated by incubating for 5 minutes in decreasing concentrations of ethanol from 100%, to 95%, to 80%. The slides were rinsed in running tap water for around 5 minutes and nuclei were stained with the Gill hematoxylin for 5 minutes. After washing with running tap water, the slides were incubated in 0.3% acid alcohol for 5 seconds and then rinsed in running tap water for another 5 minutes, followed by 10 seconds of rinse in Scott's tap water. After this, the slides were stained with eosin for around 2 minutes and dehydrated by rinsing in increasing concentrations of ethanol from 80% to 95% to 100%. After incubation in xylene for 4 minutes, the slides were mounted with coverslip using Permount and left it to dry for overnight.

2.17 Tissue microarray, immunohistochemistry and staining scoring

2.17.1 Tissue Microarray

The Tissue Microarray (TMA) for analyses between FOXO3a expression and BRCA mutation status was a kind gift from our Korean collaborator, Prof. Gyungyub Gong (Department of Hospital Pathology, The Catholic University of Korea College of Medicine, Seoul, Korea). The TMA contains 308 cases of breast cancer cases including 62 cases with BRCA1 mutation, 96 cases with BRCA2 mutation and 150 cases of wild-type BRCA. Each case was constructed as duplicate or triplicate in the TMA and average score was taken as the score for this case.

For the TMA analysis of BQ323636.1 Two hundred and thirty-eight cases of breast cancer diagnosed between the years 1992 to 2008 with clinical follow up data were retrieved from the records of the Department of Pathology, Queen Mary Hospital of Hong Kong, with approval by the Institutional Review Board of The University of Hong Kong (UW 08-147). Histological sections of all cases were reviewed by the pathologist, the representative paraffin tumour blocks were chosen as donor block for each case and the selected areas were marked for construction of TMA blocks. Each case was constructed as duplicate in the TMA and average score of the duplicate was taken as the score for this case.

2.17.2 Immunohistochemistry

The TMA sections were deparaffinized and rehydrated by incubation with xylene and decreasing concentrations of ethanol. Antigen retrieval was performed by heating using pressure cooker in EDTA buffer. The slides were immersed into 3% H₂O₂/methanol for 10 min at room temperature to quench endogenous peroxidase. After rinsing in 0.05% Tween in PBS (PBST) twice, anti-FOXO3a (1:850, Millipore), anti-EZH2 (1:300, Diagenode) or anti-BQ323636.1 (1:50, homemade, US Provisional license patent no. UHK.198P) was added to each section and incubated at 4°C overnight. The slides were then washed in PBST and incubated with DAKO EnVision+System-HRP-labelled Polymer Anti-Rabbit at room temperature in dark for 30 min. After washing, Chromogen DAB/substrate reagent was added onto the slides and the slides incubated further for several minutes. Finally, the slides were dehydrated and mounted. Aperio ScanScope ® system (Aperio technology, USA) was used to visualize and assess for FOXO3a, EZH2 and BQ323636.1 expression. The TMA slides were scanned by ScanScope scanners and individual stained TMA spots were assessed in computer screen with the use of Aperio's image viewer, ImageScope.

2.17.3 Semi-quantitative scoring

To avoid subjectivity in evaluation, the intensities and percentages of the staining were scored by two independent individuals (myself and Ms. Ellen Man) in a semi-quantitative way and the average was taken.

Cytoplasm and nucleus expression of FOXO3a and EZH2 was scored as previously described (Chen, Gomes et al. 2010). In brief, the intensity or percentage of the staining were assessed separately in cytoplasm and nucleus. The intensity was scored as 0= none, 1= weak, 2=moderate, 3=strong. The percentage was scored as 1= less than 25%, 2= 25% to 50%, 3= 50% to 75% and 4= more than 75%. Cytoplasm/nucleus score was calculated as intensity * percentage and total score was calculated as cytoplasm score + nucleus score.

BQ323636.1 expression level was scored according to the H-score system which takes into account the intensity of the staining and percentage of each intensity (Detre, Saclani Jotti et al. 1995). $H\ score = (1 \times \% \text{ of cells stained at intensity category 1}) + (2 \times \% \text{ of cells stained at intensity category 2}) + (3 \times \% \text{ of cells stained at intensity category 3})$. The cutoff was set as the median of the scores, which was 130.

2.18 Statistical analyses

Statistical analyses were conducted using SPSS (IBM Chicago, USA, version 17) or Excel (Microsoft). Data from tissue microarray were analyzed in SPSS. Where appropriate, the data were dichotomized into two groups including high or low expression using median expression level as cutoff. The correlations between FOXO3a expression levels and BRCA mutation status were studied by Mann-Whiney U Rank test. The correlations between expression levels of splice variant BQ323636.1 and tamoxifen responsiveness of the patients were analyzed by Chi-square tests. The expression levels of BQ323636.1 were compared between different groups using Mann-Whiney U Rank test. Survival analyses were done by Kaplan–Meier estimates followed by Log-rank test, and Cox regression model. Results from other experiments were compared by two-tailed students' t-test in Excel, if applicable. P values of less than 0.05 were considered statistically significant. In the figures, 'ns' indicates no statistical significance, '*' indicates $P < 0.05$, '**' indicates $P < 0.01$ and '***' indicates $P < 0.001$.

Chapter 3 BRCA1 regulates FOXO3a expression indirectly through an EZH2-mediated epigenetic mechanism

3.1 Introduction

3.1.1 The tumour-suppressive role of BRCA1

As introduced in chapter 1, breast cancer is the most common type of cancer amongst women worldwide. BRCA1 dysregulation and/or BRCA1 mutation are closely associated with higher risk of breast cancer in familial cases. Accordingly, reduced BRCA1 expression or mutation has also been frequently reported in sporadic breast cancer (Wilson, Ramos et al. 1999, Yang, Sakurai et al. 2001, Janatova, Zikan et al. 2005). BRCA1 is a multifunctional tumour suppressor participating in various cellular processes such as DNA damage response, transcription regulation, cell cycle progression and apoptosis *etc.* It plays a key role in mammary tumourigenesis. BRCA1 silencing or mutation is also associated with the development of basal-type breast cancer phenotype in which the tumour cells express no ER, PR nor HER2 receptor, high metastasis and poor prognosis (Buckley, Nic An tSaoir et al. 2013). It has been reported that germline mutation of *Brca1* in mouse model could cause luminal-to-basal mammary transformation of the breast tumours (Bai, Smith et al. 2013) and

knockdown of BRCA1 enhanced tumour formation potential *in vivo* (Bai, Chan et al. 2014).

The mechanisms underlying BRCA1 loss/mutation-associated tumour aggressiveness are not clearly understood. There are a large number of studies in the literature proposing multiple mechanisms through which BRCA1 could exert its tumour-suppressive role. For instance, inactivation of BRCA1 has been shown to induce malignant cell behaviour including accelerated cell proliferation, enhanced anchorage-independent growth and suppressed expression of negatively proliferation inhibitors p21^{Waf1/Cip1} and p27^{kip1} (Promkan, Liu et al. 2009). In this context, BRCA1 can transcriptionally either regulate or co-regulate p27^{kip1} in cooperation with FOXA1 (Somasundaram 2003, Williamson, Wolf et al. 2006). BRCA1 also regulates expression of survivin, an inhibitor of cell division and apoptosis (Kennedy, O'Driscoll et al. 2003), through regulating the expression of the NAD⁺-dependent deacetylase sirtuin-1 (SIRT1) (Wang, Zheng et al. 2008). BRCA1 could cooperate with GATA3 to repress expression of FOXC1 that is important for maintaining of triple-negative cell line growth (Tkocz, Crawford et al. 2012). In addition, it has been reported that BRCA1 can negatively regulate the oncogenic PI3K-AKT pathway by binding to pAKT and inducing ubiquitination and degradation of AKT protein, resulting in FOXO3a hypophosphorylation and induction of FOXO3a activity (Xiang, Ohashi et al. 2008).

3.1.2 The tumour-suppressive role of FOXO3a

The transcription factor FOXO3a (Forkhead Box O3a) is a member of O subclass of forkhead box transcription factors family and is a key negative regulator of cell proliferation and survival (Myatt and Lam 2007). It is a well-characterised tumour suppressor. Active FOXO3a is localized in the nucleus where it transcriptionally regulates the genes that are important in a myriad of cellular processes such as cell cycle progression, apoptosis, angiogenesis, oxidative stress response and therapeutic drug response. FOXO3a can transcriptionally promote expression of p27^{kip1} (Dijkers, Medema et al. 2000). Moreover, FOXO3a represses vascular endothelial growth factor (VEGF) expression directly through transcriptional regulation and indirectly through interacting and inhibiting of FOXM1 (Karadedou, Gomes et al. 2012). FOXO3a is also able to regulate mitochondrial activity and reactive oxygen metabolism through inhibiting functions of c-Myc (Ferber, Peck et al. 2012). FOXO3a also plays a central role in mediating cytotoxic effects of doxorubicin, as doxorubicin treatment caused phosphorylation of FOXO3a at Ser-7 and subsequently nuclear localization and activation (Ho, McGuire et al. 2012). The anti-proliferative activity of tyrosine kinase inhibitor in chronic myeloid leukaemia is also mediated by FOXO transcription factors including FOXO1, FOXO3a and FOXO4 (Pellicano, Scott et al. 2014).

As a result, FOXO3a down-regulation or inactivation is often reported in cancers and is associated with poorer patients survival. Decreased expression of FOXO3a both at mRNA and protein level is associated with poor prognosis in primary gastric adenocarcinoma patients (Yang, Zhao et al. 2013). Loss of FOXO3a

expression is a hallmark of the early stages of high-grade pelvic serous carcinogenesis (Levanon, Sapoznik et al. 2014). Conversely, FOXO3a high expression has been reported to be associated with low histological grade, low tumour stage, lymph node negativity and better survival in breast cancer patients (Jiang, Zou et al. 2013) and shown to suppress the oestrogen-dependent breast cancer tumourigenesis *in vivo* (Zou, Tsai et al. 2008). In addition, down regulation of FOXO3a activity is often reported in cancer and has been shown to promote tumourigenesis (Hu, Lee et al. 2004, Yang, Zong et al. 2008).

The regulatory mechanisms of FOXO3a expression are not well understood. The majority of the studies focused on regulation of FOXO3a functional activities and ubiquitination-mediated degradation by various kinases. FOXO3a is also the central mediator of the pro-proliferative PI3K-AKT pathway in which AKT causes the phosphorylation, inactivation, nuclear exclusion and subsequently degradation of FOXO3a (Brunet, Bonni et al. 1999). Other kinases such as IkappaB kinase (IKK) (Hu, Lee et al. 2004) and ERK kinase (Yang, Zong et al. 2008) can also phosphorylate and down-regulate FOXO3a activity in a similar fashion, and FOXO3a is the key player through which these kinases exert their oncogenic effects in promoting cell growth and tumourigenesis. Recent studies also suggested that FOXO3a expression is regulated by its DNA methylation status in mouse embryonic fibroblasts (MEFs) (Mori, Nada et al. 2014) and hypomethylating agents can reactivate FOXO3a in acute myeloid leukaemia (Thepot, Lainey et al. 2011).

3.1.3 The oncogenic role of EZH2

EZH2 (enhancer of zeste homolog 2) is the catalytic subunit of the polycomb-repressive complex 2 (PRC2), and a methyltransferase which specifically catalyses the addition of methyl groups to histone 3 lysine 27 (H3K27). Trimethylation of H3K27 (H3K27me3) by EZH2 serves as an epigenetic code for further recruitment of other polycomb complexes, DNA methyltransferases (DNMTs) including DNMT1, DNMT3a and DNMT3b, and histone deacetylases (HDACs), leading to chromatin condensation and transcription suppression (Sauvageau and Sauvageau 2010) (Vire, Brenner et al. 2006).

EZH2 is recognized as an oncogene whose overexpression is often reported in multiple malignancies. EZH2 is up-regulated in hormone-refractory, metastatic prostate cancer and depletion of EZH2 inhibited cell proliferation (Varambally, Dhanasekaran et al. 2002). EZH2 overexpression is associated with cancer metastasis and poor survival in breast cancer patients (Kleer, Cao et al. 2003). EZH2 regulates cancer invasion and progression by regulating a number of genes that are important in such functions. Trimethylation of H3K27 by EZH2 suppresses the expression of RUNX3, a significant tumour suppressor important in regulating cell cycle progression and apoptosis (Fujii, Ito et al. 2008). It is also reported that EZH2 suppressed expression of E-cadherin that is related to invasiveness of epithelial tumours (Yoo and Hennighausen 2012). EZH2 also regulates tumour invasion by suppressing the expression of metastasis suppressor RKIP in breast and prostate cancer (Ren, Baritaki et al. 2012). Recently, our group has identified FOXA1 that is transcriptionally repressed by EZH2 expression and such regulation involves BRCA1 hence might be important for

development of triple-negative breast cancer phenotype (Gong, Fujino et al. 2014). Interestingly, BRCA1 has also been shown to directly interact with EZH2 and negatively regulate functions of EZH2 in both mouse embryonic stem cells and human breast cancer cells (Wang, Zeng et al. 2013). Another line of evidence suggesting the interplay between BRCA1 and EZH2 in breast cancer is the observation that growth of *Brca1*-deficient mouse mammary tumours is dependent of EZH2 expression and is sensitive to PRC2 inhibition (Puppe, Drost et al. 2009). Therefore, decreased BRCA1 expression could result in enhanced EZH2 activity which, in turn, induces aggressiveness of breast cancer.

Despite the small number of EZH2-regulated genes identified, its functional targets in cancers remain largely unknown. More importantly, its interplay with BRCA1 and the functional significance in regulating breast cancer initiation and progression is not fully explored.

3.1.4 The hypothesis

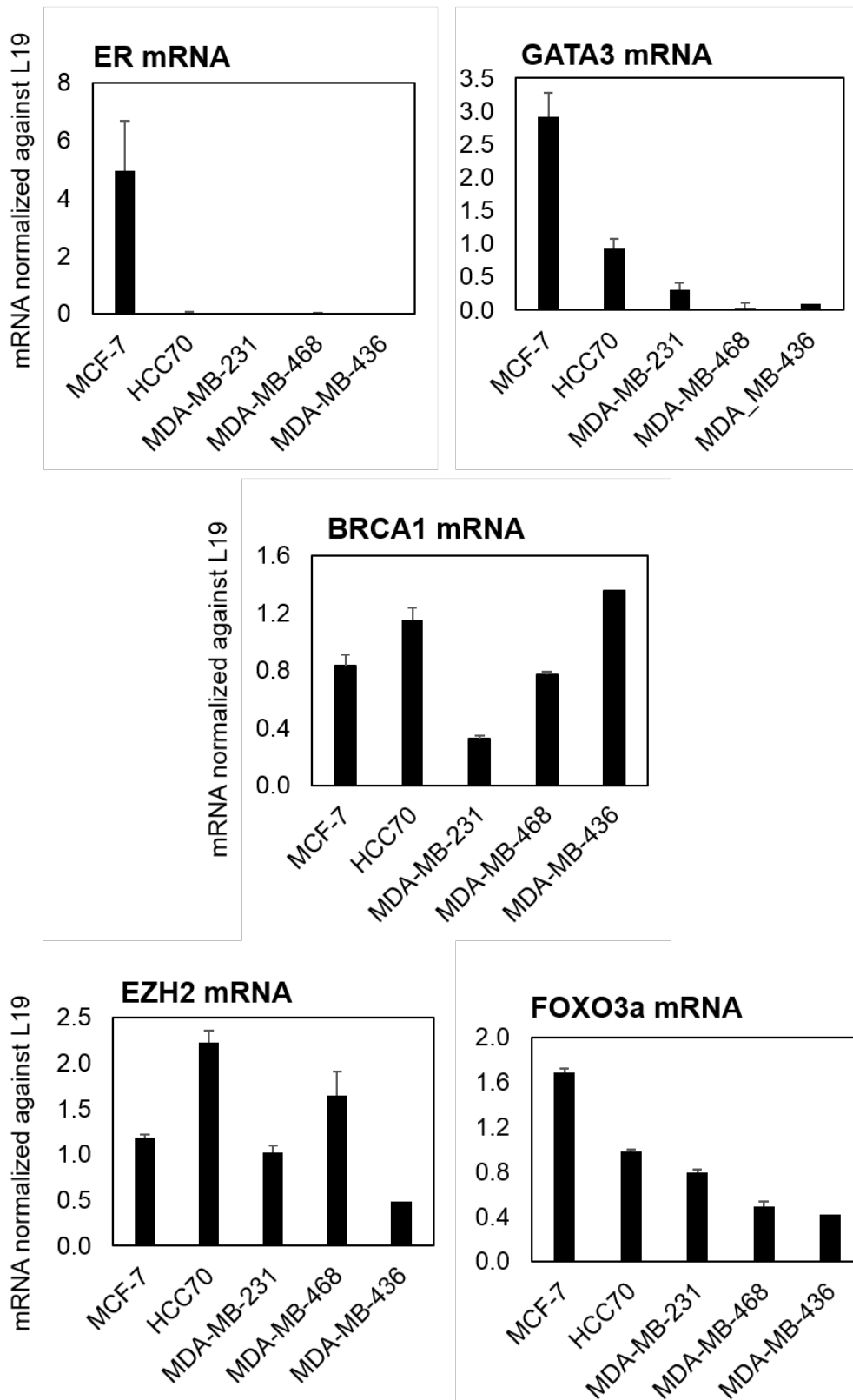
Since FOXO3a expression is regulated by epigenetic mechanism which involves EZH2-H3K27me3 and BRCA1 could interfere with functions of EZH2, it was hypothesized that BRCA1 could regulate expression of FOXO3a indirectly through inhibiting EZH2. This study can bridge BRCA1 and FOXO3a, the two core tumour suppressors in breast cancer, and the findings would contribute to better understanding of the tumour suppressor role of BRCA1 and the regulation of FOXO3a in breast cancer.

3.2 Results

3.2.1 FOXO3a expression is correlated with competency of BRCA1 in breast cancer cell lines

In order to unveil the correlation between BRCA1 and FOXO3a expression, western blot as well as RT-qPCR analysis was performed using a panel of breast cancer cell lines, consisting of the luminal-type MCF-7 cells (wild-type BRCA1) and the basal-type lines HCC70 (BRCA1 low), MDA-MB-231 (BRCA1 low), MDA-MB-436 (BRCA1 mutated) and MDA-MB-468 (BRCA1 mutated). Consistent with their phenotypes, ER α and GATA3, which are molecular markers for luminal phenotype, were expressed in MCF-7 but not in the basal-type breast cancer cell lines (Figure 3.1). Moreover, despite the great heterogeneity amongst the cell lines, FOXO3a expression was found to be closely correlated with levels of functional BRCA1 in the cell lines, with highest FOXO3a expression in MCF-7 cells. The mRNA levels were also quantified by RT-qPCR and results were normalized against the housekeeping gene L19. There was significant higher FOXO3a expression at both the mRNA and the protein levels in MCF7 compared with the basal-type cell lines (Figure 3.1A and B, respectively). Consistent with our previous results (Gong, Fujino et al. 2014), the Polycomb protein EZH2 was found to be ubiquitously expressed in all five cell lines (Figure 3.1). These results suggest the possible correlation between BRCA1 and FOXO3a expression in breast cancer. It was also noted that there is a discrepancy between the BRCA1 mRNA and protein level, suggesting that expression of BRCA1 was significantly regulated at post-transcriptional level.

(A)



(B)

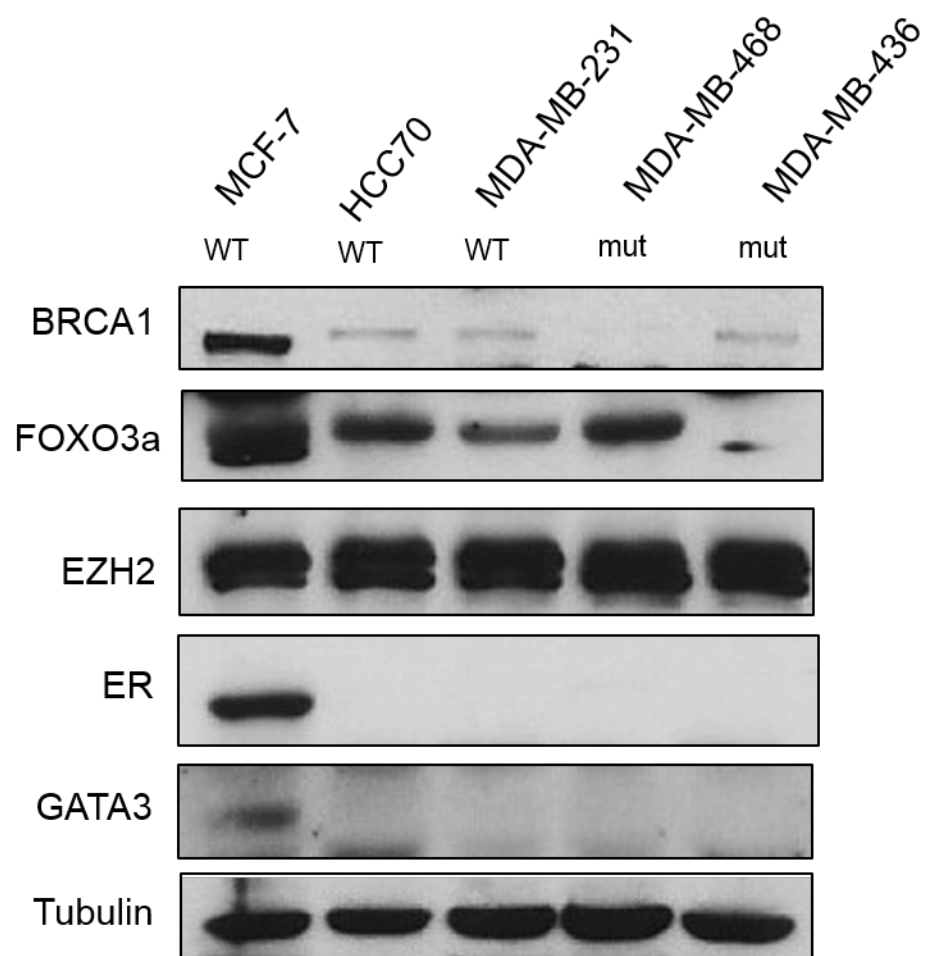
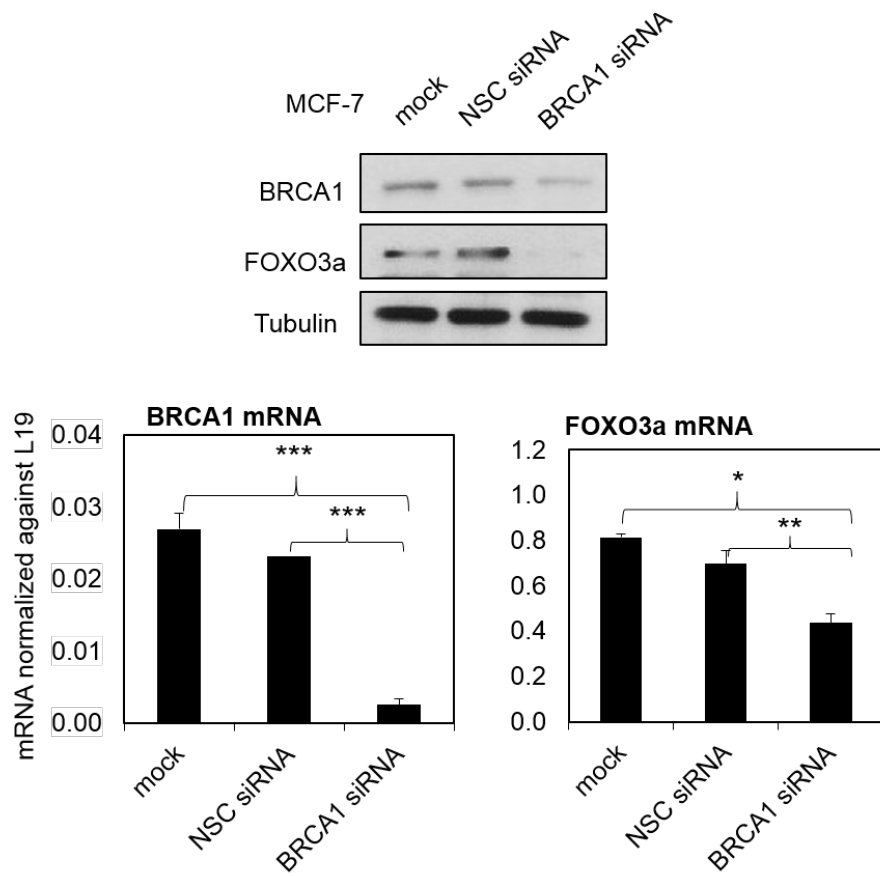


Figure 3.1. BRCA1 correlates with FOXO3a expression at both **(A)** the protein and **(B)** the mRNA level in a panel of breast cancer cell lines.

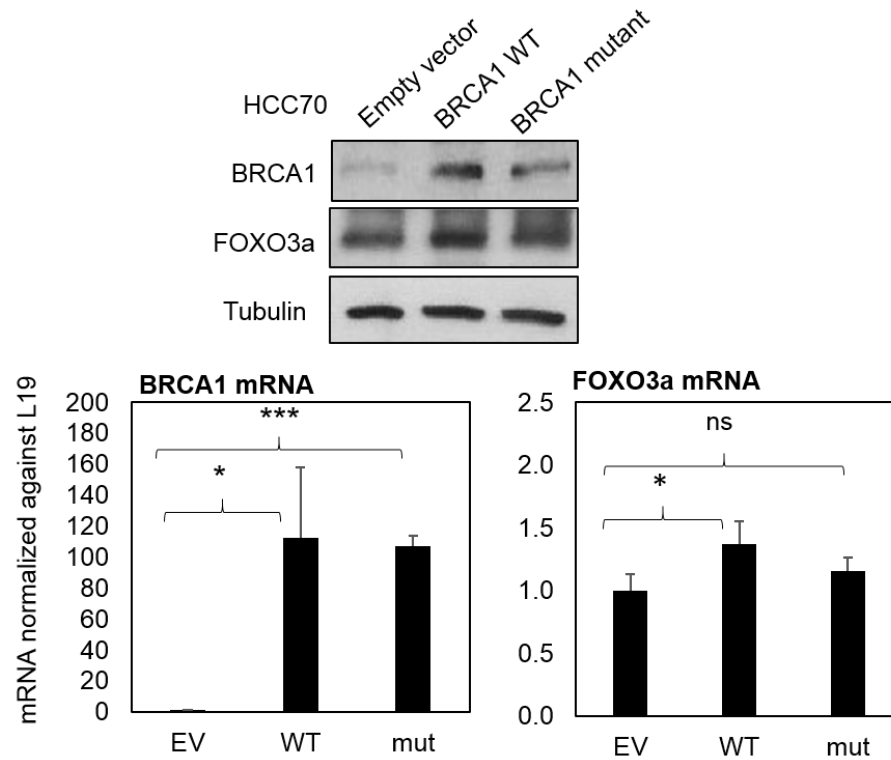
3.2.2 BRCA1 regulates FOXO3a expression

The correlation between BRCA1 and FOXO3a expression shown previously suggested a possibility that BRCA1 might regulate FOXO3a expression. To test this hypothesis, BRCA1 was depleted by BRCA1-specific siRNA pool in MCF-7 cells in which BRCA1 is functionally active, and western blot and RT-qPCR were performed to study the effects of BRCA1 depletion on the expression of FOXO3a. As shown in Figure 3.2A, when compared with the non-silencing control (NSC) and the mock transfected MCF-7 cells, BRCA1-silencing by specific siRNA caused a significant decrease in FOXO3a expression at both the protein and the mRNA levels in MCF-7 cells, supporting the notion that BRCA1 regulates FOXO3a expression. Consistently, overexpression of BRCA1 by transient transfection in basal-type cell lines HCC70 (low BRCA1) and MDA-MB-468 (mutated BRCA1) significantly enhanced the expression of FOXO3a at both the protein and mRNA levels (Fig. 3.2B and Fig. 3.2C, respectively), as revealed by western blot and RT-qPCR analyses. In addition, overexpression of BRCA1 mutant did not significantly promote expression of FOXO3a either. This BRCA1-mutant plasmid carries a cancer-predisposing point mutation C61G in the RING finger domain of BRCA1, which is expected to impair the normal function of BRCA1. Taken together these results suggest that BRCA1 regulates FOXO3a expression, at least in part, at the transcriptional level.

(A)



(B)



(C)

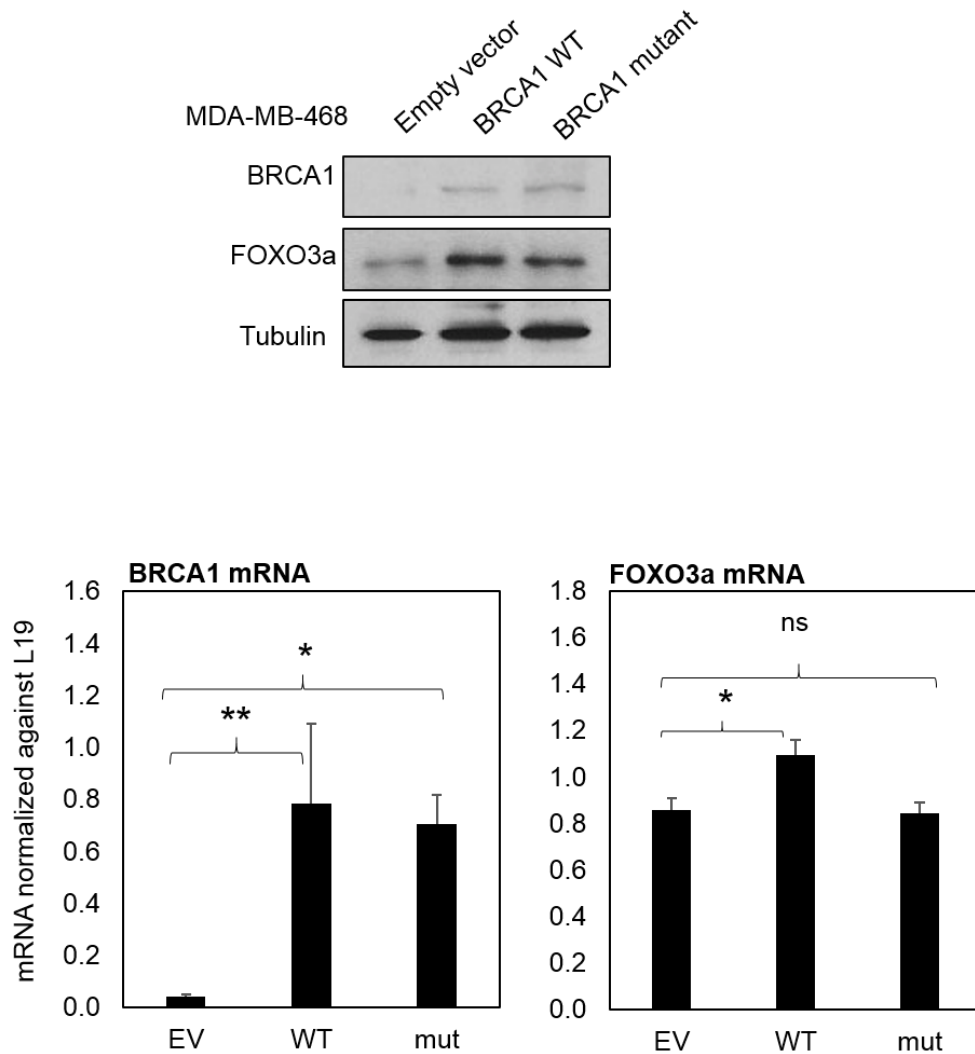


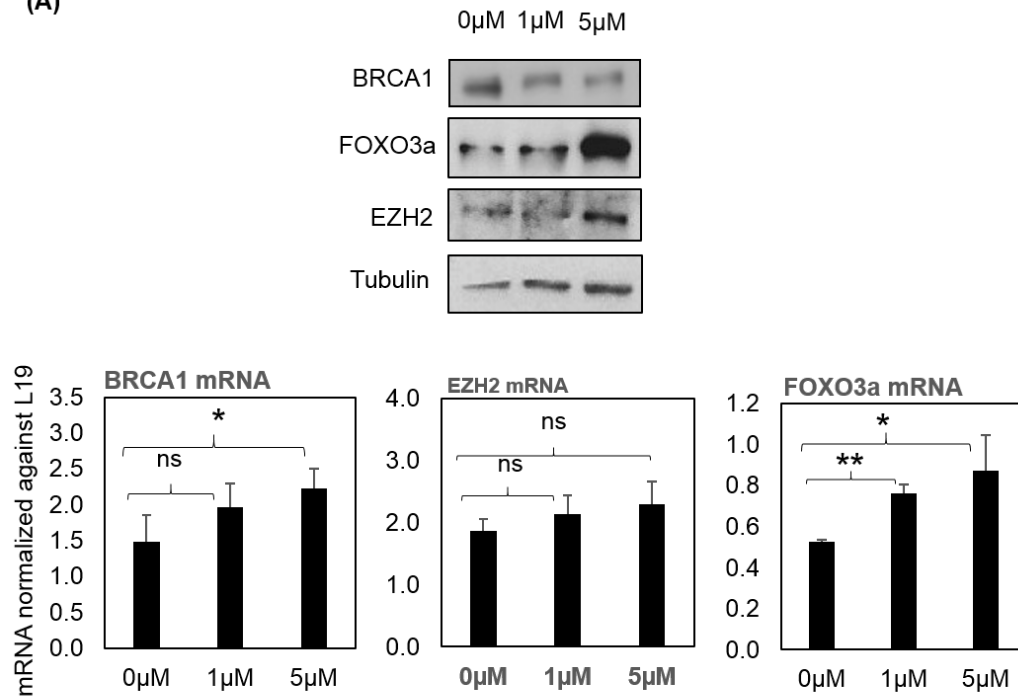
Figure 3.2. BRCA1 regulates expression of FOXO3a, at least partially, at transcription level. **(A)** BRCA1 knockdown using siRNA in MCF-7 cells suppressed the expression of FOXO3a. **(B)** Ectopic overexpression of BRCA1 wild-type (WT) but not mutant (mut) induced FOXO3a expression, comparing with empty vector (EV) in HCC70 cells. **(C)** Over-expression of BRCA1 WT but not mut induced FOXO3a expression in MDA-MB-468. These experiments have been repeated three times and the representative western blots were shown and RT-qPCR data were expressed as mean+SD, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, no significance.

3.2.3 5'-aza-dC treatment induces FOXO3a expression in basal type cell lines

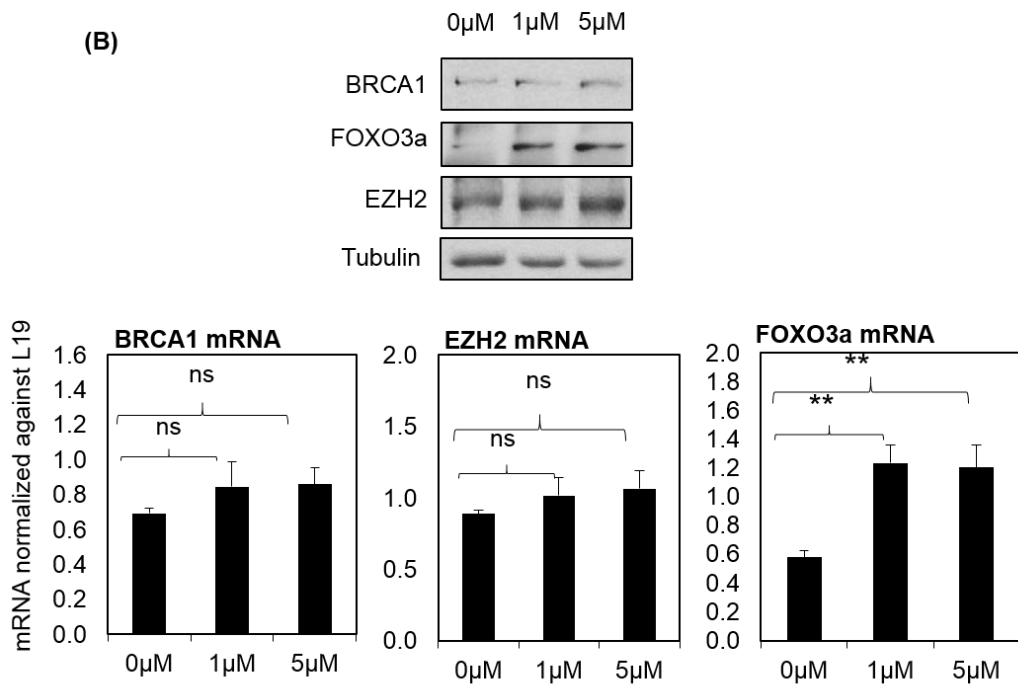
As it has been previously indicated that FOXO3a expression might be regulated by epigenetic mechanism (Mori, Nada et al. 2014), and BRCA1 has been shown to interact and interfere with EZH2-mediated methylation (Wang, Zeng et al. 2013), the correlation between FOXO3a methylation and FOXO3a expression was further studied in the presence or absence of wild-type BRCA1.

Breast cancer cell lines HCC70 (basal type), MDA-MB-468 (basal type) and MCF-7 (luminal type) were treated with various concentrations of 5'-aza-dC (0 μ M, 1 μ M and 5 μ M) for 72h. After treatment, the expression levels of BRCA1, FOXO3a and EZH2 were assessed by both western blot and RT-qPCR. In HCC70, there was significant increase in FOXO3a mRNA expression after cells being treated with 1 μ M and 5 μ M of 5'-aza-dC ($p < 0.05$, students' t-test) whilst the FOXO3a protein expression was significantly induced by the treatment of 5 μ M 5'-aza-dC (Fig. 3.3A). In agreement, there was also significant increase of FOXO3a expression in MDA-MB-468 cells after being treated with 1 μ M and 5 μ M of 5'-aza-dC (both $p < 0.01$, students' t-test) (Fig. 3.3B). On the contrary, the expression levels of FOXO3a were not affected by the treatment of 5'-aza-dC in the MCF-7 cells (Fig. 3.3C), indicating that, FOXO3a expression is not regulated by DNA methylation mechanism in the presence of BRCA1. Collectively, these data has highlighted the functional importance of BRCA1 in promoting FOXO3a expression through suppressing FOXO3a methylation in breast cancer.

(A)



(B)



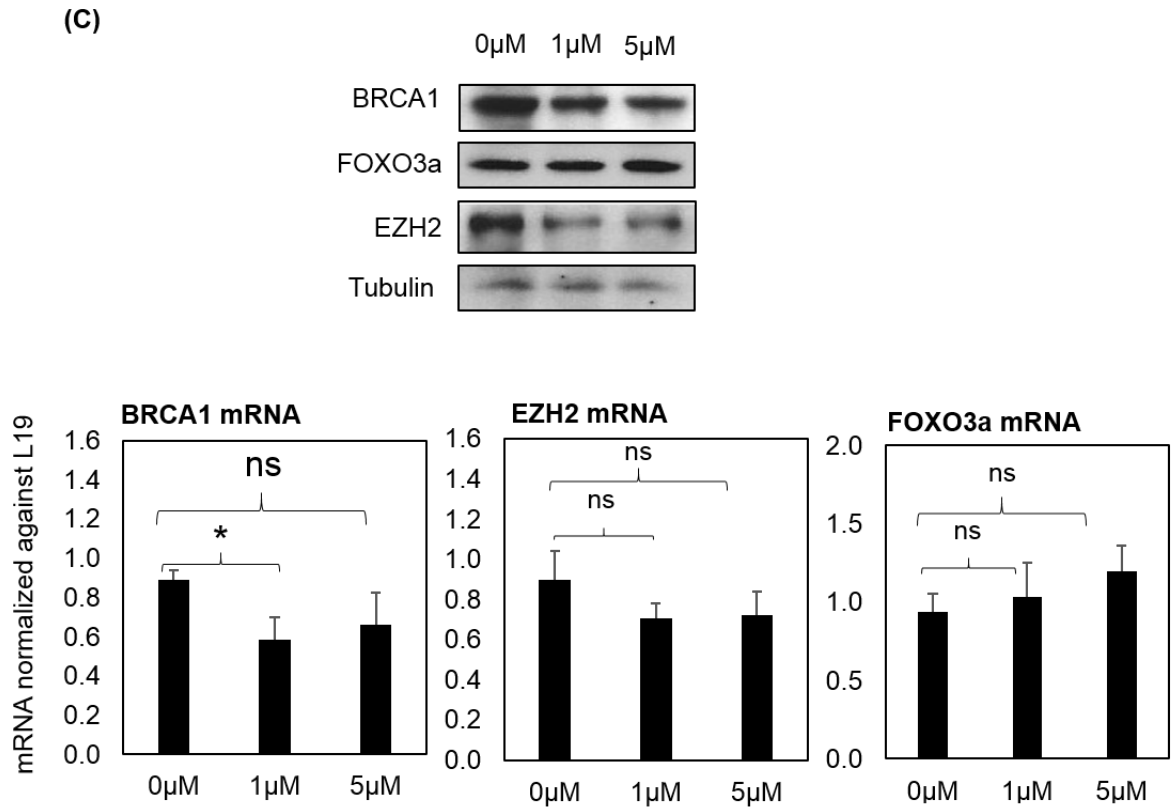


Figure 3.3. 5'-aza-dC treatment induces FOXO3a expression in HCC70, MDA-MB-468 but not in MCF-7. **(A)** In HCC70, FOXO3a expression was significantly induced by 5'-aza-dC treatment at the concentrations of 1 μM and 5 μM at the mRNA levels and of 5 μM at the protein levels. **(B)** FOXO3a mRNA and protein expression was induced after treatment with 1 μM and 5 μM of 5'-aza-dC in MDA-MB-468 cells. **(C)** 5'-aza-dC treatment did not alter the expression levels of FOXO3a in MCF-7. These experiments have been repeated three times and the representative western blots were shown and RT-qPCR data were expressed as mean±SD, *, p<0.05; **, p<0.01; ns, no significance.

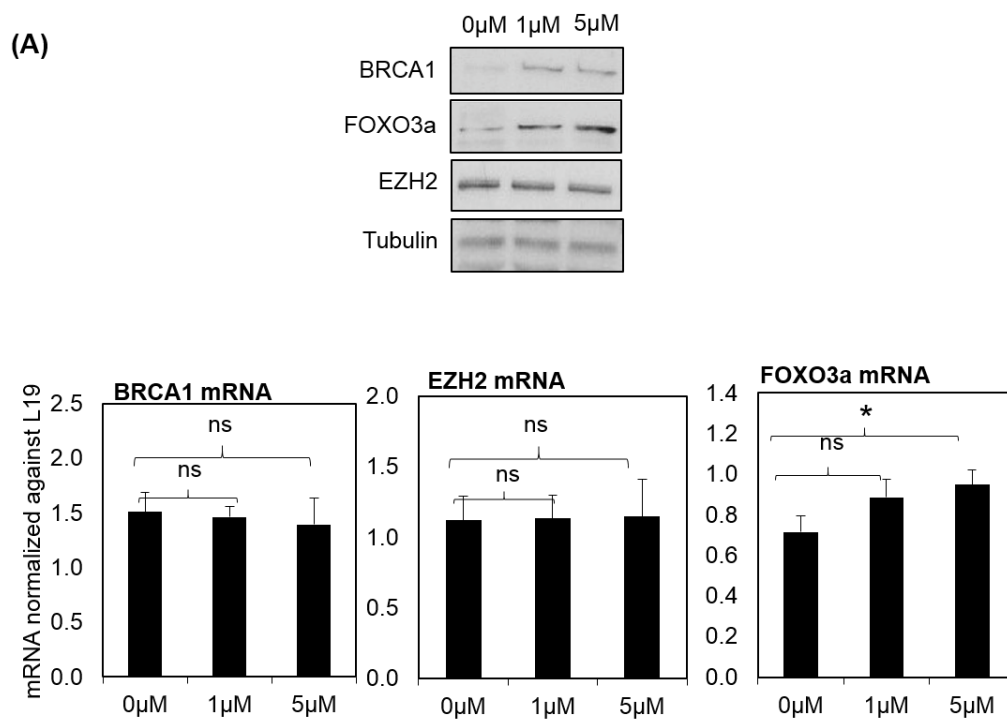
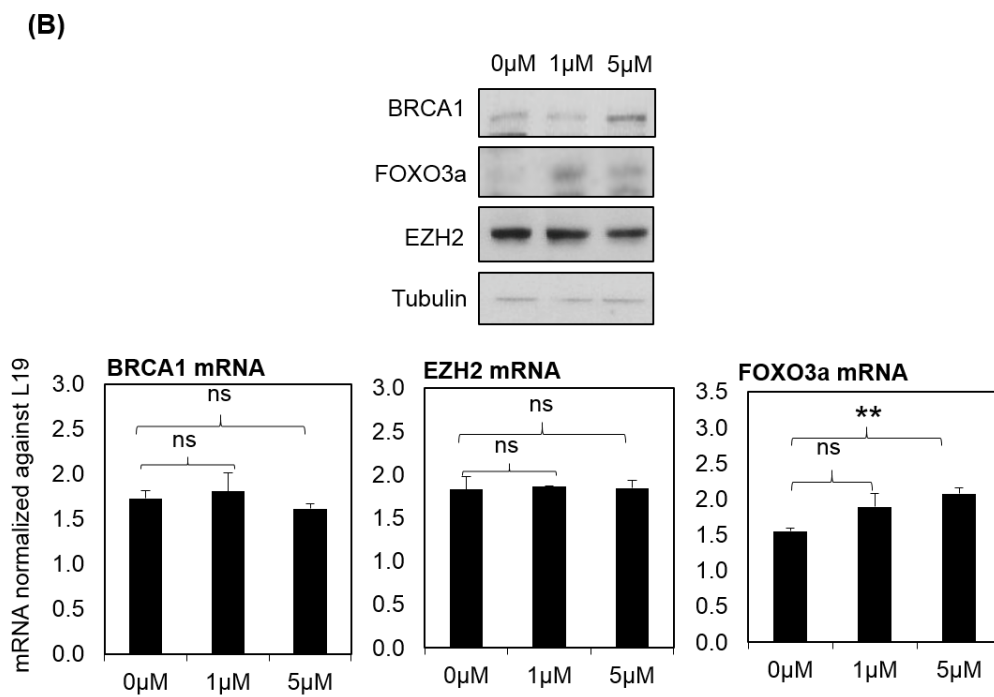
3.2.4 Inhibition or depletion of EZH2 induces FOXO3a expression

Next, the mechanism through which BRCA1 regulates FOXO3a methylation and the cofactors involved were explored. It has been shown in a previous study that BRCA1 interacts with EZH2 to inhibit its repression and histone 3 lysine 27 trimethylation (H3K27me3) on genes targeted by the PRC2 repressive complex in mouse embryonic stem and human breast cancer cells (Wang, Zeng et al. 2013). Our group has previously shown that BRCA1 inhibits promoter methylation and chromatin silencing of FOXA1 through interaction with EZH2 (Gong, Fujino et al. 2014). These findings raised the possibility that BRCA1 regulates FOXO3a expression through inhibition of EZH2 and histone 3 lysine 27 tri-methylation.

To test this idea, HCC70, MDA-MB-468 and MCF7 were treated with GSK126, a highly selective inhibitor of EZH2 methyltransferase activities (McCabe, Ott et al. 2012) and its effects on FOXO3a expression were studied by western blot and RT-qPCR. It was found that FOXO3a mRNA and protein levels were significantly induced by treatment with 5 μ M of GSK126 in both the BRCA1-low basal-type cell HCC70 and BRCA1-mutated basal-type cell MDA-MB-468 ($p < 0.05$ and $p < 0.01$ students' t-test, Fig. 3.4A and Fig. 3.4B, respectively). In comparison, FOXO3a expression was not affected by GSK126 treatment in MCF7 cells which expressed high level of wild-type BRCA1 (Fig. 3.4C). These data indicate that EZH2 is involved in the repression of FOXO3a expression in the BRCA1-deficient cells (HCC70 and MDA-MB-468) but not in the BRCA1-competent MCF-7 cells.

To further confirm this finding, EZH2 was depleted in the HCC70 and MCF7

cells by siRNA. The knockdown efficiency of EZH2 after 72 h was confirmed by western blot and qRT-PCR analysis. It was found that, in HCC70 cells, EZH2 depletion significantly induced FOXO3a expression both at the protein and mRNA levels when compared with the non-silencing controls as well as with the mock transfected cells (Fig. 3.5A). However, in MCF-7 cells, EZH2 knockdown did not affect FOXO3a expression (Fig. 3.5B). This result, together with the result of GSK126 inhibitor treatment, support the idea that EZH2 is functionally active in negatively regulating FOXO3a transcription in BRCA1-deficient breast cancer cells, while such activity is repressed by BRCA1 in BRCA1-competent cells.



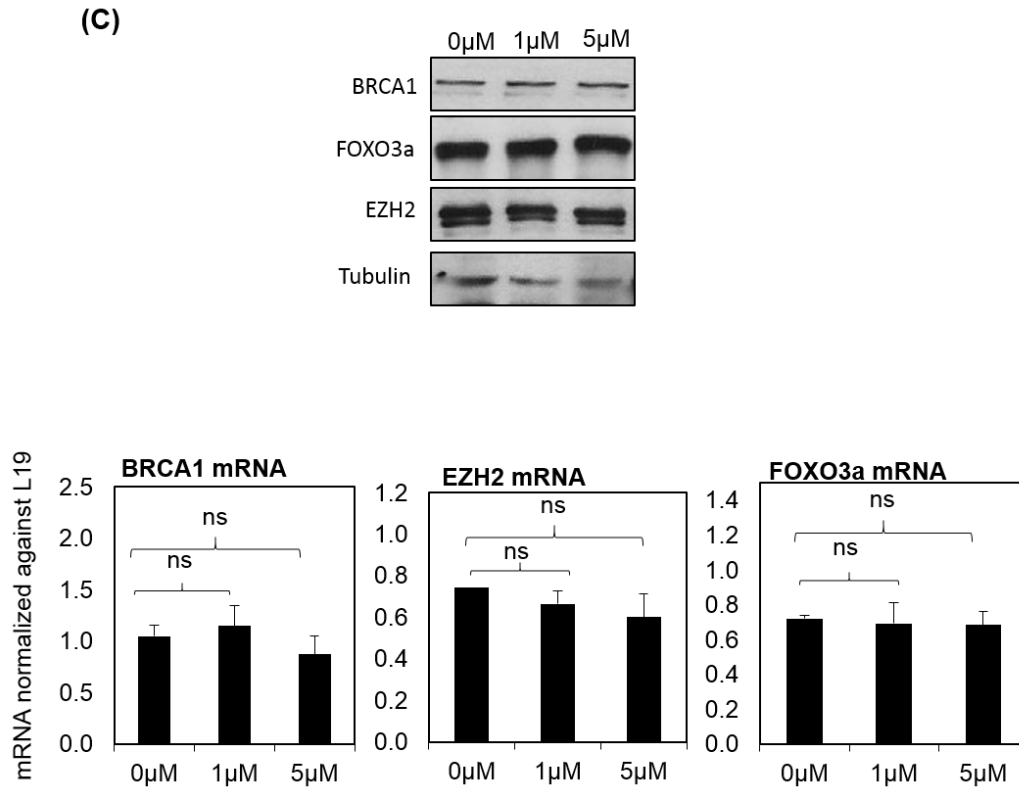


Figure 3.4. Inhibition of EZH2 induced FOXO3a expression in basal type cell lines. **(A)** GSK126 treatment significantly induced FOXO3a expression in HCC70 cells. **(B)** GSK126 treatment significantly induced FOXO3a expression in MDA-MB-468 cells. **(C)** GSK126 could not induce FOXO3a expression in MCF-7 cells. These experiments have been repeated three times and the representative western blots were shown and RT-qPCR data were expressed as mean+SD, *, $p < 0.05$; **, $p < 0.01$; ns, no significance.

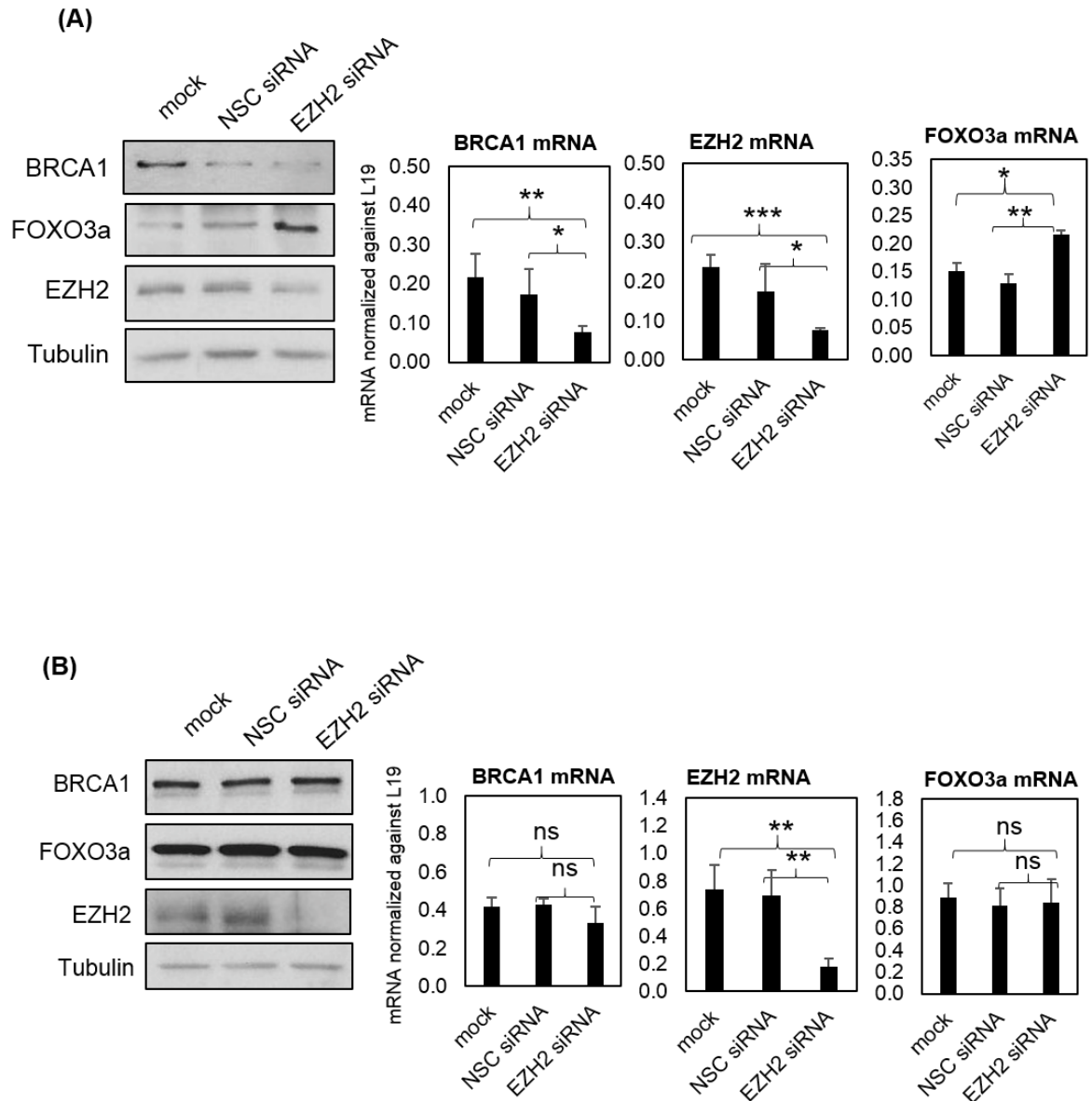


Figure 3.5. Depletion of EZH2 induced FOXO3a expression in HCC70 but not in MCF-7. **(A)** Depletion of EZH2 by siRNA in HCC70 significantly induced FOXO3a expression. **(B)** Depletion of EZH2 by siRNA in MCF-7 did not affect FOXO3a expression. These experiments have been repeated three times and the representative western blots were shown and RT-qPCR data were expressed as mean+SD, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, no significance.

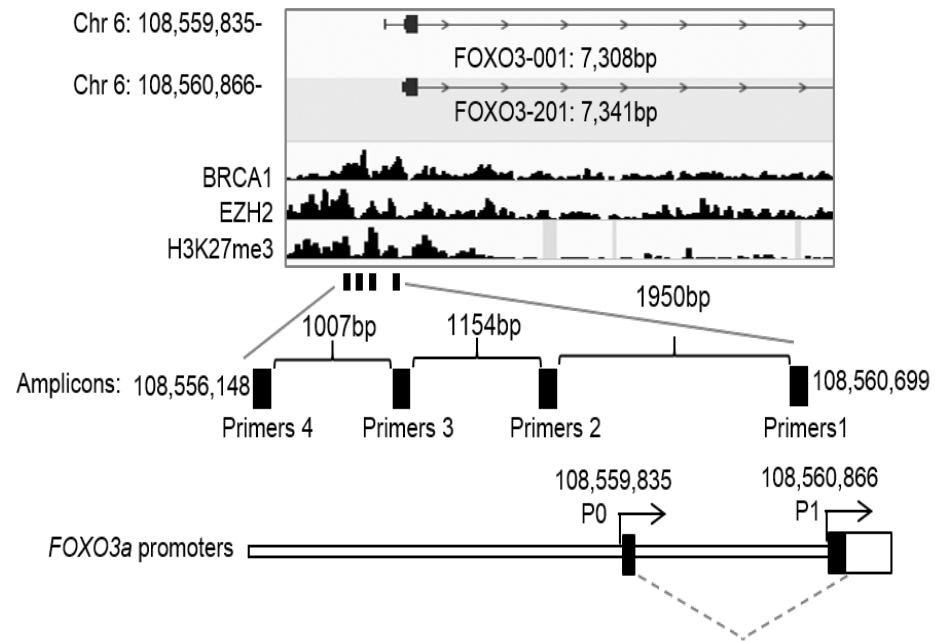
3.2.5 FOXO3a promoter is enriched with EZH2 and H3K27me3 in basal-type cells

The data presented above indicated that the functions of EZH2 to suppress the expression of FOXO3a is inhibited by BRCA1 in breast cancer. Previously, it has been shown that EZH2 is capable of causing trimethylation of histone 3 lysine 27 and, subsequently triggering the recruitment of DNA methyltransferases (DNMTs) including DNMT1, DNMT3a and DNMT3b to induce DNA methylation at target genes (Vire, Brenner et al. 2006). Collectively, these and the present findings suggest the possibility that EZH2 induces H3K27 trimethylation which facilitates the recruitment of DNMTs to promote DNA methylation at the FOXO3a promoter in BRCA1-deficient cells.

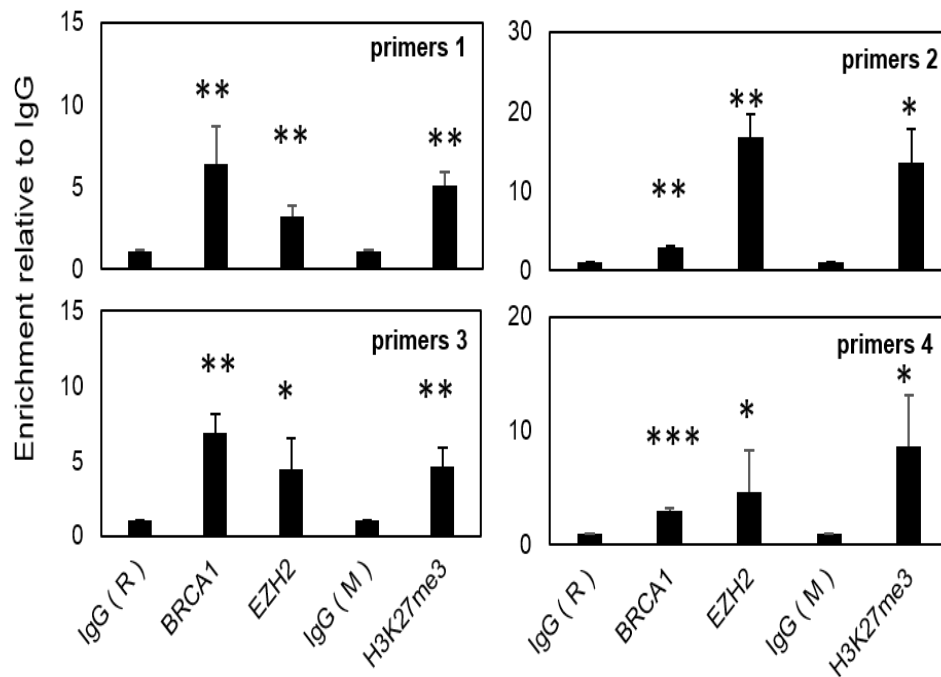
To test this conjecture, chromatin immunoprecipitation (ChIP) analysis was employed to investigate the occupancy of BRCA1, EZH2, DNMT1, DNMT3a, DNMT3b, and H3K27me3 at the human FOXO3a promoter region in BRCA1-low HCC70 cells and BRCA1-mutated MDA-MB-468 cells as well as the BRCA1-competent MCF-7 cells and recruitment to the endogenous *FOXO3a* promoter was examined by RT-qPCR following ChIP. *In Silico* analysis of the transcription factor ChIP-seq datasets in MCF-7 cell available in ENCODE project repository (Consortium 2004) (done by Dr. Ana R Gomes, Department of Surgery and Cancer, Imperial College London) identified two putative binding regions for EZH2 and BRCA1, respectively, located at around 1000 bp apart from each other on the FOXO3a promoter region (Fig. 3.6a). Primers for ChIP-qPCR were designed to amplify across each predicted EZH2 or BRCA1 binding regions on the FOXO3a promoter. The RT-qPCR results showed that the BRCA1

and EZH2 binding sites of the FOXO3a promoter were enriched for BRCA1, EZH2 and H3K27me3 in the BRCA1-deficient HCC70 (Fig. 3.6b) and MDA-MB-468 (Fig. 3.6c) cells, as detected by all four sets of primers, denoted by the significant enrichment of protein binding comparing to the IgG control. In contrast, in MCF7, although there was enrichment of BRCA1 and EZH2 to the BRCA1 binding site on FOXO3a promoter, there was no significant enrichment of H3K27me3 to FOXO3a promoter (Fig. 3.6d). These results further support the notion that BRCA1 inhibits EZH2 to recruit H3k27me3 to the promoter of FOXO3a. The recruitment of DNMTs to FOXO3a was also studied and compared in the three cell lines by ChIP-qPCR. Consistently, deposit of DNMT1, DNMT3a and DNMT3b to FOXO3a promoter were detected in HCC70 (Fig. 3.7a) and MDA-MB-468 (Fig. 3.7b) but not in MCF7 (Fig. 3.7c). All together, these results support the hypothesis that BRCA1 inhibits functions of EZH2 in the trimethylation of H3K27 and assembly of DNMTs to FOXO3a promoter.

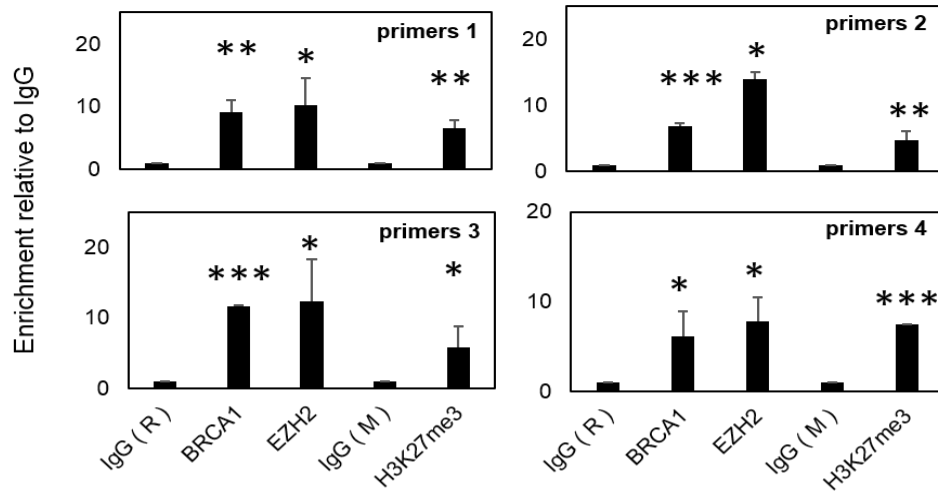
(A) Predicted binding sites of BRCA1 and EZH2 in FOXO3a promoter



(B) HCC70



(C) MDA-MB-468



(D) MCF7

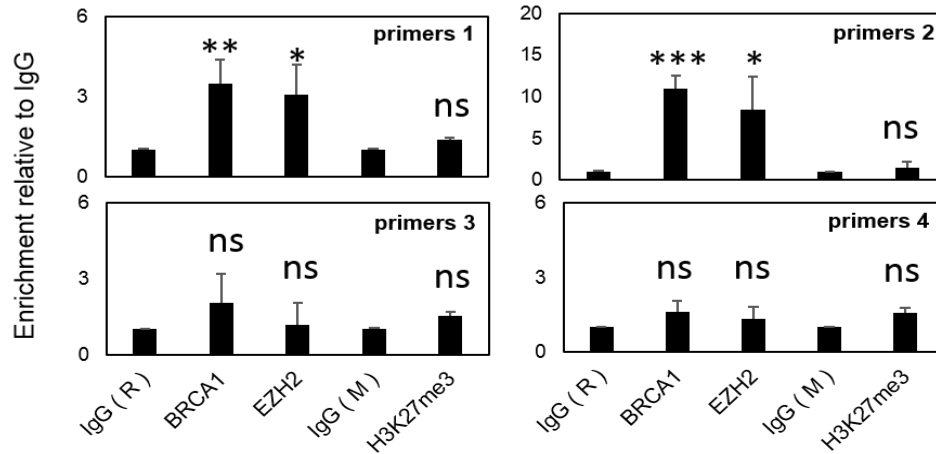
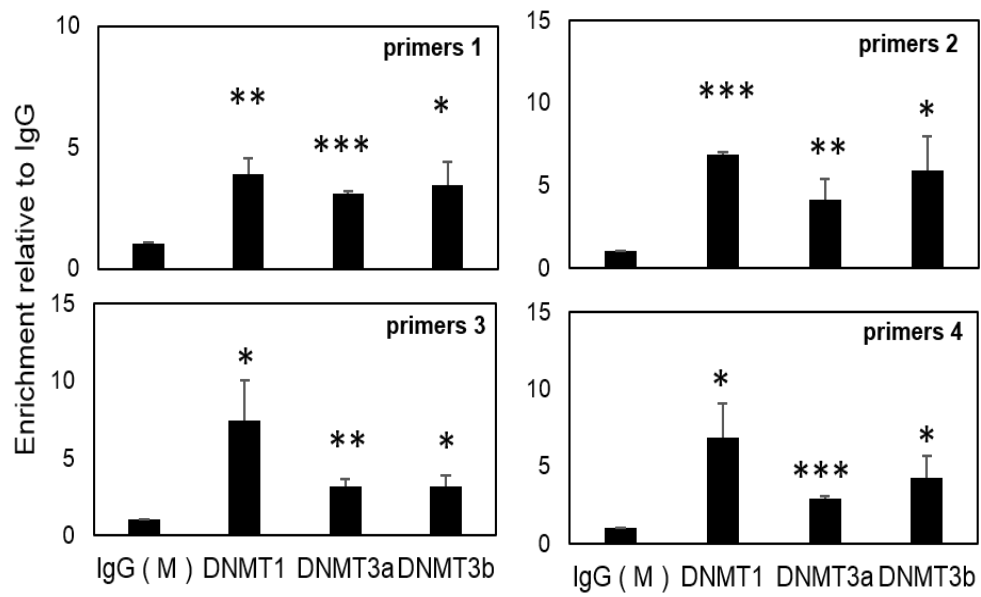
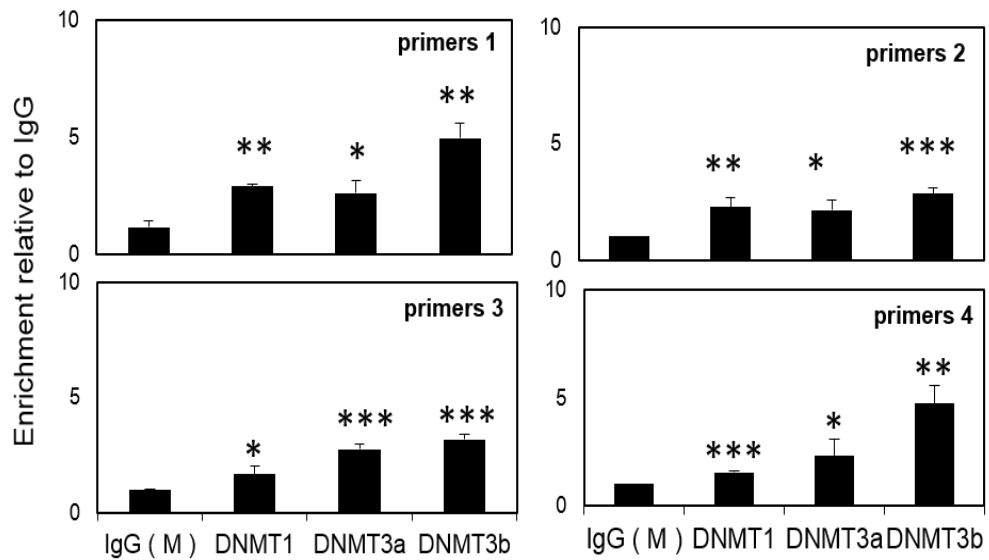


Figure 3.6. H3K27me3 occupancy of FOXO3a promoter was detected in basal type cell lines but not in MCF-7. **(A)** The predicted binding sites of EZH2 and BRCA1 to FOXO3a promoter BRCA1, EZH2, and H3K27me3 bind to the FOXO3a promoter in **(B)** HCC70 and **(C)** MDA-MB-468 cells as revealed by ChIP-qPCR analyses. **(D)** In MCF-7, BRCA1 and EZH2 bind to FOXO3a promoter but H3K27me3 does not. These experiments have been repeated three times and the RT-qPCR data were expressed as mean+SD relative to IgG control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ns, no significance, compared with IgG control, students' t-test.

(A) HCC70



(B) MDA-MB-468



(C) MCF7

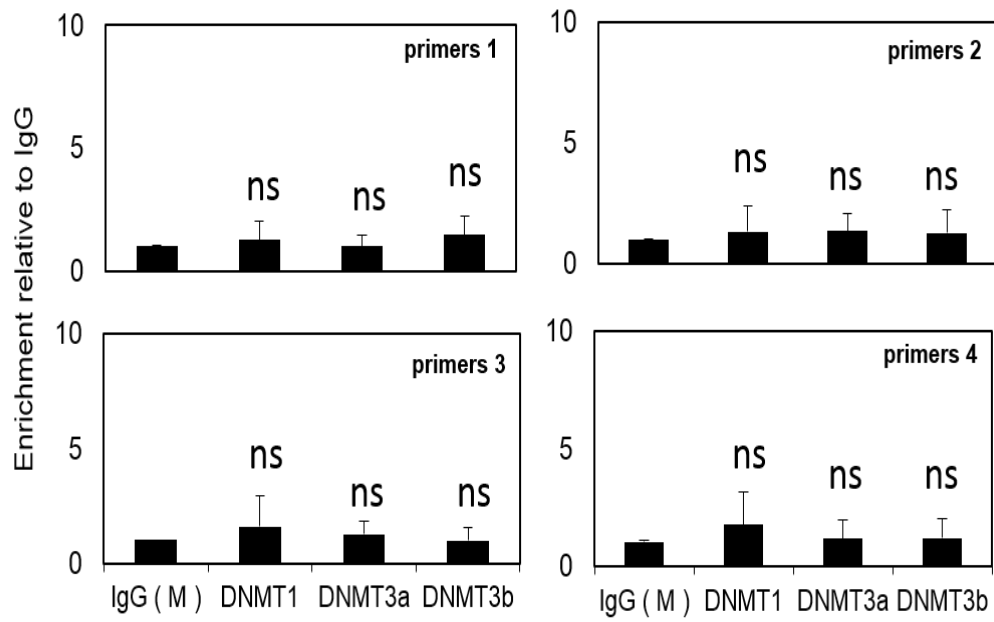


Figure 3.7. DNMT1, DNMT3a and DNMT3b bind to FOXO3a promoter in basal type cell lines **(A)** HCC70 and **(B)** MDA-MB-468 but not in **(C)** MCF-7 as analysed by ChIP-qPCR. These experiments have been repeated three times and the RT-qPCR data were expressed as mean+SD relative to IgG control. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ns, no significance, compared with IgG control, students' t-test.

3.2.6 BRCA1 represses the deposit of H3K27me3 and DNMTs to FOXO3a promoter

To establish further the role of BRCA1 in regulating EZH2-induced deposition of histone mark H3K27me3 and DNMTs on the FOXO3a promoter, BRCA1 was silenced using siRNA in MCF-7 cells which express high level of wild-type BRCA1. Cells were harvested for ChIP-qPCR analysis 72 h after transfection.

It was found that, when compared with the NSC siRNA-transfected controls, depletion of BRCA1 induced an accumulation of H3K27me3 on FOXO3a promoter, as detected by all four pairs of primers, the difference being statistically significant using primers 1, 3 and 4 ($p < 0.05$, students' t-test, Fig. 3.8). In addition, knockdown of BRCA1 using siRNA induced the recruitment of DNMT1, DNMT3a, and DNMT3b to FOXO3a promoter (Fig. 3.9) and such changes were detected using all four sets of primers with the difference between statistically significant using primers 1, 2 and 3. These results are in line with the hypothesis that wild-type BRCA1 plays a crucial role in suppressing EZH2-mediated recruitment of H3K27me3 and DNMTs to methylate FOXO3a promoter and repress its expression.

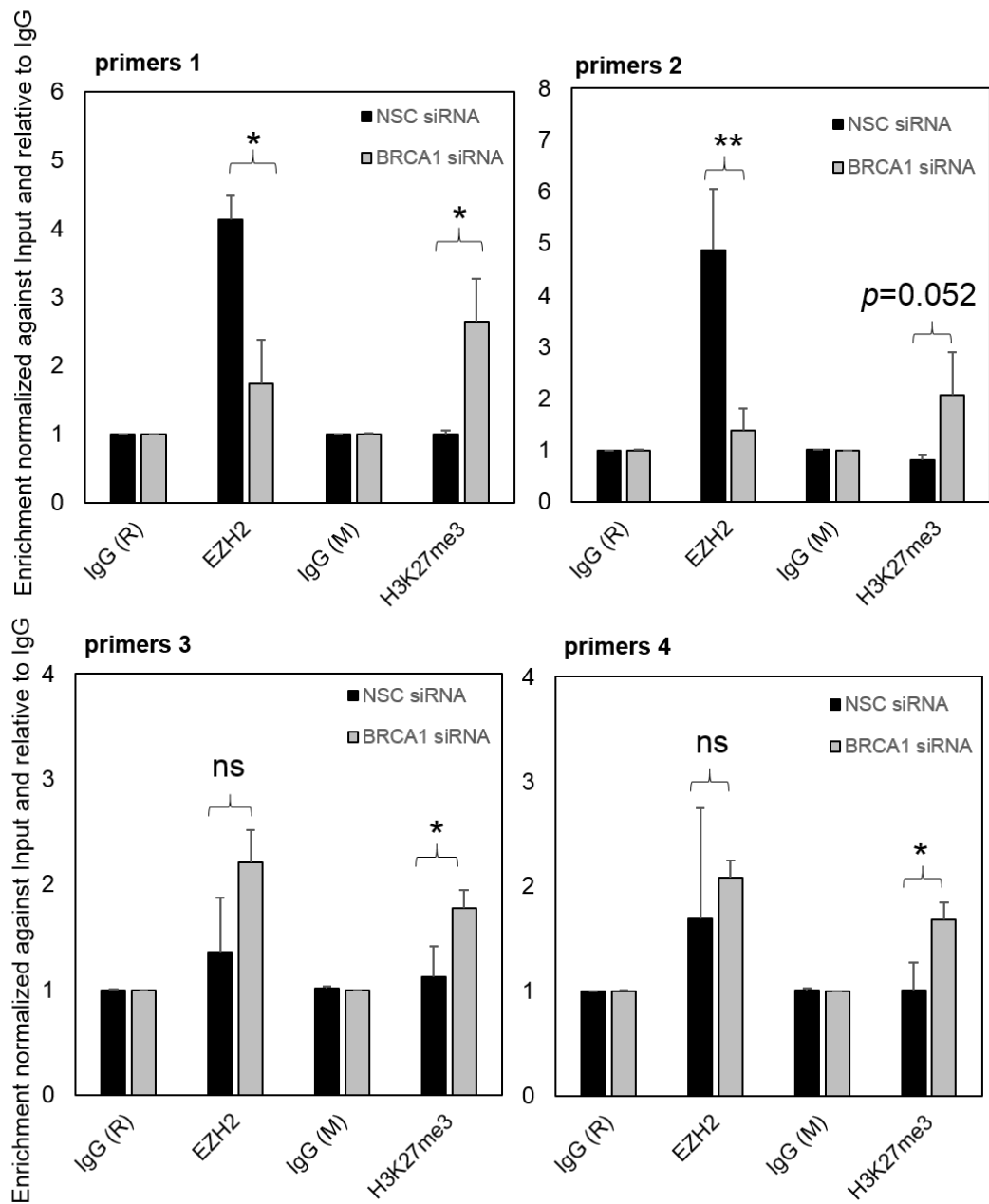


Figure 3.8. Silencing of BRCA1 in MCF-7 promoted binding of H3K27me3 to FOXO3a promoter in MCF-7. These experiments have been repeated three times and the RT-qPCR data were expressed as mean+SD relative to IgG control. *, $p<0.05$; **, $p<0.01$; ns, no significance.

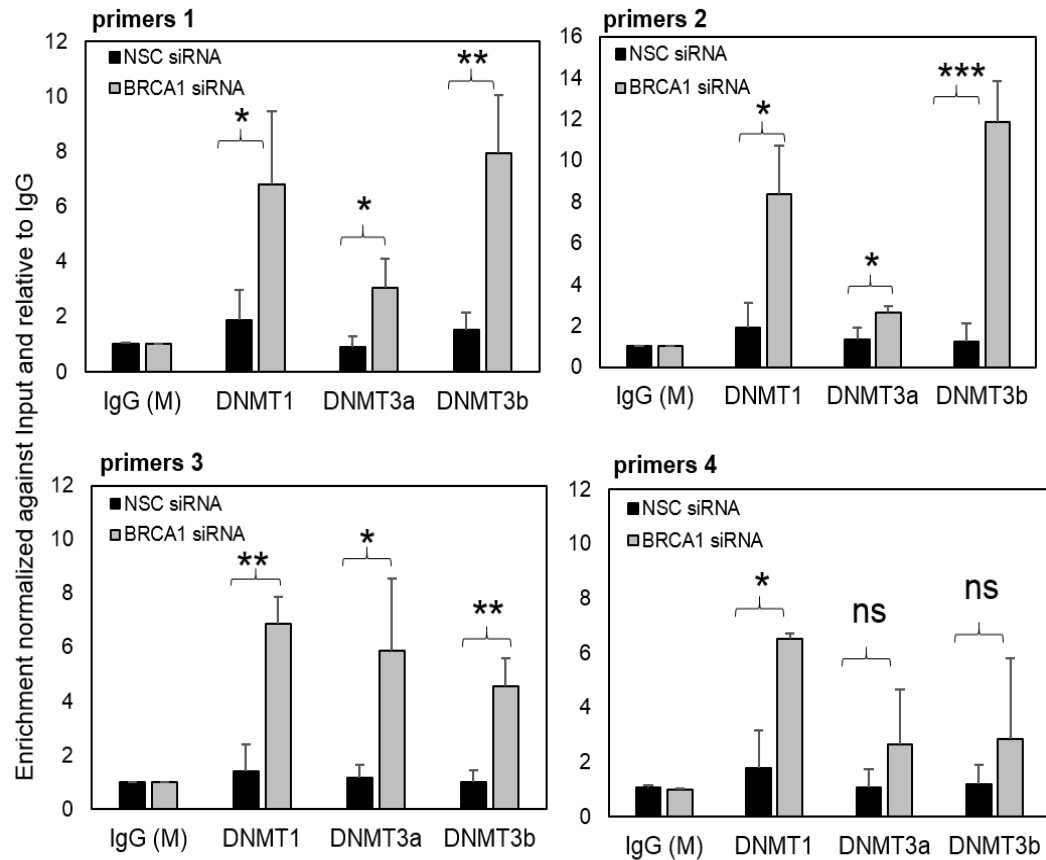


Figure 3.9. Silencing of BRCA1 in MCF-7 promoted binding of DNMTs to FOXO3a promoter in MCF-7, which were detected in all four sets of primers. These experiments were repeated three times and the RT-qPCR data expressed as mean+SD relative to IgG control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, no significance.

3.2.7 BRCA1 suppresses cell proliferation at least partially through FOXO3a

To establish the functional significance of the BRCA1-FOXO3a regulatory axis in breast cancer, further investigation was done to evaluate if the anti-proliferative function of BRCA1 is mediated through FOXO3a. To this end, HCC70 cells were transfected with either FOXO3a siRNA or NSC siRNA together with BRCA1 expression vector or the control empty vector (pcDNA3). Knockdown FOXO3a and overexpression of BRCA1 was confirmed by western blot (Fig. 3.10a). Consistent with their tumour suppressor functions, FOXO3a depletion by siRNA (orange line) accelerated cell proliferation when compared with the control cells transfected with NSC siRNA and empty vector (NSC+EV, blue line) while BRCA1 overexpression (purple line) suppressed the cell proliferation (Fig. 3.10b). Importantly, BRCA1 overexpression failed to effectively suppress cell proliferation when FOXO3a was depleted by siRNA (green line). These results suggest that anti-proliferative function of BRCA1 was achieved, at least partially, through FOXO3a.

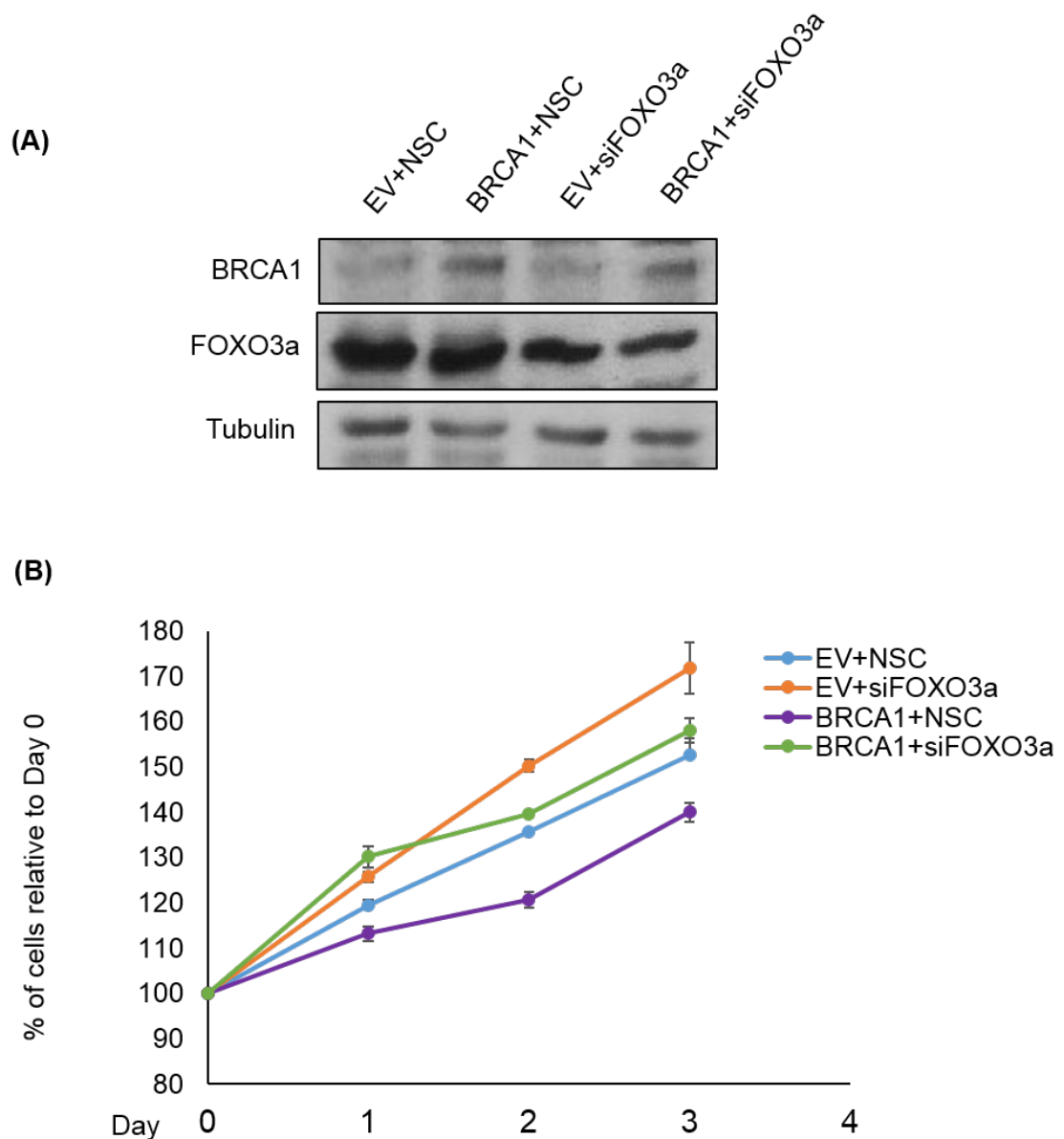


Figure 3.10. BRCA1 suppresses cell proliferation, at least partially, through FOXO3a. (A) FOXO3a was depleted by siRNA in HCC70 cells and BRCA1 or the expression vector alone was transfected into the cells. (B) BRCA1 overexpression failed to suppress cell proliferation when FOXO3a was depleted. These experiments were repeated three times and the data presented as mean $\pm$ SD.

3.2.8 FOXO3a is hyper-methylated and down-regulated in BRCA1-mutated breast tumours

Having established that BRCA1 positively regulates FOXO3a expression through modulation of the ability of EZH2 to mediate FOXO3a methylation and silencing in breast cancer cell lines, I next investigated the association between FOXO3a methylation and BRCA1 mutation status in a published methylated DNA immunoprecipitation (MeDIP) dataset covering 10 kb around 25,500 transcription start sites using familial breast tumour samples collected by kConFab (The Kathleen Cuninghame Foundation Consortium for Research into Familial Breast Cancer, Melbourne, Australia) (Flanagan, Cocciardi et al. 2010). The data was extracted from the Gene Expression Omnibus (GEO) (accession number is GSE17125) and analysed in SPSS. There are 33 samples in the cohort and, 11 of which harbour BRCA1-mutation, 8 samples are with BRCA2 mutation and 14 samples are non-BRCA1/2 mutated (BRCAx). Statistical analysis revealed that there were significant higher FOXO3a methylation levels in BRCA1-mutated tumours, when compared with BRCA2, BRCAx (non-BRCA1/2) or non-BRCA1 mutated (BRCA2/x) tumours (student's t-tests, $p=0.019$, $p=0.053$ and $p=0.026$, respectively) (Fig. 3.11).

To further confirm the correlation between FOXO3a expression and BRCA1 mutation status, IHC of FOXO3a was performed in the TMA constructed with 308 cases of Korean breast cancer samples with known mutation status of BRCA1 and BRCA2 (Fig. 3.12A). The representative images of the IHC staining of BRCA1-mutated, BRCA2-mutated or wild-type samples were shown in figure 3.12B. FOXO3a was found to be expressed both in cytoplasm and nucleus. The

expression levels of cytoplasmic and nuclear FOXO3a were scored in a semi-quantitative way as described in 2.17. FOXO3a expression level was represented as sum of cytoplasmic and nuclear FOXO3a score and was used for statistical analyses.

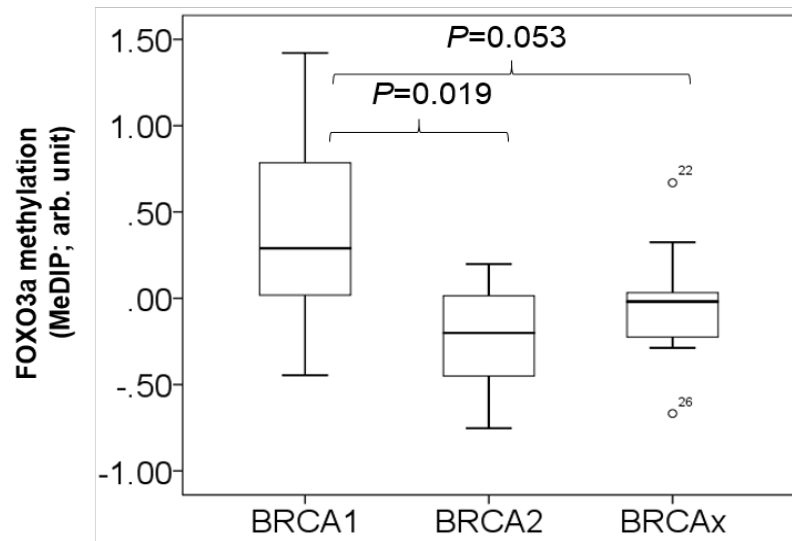
As shown in Figure 3.13, it was found that, although not statistically significant, the mean FOXO3a protein expression levels were lower in BRCA1 mutated samples when compared with BRCA2 mutated, non-mutated or non-BRCA1 mutated (including BRCA2 mutated or wild-type samples) (Mann Whitney U Rank test, $p=0.124$, $p=0.344$ and $p=0.19$, respectively). Since EZH2 could also affect FOXO3a expression, especially in BRCA1-mutated samples, therefore, it would be interesting to further investigate and compare the correlations between FOXO3a expression and BRCA mutation status in samples adjusted for their EZH2 expression levels.

As shown in figure 3.12B, EZH2 was expressed in both the cytoplasm and the nucleus. The expression levels of EZH2 in the cytoplasm were similar among all the samples with almost all the cell cytoplasm showing intermediate intensity, while the nuclear EZH2 expression varied among samples. Moreover, EZH2 is mainly functionally active in the nucleus to catalyse methylation of H3K27, and, therefore only nuclear EZH2 expression was taken into account when analysing the data. It was found that, in patients who expressed low nuclear EZH2 (using median score as cut-off), there was no significant difference in terms of FOXO3a expression levels between BRCA1 and BRCA2-mutated samples, between BRCA1 and wild-type samples, and between BRCA1 and non-BRCA1-mutated samples (Figure 3.14, Mann Whitney U Rank test, $p=0.072$, $p=0.507$, and

p=0.21, respectively). In contrast, in patients who expressed high nuclear EZH2, FOXO3a expression was significantly lower in BRCA1-mutated samples when compared with BRCA2-mutated samples, and with non-BRCA1 mutated samples (Figure 3.15, Mann Whitney U Rank test, p=0.002 and p=0.017, respectively). These results indicate that the regulation of FOXO3a by BRCA1 requires EZH2.

Collectively, these *in vivo* data provided strong evidence for the *in vitro* finding that BRCA1 positively regulates FOXO3a expression by suppressing the function of EZH2 to modulate trimethylation of H3K27 and promoter methylation of FOXO3a.

(A)



(B)

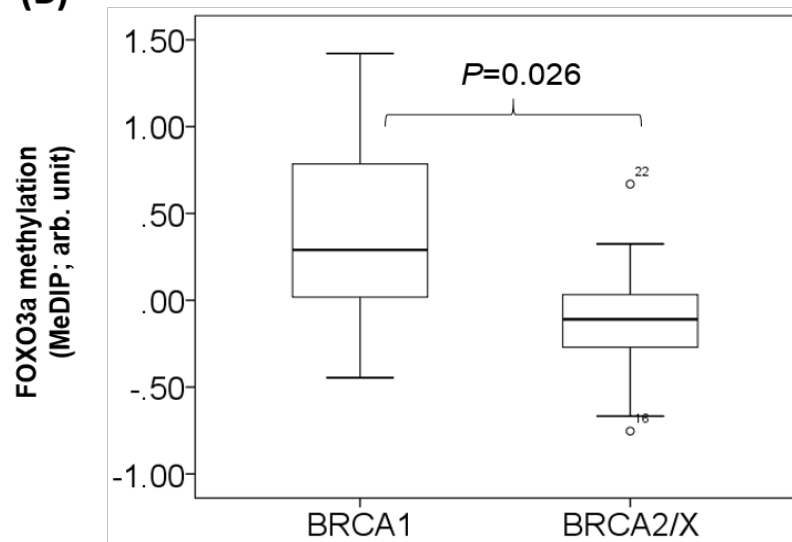


Figure 3.11 In 33 familial breast tumour samples, FOXO3a methylation scores were significantly higher in BRCA1 mutated samples comparing with **(A)** BRC2 or BRCAx ($p=0.019$ and $p=0.053$, respectively, students' t-test) or **(B)** BRCA2/x ($p=0.026$, students' t-test).

(A)

Mutation	Number of samples	%
BRCA1	62	20.1
BRCA2	96	31.2
wildtype	150	48.7
Total	308	100

(B)

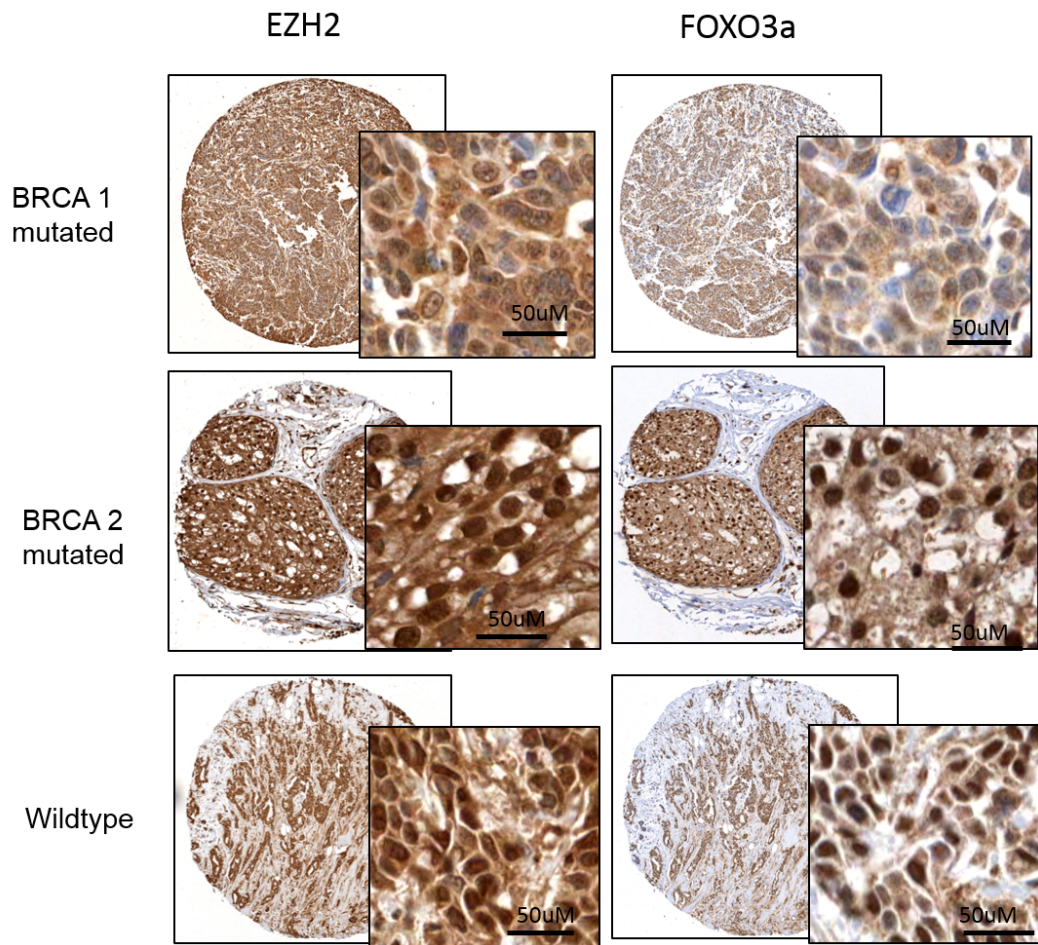
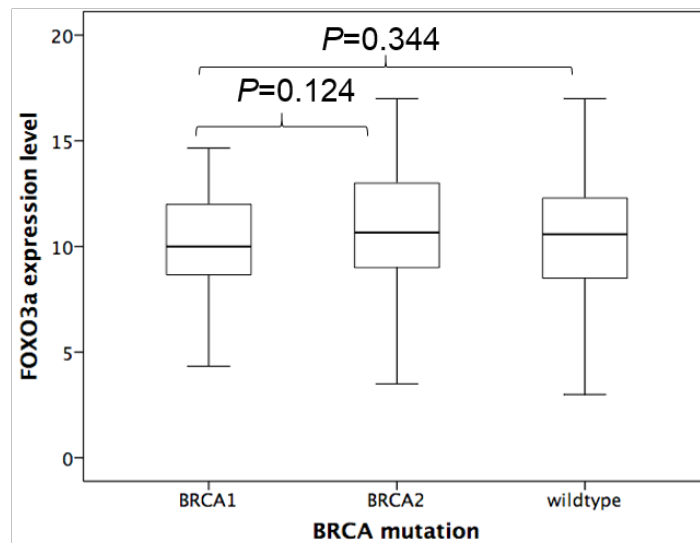


Figure 3.12 (A) BRCA mutation status of TMA constructed with 308 cases of Korean breast cancer samples. **(B)** The representative images of the IHC staining of BRCA1-mutated, BRCA2-mutated or wild-type samples.

(A)



(B)

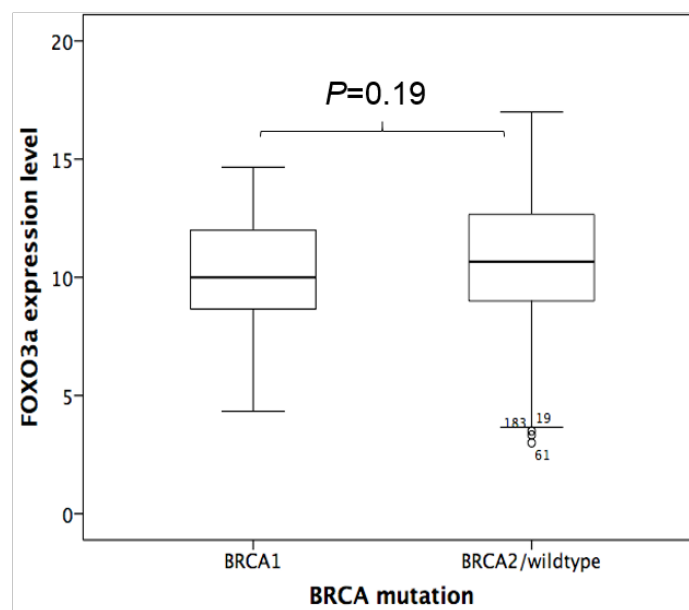
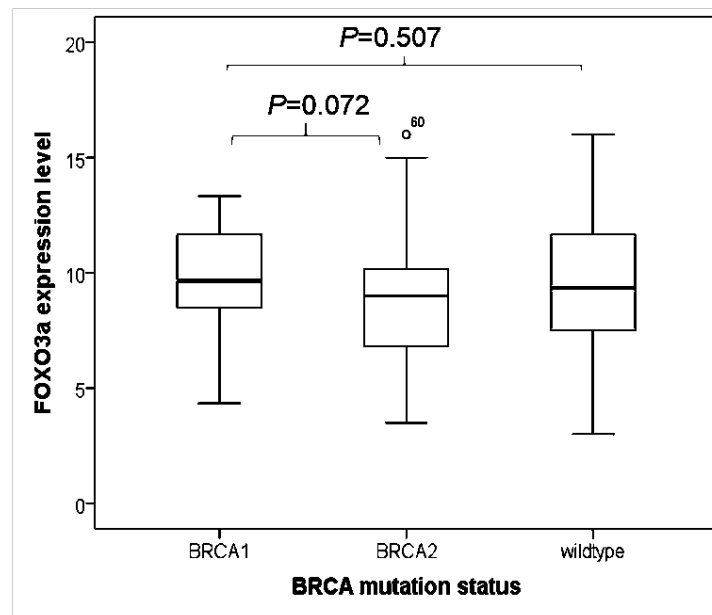


Figure 3.13 Comparison of FOXO3a expression levels with different BRCA mutation status by Mann Whitney U rank test using all samples. **(A)** Comparison of FOXO3a expression level between BRCA1-mutated samples and BRCA2-mutated/wild-type samples. **(B)** Comparison of FOXO3a expression level between BRCA1-mutated samples and non-BRCA1 mutated samples.

(A)



(B)

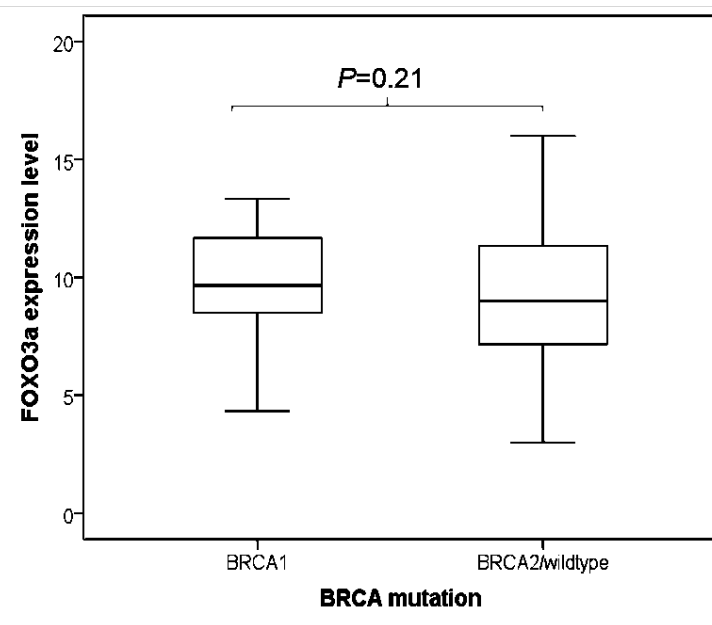


Figure 3.14 Comparison of FOXO3a expression levels with different BRCA mutation status compared using samples that express low levels of nuclear EZH2 by Mann Whitney U rank test. **(A)** Comparison of FOXO3a expression level between BRCA1-mutated samples and BRCA2-mutated/wild-type samples. **(B)** Comparison of FOXO3a expression level between BRCA1-mutated samples and non-BRCA1 mutated samples.

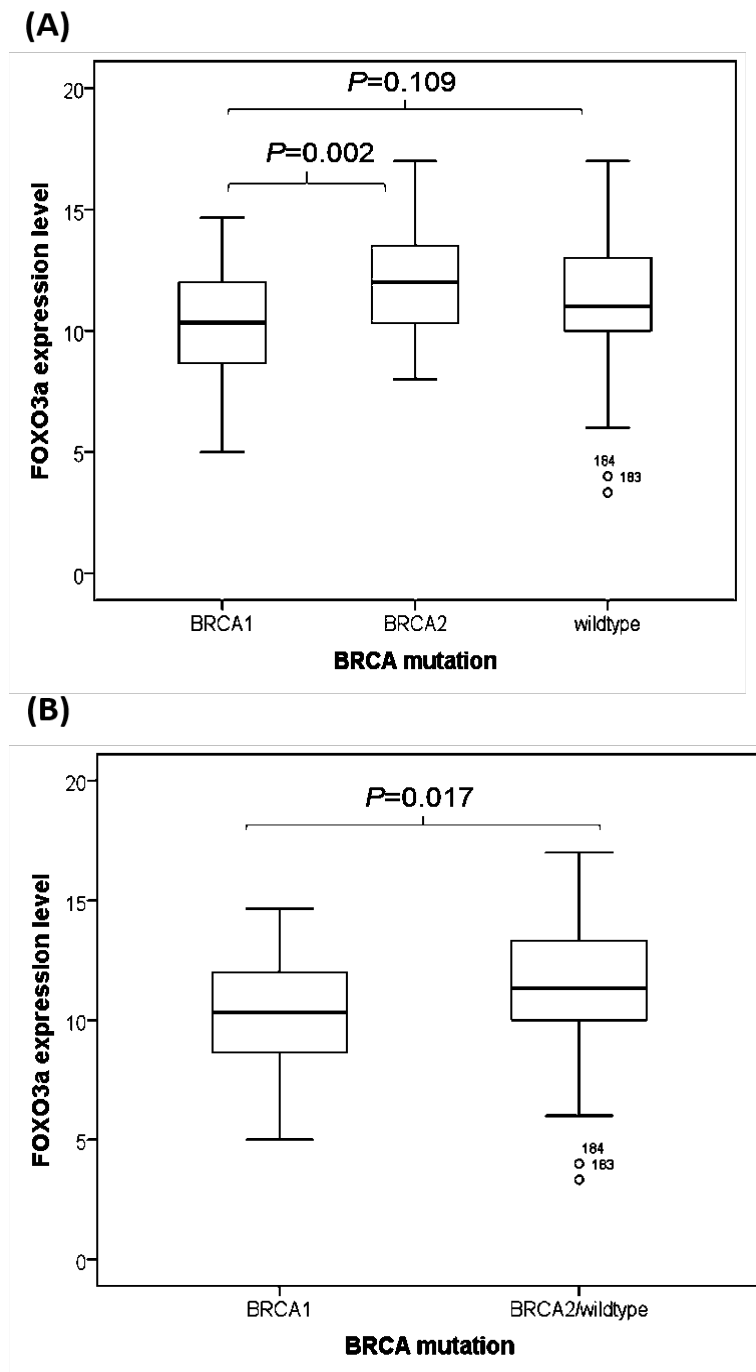


Figure 3.15 Comparison of FOXO3a expression levels with different BRCA mutation status compared using samples that express high levels of nuclear EZH2 by Mann Whitney U rank test. **(A)** Comparison of FOXO3a expression level between BRCA1-mutated samples and BRCA2-mutated/wild-type samples. **(B)** Comparison of FOXO3a expression level between BRCA1-mutated samples and non-BRCA1 mutated samples.

3.3 Discussion

The breast cancer susceptibility gene BRCA1 is an important tumour suppressor gene in breast cancer. Mutations in BRCA1 predispose women to a high lifetime risk of breast and ovarian cancer (Narod and Foulkes 2004). BRCA1 is a tumour suppressor that plays an important role in regulation of mammary epithelial cell differentiation and breast tumourigenesis. Deficiency of BRCA1 is often associated with higher risk of breast cancer and basal-like phenotype. Although it has been reported that BRCA1 imposes its tumour suppressive role by regulating DNA damage repair and cell cycle checkpoint, the exact reason why BRCA1 deficiency would cause more aggressive breast cancer phenotype remains largely unknown.

In this study, it is shown that BRCA1 regulates FOXO3a expression through suppressing EZH2-induced H3K27 trimethylation and DNMT1/3a/3b recruitment to FOXO3a promoter in human breast cancer. Following treatment with 5'-aza-dC, FOXO3a expression was restored in basal but not in BRCA1-competent MCF-7 cells (Figure 3.3), supporting the hypothesis that FOXO3a is silenced by hyper-methylation in the BRCA1-deficient basal cell lines. The data also reveal that the expression of FOXO3a is negatively regulated by EZH2 in basal cell lines where BRCA1 is down-regulated or mutated but not in BRCA1-competent MCF-7 cells (Figure 3.4 and 3.5), suggesting that, in the presence of BRCA1, EZH2 is functionally inactive in regulating FOXO3a. Consistently, there has been evidence suggesting the regulation of FOXO3a expression by DNA methylation. For example, FOXO3a expression is regulated by its DNA methylation status in mouse embryonic fibroblasts (MEFs) (Mori, Nada et al. 2014) and

hypomethylating agents including Aza-cytidine and Decitabine can restore FOXO3a in acute myeloid leukaemia (AML) patients (Thepot, Lainey et al. 2011). FOXO3a has also been identified as one of the target genes regulated by the EZH2-H3K27me3-dependent transcriptional network in hepatocellular carcinoma (Gao, Xu et al. 2014). Interestingly, FOXO3a has also been reported to be a transcriptional regulator of DNMT3b in lung cancer (Yang, Tang et al. 2014).

In addition, changes in BRCA1 expression were also observed following 5'-aza-dC treatment (Fig. 3.3) or EZH2 depletion (Fig. 3.5), which indicates that BRCA1 is regulated by epigenetic mechanisms. There have been multiple evidences in accordance with this. For instance, BRCA1 promoter hypermethylation was reported in triple negative breast cancer tumours (Yamashita, Tokunaga et al. 2015). EZH2 knockdown could up-regulate BRCA1 protein expression (Gonzalez, Li et al. 2009) and EZH2 overexpression induced nuclear export of BRCA1 (Gonzalez, DuPrie et al. 2011).

EZH2 is known to be able to epigenetically suppress the expression of target genes through modulating both histone and DNA methylation. Accordingly, in addition to regulating tri-methylation of H3K27, EZH2 also directly recruits DNA methyltransferases (DNMTs), including DNMT1, DNMT3a and DNMT3b, to enhance promoter methylation and gene silencing. BRCA1 is also capable of binding to EZH2 and negatively regulate the activity of PRC2 complex (Wang, Zeng et al. 2013). Furthermore, our group has previously showed that both BRCA1 and DNMT3b bind to EZH2 but do not exist in the same complexes in breast cancer cells (Gong, Fujino et al. 2014). It is, therefore, reasonable to suggest that BRCA1 can bind to EZH2, a subunit of the PRC2 complex, and

restrain its methyltransferase activity to promote the transcription of FOXO3a indirectly. In agreement with the hypothesis, the ChIP-qPCR assays showed that DNMT1, DNMT3a and DNMT3b and H3K27me3 are recruited to the promoter region of FOXO3a in BRCA1-low or -mutated cell lines but not in MCF-7 where the wildtype BRCA1 is expressed at high level (Figure 3.6 and 3.7). Furthermore, depletion of BRCA1 by siRNA in MCF-7 induces the deposition of H3K27me3, DNMT1, DNMT3a and DNMT3b onto the FOXO3a promoter (Figure 3.8 and 3.9).

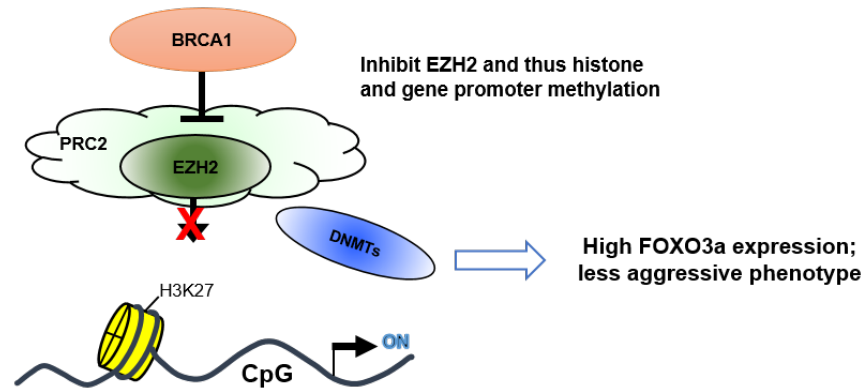
These *in vitro* results are further corroborated by the *in vivo* correlation between BRCA1 mutation and FOXO3a promoter methylation / protein expression. FOXO3a methylation levels were significantly higher in BRCA1-mutated tumours, compared to BRCA2, BRCAx (non-BRCA1/2) and non-BRCA1 mutated (BRCA2/x) tumours (Figure 3.11). More interestingly, in patients who expressed low EZH2, there is no significant difference in FOXO3a protein expression level when comparing BRCA1-mutated samples with BRCA2-mutated, wildtype or non-BRCA1 mutated samples (Figure 3.14). However, there is statistically significant difference in FOXO3a expression between BRCA1 mutated and BRCA2-mutated / non-BRCA1 mutated samples (Figure 3.15). These results indicate that BRCA1 is not important for the regulation of FOXO3a expression when EZH2 expression/activity is low, in other words, the role of BRCA1 in positively regulating FOXO3a expression is mediated through EZH2, consistent with the previous *in vitro* results showing that BRCA1 promotes FOXO3a expression through relieving the suppressive function of EZH2.

FOXO3a is a *bona fide* tumour suppressor that negatively regulates cell proliferation and cancer progression by regulating the expression of genes involved in differentiation, apoptosis, cell cycle regulation, DNA damage repair, oxidative stress response, metastasis and angiogenesis (Lam, Brosens et al. 2013). FOXO3a can also antagonize functions of FOXM1, which is a potent oncogene that have a central role in promoting cell differentiation, migration, invasion, angiogenesis, stem cell renewal and DNA damage repair (Lam, Brosens et al. 2013). As a result, FOXO3a expression is a good prognostic marker in breast cancer (Habashy, Rakha et al. 2011, Jiang, Zou et al. 2013). FOXO3a is also a molecular target of multiple clinically available or potential anti-cancer therapeutics and, its deregulation can often cause drug resistance. Doxorubicin treatment has been shown to cause the phosphorylation of FOXO3a by stress-activated p38 MAP kinase and subsequently nuclear localization and activation of FOXO3a (Ho, McGuire et al. 2012). Another recent study showed that low dose of metformin, which is the active metabolite of a topoisomerase-1 inhibitor derivative, suppresses breast and ovarian cancer growth both *in vitro* and *in vivo* in a FOXO3a-dependent manner (Hu, Chung et al. 2014). As FOXO3a is such an important tumour suppressor and the efficacy of the anti-cancer drugs often depends on FOXO3a, it is crucial to understand how FOXO3a expression is regulated in cancer. Past studies have reported that the FOXO3a activity/expression is mainly regulated by multiple kinases that could phosphorylate FOXO3a, which subsequently lead to nuclear exclusion and ubiquitination/degradation in the cytoplasm. This study reports a novel regulatory mechanism of FOXO3a by BRCA1 in breast cancer where BRCA1 indirectly regulates FOXO3a expression through interfering with EZH2-H3K27me3

interaction with FOXO3a promoter. This could play an important role in breast cancer progression and contribute to the understanding of the tumour suppressive role of BRCA1. In agreement, the current results also show that BRCA1 suppresses proliferation of basal type breast cancer cell line HCC70 at least partially through FOXO3a (Fig. 3.10), as depletion of FOXO3a by siRNA compromised the cell proliferation suppression induced by BRCA1 overexpression. The result suggests that the tumour suppressive role of BRCA1 is, at least, partially achieved through regulating FOXO3a expression in breast cancer. Interestingly and consistently, a recent Phase 1/1b trial of olaparib and carboplatin in BRCA1 or BRCA2 mutation-associated breast or ovarian cancer has revealed that FOXO3a expression is associated with responsiveness (Lee, Hays et al. 2014)

To summarize, the current study demonstrates that BRCA1 regulates FOXO3a expression through inhibiting the activity of EZH2 in breast cancer, whereas depletion or mutation of BRCA1 would lead to down-regulation of FOXO3a indirectly by activating of EZH2 to induce trimethylation of H3K27 and recruitment of DNMTs to methylate and silence FOXO3a promoter. The proposed mechanism is illustrated in Figure 3.16. In the presence of wild-type BRCA1, the EZH2 functions of histone and gene promoter methylation is suppressed, resulting in active transcription and high expression level of FOXO3a. Upon BRCA1 mutation or loss, EZH2 becomes functionally active in trimethylating H3K27 and recruiting DNMTs to repress FOXO3a expression, which could contribute to the development of more aggressive tumour behaviour. The findings from this study contribute to the better understanding of the tumour suppressor role of BRCA1 in breast cancer.

(A). BRCA1 (+)



(B). BRCA1 (-) or mutation

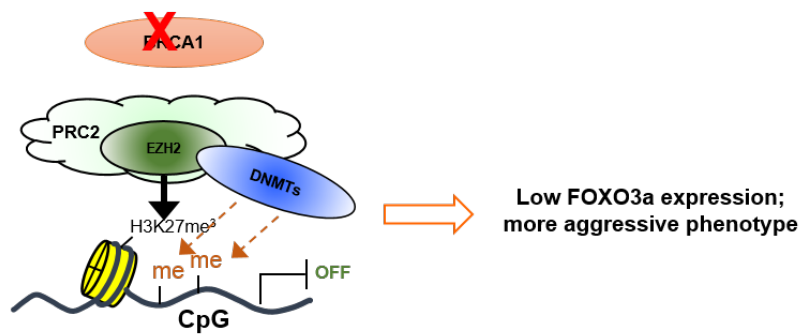


Figure 3.16 The proposed mechanism of how BRCA1 interfere with EZH2 to regulate FOXO3a expression and suppress tumour growth. **(A)** When wild-type BRCA1 is expressed, the EZH2 functions in modulating histone and gene promoter methylation in breast cancer are suppressed, resulting in active transcription and high expression level of FOXO3a. **(B)** Upon BRCA1 mutation or loss, EZH2 becomes functionally active in repressing FOXO3a expression, leading to low FOXO3a level.

As mentioned previously, BRCA1 loss or mutation is often associated with the triple negative breast cancer phenotype that expresses no ER and therefore *de novo* resistant to endocrine therapy such as tamoxifen. It has also been shown that decreased BRCA1 expression could cause tamoxifen resistance in breast cancer by altering the interaction between ER and its co-regulators (Wen, Li et al. 2009). Tamoxifen stands as the first line breast cancer adjuvant therapy, serving to prevent cancer progression, relapse and metastasis. However, tamoxifen resistance remains prominent issue in the clinic jeopardizing patients' survival. Molecular mechanisms related to tamoxifen resistance will be discussed and studied in detail in the following chapter.

Chapter 4 A splice variant of NCOR2 induces tamoxifen resistance in breast cancer through competing and inhibiting functions of NCOR2 and is a predictive marker of tamoxifen response

4.1 Introduction

4.1.1 NCOR2 in breast cancer

As introduced in chapter 1, breast cancer is the most common cancer affecting women worldwide. Oestrogen receptor (ER) signalling pathway is a fundamental pro-proliferative pathway in the context of breast cancer. Upon its activation by binding with oestrogen, ER activates target gene transcription and cell growth either directly through its genomic pathway or indirectly through a non-genomic pathway that involves PI3K/AKT signalling cascade. Most of the well-characterized ER-target genes are oncogenic that could promote cancer cell proliferation or resistance to apoptosis. For example, binding of oestrogen to ER stimulates the expression of cyclin D1 which is one of the master regulators of cell cycle progression (Eeckhoute, Carroll et al. 2006, Yamaga, Ikeda et al. 2013). The anti-apoptotic Bcl2 that contributes to apoptosis resistance and cell

survival in various types of cancer has a promoter containing the oestrogen-response element and is transcriptionally regulated by Oestrogen-ER signalling (Romano, Adriaens et al. 2010). A small secretory protein called pS2 (also known as TFF1) which can protect cells from *in vitro* induced apoptosis and is involved in resistance to doxorubin-induced cell death in breast cancer cells, is also an oestrogen-response gene (Rio and Chambon 1990, Pelden, Insawang et al. 2013).

Around 70% of breast cancer patients are ER positive and can benefit from anti-oestrogen therapy. Tamoxifen is a selective oestrogen receptor modulator (SERM) that acts as an antagonist of oestrogen in the context of breast cancer and is the most commonly prescribed anti-oestrogen drug to ER positive patients for preventing of breast cancer relapse or metastasis. In the context of breast cancer, as shown in figure 4.1, tamoxifen competes with oestrogen for binding to ER and binding of tamoxifen to ER triggers the recruitment of nuclear co-repressor 2 (NCOR2, also known as SMRT, Silencing Mediator of Retinoic acid and Thyroid hormone receptor) as well as other co-repressors, such as GPS2, TBLR1, HDAC3 to form a functional co-repressor complex, which will, in turn, halt the transcriptional activation of oestrogen-response genes and suppress the pro-proliferative ER signalling pathway (Sharma, Saxena et al. 2006, Zhang, Chang et al. 2006, Cheng and Kao 2009). In the formation of this co-repressive machinery, NCOR2 plays a core role by dimerizing with itself between regions encompassing amino acids 290-427 and 1788-1903 in an anti-parallel fashion and serving as a central platform on which the additional co-repressor proteins assemble (Varlakhanova, Hahm et al. 2011).

NCOR2 is a large protein with more than 2300 amino acid residues and consists of at least four repression domains (RD) within the N-terminal portion (denoted as RD1 to RD4) and a series of more C-terminal nuclear receptor interaction domains (denoted S1 and S2) (Nagy, Kao et al. 1999, Perissi, Staszewski et al. 1999). As represented in figure 4.2, RD1, corresponding to amino acid 1-316 is responsible for interaction with GPS2 and TBLR1. The two SANT motifs located in RD2 are critical components of deacetylase activation domain that bind and activate HDAC3. RD3 is important for interaction with HDAC4 and HDAC5 while RD4 is also for interaction with HDAC3, GPS2 and TBLR1 (Sharma, Saxena et al. 2006, Zhang, Chang et al. 2006, Cheng and Kao 2009). The nuclear receptor interaction domains at the C-terminus of the NCOR2 protein is responsible for interaction with various nuclear receptors such as oestrogen receptor and androgen receptor (Lavinsky, Jepsen et al. 1998, Liao, Chen et al. 2003).

Alternative splicing of NCOR2 during premature mRNA processing is commonly reported, generating multiple co-repressor isoforms with conserved repression domains but different affinities for different nuclear receptors (Malartre, Short et al. 2004, Goodson, Jonas et al. 2005). These isoforms are often found to be expressed in a tissue-specific manner and none of them has been reported in breast tissues. Alternative splicing of NCOR2 is thought to be the means of creating functional diversity, allowing the customization of its co-repressor functions in different cells (Goodson, Jonas et al. 2005).

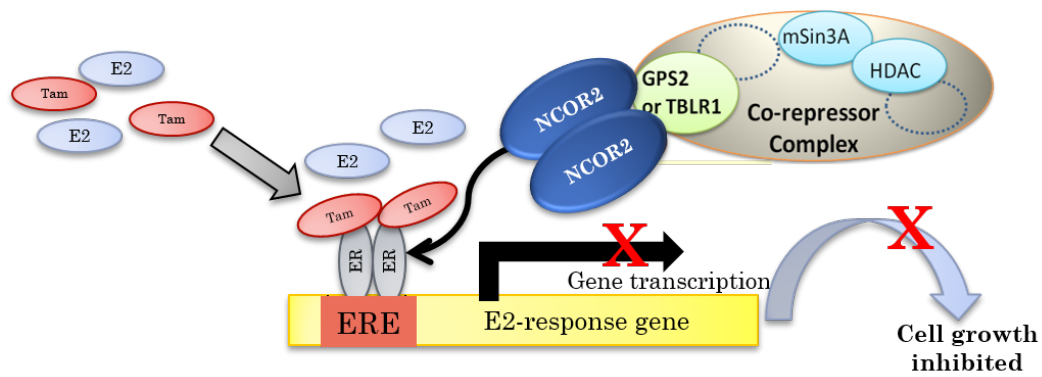


Figure 4.1 The molecular cascade followed by tamoxifen treatment. Tamoxifen competes with E2 for binding to ER. Binding of tamoxifen to ER would trigger the recruitment of NCOR2 as well as other repressor proteins to suppress the transcription activation of oestrogen-response gene.

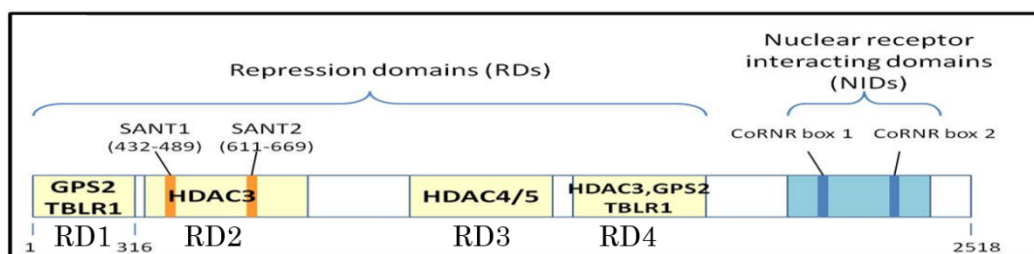


Figure 4.2 The functional domains of NCOR2 protein. NCOR2 protein contains four repression domains responsible for interacting with other co-repressor proteins and two nuclear receptor interacting domains responsible for interacting with nuclear receptors e.g. ER.

4.1.2 The splice variant, BQ323636.1, in tamoxifen resistance

Alternative splicing is an important posttranscriptional mechanism to cope with the need for gene diversity, yielding multiple protein products from a single gene. Many studies have reported that alternatively spliced variants are differentially expressed in samples of tumours including those of breast, ovarian and prostate, when comparing with normal tissues (Malartre, Short et al. 2004, Klinck, Bramard et al. 2008, Venables, Klinck et al. 2008).

By SpliceArray profiling, our group has reported a novel splice variant of NCOR2, named BQ323636.1, with exon 11 skipping during mRNA processing, resulting in an early translation stop codon and a severely truncated protein product. It retains only the RD1 domain (Zhang, Gong et al. 2013) compared with its wild type and, is therefore, predicted to be unable to interact with nuclear receptors.

In a cohort of 77 breast cancer patients, BQ323636.1 overexpression at mRNA level was significantly associated with tamoxifen resistance, which was defined as patients who had been treated with tamoxifen in the adjuvant setting but who subsequently developed relapse or distant metastasis. These patients generally demonstrated poor overall and disease-free survival (Zhang, Gong et al. 2013). These findings led us to postulate that this splice variant could serve as a biomarker for prediction of tamoxifen response. However, before any definite conclusions can be reached, the underlying molecular mechanism need to be characterized and the findings need to be validated using *in vivo* models.

As this splice variant is capable of modulating transcription of luciferase reporter containing oestrogen-response element (ERE) in the promoter region in a way

opposite to NCOR2 (Zhang, Gong et al. 2013), which suggests the possibility that it confers tamoxifen resistance through competing with and counteracting the repressive functions of its wild-type. Moreover, although RT-qPCR analysis showed that BQ323636.1 is expressed only at a level of 2% to 5% of its wild-type in the cell lines, western blot analysis using an antibody which can recognise both NCOR2 and BQ32363.1 showed the protein level of this variant was comparable to that of its wild-type (Zhang, Gong et al. 2013), suggesting that BQ323636.1 is significantly regulated by translational or post-translational mechanisms. NCOR2 protein is regulated by proteasome-mediated degradation and a phosphorylation site at Ser2410 on NCOR2 was found to be the recognition motif for nuclear exportation of NCOR2 and subsequent proteasome-mediated degradation (Hoberg, Popko et al. 2006). Since this phosphorylation site is deleted in BQ323636.1, it lead to the postulation that BQ323636.1 could be protected from proteasome-mediated degradation hence higher protein stability.

4.1.3 Research Initiatives and the hypothesis

In the adjuvant setting, almost all patients who are ER positive will be treated with tamoxifen for prevention of disease relapse and metastasis. Despite the success of tamoxifen, resistance is an outstanding problem with up to 50% of non-responding patients and many of the initial responders subsequently experience relapse (Ring and Dowsett 2004). However, there is no clinically in-use biomarker for prediction of patients' response to tamoxifen and the mechanisms for developing tamoxifen resistance are still not well understood.

The splice variant of NCOR2, BQ323636.1, could be a predictive marker for tamoxifen response in breast cancer patients. However, the previous study only investigated the association of BQ323636.1 with tamoxifen resistance at the mRNA level since there was no antibody available to directly distinguish this isoform from its wild-type except through difference in molecular size as seen in Western blot. Thus it became important for us to generate a BQ32363.1-specific antibody in order to properly validate our previous findings at protein level. This antibody could be readily used for immunohistochemical staining on human breast cancer tissue sections, to facilitate the prediction of tamoxifen response. Furthermore, a BQ323636.1-specific antibody would be indispensable for further *in vitro* mechanistic and *in vivo* studies.

Moreover, the molecular mechanism through which BQ323636.1 could confer resistance to tamoxifen remains unknown. Based on our previous observations, it was hypothesized that BQ323636.1 could functionally compete with NCOR2 in modulating tamoxifen-induced suppression of ER-target genes.

4.2 Results

4.2.1 BQ323636.1 is overexpressed in tamoxifen resistant breast cancer cells

The monoclonal anti-BQ323636.1 antibody was generated by a German company named InVivo Biotech, and the protocol for antibody generation and quality control is attached in Appendix I. The epitope of this antibody mapped to the last 11 amino acid residues on BQ323636.1 protein which distinguish the variant from its wild-type.

The expression level of BQ323636.1 was examined by RT-qPCR and western blot using two pairs of cell lines: MCF7 (ER+ve, Tamoxifen sensitive) vs LCC2 (ER+ve, Tamoxifen resistant) and ZR75 (ER+ve, Tamoxifen sensitive) vs AK47 (ER-ve, Tamoxifen resistant). It was found that, BQ323636.1 overexpression was only detected at the mRNA level in AK47 compared with ZR75 but not in the MCF7/LCC2 pair (Figure 4.3A). However, at the protein level, BQ was found to be overexpressed in both LCC2 and AK47 compared with MCF7 and ZR75, respectively (Figure 4.3B). The finding supports the notion that BQ323636.1 is involved in tamoxifen resistance and the discrepancy between mRNA and protein expression level of BQ323636.1 suggests that it is also regulated by post-transcriptional mechanisms.

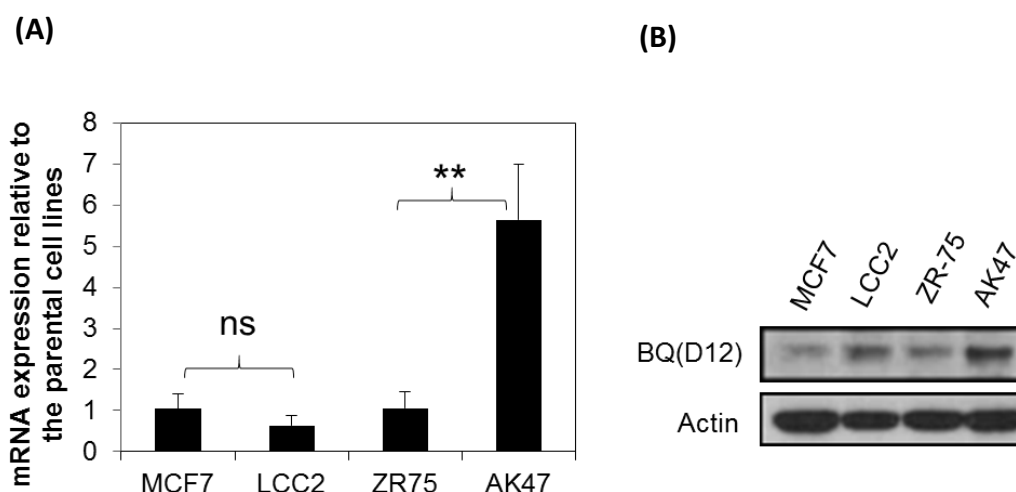


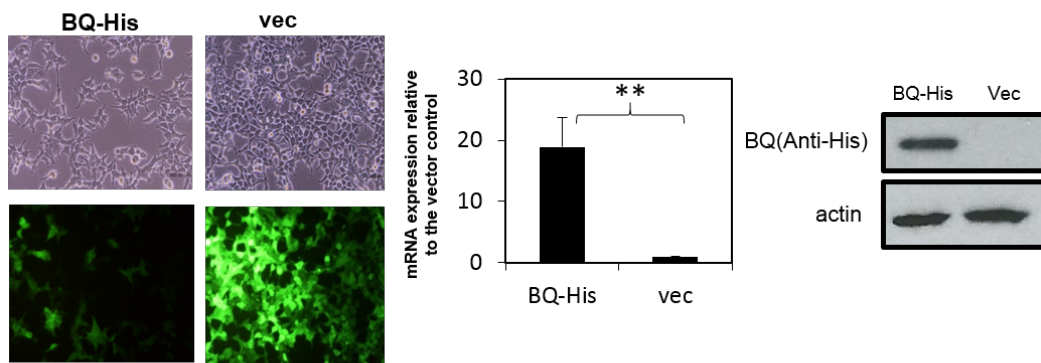
Figure 4.3 Expression levels of BQ323636.1 examined by RT-qPCR and western blot analyses. **(A)** At the mRNA level, differential expression of BQ323636.1 was only detected in the ZR75/AK47 cell line pair. **(B)** BQ323636.1 was over-expressed at the protein level in Tamoxifen-resistant cell lines (LCC2 and AK47) when compared to their parental tamoxifen-sensitive cell lines (MCF7 and ZR-75), respectively. Actin was used as loading control. The experiments were repeated three times independently and the results represented as mean+SD. The statistical difference was tested by two-tailed students' t test. **, $p < 0.01$; ns, no significance.

4.2.2 BQ323636.1 overexpression induced tamoxifen resistance in breast cancer cell lines

To confirm the role of BQ323636.1 in conferring tamoxifen resistance *in vitro* and *in vivo*, BQ323636.1 was stably overexpressed using lentiviral transduction system in tamoxifen sensitive cell lines MCF7 and ZR75 which express low levels of BQ323636.1 compared with their tamoxifen resistant counterparts. Controls were generated by transducing the cells with empty vector alone. Cells that were successfully infected were marked by GFP and were selected by puromycin. Overexpression of BQ323636.1 was confirmed by RT-qPCR and western blot (Fig. 4.4).

The responses of ZR75-BQ/ZR75-vec (vector) and MCF7-BQ/MCF7-vec to tamoxifen treatment were studied using MTT assay. It was found that, as shown in figure 4.5, overexpression of BQ323636.1 (blue lines) could significantly induce resistance to tamoxifen in both ZR75 and MCF7 cell lines, marked by enhanced viability of cells under treatment of a series of tamoxifen concentrations when compared with the vector controls (orange lines). This result is consistent with our previous finding using transient transfection (Zhang, Gong et al. 2013) and further strengthens the role of BQ323636.1 in conferring tamoxifen resistance.

(A) ZR75



(B) MCF7

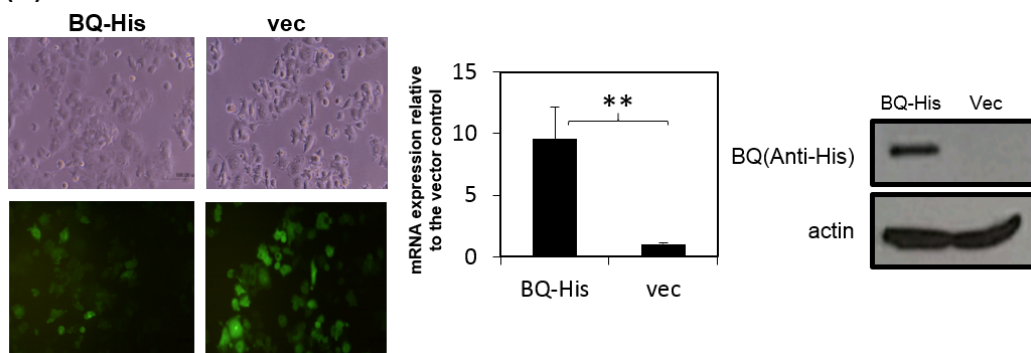


Figure 4.4 BQ323636.1-overexpressing stable cell lines were produced using the lentiviral system. BQ-His refers to the BQ323636.1-overexpression cell line while vector refers to the cell line transduced with empty vector as control. Cells that were successfully transduced were labelled with GFP. The overexpression of BQ323636.1 was also confirmed at mRNA level and protein level. RT-qPCR result was presented as mean+SD. **, $p < 0.01$.

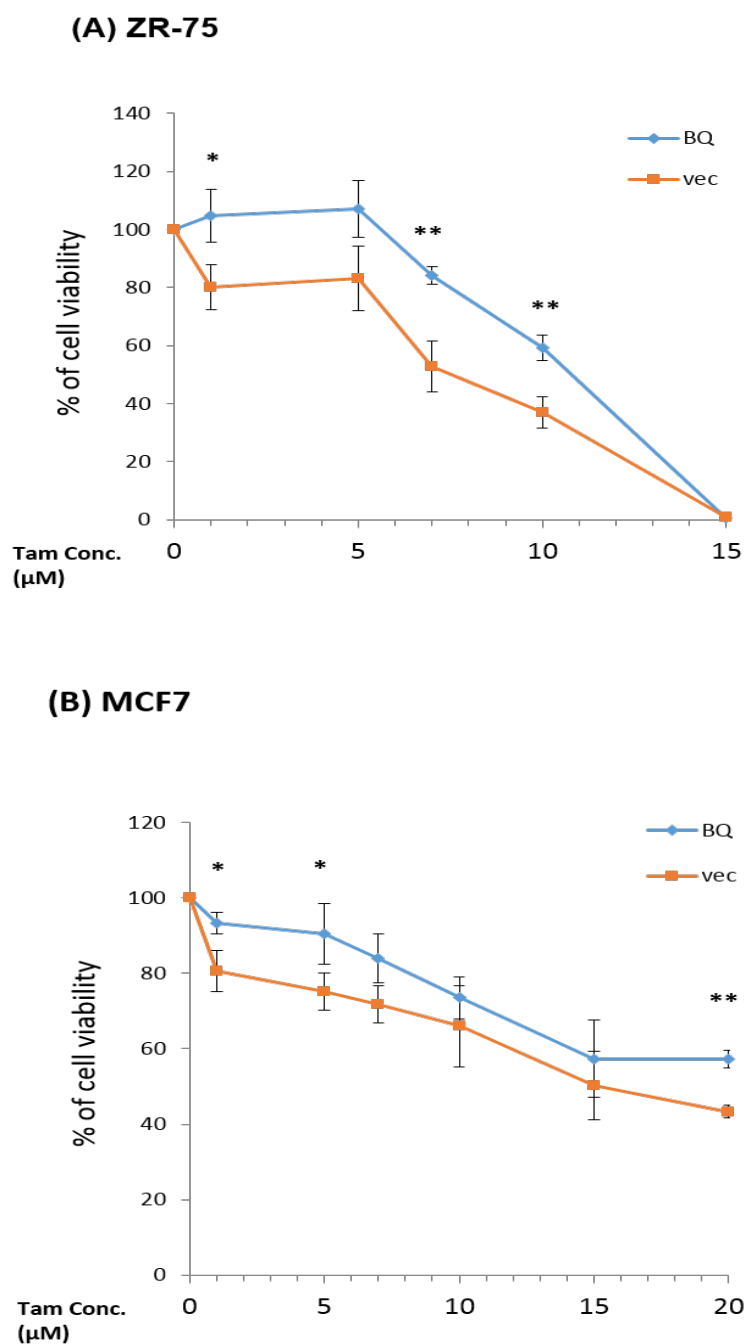


Figure 4.5 BQ323636.1-overexpression promotes tamoxifen resistance. **(A)** In ZR75, cell viability was significantly higher under the concentrations of 1μM, 7μM and 10μM. **(B)** In MCF7, cell viability was significantly higher under the concentrations of 1μM, 5μM and 20μM. The experiments have been repeated three times independently and result was presented as mean±SD. *, $p<0.05$, **, $p<0.01$.

4.2.3 BQ323636.1 overexpression induced tamoxifen resistance in vivo

The causal role of BQ323636.1 in inducing tamoxifen resistance was further confirmed *in vivo* using an orthotopic mouse model established by inoculating the stable cell lines into mammary fat pad of 6 to 7 weeks old female nude mice. Tumours were allowed to grow until the sizes were palpable, at which time (day 0), mice were randomized into treatment and control groups. Mice in the treatment group received tamoxifen treatment daily for 6 consecutive days per week while mice in the control group received solvent (peanut oil) oil. Tumour growth was expressed as percentage of tumour volume relative to day 0.

As shown in figure 4.6A, ZR75-vec (vector transfected only) tumours were sensitive to tamoxifen treatment (orange line) as marked by significant reduction of tumour growth when compared with controls (blue line). However, when BQ323636.1 was overexpressed (Fig 4.6B), the tumours failed to respond to tamoxifen treatment, as there was no significant difference in terms of tumour growth rate when comparing between the treatment (orange line) and the control (blue line) groups. Representative pictures of H&E staining of the tumour were also shown. Similar results were also observed using MCF7 (Fig. 4.7), showing that the effects were not cell line specific. Taken together, these results provide solid proof to support the claim that BQ323636.1 overexpression induces tamoxifen resistance.

Since tamoxifen can prevent both breast cancer progression and metastasis, the metastases of GFP-labelled cancer cells was also examined using PE IVIS Spectrum in vivo imaging system (HKU Faculty core facility). The lungs and livers of mice after euthanasia were taken out and washed three times in 1xPBS

and placed in the imaging system. GFP signals (green) can be detected in the lungs of 3 out of 5 mice in ZR75-vec control mice without tamoxifen treatment, indicating potential metastasis of cancer cells to the lungs in these mice, while no GFP signals can be detected in the tamoxifen-treated ZR75-vec mice (Fig.4.8A). In comparison, GFP signals can be observed in the lungs of both ZR75-BQ control and treatment mice (1/5 and 2/5, respectively, Fig. 4.8B). The results suggest that ZR75-BQ xenografts are insensitive to tamoxifen treatment and are consistent with the previous data (Fig. 4.6). There were no GFP signals detected in the livers of any ZR75 mice or in lungs and livers of any MCF7 mice (data not shown). However, the tumour metastasis cannot be confirmed in the H&E staining of the corresponding organs. This could be because that the GFP signals emitted from the micrometastases were too minuscule to be sampled in tissue sections but were detectable as immunofluorescent signals by the machine. Since the cancer metastases cannot be confirmed by H&E, there is not enough evidence to conclude whether ZR75-BQ xenografts became tamoxifen resistant in terms of mediating metastases.

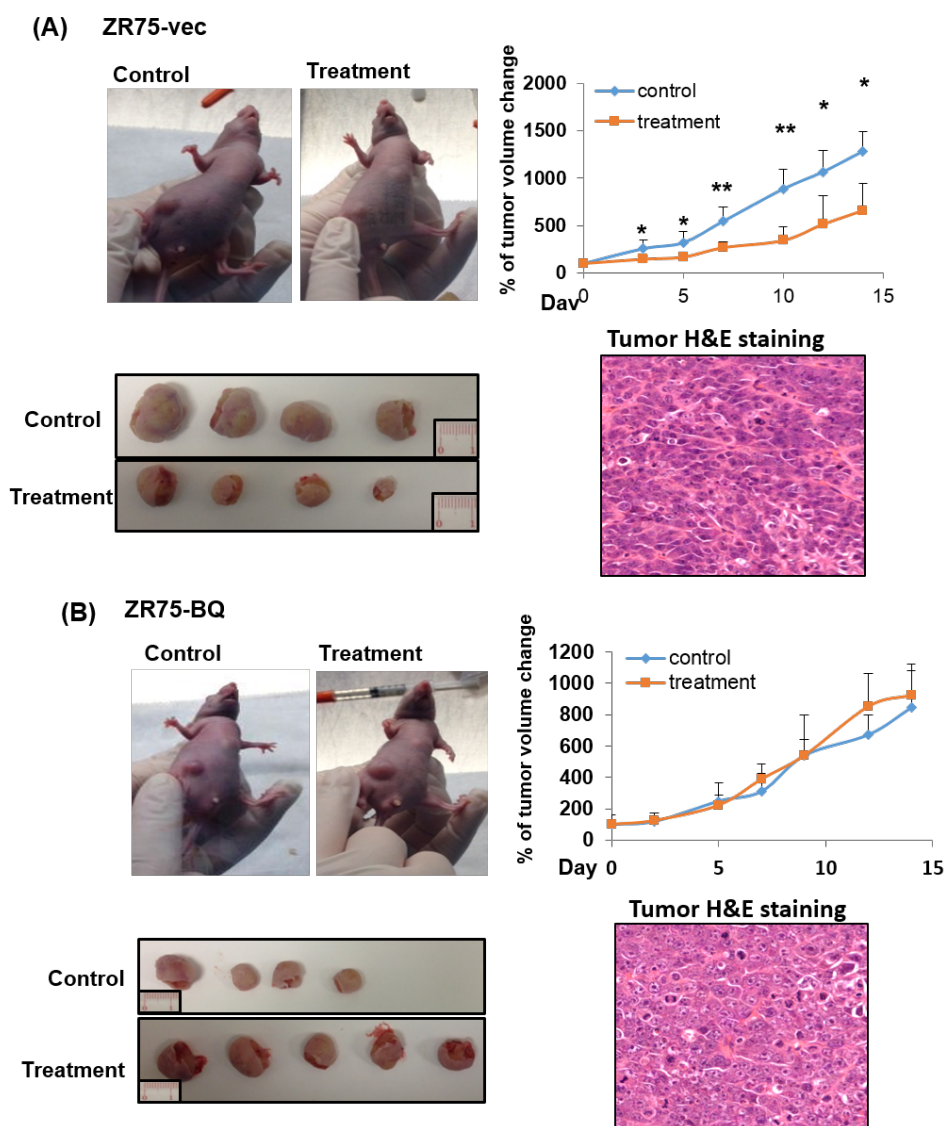
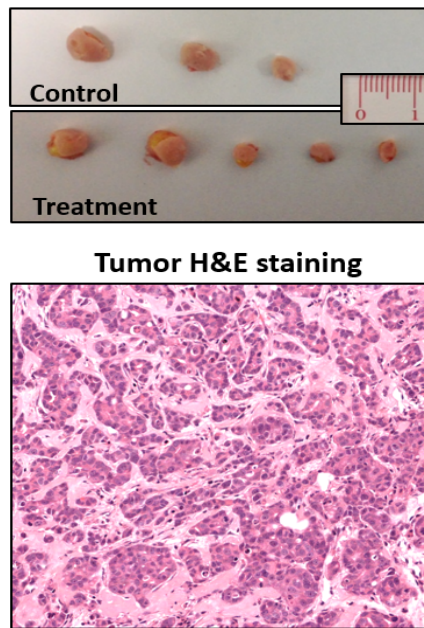
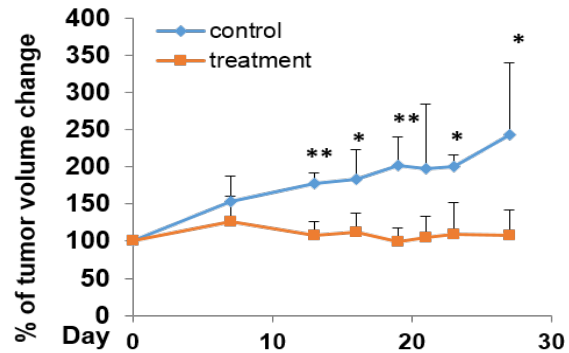


Figure 4.6 BQ323636.1 overexpression conferred resistance to tamoxifen *in vivo* using xenografts established with ZR75. **(A)** Mice bearing ZR75-vec tumours responded well to tamoxifen treatment, as expected **(B)** Mice bearing BQ323636.1-overexpressed ZR75 tumour did not respond to tamoxifen treatment. % of tumour volume change was presented as mean+SD. *, $p < 0.05$, **, $p < 0.01$.

(A) MCF7-vec



(B) MCF7-BQ

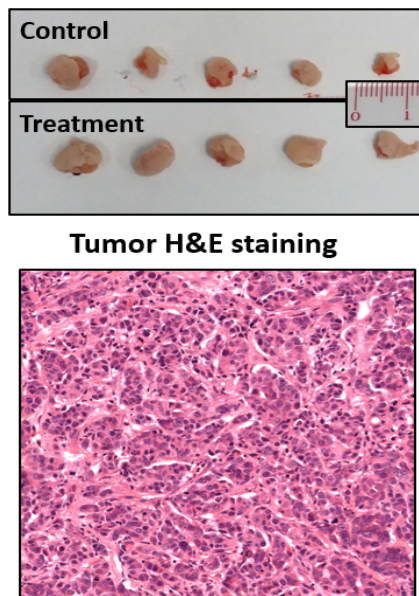
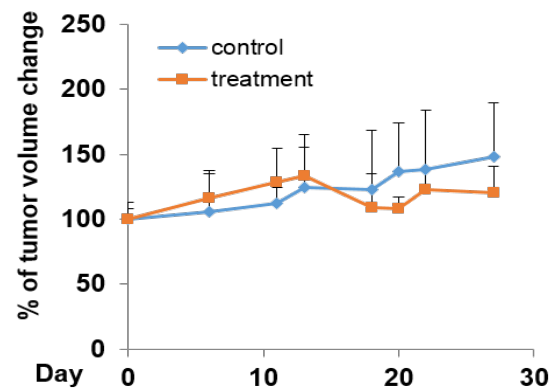
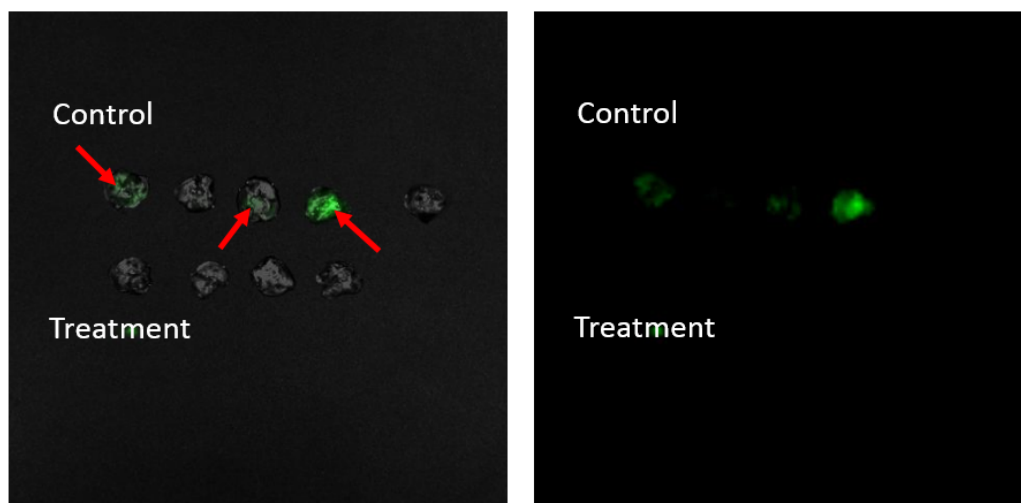


Figure 4.7 BQ323636.1 overexpression conferred resistance to tamoxifen *in vivo* using xenografts established with MCF7. **(A)** Mice bearing MCF7-vec tumours responded well to tamoxifen treatment, as expected **(B)** Mice bearing MCF7-BQ tumour did not respond to tamoxifen treatment. % of tumour volume change was presented as mean+SD. *, $p < 0.05$, **, $p < 0.01$.

(A) ZR75-vec



(B) ZR75-BQ

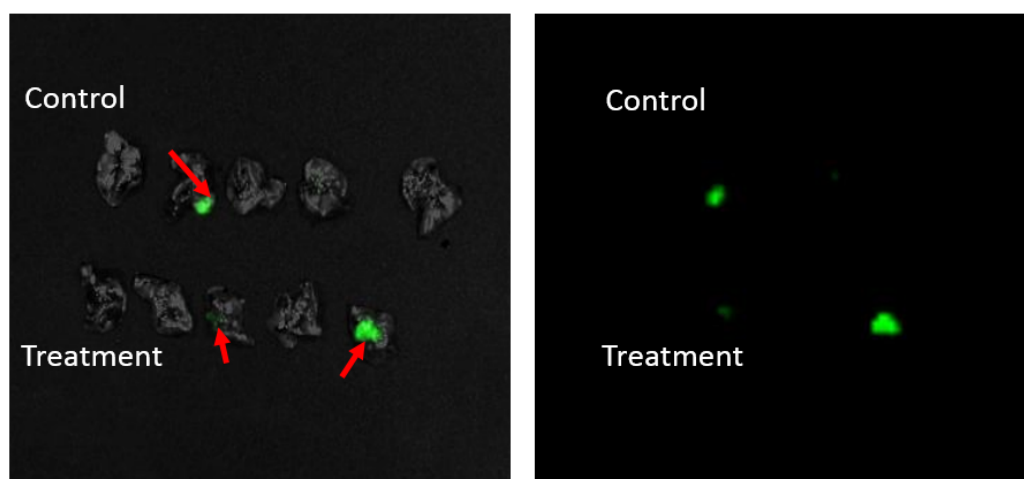


Figure 4.8 Images visualizing the GFP signals in the lungs of mice with **(A)** ZR75-vec and **(B)** ZR75-BQ xenografts by PE IVIS Spectrum in vivo imaging system (HKU Faculty core facility).

4.2.4 BQ323636.1 competes with NCOR2

Having established that BQ323636.1 overexpression induced tamoxifen resistance, the next step was to understand the mechanism. It has been observed that this splice variant could modulate transcription of ERE-containing luciferase reporter in a way opposite to its wild-type (Zhang, Gong et al. 2013). Since NCOR2 functions as a co-repressor to suppress ER-target genes in response to tamoxifen treatment, therefore it was hypothesized that BQ323636.1 could compete with NCOR2 to jeopardize the suppressive functions of NCOR2, which in turn relieves the transcription suppression imposed by tamoxifen.

To test this idea, the effects of BQ323636.1 and/or NCOR2 overexpression on transcription of ERE-containing luciferase reporter (ERE-E1b-luc) were studied and the results were expressed as relative luciferase activity normalized against the vector control (vector control was 1). Consistent to its co-repressor role in breast cancer, ectopic overexpression of NCOR2 was found to suppress luciferase activities in both ZR75 and MCF7 in all three conditions including non-treated (EtOH), treated with E2 or treated with tamoxifen (Tam). However, when BQ323636.1 was overexpressed together with NCOR2, the luciferase activities were significantly higher when compared with NCOR2 overexpression alone (Fig. 4.9), indicating that the suppression on luciferase activity imposed by NCOR2 was, at least partially, rescued.

The effects of BQ323636.1 on the expression of ER-target genes, including BCL2, cyclin D1 and pS2, under tamoxifen treatment were further studied by RT-qPCR. It was found that, although not statistically significant for all the genes tested, BQ323636.1 overexpression generally relieved the tamoxifen-induced

transcription suppression of these ER-target genes (Fig. 4.10). Collectively, these data (Fig. 4.9 and Fig. 4.10) are consistent with the notion that BQ323636.1 competes with NCOR2, compromising the suppressive functions of NCOR2 in response to tamoxifen treatment. It was also observed that there was significant induction at mRNA levels of both NCOR2 and BQ323636.1 (Fig. 4.10) under tamoxifen treatment.

It was of great interest to me to understand how BQ323636.1 could interfere with the functions of NCOR2. From these and previous results (Zhang, Gong et al. 2013), BQ323636.1 does not appear to exert a dominant negative effect on NCOR2, but instead, competes with NCOR2. It has been reported that NCOR2 functions as dimers in the co-repressor complex by binding to itself in an anti-parallel fashion (aa290-427 binds to aa1788-1903) (Varlakhanova, Hahm et al. 2011), serving as a central dock for the further recruitment of other co-repressor proteins. As BQ323636.1 protein retains only the RD1 portion of NCOR2, theoretically, it would have lost the ability to interact with ER and with other co-repressor proteins such as HDACs. Therefore, it was hypothesized that BQ323636.1 could bind to NCOR2, forming a defective platform to which other co-repressor proteins cannot be fully recruited.

To test this idea, co-immunoprecipitation was firstly performed to study the interactions among ER, NCOR2 and BQ323636.1. As shown in figure 4.11A, the interaction between NCOR2 and ER was confirmed in ZR75 and to a lesser extent in MCF7. More importantly, it was found that BQ323636.1 interacted with NCOR2 but not with ER, consistent with the prediction and supporting the notion that BQ323636.1 binds to NCOR2. Next, the effects of BQ323636.1

overexpression on the interactions between NCOR2 and the co-repressor proteins (HDAC3 and TBLR1) were studied by Co-IP. To this end, the effects of BQ323636.1 on the expression levels of NCOR2, HDAC3 and TBLR1 were firstly examined by western blot. It was observed that BQ323636.1 overexpression did not alter the expression levels of NCOR2, HDAC3 and TBLR1 (Fig. 4.11B). Co-IP further revealed that BQ323636.1 overexpression suppressed the interaction between HDAC3 and NCOR2 and between HDAC3 and TBLR1 (Fig. 4.11C). IgG was used as a negative control in this set of experiments. The experiments were repeated three times and band intensities were quantified using ImageJ. The band intensities of NCOR2 or TBLR1 were divided by the total HDAC3 that had been pulled down and the ratios were represented as relatively to the vector. As shown in figure 4.11D, the results confirmed that overall there was significantly less binding of NCOR2 or TBLR1 to HDAC3 when BQ323636.1 was overexpressed. Collectively, these data suggest that BQ323636.1 binds to NCOR2, which could in turn suppress the formation of co-repressor complex.

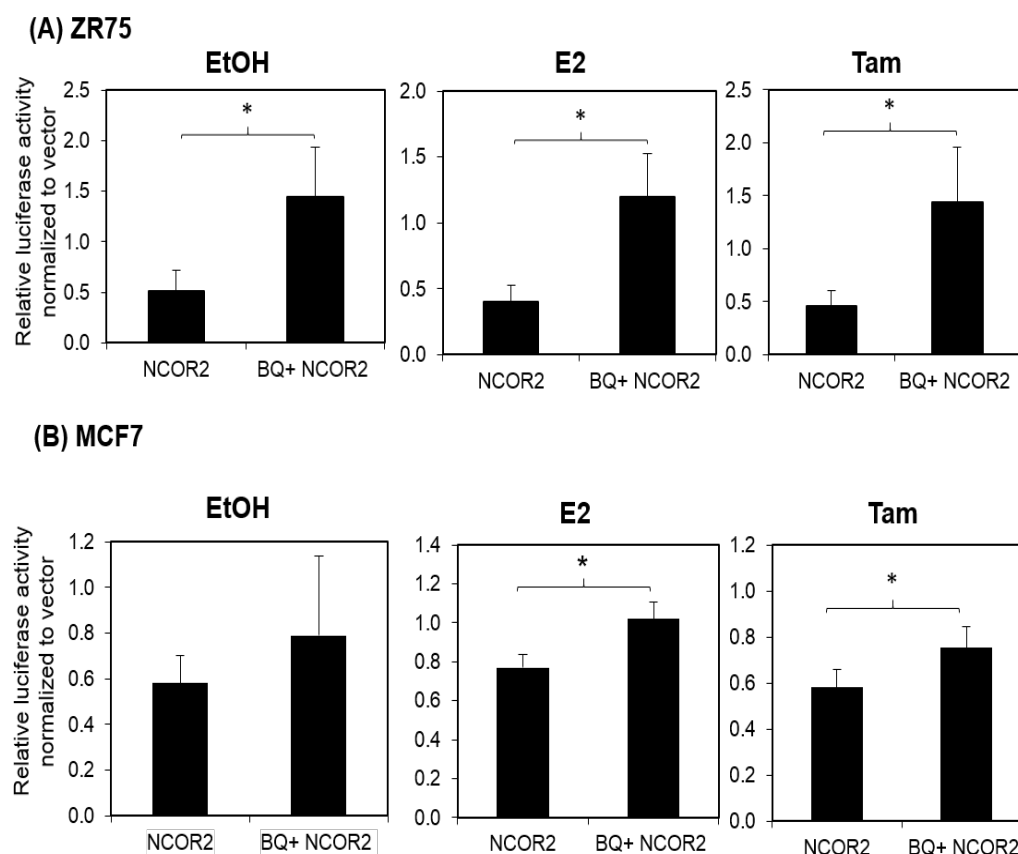
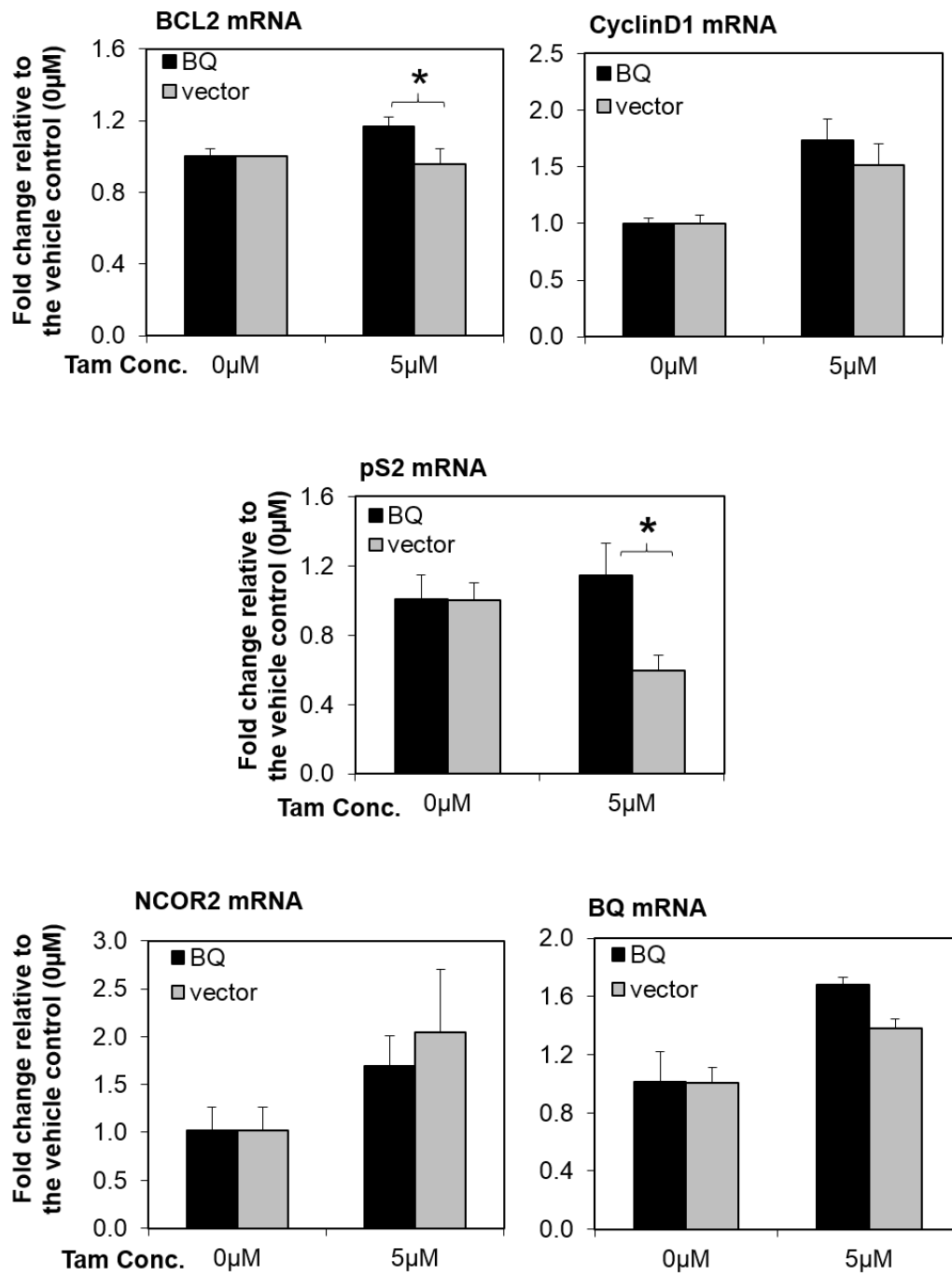


Figure 4.9 BQ323636.1 competes with NCOR2 in regulating transcription of ERE-E1b-luc in both **(A)** ZR75 and **(B)** MCF7 as measured by luciferase activity assay. The experiments have been repeated three times independently and the results were represented as mean+SD. *, $p < 0.05$.

(A) ZR75



(B) MCF7

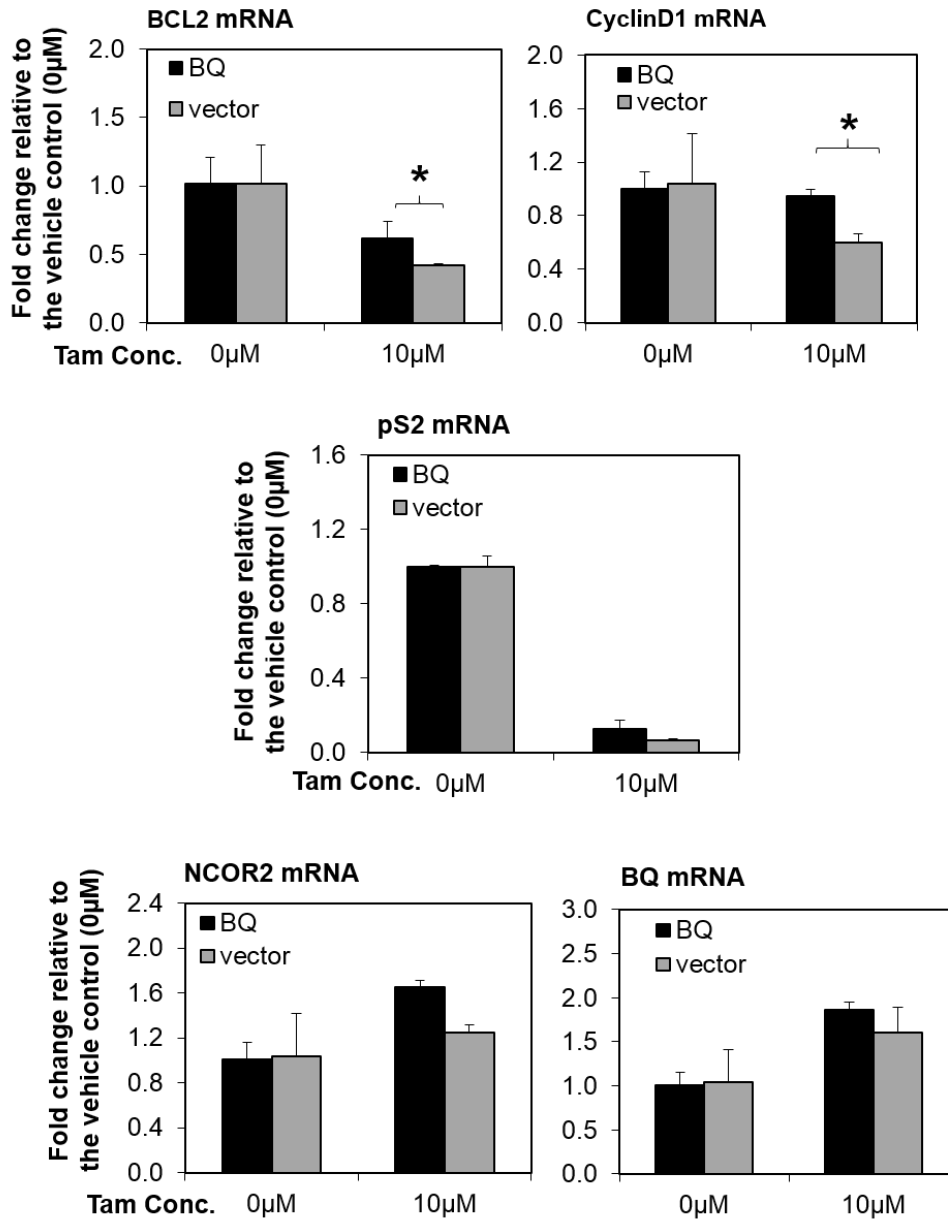
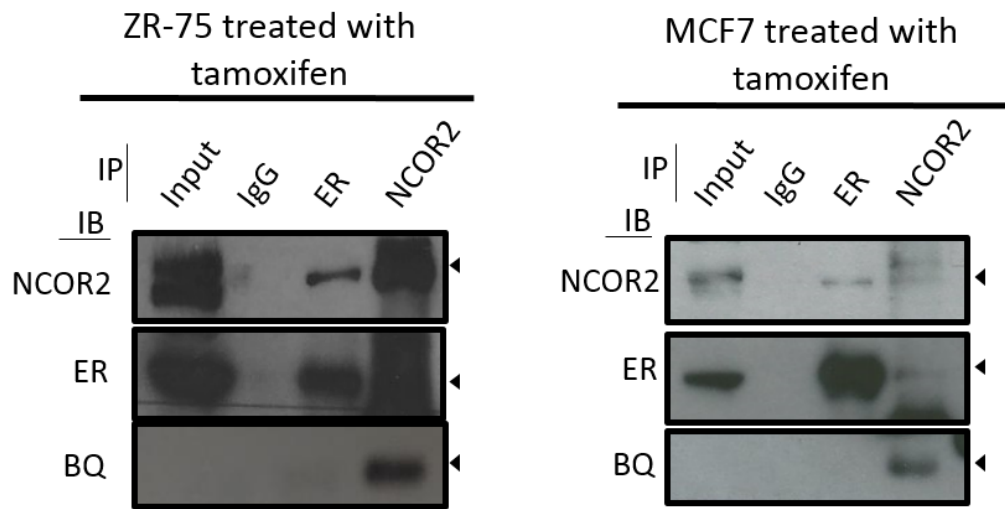
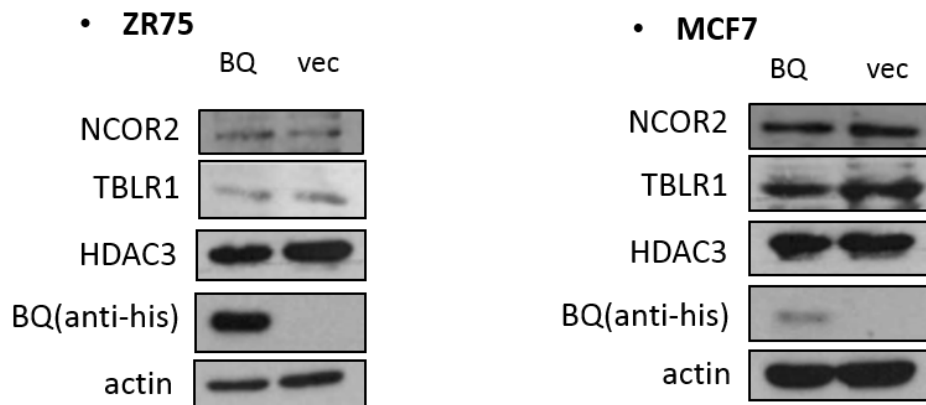


Figure 4.10 BQ323636.1 overexpression partially rescued the tamoxifen-induced transcription suppression of ER-target genes in both (A) ZR75 and (B) MCF7 cells. The experiments have been repeated three times independently and the results were represented as mean+SD. *, $p < 0.05$.

(A)



(B)



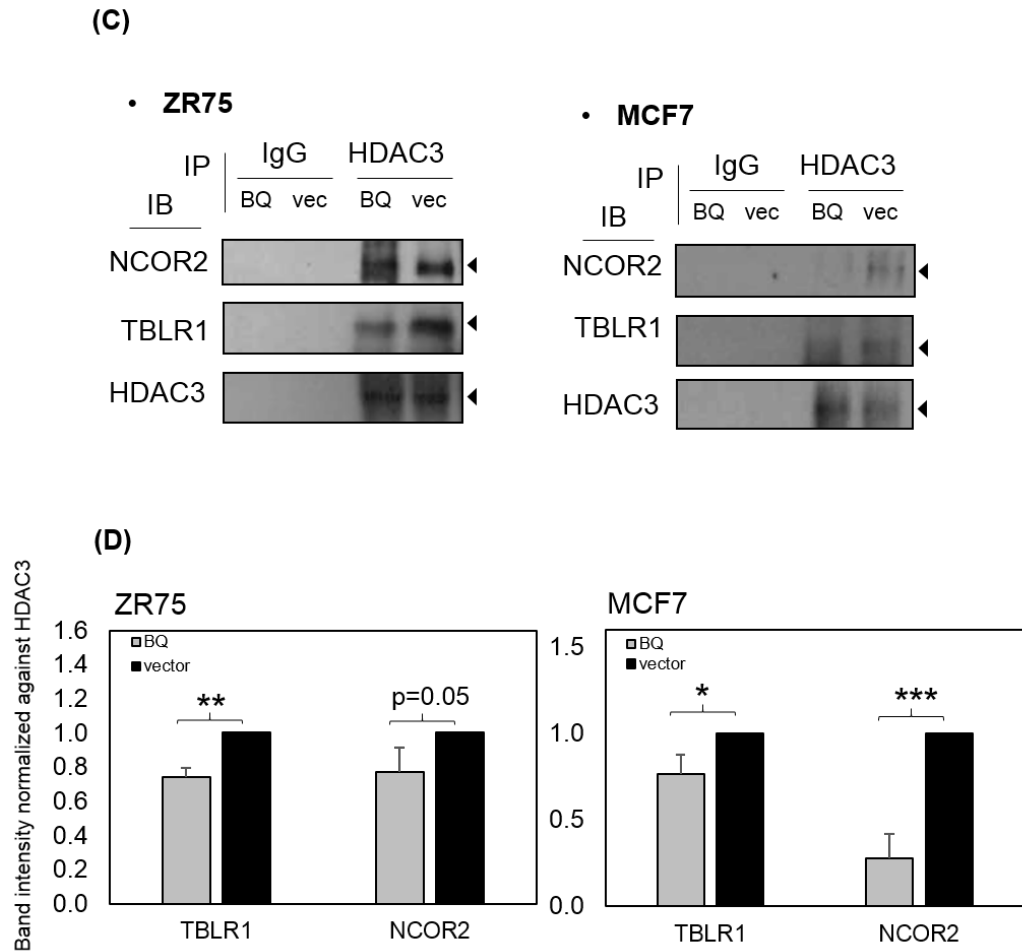


Figure 4.11 BQ323636.1 binds to NCOR2 and suppresses formation of co-repressor complex. **(A)** Co-IP showed that the interaction between BQ323636.1 and NCOR2. **(B)** BQ323636.1 overexpression did not affect the expression levels of NCOR2, TBLR1 nor HDAC3. **(C)** Overexpression BQ suppressed the interaction between HDAC3 and NCOR2 and between HDAC3 and TLBR1. **(D)** Band intensities were quantified by ImageJ, ratios of NCOR2/HDAC3 and TBLR1/HDAC3 were calculated and results were represented as mean+SD. The experiments have been repeated three times independently. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

4.2.5 BQ323636.1 protein has higher stability than NCOR2 protein

Evidence (Fig. 4.3, (Zhang, Gong et al. 2013)) suggests that BQ323636.1 expression is regulated at post-transcriptional level and it was hypothesized that, compared with NCOR2, BQ323636.1 was less susceptible to proteasome-mediated degradation because of the loss of phosphorylation site Ser2410.

To study the proteasome-mediated degradation of BQ323636.1, MCF7 and ZR75 were treated with 5 μ M of MG132, a potent proteasome inhibitor, in a time course as indicated in figure 4.12A. It was found that, MG132 treatment could cause the accumulation of NCOR2 but not BQ323636.1, suggesting that NCOR2 protein is actively degraded through the proteasome-mediated degradation pathway, whilst BQ323636.1 is not. To gain definitive knowledge for the turnover of these two proteins in the cells, pulse-chase analyses were performed to determine the half-lives. MCF7 cells were transiently labelled with ^{35} S-methionine and the degradation of the labelled proteins were chased through a course of time as indicated in figure 4.12B. NCOR2 degraded continuously along the time while BQ323636.1 protein stayed relatively constant after an initial decrease from 0 h to 2 h. The half-life of NCOR2 was around 4 h while that of BQ323636.1 was undetermined using the time course. Overall, these data suggest the BQ323636.1 protein has a higher stability compared to the NCOR2, probably because of the impaired proteasome-degradation pathway.

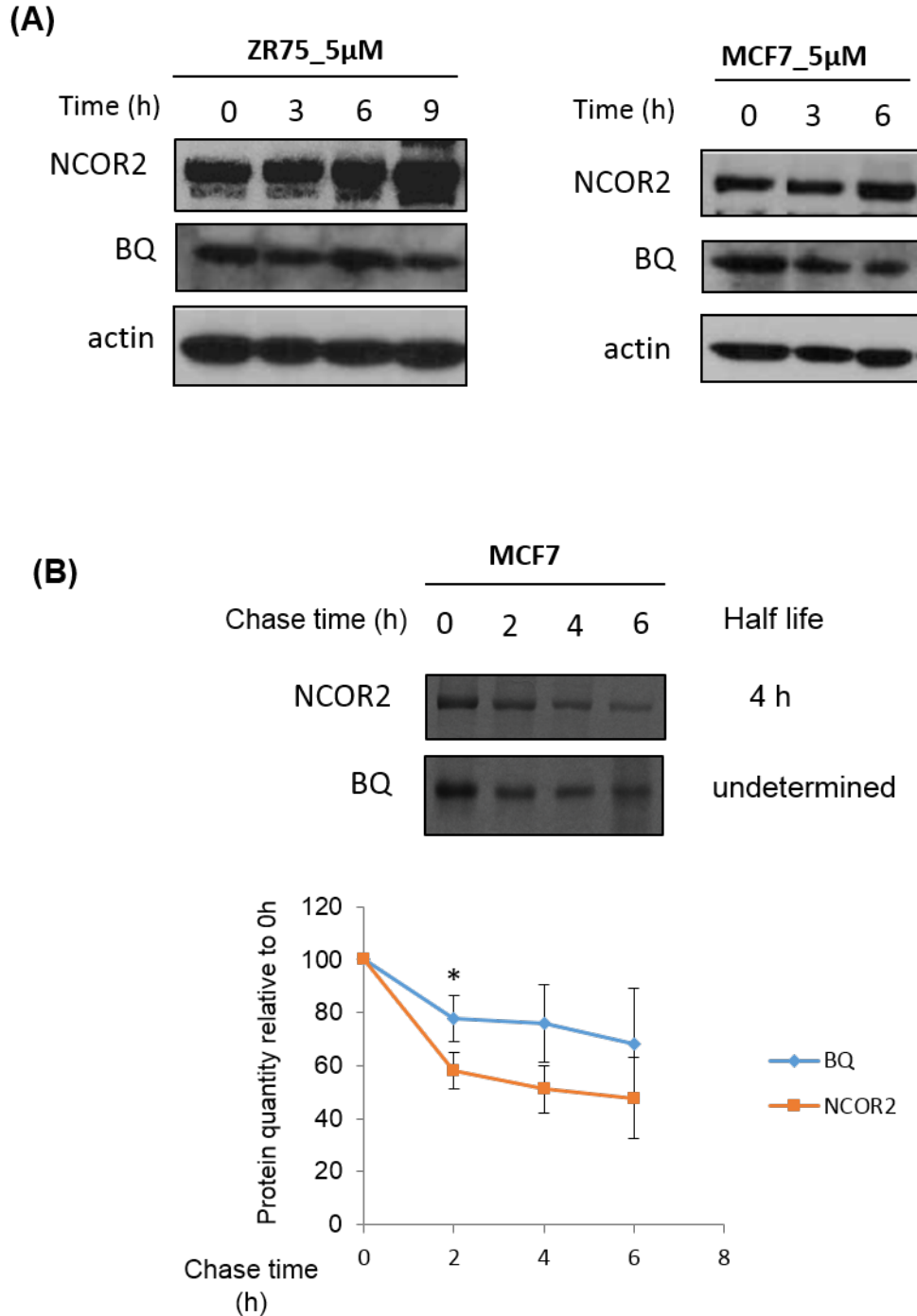


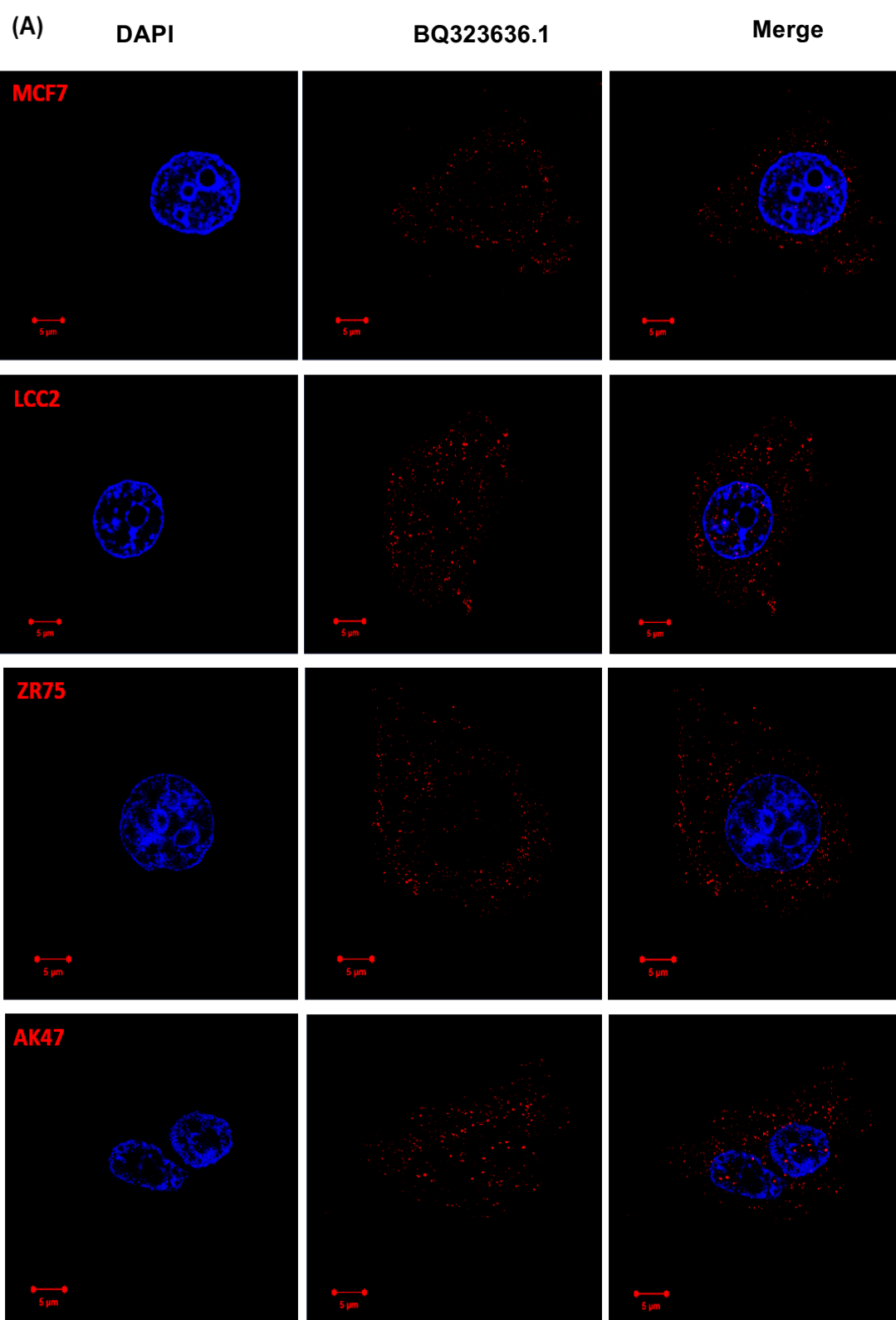
Figure 4.12 (A) BQ323636.1 protein did not accumulate following MG132 treatment while NCOR2 did. (B) BQ323636.1 degraded slower than NCOR2 and had a longer half-life in the cell. The experiments were repeated three times with band intensities quantified by ImageJ and normalized against the intensities at 0 h. Results were represented as mean±SD. *, $p < 0.05$.

4.2.6 Tamoxifen resistant cell lines expressed higher nuclear BQ323636.1

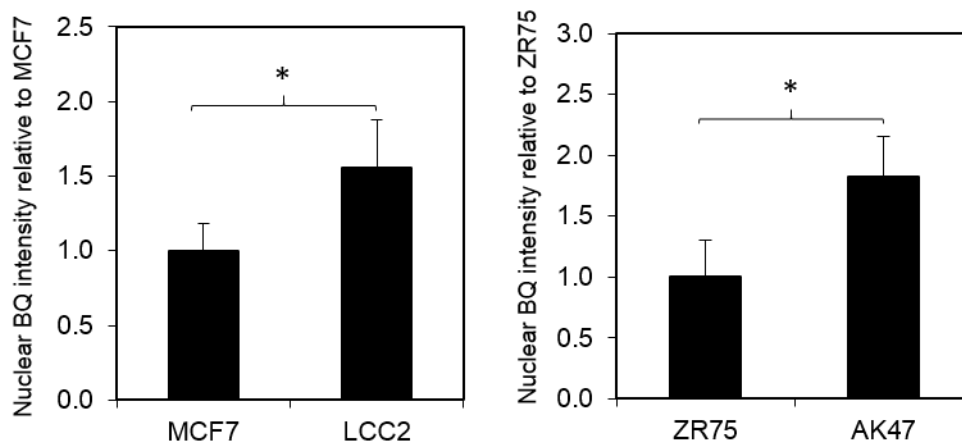
NCOR2 is a co-repressor that localized in the nucleus (THE HUMAN PROTEIN ATLAS), and it would be expected that the splice variant BQ323636.1 would similarly be localized in the nucleus.

The subcellular localization of BQ323636.1 in breast cancer cell lines was studied by immunofluorescent staining followed by confocal microscopy. Pictures of at least three independent fields were taken and the representative images shown in figure 4.13A. Accordingly, BQ323636.1 was found to be expressed in both cytoplasm and nucleus. It was also noted that, not only BQ323636.1 was expressed at higher levels in the tamoxifen resistant cell lines (LCC2 and AK47) when compared with their parental tamoxifen sensitive cell lines (MCF7 and ZR75), but nuclear localization was observed more distinctly in tamoxifen resistant cell lines LCC2 and AK47. This observation was further confirmed by the quantification of fluorescent intensities of nuclear BQ323636.1 in the cell lines (Fig. 4.13B). Results from subcellular fractionation experiments (Fig. 4.13C) also support the notion that LCC2 and AK47 expresses higher level of nuclear BQ323636.1 compared to their tamoxifen sensitive parental cell lines.

Based on the existing knowledge of NCOR2 and my previous results, when present in the nucleus, BQ323636.1 is likely to be functionally active where it can bind to NCOR2 and suppress the formation of co-repressor complexes. It is not yet clear why BQ323636.1 is expressed in both cytoplasm and nucleus. Moreover, it will be very interesting to study further what regulates the shuttling of BQ323636.1 between nucleus and cytoplasm, which will be further discussed in section 4.3.



(B)



(C)

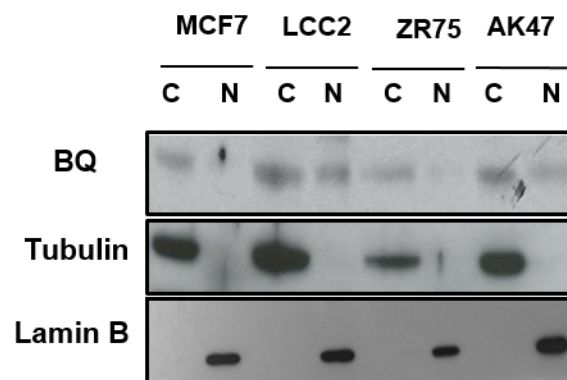


Figure 4.13 Tamoxifen resistant cell lines expressed higher levels of nuclear BQ323636.1. **(A)** Representative images of immunofluorescent staining of BQ323636.1, captured by confocal microscope. **(B)** Quantification of fluorescent intensities of BQ323636.1 in the nuclear compartment for each cell line. At least three images were captured independently and the results expressed as mean+SD. *, $p < 0.05$. **(C)** Subcellular fractionation further confirmed that BQ323636.1 was expressed at higher level in the nucleus of tamoxifen resistant cell lines. Tubulin (cytoplasm) and lamin B (nucleus) were used as the control of fractionation efficiency.

4.2.7 BQ323636.1 nuclear overexpression is associated with tamoxifen resistance in breast cancer patient samples and is a prognostic marker

To validate the predictive value of BQ323636.1 for tamoxifen resistance, immunohistochemical (IHC) staining was performed on TMA constructed from 238 cases of primary breast cancer and the expression levels of BQ323636.1 were scored as described in section 2.17. Tamoxifen resistance is defined as patients who have been treated with tamoxifen in the adjuvant setting but subsequently developed disease relapse or distant metastases. The clinicopathological data of the cohort were shown in Table 4.1. Representative images of IHC staining are shown in figure 4.14A. It was found that, all the patients uniformly expressed similar levels of BQ323636.1 in the cytoplasm while the level of nuclear BQ323636.1 was heterogeneous that varied from patient to patient. Hence, semi-quantitative assessment of nuclear BQ323636.1 expression levels was performed according to the H-score system which takes into account the intensity of the nuclear staining and percentage of cells stained at each intensity category, as described in Section 2.17.

Statistical analysis revealed that high nuclear expression of BQ323636.1 significantly correlated with tamoxifen resistance (Chi-square test, $p < 0.001$) and, consistently, expression level of nuclear BQ323636.1 was also significantly higher in tamoxifen resistant patients compared to tamoxifen sensitive patients (Fig. 4.14B, Mann-Whitney U Rank test, $p < 0.01$). The sensitivity of BQ323636.1 for prediction tamoxifen resistance is 67.8% while the specificity is 78.8%. Positive predictive value is 63.3% while the negative predictive value is 82%. Consistent with its association with tamoxifen resistance, for the patients who

have received tamoxifen treatment (n=94, only 79 cases with assessable BQ323636.1 expression), nuclear BQ323636.1 overexpression was also significantly associated with disease relapse (Chi-square test, $p=0.007$, Figure 4.15A) and with metastasis (Chi-square test, $p<0.001$, Figure 4.15B).

Survival analysis also showed that tamoxifen-treated patients with high BQ323636.1 nuclear expression (green line) had significant poorer survival compared with those with low BQ323636.1 nucleus expression (blue line) (Fig. 4.16, Log-rank test, $p=0.026$ for overall survival and $p=0.006$ for disease-specific survival, respectively). The risk ratio (RR) of BQ323636.1 calculated by univariate cox-regression model was 2.216 for overall survival (Table 4.2A, $p=0.03$, 95% confidence interval (1.078, 4.555)) and was 3.706 for disease-specific survival (Table 4.2B, $p=0.01$, 95% confidence interval (1.376, 10.048)). Multivariate cox-regression analyses showed that after being adjusted with tumour stage, tumour grade, progesterone receptor (PR) status, nuclear BQ323636.1 overexpression remained to be significantly associated with higher risk of death (Table 4.2, overall survival: $RR=9.541$, $p=0.006$; disease-specific survival: $RR=8.419$, $p=0.036$). The results indicate that BQ323636.1 overexpression is an independent prognostic marker for patients who had received tamoxifen treatment, consistent with its role in tamoxifen resistance.

Table 4.1 Clinicopathological data of the 238 breast cancer cases.

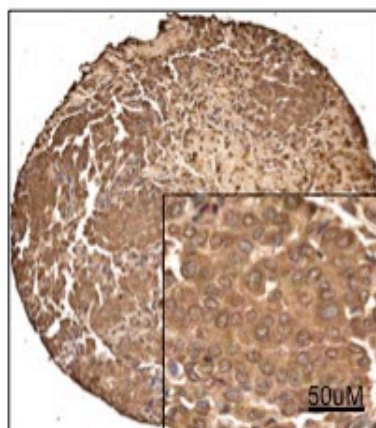
Clinicopathological characteristics	Number of patients (%)
Tumor grade	
Low (1 or 2)	84 (35.3)
High (3)	118 (49.6)
Missing	36 (15.1)
Tumor stage	
Low (1 or 2)	119 (50)
High (3 or 4)	25 (10.5)
Missing	94 (39.5)
Lymph node metastasis at diagnosis	
Positive	114 (47.9)
Negative	98 (41.2)
Missing	26 (10.9)
Estrogne receptor status	
Positive	120 (50.4)
Negative	54 (22.7)
Missing	64 (26.9)
Progesterone receptor status	
Positive	94 (39.5)
Negative	76 (31.5)
Missing	68 (28.6)
HER2 status	
Positive	40 (16.8)
Negative	71 (29.8)
Missing	127 (53.4)
Tamoxifen treatment	
Treated	94 (39.5)
Sensitive	60 (63.8)
Resistant	30 (31.9)
Missing	4 (4.3)

(A)

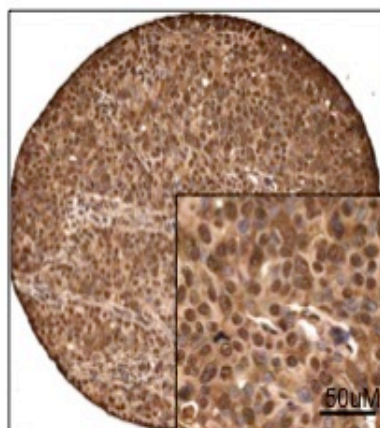
nucleus Low

nucleus high

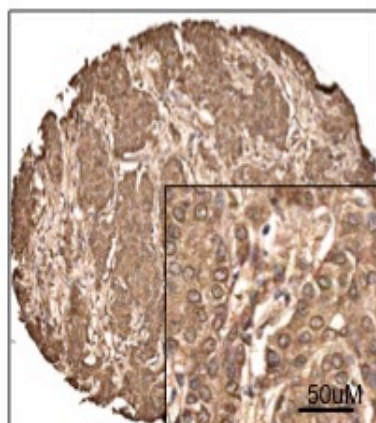
01-13241



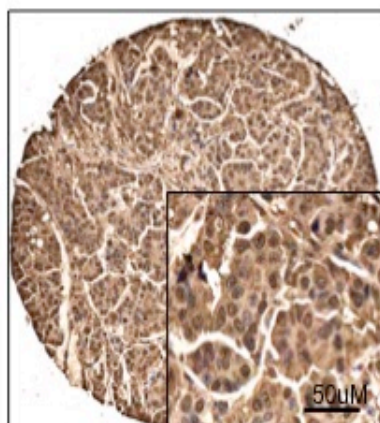
95-01896



93-04622



00-10482



(B)

BQ expression level * Tam response status
Crosstabulation

Count		Tam response status		Total
		sensitive	resistant	
BQ expression	Low	41	9	50
	High	11	19	30
Total		52	28	80

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	16.938 ^a	1	.000		
Continuity Correction ^b	15.004	1	.000		
Likelihood Ratio	17.023	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	16.726	1	.000		
N of Valid Cases	80				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 10.50.

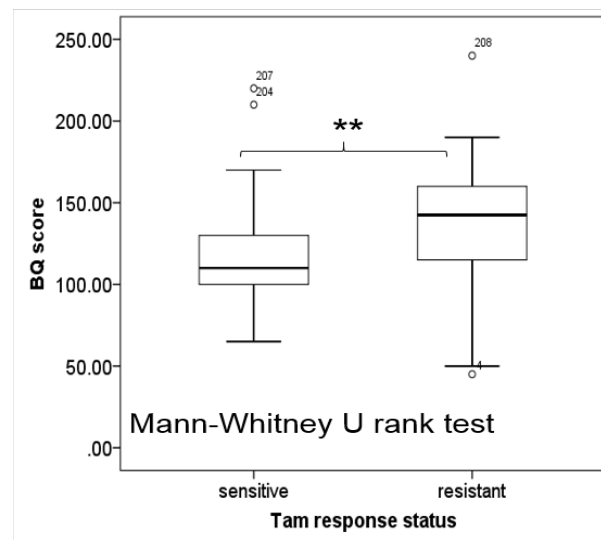


Figure 4.14 (A) Representative images of BQ323636.1 IHC staining in human breast TMA show two cases with high nuclear BQ323636.1 expression and two cases with low nuclear BQ323636.1 expression. The accession numbers of the samples are provided. **(B)** Nuclear BQ323636.1 overexpression was significantly correlated with tamoxifen resistance.

(A) Disease relapse

BQ expression level * relapse Crosstabulation

Count				
		relapse		Total
		yes	no	
BQ expression	Low	6	44	50
	High	11	18	29
Total		17	62	79

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	7.308 ^a	1	.007		
Continuity Correction ^b	5.853	1	.016		
Likelihood Ratio	7.090	1	.008		
Fisher's Exact Test				.010	.008
Linear-by-Linear Association	7.215	1	.007		
N of Valid Cases	79				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.24.

(B) Metastasis

BQ expression level * Metastasis Crosstabulation

Count				
		Metastasis		Total
		yes	no	
BQ expression	Low	8	42	50
	High	17	12	29
Total		25	54	79

Chi-Square Tests

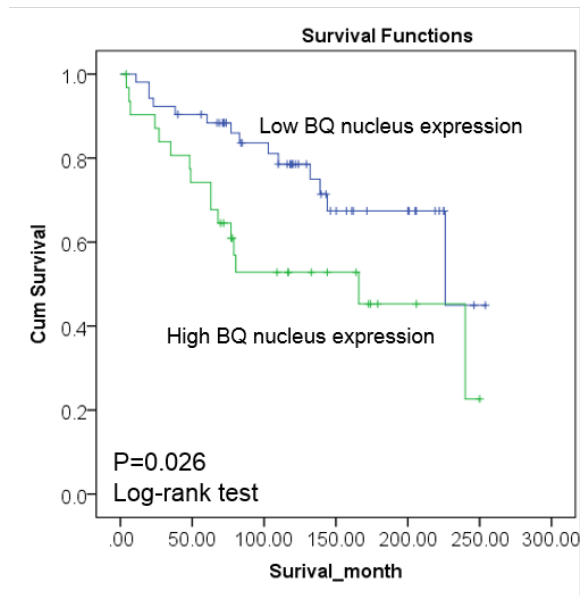
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	15.414 ^a	1	.000		
Continuity Correction ^b	13.506	1	.000		
Likelihood Ratio	15.316	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	15.218	1	.000		
N of Valid Cases	79				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 9.18.

Figure 4.15 The correlations between BQ323636.1 and (A) disease relapse (B)

distant metastasis by Chi-square tests.

(A) Overall survival



(B) Disease-specific survival

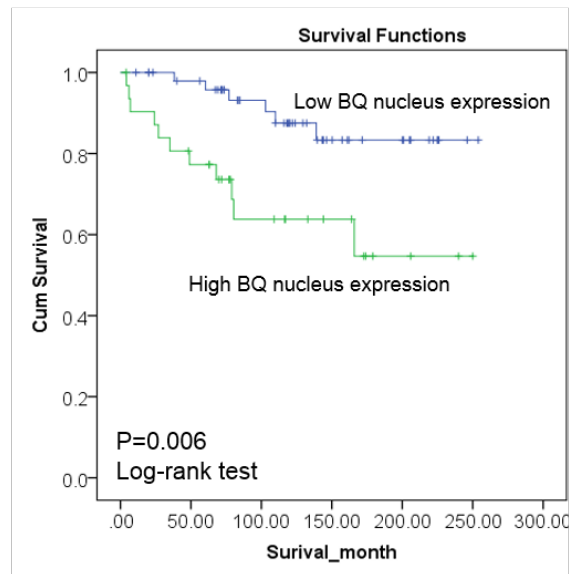


Figure 4.16 Kaplan-Meier estimate of (A) overall survival and (B) disease-specific survival of tamoxifen-treated patients (n=94) using nuclear BQ323636.1 scores dichotomized by the median.

Table 4.2 Cox regression analysis of **(A)** overall survival and **(B)** disease-specific survival using patients who had received tamoxifen treatment. $p < 0.05$ was considered as statistically significant and was highlighted in red.

(A) Overall survival

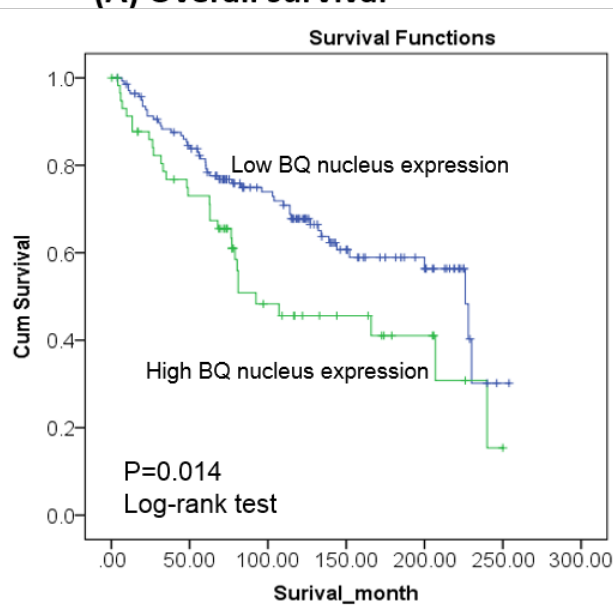
Clinical-pathological parameters	Univariate analysis		multivariate analysis	
	RR (95% CI)	<i>p</i>	RR (95% CI)	<i>p</i>
Age (n=94)	1.639 (0.801, 3.355)	0.176		
T-stage (n=94)	7.757 (2.080, 28.919)	0.002	2.378 (0.436, 12.978)	0.137
Lymph-node involvement	1.335 (0.637, 2.794)	0.444		
Tumor-Grade	1.918 (0.923, 3.987)	0.081	1.054 (0.248, 4.483)	0.943
PR status	0.254 (0.105, 0.615)	0.002	0.090 (0.019, 0.441)	0.003
HER2 status	1.873 (0.621, 5.649)	0.265		
BQ nucleus score	2.216 (1.078, 4.555)	0.03	9.541 (1.896, 48.014)	0.006

(B) Disease-specific survival

Clinical-pathological parameters	Univariate analysis		multivariate analysis	
	RR (95% CI)	<i>p</i>	RR (95% CI)	<i>p</i>
Age (n=94)	1.394 (0.559, 3.473)	0.476		
T-stage (n=94)	5.806 (1.219, 27.662)	0.027	1.549 (0.152, 15.778)	0.712
Lymph-node involvement	1.734 (0.626, 4.806)	0.29		
Tumor-Grade	4.400 (1.447, 13.374)	0.009	1.607 (0.271, 9.539)	0.602
PR status	0.183 (0.066, 0.505)	0.001	0.072 (0.010, 0.512)	0.009
HER2 status	2.247 (0.637, 7.927)	0.208		
BQ nucleus score	3.706 (1.367, 10.048)	0.01	8.419 (1.145, 61.886)	0.036

Survival analyses were also performed on all patients regardless of their ER status and tamoxifen treatment status. It was found that high BQ323636.1 nuclear expression significantly correlated with poor overall as well as disease-specific survival (Fig. 4.17, Log-rank test, $p=0.014$ and $p=0.013$, respectively). The risk ratio (RR) of BQ323636.1 calculated by univariate cox-regression model was 1.753 for overall survival (Table 4.3A, $p=0.15$) and was 2.035 for disease-specific survival (Table 4.3B, $p=0.016$). Moreover, nuclear BQ323636.1 overexpression remained to be an independent poor prognostic marker for overall survival in multivariate cox regression model (Table 4.3, $RR=2.18$, $p=0.03$). The results suggest that, besides being involved in tamoxifen resistance, BQ323636.1 overexpression might contribute to a more overall aggressive behaviour of the disease.

(A) Overall survival



(B) Disease-specific survival

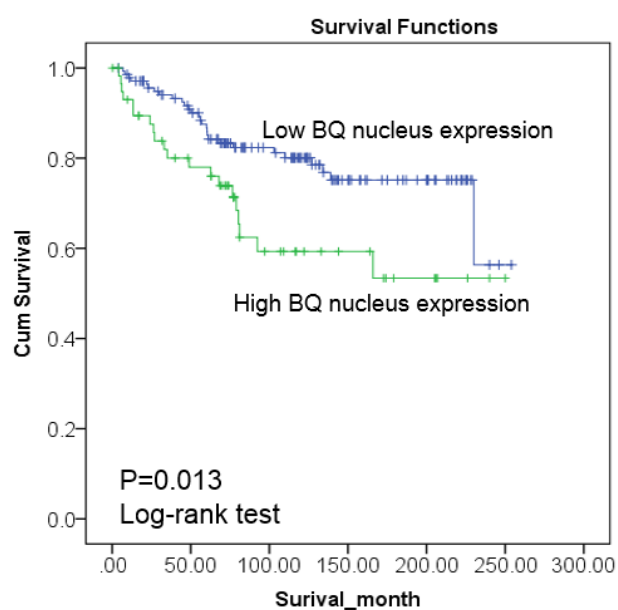


Figure 4.17 Kaplan-Meier estimate of (A) overall survival and (B) disease-specific survival of all patients (n=238) using nuclear BQ323636.1 scores dichotomized by the median.

Table 4.3 Cox regression analyses of **(A)** overall survival and **(B)** disease-specific survival using all the patients. $p < 0.05$ was considered as statistically significant and was highlighted in red.

(A) Overall survival

Clinical-pathological parameters	Univariate analysis		multivariate analysis	
	RR (95% CI)	<i>p</i>	RR (95% CI)	<i>p</i>
Age (n=238)	1.422 (0.932, 2.171)	0.103		
T-stage (n=238)	3.14 (1.668, 5.910)	<0.001	3.277 (1.545, 6.952)	0.002
Lymph-node involvement	1.469 (0.933, 2.315)	0.097	2.598 (1.145, 5.897)	0.002
Tumor-Grade	0.855 (0.538, 1.357)	0.506		
Estrogen receptor status	1.077 (0.598, 1.940)	0.805		
PR status	0.754 (0.446, 1.274)	0.291		
HER2 status	0.97 (0.467, 2.015)	0.936		
triple negative	1.01 (0.454, 2.247)	0.98		
BQ nucleus score	1.753 (1.113, 2.762)	0.015	2.180 (1.079, 4.407)	0.03

(B) Disease-specific survival

Clinical-pathological parameters	Univariate analysis		multivariate analysis	
	RR (95% CI)	<i>p</i>	RR (95% CI)	<i>p</i>
Age (n=238)	0.773 (0.455, 1.314)	0.342		
T-stage (n=238)	3.619 (1.799, 7.280)	<0.001	3.432 (1.458, 7.931)	0.004
Lymph-node involvement	2.003 (1.115, 3.601)	0.02	2.697 (1.058, 6.876)	0.038
Tumor-Grade	1.619 (0.888, 2.951)	0.116		
Estrogen receptor status	0.836 (0.444, 1.573)	0.578		
PR status	0.555 (0.303, 1.014)	0.055		
HER2 status	1.030 (0.455, 2.335)	0.943		
triple negative	1.198 (0.499, 2.872)	0.686		
BQ nucleus score	2.035 (1.145, 3.618)	0.016	1.941 (0.872, 4.322)	0.104

The correlations between BQ323636.1 expression and other clinicopathological parameters including were tumour stage (T stage), lymph node metastasis at diagnosis (positive LN), tumour grade, PR status, ER status and HER2 status were studied by Chi-square tests using SPSS (Table 4.4). Receptor statuses and positive LN were dichotomized as either positive or negative while T stage was dichotomized as low (stage 1 and 2) or high (stage 3 and 4), and tumour grade as low (grade 1 and 2) or high (grade 3). It was also found that, in the cohort of patients who had received tamoxifen treatment (i.e. ER positive), nuclear BQ323636.1 overexpression was not significantly correlated with all the clinicopathological parameters tested, with only marginal positive correlation with T stage and tumour grade ($p=0.087$ and $p=0.085$, respectively). Nuclear BQ323636.1 overexpression was found to be significantly correlated with ER status in all the patients (Table 4.4B, correlation coefficient=0.196, $p=0.016$) but not with other clinicopathological parameters.

Table 4.4 The correlations between BQ323636.1 nuclear scores and other clinicopathological parameters using the cohort of (A) tamoxifen treated patients only, and (B) all patients. Chi-square test, $p < 0.05$ was considered as statistically significant and was highlighted in red.

(A) Tam treated patients only

	correlation coefficient	p value
T stage	0.242	0.087
Positive LN	-0.053	0.65
tumor grade	0.198	0.085
PR status	-0.112	0.386
HER2 status	0.123	0.404

(B) All patients

	correlation coefficient	p value
T stage	0.157	0.081
Positive LN	0.04	0.594
tumor grade	-0.031	0.689
ER status	0.196	0.016
PR status	0.13	0.117
HER2 status	-0.114	0.189

4.2.8 BQ323636.1 overexpression activates NRF2-antioxidant pathway

Previous results showed that BQ323636.1 overexpression was associated with poor overall survival. It was observed that BQ323636.1-overexpressed mouse xenografts grew faster than the control (data not shown), highlighting the possibility that BQ323636.1 overexpression favours tumour growth. It has been reported that NCOR2 binds to Nuclear factor erythroid 2-related factor 2 (NRF2) which suppresses the transcription of antioxidant and detoxification enzymes (Ki, Cho et al. 2005, Kratschmar, Calabrese et al. 2012). Since we have shown that BQ323636.1 interferes with suppressive functions of NCOR2 in breast cancer (Fig. 4.8 to Fig. 4.10), it was then hypothesized that BQ323636.1 might also interfere with NCOR2-NRF2 interaction, which could in turn modify the expression of downstream antioxidant and detoxification enzymes, resulting in decreased level of reactive oxygen species and enhanced cancer cell survival.

To test this possibility, the expression levels of three enzymes regulated by NRF2 including NQO1, GCLC and GCLM were examined by RT-qPCR and results compared between BQ323636.1 overexpressed cells and the controls. As shown in Fig. 4.18, BQ323636.1 overexpression promoted the expression of NQO1, GCLC and GCLM, suggesting that BQ323636.1 overexpression could activate antioxidant pathway. The expression level of NRF2 was not affected, indicating that such modulation was achieved through affecting the functions rather than the expression of NRF2.

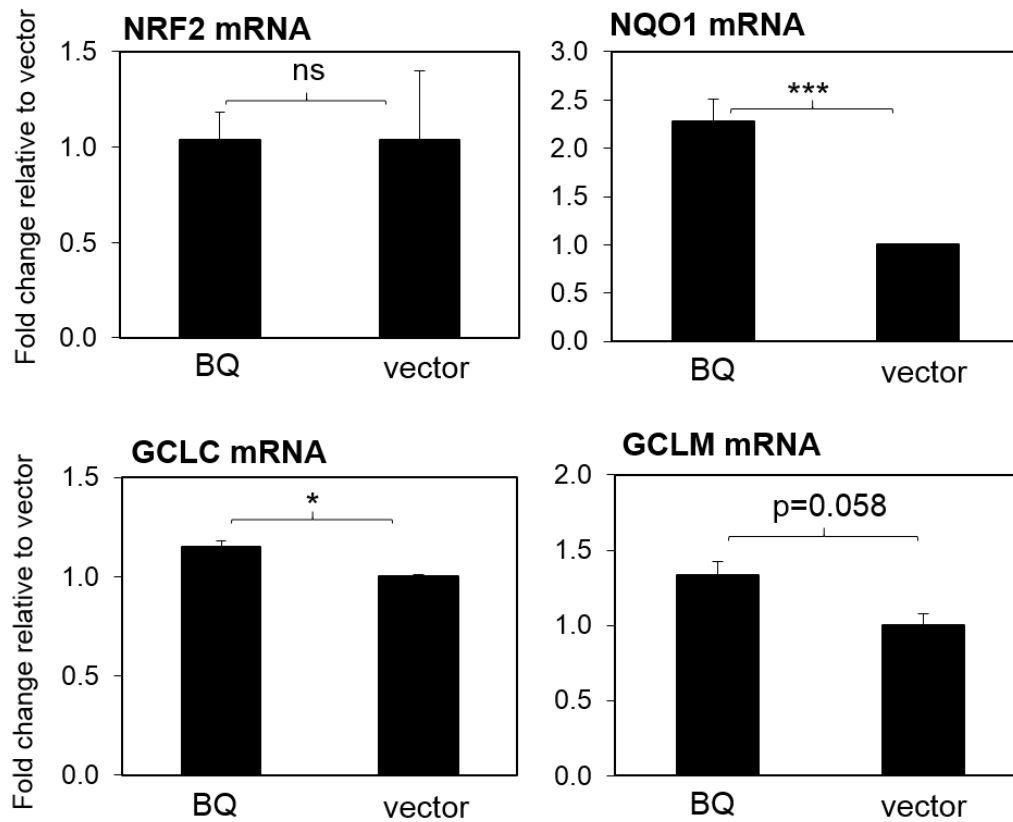


Figure 4.18 BQ323636.1 overexpression promoted the expression of NQO1, GCLC and GCLM while did not affect expression of NRF2. The experiments were repeated three times and results represented as mean+SD. Significance: *, $p < 0.05$, ***, $p < 0.001$, ns, no significance.

4.3 Discussion

The oestrogen (ER) signalling pathway is an important and fundamental pro-proliferative pathway in breast cancer. Upon its activation by binding with oestrogen, ER activates target gene transcription and cell growth either directly through its genomic pathway or indirectly through a non- genomic pathway that involves the PI3K/AKT signalling. About 70% of breast cancer patients are ER α positive and can be treated with endocrine therapy. Tamoxifen is a selective oestrogen receptor modulator (SERM) that acts as antagonist of oestrogen in breast tissue and is the most commonly prescribed anti-oestrogen drug given to ER+ patients for the prevention of breast cancer relapse or metastasis. The binding of tamoxifen to ER triggers the recruitment of NCOR2 as well as other co-repressors such as GPS2, TBLR1, HDAC3 *etc.*, and suppresses the pro-proliferative ER signalling pathway (Sharma, Saxena et al. 2006, Zhang, Chang et al. 2006, Cheng and Kao 2009). Despite the relative safe and significant anti-neoplastic activities of tamoxifen in preventing disease relapse and improving patients' survival, resistance to tamoxifen is an outstanding issue with up to 50% of non-responding patients and many of the initial responders experience relapse (Ring and Dowsett 2004).

The mechanisms underlying tamoxifen resistance have been extensively studied but not yet well understood. More importantly, there has been no available robust biomarker to discriminate ER positive patients who will be sensitive to tamoxifen from those who will be resistant. Therefore, almost all the patients with positive ER α status will be prescribed with tamoxifen. Some of patients are resistant to

this drug but clinicians can only document drug failure when cancer reoccurs and/or metastasizes.

In 2006, the US Food and Drug Administration (FDA) recommended to include information on *CYP2D6* genotypes to predict patient outcomes for tamoxifen treatment, but a consensus on whether genotyping should be required or considered optional was not reached (de Souza and Olopade 2011). Since then, many more clinical studies addressing the relationship between *CYP2D6* and tamoxifen resistance have been published but the results have been inconsistent and conflicting. *CYP2D6* is a metabolic enzyme that catalyses 4-hydroxylation of tamoxifen (Dehal and Kupfer 1997, Collier, Krebsfaenger et al. 2002). In terms of mortality, some studies showed that *CYP2D6* genotype is associated with shorter relapse free survival and disease-free survival while others could not find such associations (Dezentje, Guchelaar et al. 2009, Hoskins, Carey et al. 2009). Another fourteen studies examined the association between *CYP2D6* and disease recurrence but the majority of them did not report statistically significant relationships. Therefore, there are not enough solid data to justify the implementation of individual *CYP2D6* genotyping for adjuvant tamoxifen treatment of breast cancer and it is still being questioned whether *CYP2D6* can serve as a robust biomarker for predicting tamoxifen resistance.

BQ323636.1 is a splice variant of NCOR2 identified by our group with exon 11 skipping during mRNA splicing, resulting in an early translation stop codon and a truncated protein product retaining only the N-terminal fragment compared to its wild-type (Zhang, Gong et al. 2013). In this study, *in vitro* and *in vivo* evidences were presented to support that BQ323636.1 overexpression could induce

tamoxifen resistance. Moreover, the functions of BQ323636.1 in regulating ER signalling pathway were studied. BQ323636.1 overexpression rescued the suppressive effect of NCOR2 overexpression exerted on the transcription of an ERE-containing luciferase reporter (Fig. 4.9) and compromised the inhibitory effects of tamoxifen on ER-target gene expressions (Fig. 4.10). Such effects can be explained by the fact that BQ323636.1 bound to NCOR2 but not to ER in the cells (Fig. 4.10A) and BQ323636.1 overexpression inhibited the interaction between HDAC3 and NCOR2, and between HDAC3 and TBLR1 (Fig. 4.10C and D), indicating the suppressed formation of co-repressor complex. Based on these experimental evidence, the mechanism of how BQ323636.1 induces tamoxifen resistance is proposed as follows. When BQ323636.1 is not over-expressed (Fig. 4.19A), in response to tamoxifen treatment, NCOR2 dimerises with itself in an anti-parallel fashion, serving as a dock for further recruitment of other co-repressor proteins such as TBLR1, HDACs and GPS2 to form a complete and functional co-repressor complex which will bind to ER to suppress gene transcription and halt cell growth. In contrast, when BQ323636.1 is present (Fig. 4.19B), it binds to NCOR2, forming a faulty dock to which other co-repressor proteins cannot be fully recruited, resulting in a defective co-repressor complex which cannot bind to ER or is unable to fully suppress gene transcription. Hence the cells become inert to tamoxifen treatment.

Although BQ323636.1 is a splice variant of NCOR2 whose expression at mRNA level is 2% to 5% of NCOR2, it was found that BQ323636.1 protein is expressed at similar level to NCOR2 (Zhang, Gong et al. 2013). This could be due in part to BQ323636.1 is not actively degraded through the proteasome-mediated pathway and has a longer half-life in the cell compared with that of NCOR2 (Fig. 4.12).

This mechanism could help to maintain protein expression of BQ323636.1 at a functionally significant level in the cell. The fact that NCOR2 functions as dimmer and that BQ323636.1 binds to NCOR2 could amplify the functional importance of the splice variant in the cells.

The mechanism

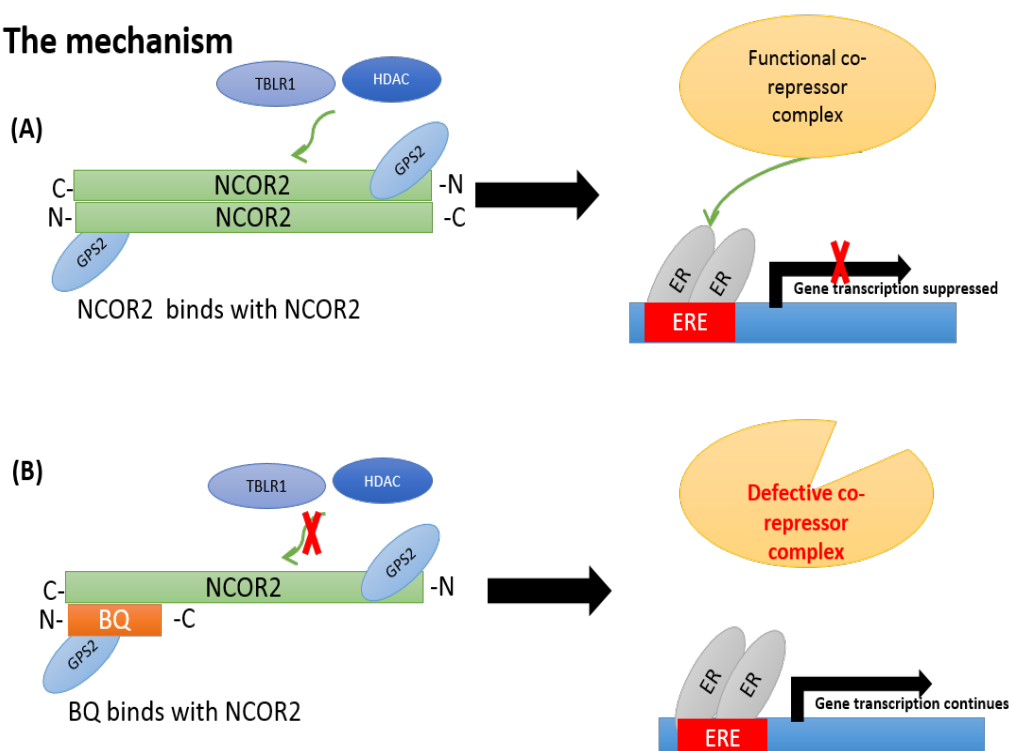


Figure 4.19 The proposed mechanism of how BQ323636.1 could induce tamoxifen resistance in breast cancer.

Statistical analysis using TMA constructed from 238 cases of human breast cancers showed that, for patients who had been treated with tamoxifen (n=94), BQ323636.1 nuclear overexpression is significantly associated with tamoxifen resistance (Chi-square test, $p < 0.0001$), which is defined as disease relapse or

metastasis after being treated with tamoxifen. The predictive values of BQ323636.1 for tamoxifen response were assessed by four parameters: sensitivity, specificity, positive predictive value and negative predictive value. These parameters (67.8%, 78.7%, 63.3% and 82%, respectively) are however less than optimal. On the one hand, they could be improved using an expanded cohort. On the other hand, the strong cytoplasmic staining may have interfered with scorer's interpretation of nuclear BQ323636.1 expression. Unlike NCOR2 which is localized only in the nucleus, BQ323636.1 was found to be expressed in both the cytoplasm and the nucleus (Fig. 4.13 and Fig. 4.14). This raises a very interesting question as to understand what the functional differences between cytoplasmic and nuclear BQ323636.1 are, and what controls the shuttling of this protein between the cytoplasm and the nucleus. By investigating such questions, it may be possible to generate an antibody that specifically recognizes nuclear BQ323636.1, thus improving the accuracy of tamoxifen response prediction. Phosphorylation is a key regulatory step redirecting cellular localization between the cytoplasm and nucleus of many proteins. For example, nuclear iASSP, which is enriched in melanoma metastasis and associated with poor patient survival, is the phosphorylated form of iASSP and phosphorylation by cyclin B1/CDK1 on Ser-84 and Ser-113 triggers iASSP to enter nucleus (Lu, Breysens et al. 2013). Phosphorylation of FOXO3a on Ser-7 by p38 MAPK also triggers the translocation of FOXO3a from cytoplasm to nucleus upon doxorubicin treatment (Ho, McGuire et al. 2012). Therefore, it is postulated that phosphorylation profile determines the subcellular localisation of BQ323636.1, and phosphorylation could signal BQ323636.1 to enter the nucleus from the cytoplasm or export into the cytoplasm from the nucleus.

In the TMA, nuclear BQ323636.1 overexpression is only found to be positively correlated with ER status (Table 4.3B) but not with other clinicopathological parameters, which is inconsistent with our previous findings at mRNA level (Zhang, Gong et al. 2013). Previously, it was found that BQ323636.1 mRNA level is negatively correlated with ER and PR status, with BQ323636.1 mRNA level being higher in ER or PR negative patients. The discrepancy highlights the possible post-transcriptional regulatory mechanism of BQ323636.1 expression (such as deregulated protein degradation as presented in this thesis) that could cause the discordant expression level of mRNA and protein. Since protein expression level is a more accurate indicator of its functional activity, it further supports the importance of studying BQ323636.1 at protein level in predicting tamoxifen resistance.

It was also found that BQ323636.1 overexpression is an overall poor prognostic marker and appeared to favour tumour growth *in vivo*. This could be due to its ability to modulate the NRF2/antioxidant pathway (Fig. 4.18). Proper intracellular oxidation and reduction levels are vital for maintaining homeostasis in living cells. Oxidative stress generated from a variety of environmental pollutants and toxicants elevates the production of reactive oxygen species (ROS) which contribute to the perturbation of homeostasis and cause DNA damage ultimately resulting in cardiovascular and neurodegenerative diseases and the initiation and progression of cancer (Waris and Ahsan 2006). However, in the context of cancer, excess ROS can cause cancer cell damage and death and high levels of ROS limit tumour formation. As such, reduced intracellular ROS levels through activation of antioxidant signalling pathway can promote cell transformation and tumorigenic phenotypes (Schafer, Grassian et al. 2009).

NRF2 belongs to the Cap 'N' Collar family of transcription factors that contain a highly conserved basic leucine zipper structure (Moi, Chan et al. 1994). It is the key transcriptional factor that drives the expression of detoxification enzyme. Under normal conditions, NRF2 is constantly degraded by KEAP1, a negative regulator of NRF2, via the ubiquitin-proteasome pathway. However, KEAP1 is inactivated upon oxidative stress leading to the accumulation of NRF2 in the nucleus and the subsequent induction of various antioxidants and phase II cytoprotective and detoxifying enzymes such as NAD(P)H: quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), haeme oxygenase-1 (HO-1) and glutathione S-transferases (GSTs) (Thimmulappa, Mai et al. 2002, Kansanen, Kuosmanen et al. 2013, Namani, Li et al. 2014). Deregulation of NRF2 and the antioxidant pathways have been extensively reported in cancer. Up-regulation of NRF2 has been reported in several malignant tumours and associations between NRF2 accumulation and adverse patient outcomes have been documented in cancers of ovarian and lung (Solis, Behrens et al. 2010, Konstantinopoulos, Spentzos et al. 2011). Elevated NQO1 has also been reported in many human tumours, including breast cancer, melanoma, lung cancer, and pancreatic cancer (Siegel, Franklin et al. 1998, Awadallah, Dehn et al. 2008, Garate, Wani et al. 2010) and is associated with poor patient survival and breast cancer progression (Yang, Zhang et al. 2014).

Besides the regulation by KEAP1, the interaction of NRF2 with a number of nuclear receptors can affect the transcriptional activity of NRF2 (Namani, Li et al. 2014). In particular, NCOR2 has been reported to suppress NRF2's transcriptional activation activity through activation of glucocorticoids receptor

(GR), followed by recruitment of NCOR2 to the Neh4/5 transactivation domains of NRF2 and subsequent inactivation of NRF2 in transcription induction (Ki, Cho et al. 2005). On the other hand, BQ323636.1 is found to be able to bind to NCOR2 and modulate suppressive function of NCOR2 in response to tamoxifen treatment (Fig. 4.9 to Fig. 4.11). Therefore, it is hypothesized that, as illustrated in figure 4.20, BQ323636.1 overexpression could promote breast tumour growth through relieving the suppressive role of NCOR2 imposed on NRF2/antioxidant pathway, which subsequently promotes the expression of antioxidant enzymes, resulting in reduced levels of intracellular ROS and enhanced tumour cell survival.

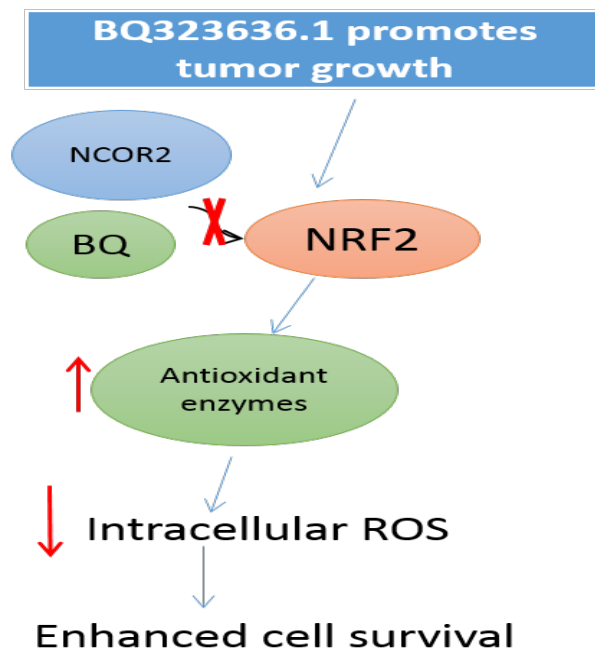


Figure 4.20 Proposed mechanism of how BQ323636.1 could interfere with antioxidant pathway and enhance tumor cell survival.

To conclude, the project has provided *in vitro* and *in vivo* evidence to support the notion that BQ323636.1 overexpression confer tamoxifen resistance and can be used as a predictive marker for patients' response to tamoxifen treatment. The molecular mechanism through which BQ323636.1 could induce tamoxifen resistance has also been studied and proposed. Some interesting aspects of this splice variant have arisen during my investigation including nucleus-cytoplasm shuttling and its role in promoting tumour growth, which will require future efforts to address.

Chapter 5 Final discussion and future aspects

Breast cancer is the most common female cancer. In Hong Kong, its incidence has been rising steadily over the last few decades, with age-standardized incidence and mortality rates of 61.2 and 9.5 per 100,000 respectively in 2012 (Hong Kong Cancer Registry, 2013). Worldwide, according to WHO, an estimated 1.67 million new cases were diagnosed in 2012, accounting for 25% of all cancers.

Tumourigenesis in the breast might arise from multiple mechanisms, of which BRCA1-loss/mutation is the best recognized and most extensively studied mechanism. BRCA1 mutations or loss of expression are closely associated with incurrence of both hereditary and sporadic breast cancer. BRCA1-associated tumours often display basal-like phenotype that expresses no receptors and is the most aggressive type of breast cancer with fewest treatment options and poorest survival. BRCA1 is a tumour suppressor with multifaceted roles such as DNA damage repair, maintenance of genome stability, regulation of transcription and ubiquitination. Although extensively studied, the roles of BRCA1 in tumourigenesis are not fully understood. A recent study has uncovered novel role of BRCA1 in epigenetic regulation of gene expression (Wang, Zeng et al. 2013), which introduces an intriguing research focus on understanding the BRCA1-mediated epigenetic regulation in breast tumourigenesis.

Breast cancer patients may be treated with neo-adjuvant and adjuvant therapy including endocrine therapy (e.g. tamoxifen), targeted anti-HER2 therapy (e.g. Herceptin) and various forms of systemic chemotherapy. Tamoxifen is often the

first-line endocrine therapeutic agent with the widest spectrum of FDA-approved clinical applications and has significantly improved survival of ER-positive patients over the past 20 years. Tamoxifen antagonises the functions of oestrogen, binding of which to ER would induce recruitment of co-repressor proteins and suppress ER-target gene transcription. However, resistance to tamoxifen has become a major obstacle, undermining the success rate of the treatment. Patients can be *de novo* resistant to tamoxifen even though ER is expressed, or they may acquire resistance after a period of time following treatment. The mechanisms underlying tamoxifen resistance have been intensively studied but not yet fully understood. More importantly, there is no reliable biomarker that can predict tamoxifen response. Such predictive marker can help clinicians to optimize treatment regimen for the patients to maximise the benefits and minimise the time and money wasted.

Therefore, in my PhD study, I have aimed to characterise novel molecular mechanisms that are involved in tumourigenesis and tamoxifen resistance focusing on: 1) understanding the role of BRCA1 in regulation of FOXO3a through EZH2-mediated epigenetic mechanism and its implication in tumourigenesis; 2) studying the role as well as the mechanism of a splice variant of NCOR2 in conferring tamoxifen resistance and its potential as a predictive marker for tamoxifen response.

In chapter 3, the data demonstrates that BRCA1 regulates FOXO3a expression in breast cancer, where loss or mutation of BRCA1 would lead to down-regulation of FOXO3a indirectly by activating of EZH2 to induce trimethylation of H3K27 and recruitment of DNMTs to methylate and silence the FOXO3a promoter.

Consistent with what has been suggested by other colleagues in the field (Wang and Huang 2013), the role of BRCA1 in regulating EZH2 activity to induce histone trimethylation and promoter methylation in breast cancer is highlighted by the findings which showed that EZH2 only regulated FOXO3a expression in basal-like cell lines with loss or mutations of BRCA1 and deposition of the repressive histone mark H3K27me3 as well as DNMTs (DNMT1, DNMT3a and DNMT3b) to FOXO3a promoters were only found in these cell lines. The BRCA1-FOXO3a regulatory axis might be important in BRCA1-associated tumourigenesis, as results showed that the tumour suppressive role of BRCA1 is, at least, partially achieved through regulating FOXO3a expression in breast cancer. The *in vitro* data was further supported by the *in vivo* findings that showed significant higher levels of FOXO3a promoter methylation and lower levels of FOXO3a protein level in BRCA1-mutated patients as well as the significant negative correlation between EZH2 and FOXO3a protein expression levels.

The current study has only characterised FOXO3a as a target gene regulated by the BRCA1-EZH2 axis with the range of genes downstream of this regulatory mechanism still largely unknown. To fully understand and appreciate the functional significance of BRCA1 in modulating epigenetics through EZH2, in the future, it would be worthwhile to identify at whole scale the genes that are regulated by the BRCA1-EZH2 axis. To achieve this, chromatin-immunoprecipitation can be performed to pull down DNAs bound to EZH2/H3K27me3/DNMTs followed by quantification and sequencing using cell lines, or more significantly, using human BRCA1-mutated and non-mutated tumour samples. Genes with differential occupancy of

EZH2/H3K27me3/DNMTs at the promoter between BRCA1-mutated and non-mutated samples are the ones that are most likely to be regulated by the BRCA1-EZH2 axis. Followed by identification of potential target genes, validation should be performed in a way similar to what have been presented in this thesis and functional significances can be further studied.

The regulatory axis of BRCA1-EZH2-FOXO3a reported in the thesis has important implication in the designing of therapeutic approach for breast cancer treatment in the future. For example, the tumour suppressive role of BRCA1 is found to be partially achieved through FOXO3a, which highlights the importance of taking into consideration of the FOXO3a expression level when therapeutically targeting BRCA1 in breast cancer. Additionally, there have been numerous studies showing potent anti-tumour effects induced by chemical inhibition of EZH2 using small molecules such as GSK126, GSK343 and EPZ-6438, and several clinical trials underway. However, my current results suggest that, in the context of breast cancer, the situation may be more complicated, as the results show that EZH2 is only functionally active in repressing FOXO3a expression when BRCA1 is deficient. Hence, in the future, it might be useful to use BRCA1 as a marker to screen breast cancer patients for tumours that are functionally dependent on EZH2 and are most likely be able to benefit from treatments using EZH2 inhibitors.

In chapter 4, it is shown that the splice variant of NCOR2 named BQ323636.1 can confer resistance to tamoxifen both *in vitro* and *in vivo*. This could be achieved through binding of BQ323636.1 to NCOR2 to inhibit the assembly of co-repressor complex and, consequently, tamoxifen-induced transcriptional

repression of ER-target genes. Nuclear BQ323636.1 was also found to be expressed at significant levels in tamoxifen resistant cell lines and patients. Moreover, nuclear BQ323636.1 overexpression is significantly correlated with tamoxifen resistance, disease relapse/metastasis and survival in tamoxifen-treated human breast cancer samples, supporting its role in predicting tamoxifen resistance. Results also showed that BQ323636.1 expression is significantly regulated at post-transcriptional level with impaired proteasome-mediated ubiquitination-degradation pathway and higher protein stability, which could help to maintain protein expression of BQ323636.1 at a functionally significant level in the cell. The fact that NCOR2 functions as dimers and that BQ323636.1 binds to NCOR2 could further amplify the functional importance of this splice variant in the cells. Some interesting data were also noted in this project including the subcellular localization of BQ323636.1 and the role of BQ323636.1 in regulating anti-oxidant signalling pathway and predicting patients' survival regardless of their ER status.

As BQ323636.1 is expressed in both nucleus and cytoplasm but only the nuclear BQ323636.1 provides functional significance in terms of predicting tamoxifen response and patients' survival, one future research direction could be to study the difference between cytoplasmic and nuclear fraction of BQ323636.1 protein and to understand the mechanism that controls the subcellular localization of BQ323636.1. As post-translational modification especially phosphorylation is the most common mechanism that regulates the shuttling of protein between the two cellular compartments, in the future, the post-translational modifications between the cytoplasmic and nuclear BQ323636.1 can be studied and compared by high-performance liquid chromatography-mass spectrometry followed by

protein sequencing to identify the specific modification(s) that distinguish the nuclear BQ323636.1 from the cytoplasmic one. The results could facilitate the generation of antibody specific nuclear BQ323636.1, which would make the interpretation of IHC results easier without interference from cytoplasmic staining and possibly improve the sensitivity and specificity of using BQ323636.1 for prediction of tamoxifen resistance. Moreover, the kinases/genes that target the post-translational modification(s) for BQ323636.1 nuclear localization could be studied, which would lead to identification of novel therapeutic agents targeting BQ323636.1 nuclear localization to combat tamoxifen resistance.

In addition to targeting translocation of BQ323636.1 to prevent activation of this splice variant, another approach can be targeting its synthesis. Since BQ323636.1 is generated by alternative splicing during mRNA processing and only differs from NCOR2 at the last 33 nucleotides, designing small interference RNA to effectively deplete BQ323636.1 without affect expression levels of NCOR2 is technically challenging. Alternatively, efforts can be devoted to identify the mechanisms responsible for such alternative splicing, which can possibly be due to mutations in the NCOR2 gene that have caused the aberrant regulation of splicing by RNA splicing factors. Followed by identification of such mutations, CRISPR-Cas9 technology can be employed to edit and correct the mutated sites to eliminate the production of BQ323636.1.

Another future research direction could be to study the role of BQ323636.1 in regulating NRF2-mediated anti-oxidant signalling pathway and its implication in breast cancer tumourigenesis. Since NCOR2 has been shown to interact and

inhibit the functions of NRF2 in inducing expression of anti-oxidant enzymes (Ki, Cho et al. 2005) and, in the thesis, data has shown that BQ323636.1 binds to NCOR2 and inhibits suppressive functions of NCOR2 in regulating of ER-signalling pathway, BQ323636.1 could play a similar role in regulating NCOR2-NRF2 axis, serving as the mechanism through which BQ323636.1 could regulate expression levels of anti-oxidant genes downstream of NRF2 as shown in the thesis. These speculations are of no doubt interesting and will require intensive research efforts to address carefully in the future. Characterizing the oncogenic role of BQ in antioxidant response signalling pathway in breast cancer will strengthen the therapeutic value of BQ323636.1 not only as a marker/target for combating tamoxifen resistance but also as a target for prevention of cancer progression, and provide empirical proof to drive the search for BQ323636.1 inhibitor to benefit breast cancer patients in the future.

In summary, my PhD study has characterized two novel molecular mechanisms that are involved in breast cancer tumourigenesis/progression and tamoxifen resistance. The results not only contribute to the better understanding of the molecular basis of breast cancer and tamoxifen resistance but also provide important clinical implication for better management of the breast malignancy.

Appendix I The brief protocol for production of BQ323636.1-specific antibody

Generation of monoclonal antibody specific to BQ323636.1

Immunogen: Peptid 3781.1 Biosyntan GmbH QRTWRSRCASWP-OH

Peptide-BSA-Conjugate (BSA: Fraction V, Pierce; Cross-Linker: Sulfo-MBS, Pierce)

Host: 8-week-old female BALB/c mice

Immunisation procedures:

Immunisation (Day)	Injection	Doses (µg/mouse)	Adjuvant
0	Priming (i.p.)	109	TiterMax Gold Adjuvant
14	Boost 1 (i.p.)	109	Complete Freund's Adjuvant
21	Boost 2 (i.p.)	54	Incomplete Freund's Adjuvant
28	Boost 3 (i.p.)	54	Incomplete Freund's Adjuvant
35	Boost 4 (i.p.)	54	PBS
38	Fusion	Mouse 42	

The presence of the desired antibody was detected in the sera of the recipients prior to the final booster dose using the enzyme-linked immunosorbent assay (ELISA) with the uncoupled Peptide 3781 as immobilized antigen. For fusion experiment mouse 42 was used.

Date of fusion: 04.10.2013 **Fusion number:** 577

Parent cell fusion line: SP2/0 (non secreting mouse myeloma)

Fusion method: Two days before fusion mouse (Balb/c) peritoneal macrophages for use as feeder cells were prepared and seeded into the wells of four 96-well cell culture plates. For fusion procedures, 6×10^7 spleen cells from the immunised mouse and 2×10^7 cells from mouse myeloma cell line SP2/0 were incubated with 1.2ml of polyethylene glycol 1450 (50% in 10% DMSO; Sigma) for 30sec. at 37°C. After washing, the cells were seeded in the four 96-well cell culture plates. Hybrid clones were selected by growing in HAT medium [RPMI 1640 culture medium (Biochrom) supplemented with 20% fetal calf serum (PAN) and HAT Supplement (50x; PAN)]. After two weeks the HAT medium was replaced with HT Medium for three passages followed by returning to the normal cell culture medium.

Screening / Cloning / Recloning:

The cell culture supernatants were primary screened for antigen specific IgG antibodies two weeks after fusion. The presence of antigen specific antibodies in the culture supernatants was measured by its binding to the uncoupled Peptide 3781 attached directly to the wells of 96-well microtitre plates (100ng/well). The antibody binding was quantified by adding the relevant anti-species immunoglobulin to which an enzyme is bound, followed by a chromogenic substrate to that enzyme. Fresh culture medium and a dilution of the polyclonal mouse antiserum were used as negative or as positive controls. 12 specific antibody producing hybridoma colonies were found to be high positive. The selected 12 cell populations were transferred into 24-well plates for cell propagation and were then tested again. 4 cell populations with the highest antibodies titres were selected for cloning and re-cloning using the limiting-

dilution technique. 4 cell lines producing specific monoclonal antibodies were characterized and frozen. For the isotype characterization the Mouse Monoclonal Antibody Isotyping Kit (Roche) was used.

Cell culture medium: RPMI 1640 Medium [(1x), w 2.0 g/l NaHCO₃, w stable Glutamine (Biochrom AG; Catalog No.: FG 1415)] with 20% FCS (PAN Biotech GmbH; Cat.-No.: 1302-P283004; Lot No.: P283004; Origin: Australia); Gentamicin (PAA Laboratories GmbH; Best.-Nr. P11-004; 50µg/ml)

Optimal growth: At a temperature of 37°C and 5% CO₂

Storage of cells/Freezing: Approximately 3 x 10⁶ cells in 750µl fresh cell culture medium were pipette into a cryotube. 750µl of freezing medium (80% FCS and 20% DMSO (PAN)) were added. Using a Nalgene Cryo Freezing Container the cryotubes were placed in a -80°C freezer immediately and transferred to liquid N₂ within 24 hours for long-term storage.

Storage of cells/Thawing: The cryotubes were quickly as possible thawed in a 37°C water bath. The cells were diluted with 10ml cold medium and centrifuged at 1000 rpm for 10 min. Then the pellets were resuspended in cell culture medium and transferred into 25ml cell culture flasks.

Test for mycoplasma: The cell culture supernatants were tested for mycoplasma using an ELISA Kit (Mycoplasma detection Kit; Roche Diagnostics GmbH)

REACTIVITIES OF THE SECRETED ANTIBODIES OF THE 4 FINAL CLONES:

ELISA

The cell culture supernatants were incubated with a solid phase to which the uncoupled

Peptide 3781 has been bound by direct adsorption. During the assay any specific antibody will itself become bound to the antigen on the solid phase, and then in a second incubation is detected with a labelled anti-species antibody.

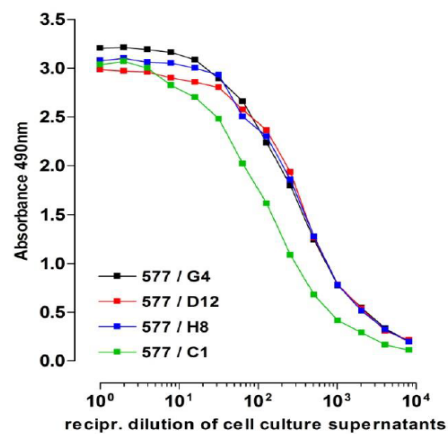
Coating: 100ng/well (well=50µl) of Peptide 3781 (diluted in PBS) overnight at 4°C;

wash plate twice with wash buffer (PBS/0.05% Tween20). Blocking: 100µl/well blocking solution (PBS/0.05% Tween20/10%NCS) for 1h at RT;

Wash plates twice with wash buffer. Incubation: 50µl/well of the cell culture supernatants serial diluted in incubation buffer (PBS/0.05%; Tween20/10%NCS) for 2h at RT; wash plates three times with wash buffer. Conjugate: 50µl/well HRP-Rabbit anti Mouse IgG; (Fc specific; Pierce; diluted in incubation buffer) for 1h at RT; wash plates three times with wash buffer. Substrate: 50µl/well enzyme substrate (OPD in citrate-hydrogenphosphate buffer) for 15min at RT. Stopping: 50µl/well stopping solution. Measure: Measure absorbance at 490nm

Substate: 3,7mM o-Phenylendiamine in Citrate/Hydrogenphosphate buffer, 0,012% H₂O₂ Citrate/Hydrogenphosphate buffer: 35mM Citric acid Monohydrate, 85mM Na₂HPO₄ x 2H₂O, pH 5,0 Stopping solution: 4N Schwefelsäure. Wash buffer: PBS/0,05% Tween20.

Four clones (C1, D12, G4, H8) with highest titre of antigen specific antibody production were selected and ELISA was applied to assess the antigen-antibody specific binding.

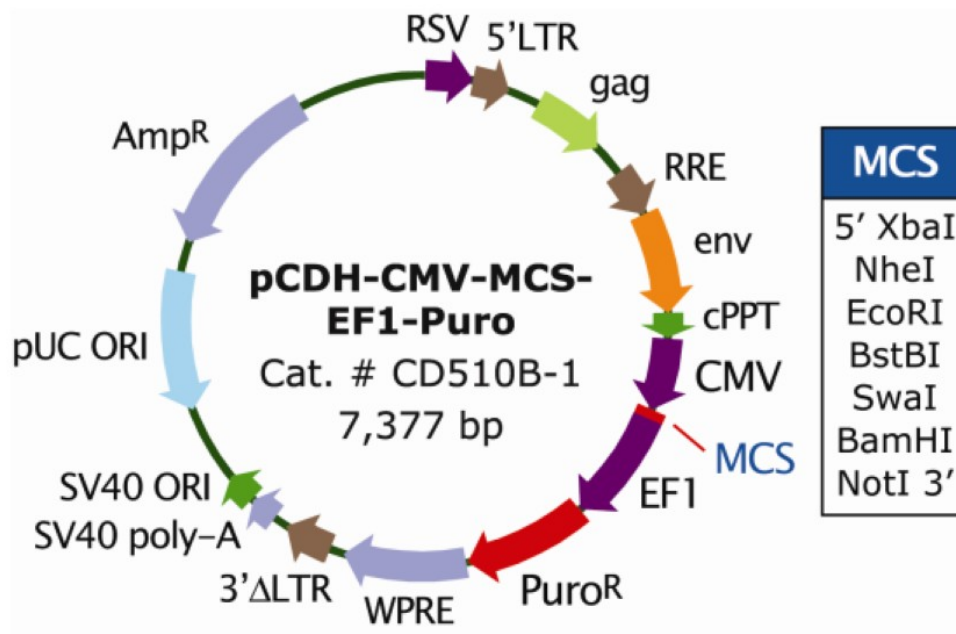


Negative control – cell culture medium:
O.D. 490nm – 0,062

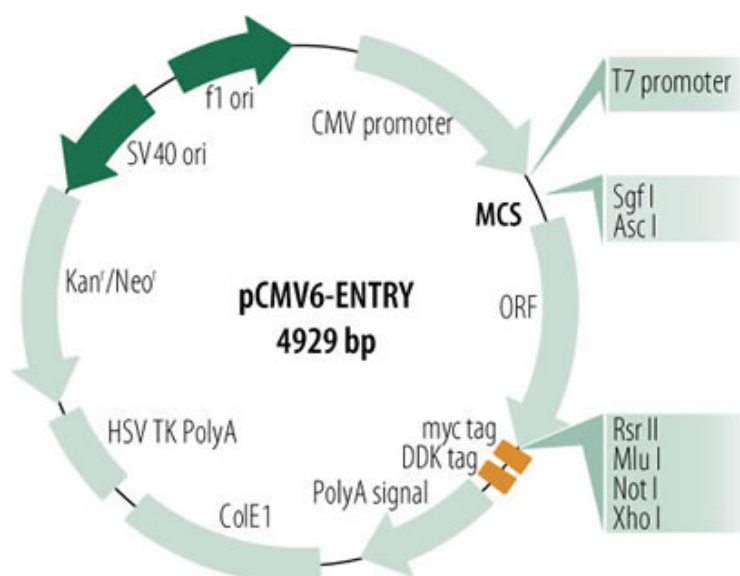
Positive control – mouse serum - mouse 42
(1:100 diluted in incubation buffer):
O.D. 490nm – 3,119

Appendix II Vector maps

1. Map of pCDH-CMV-MCS-EF1-Puro for the lentiviral transfection



2. Map of pCMV6-Entry



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