The regulation of oxidative stress in the development of drug resistance in the treatment of breast cancer

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Ph.D. THESIS

THE UNIVERSITY OF HONG KONG
2018

The regulation of oxidative stress in the development of drug resistance in the treatment of breast cancer

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

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

Abstract of thesis entitled

"The regulation of oxidative stress in the development of drug resistance in the treatment of breast cancer"

Submitted by

LEUNG Man Hong

for the Joint Degree of Doctor of Philosophy

at the University of Hong Kong and Imperial College London

in June 2018

Breast cancer is the most common type of female cancer. Reactive oxygen species (ROS) plays an important role in regulating signaling pathways that control cell survival and cell proliferation. Oxidative stress refers to the imbalance between ROS generation and ROS elimination. A number of chemotherapeutic drugs, such as taxanes, platinum compounds and anthracyclines trigger cell death via induction of oxidative stress. Development of drug resistance in cancer is associated with the adaptive response to oxidative stress. NRF2 is the main regulator of cytoprotective response to oxidative stress. Increased activity of NRF2 enhances cell growth and increases chemoresistance, providing growth advantage for malignant cells.

My study aims to identify molecular mechanisms that are involved in the development of drug resistance via modulation of oxidative stress in breast cancer. The novel molecular mechanisms through which NRF2 activity would be enhanced to confer drug resistance in breast cancer cells are explained.

FOXM1 is a well-known oncogene which contributes to all hallmarks of cancer. In Chapter 3, we hypothesized that FOXM1 and NRF2 are involved together in contributing to chemoresistance via modulation of oxidative stress. We showed that chemoresistant breast cancer cells express higher levels of FOXM1 and NRF2. These resistant cells have higher resistance to oxidative stress-mediated cell death and to ROS induction by tBHP. In drug sensitive MCF-7, we found that FOXM1 regulates NRF2 and NRF2 target genes expression. Transcriptional activation on antioxidant response element (ARE) was mediated by FOXM1 in response to epirubicin. ChIP-qPCR result

further supported that FOXM1 transcriptionally controlled NRF2 expression. However, the identified FOXM1/NRF2 axis was deregulated in chemoresistant cells. Nevertheless, the use of NRF2 inhibitor could enhance the efficacy of epirubicin treatment to treat breast cancer. Our findings showed that FOXM1 regulates the transcription of NRF2 which mediates response to epirubicin and eventual epirubicin resistance in breast cancer cells.

Our group have identified BQ323636.1 (BQ), as a novel splice variant of NCOR2 which could predict tamoxifen resistance in the treatment of breast cancer. In Chapter 4, we hypothesized that BQ could modulate oxidative stress in breast cancer. Overexpressing BQ in breast cancer cell lines could promote cell proliferation and protect cells from oxidative stress. In addition, we showed overexpression of BQ could reduce the levels of ROS. By RT-qPCR assay, several downstream NRF2 targets were found to be up-regulated in BQ overexpressing cells, suggesting that NRF2 transcriptional activity could be modulated by BQ. Luciferase reporter assay showed that NCOR2 could repress the transcriptional activity via antioxidant response element (ARE), which is the primary binding site of NRF2 in the promoter region. Furthermore, BQ could reverse the repressive effect of NCOR2 on ARE. These results suggest that BQ might modulate NRF2 activity via NCOR2. Immunoprecipitation assay indicated NCOR2 interacted with NRF2 and BQ overexpression could inhibit this interaction. Taken together, our findings suggested BQ regulates NRF2 signaling pathway via interfering with NCOR2 activity. Our findings reveal a novel role for BQ as a modulator of NRF2 and oxidative stress in breast cancer.

(Total 494 words)

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Declarations

I hereby declare that this thesis represents my own work, except where due acknowledge is made, and it 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_____

LEUNG, Man Hong

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Acknowledgement

Firstly, I would like to express my deepest gratitude to my supervisor from the University of Hong Kong Professor Ui-Soon Khoo and my supervisor from Imperial College London Professor Eric W.F. Lam. They have provided me with this special opportunity to join their teams and to pursue this Joint PhD degree.

Prof. Khoo has offered me with her enthusiasm, excellent supervisions and constant support which helped to complete the work for my PhD. She has also been very critical towards my work as well as been caring, patient and considerate. Professor Lam has supported me with his great guidance and patience throughout the whole project. He has provided me with innovative ideas and inspiring advices from the beginning of my project. They have trained me to think as a scientist and showed great concern in my academic and personal life. I am very grateful for their countless support.

I am especially thankful to Ellen Man for being extremely helpful to my project and all the post docs from Prof. Khoo's lab and Prof. Lam's lab: Frankie Tsoi, Stefania Zona, Ana Gomes, Paula Saavedra Garcia and Lukasz Boryn. Without their support and advices, my study could not be finished.

Besides, I would like to show my gratitude to lab mates from Prof.Khoo's lab: KC Mok, Jade Gong, Carmen Chau, Miao and Sophie; and from Eric's lab: Catherine Yao, Kitti Intuyod, Glowi Asiri, Jaysze Yung, Zimam Mahmud, Lavender Fan and Sathid Aimjongjun for all the help and support. All of you have provided me a fruitful academic and social environment in the 3 years of my PhD life.

Last but not least, I shall show my sincere thanks to my family and friends for their caring, patience and continuous support through all these years.

Publication

Conference Abstract

Leung, MH., Tsoi, H., Man, EPS., Lam, EWF. & Khoo, US. "The role of BQ323636.1, a novel splice variant of NCOR2, in modulation of oxidative stress in breast cancer". Poster presentation, Advances in Breast Cancer Research Conference, American Association for Cancer Research. Loews Hollywood Hotel in Hollywood, California. October 2017.

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Abbreviations

dNTP	Deoxynucleotide triphosphates
siRNA	Small-interfering RNA
ABC	ATP-binding cassette
ABCG2	ATP Binding Cassette Subfamily G Member 2
AI	Aromatase inhibitors
AKT	Protein kinase B
AP-1	Tandem activating protein 1
APS	Ammonium persulphate
ARE	Antioxidant responsive element
ASK1	Apoptosis signal regulating kinase 1
ATF3	Activating transcription factor 3
BACH	BTB Domain And CNC Homolog
Bad	Bcl-2-associated death promoter
Bax	BCL2 associated X
BCL2	B-cell lymphoma 2
BCRP	Breast cancer resistance protein
Bim	Bcl-2-like protein 11
bp	Base pair
BQ	BQ323636.1
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BSA	Bovine serum albumin
bZIP	Basic Leucine Zipper Domain
℃	Degree Celsius
CBP/300	CREB1-binding protein and p300
CDK	Cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
ChIP-Seq	ChIP-sequencing
Ct	Cycle threshold
Cu/ZnSOD / SOD1	Copper- and zinc-containing superoxide dismutase
Cul3	Cullin-3
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase

DTT	Dithiothreitol
E2F	E2 factor
EC-SOD / SOD3	Extracellular superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ER	Endoplasmic reticulum
ERK	Extracellular Signal-regulated Kinase
ERO1	Endoplasmic reticulum oxidoreductin 1
ERα	Estrogen receptor α
ESR1	Estrogen Receptor 1
F12	Nutrient Mixture F-12
FBS	Fetal bovine serum
FCS	Fetal calf serum
FdUMP	Fluoro-deoxyuridine monophosphate
FGFR1	Fibroblast growth factor receptor 1
FISH	Fluorescence in situ hybridization
FKH	Forkhead/Winged-helix domain
FOXM1	Forkhead box M1
FOXO	Forkhead Box O
GCLC	Glutamate—cysteine ligase catalytic subunit
GCLM	Glutamate-Cysteine Ligase Modifier Subunit
GDP/GTP	Guanosine diphosphate / Guanosine triphosphate
GI-GPx	Gastrointestinal glutathione peroxidase
GPS2	G protein pathway suppressor 2
GSH	Glutathione
GST	Glutathione S-transferase
GSTA3	Glutathione S-transferase α 3
GSTP	Glutathione transferase P
GSTP1	Glutathione S-Transferase Pi 1
h	Hour
H_2O	Water molecule
H_2O_2	Hydrogen peroxide
HAT	Histone acetyltransferase
HDAC	Histone deacetylases
HEK293FT	Human embryonic kidney 293 fast transform cells

HER2	Erb-b2 receptor tyrosine kinase 2
HIF-1	Hypoxia-inducible factor 1
HO-1	Heme oxygenase 1
IDC	Infiltrating ductal carcinoma
IGFR1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IKK	IκB kinase
IL-1	Interleukin-1
ILC	Infiltrating lobular carcinoma
IMEM	Improved Minimum Essential Medium
JNK	Jun N-terminal kinases
KEAP1	Kelch-like ECH-associated protein 1
KIF20A	Kinesin Family Member 20A
LB	Lysogeny broth
LCIS	Lobular carcinoma in situ
LHRH	Luteinizing hormone releasing hormone
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
min	Minute
ml	Millilitre
mM	Millimolar
MnSOD / SOD2	Manganese-dependent superoxide dismutase
Moz	Monocytic leukaemia zinc finger protein
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
mTOR	The mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCOR1	Nuclear receptor corepressor 1
NCOR2 / SMRT	Nuclear receptor co-repressor 2 / Silencing Mediator Of Retinoic Acid And Thyroid Hormone Receptor
ncRNA	Non-coding RNA
Neh	Nrf2-ECH<chicken Nrf2> homologus domain
NF-E2	Nuclear factor erythroid 2
NFE2L2 / NRF2	Nuclear factor erythroid-derived 2-like 2
NF-κB	Nuclear factor κ-B

NIK	NF-κB-inducing kinase
nm	Nanometre
nM	Nanomolar
NQO1	NAD(P)H Quinone Dehydrogenase 1
NRD	N-terminal Repressor Domian
NRF1	Nuclear factor erythroid-derived 2-like 1
NRF3	Nuclear factor erythroid-derived 2-like 3
O_2	Oxygen molecules
O_2-.	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
Oligo dT	Oligo deoxythymidine
p62 / SQSTM1	Sequestosome 1
p90RSK	90 kDa ribosomal protein S6 kinase 1
PARK7 . DJ-1	Parkinsonism Associated Deglycase
PARP	Poly ADP ribose
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDI	Protein disulfide isomerase
PERK	Eukaryotic translation initiation factor 2-alpha kinase 3
PFA	Paraformaldehyde
P-gp	P-glycoprotein
PI3K	Phosphoinositide 3-kinase
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
PR	Progesterone receptor
Prx1	Mitochondrial peroxiredoxin
Prxs	Peroxiredoxins
PTEN	Phosphatase and tensin homolog
RAR	Retinoic acid receptor
RD	Repression domain
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RXR	Retinoid X receptor
RXRα	Retinoic X receptor α
s	second
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SERD	Selective estrogen receptor downregulator

SERM	Selective estrogen receptor modulators
SMAD3	Mothers against decapentaplegic homolog 3
SOC	Super optimal broth with catabolite repression
SRB	Sulforhodamine B
TAD	Full Name
TAD	Transactivation domain
tBHP	Tert-Butyl hydroperoxide
tBHQ	Tert-butylhydroquinone
TBL-1	Transducin-Beta-Like Protein 1
TBST	Tris-buffered saline Tween-20
TCA	Trichloroacetic acid
TCGA	The Cancer Genome Atlas Network
T-DM1	Trastuzumab-DM1
TEMED	Tetraethylethyleneduanine
TMA	Tissue Microarray
TNFα	Tumor necrosis factor α
TNM staging	Tumour, Node and Metastasis staging
TRAC-1	T cell RING (really interesting new gene) protein
TRAIL	TNF-related apoptosis inducing ligand
Trx/TrxR	Thioredoxin/Thioredoxin reductase
Txn1	Thioredoxin 1
UGT1A1	UDP glucuronosyltransferase family 1 member A1
V	voltage
x g	Times Gravity
XIAP	X-Linked Inhibitor Of Apoptosis
µg	Microgram
µl	Microlitre

1 Introduction

1.1 Breast cancer

Breast cancer refers to development of malignant breast cells in breast tissue. These cells may arise from breast lobules, which are glands responsible for milk production, causing lobular carcinoma or from ducts, which are passages to drain milk from lobules to nipples, causing ductal carcinoma. Cancer cells have a potential to invade neighboring normal breast tissue. In addition, the cells may eventually metastasize into lymph nodes, and have routes going to other organs; bone, lung, liver, and brain are the common target organs for breast cancer metastasis.

Breast cancer symptoms that are common such as a new lump or mass in the breast felt by the patient or physicians, swelling of all or parts of the breast and skin irritation. Further testing are suggested with these initial symptoms like screening mammogram.

Development of breast cancer can be described according to how far cancer cells have spread beyond the original tumor. With reference to results of physical exam, biopsy, imaging tests, results of surgery and microscopy, different stages (stage 0 to stage IV) of breast cancer can be evaluated by physicians, as shown in figure 1.1 (American Joint Committee on Cancer, 2010).

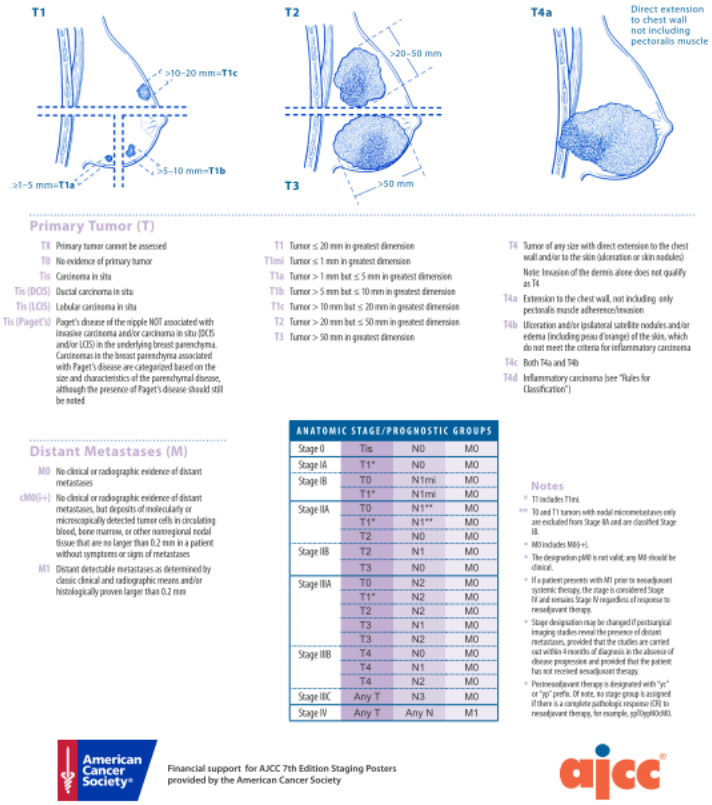


Figure 1.1 Breast cancer staging by American Joint Committee on Cancer. (American Joint Committee on Cancer, 2010)

1.1.1 Epidemiology of breast cancer

Breast cancer is one of the most common cancers in the world and the most frequent type of cancer found in women. Breast cancer can also occur in men but is much rarer than women. According to World Health Organization (WHO), there were an estimated 1.67 million new cancer cases diagnosed in 2012, which is 25% of all cancer cases. There are higher estimated incident rates in more developed regions such as Northern America and Western Europe while lower rates are found in less developed regions such as Eastern Asia and Middle Africa (Figure 1.2). Breast cancer ranked as the fifth cause of death among all type of cancers and it is the top cause of cancer death in women from less developed regions and second in women from more developed regions. As in Hong Kong, breast cancer is ranked as the first leading cancer types and third leading

cancers deaths in female. The number of invasive breast cancer case diagnosed in female has increased by 70% from 2004 to 2014 which is significantly higher than the overall 40% increase of all female cancers combined (Hong Kong Cancer Registry, 2015).

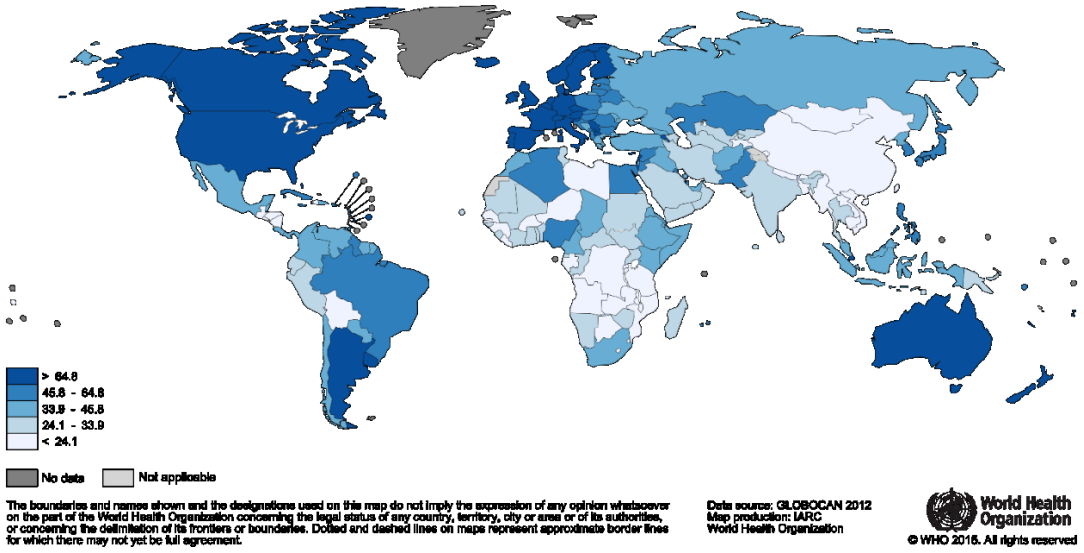


Figure 1.2 Estimated breast cancer incidence worldwide according to GLOBOCAN in 2012**.** (World Health Organization, 2015)

1.1.2 Risk factors of breast cancer

It has been estimated females would develop breast cancer for a lifetime risk of one to eight (Feuer, Wun et al. 1993, Cancer Research UK, 2012). Risk of being diagnosed with breast cancer depends on different factors. There are some risk factors that can be controlled while there are factors that are not. Several well-recognized factors will be discussed as follows.

Controllable factors are mainly related to life style. Lifestyle factors, for example, are diet, exercise, weight, intake of alcohol, smoking, use of oral contraceptives (birth

control pills) and hormonal replacement therapy (Costa and Saldanha 2017). Alcohol consumption can limit the livers' ability to control blood levels of female hormones estrogen and progesterone while oral contraceptives hormonal replacement therapy can induce levels of these hormones. Studies have shown estrogen stimulates growth of breast cells. Prolonged exposure of estrogen can increase the risk of breast cancer (Clemons and Goss, 2001).

Age is one of the factors that is non-modifiable. It is considered that cancer is an age-related disease as incidence of most cancer increases with age (White, Holman et al. 2014). The incidence of breast cancer is however relatively high at younger ages, presumably when breasts are rapidly growing. The risk of breast cancer increases with age until menopause, doubling about every 10 years (McPherson, Steel et al. 2000). Women with menstruation early in life and late menopause have higher risk of developing breast cancer. Women having natural menopause after age of 55 have twice higher risk than those who experience menopause before age of 45. Women who never had given birth in their lifetime or having first birth at late age have increased risk of breast cancer (McPherson, Steel et al. 2000).

Family history is another important risk factor of breast cancer that is not controllable. Around 10-30% of breast cancer cases are linked to hereditary factors. Germline mutations of *BRCA1* and *BRCA2* accounted for approximately 50% of total risk of hereditary breast cancer. The other 50% of cases of breast cancer are possibly contributed by a combined effects produced by gene mutations of high, moderate and low penetrance (Costa and Saldanha 2017). These inherited gene mutation are less commonly reported, such as *TP53, PTEN, CDH1, STK11* and *NF1* mutations. There are also strong evidence linking mutations in genes related to DNA-repair to higher

susceptibility of breast cancer like *PALB2, CHEK2, ATM* and *NBN* (Easton, Pharoah et al. 2015).

Among other risk factors, diet is the most leading controllable risk factor. Many studies have shown that high fat and meat intake is related to an increased risk of breast cancer as it can induce insulin resistance (Stoll 1996, Lee, Ko et al. 2004). Insulin-resistant patients have increased risk of cancer which can be due to increased generation of reactive oxygen species (ROS) (Arcidiacono, Iiritano et al. 2012). On the contrary, a rich antioxidants containing diet could prevent up to 33% incidence of breast cancer (Glade 1999). Oxidative stress and changes of trace elements are associated with breast cancer patients (Huang, Sheu et al. 1999). An imbalance between reactive oxygen species (ROS) and antioxidants level induce oxidative stress, which stimulate the development of breast cancer. It is proposed that reduction in antioxidant level and the presence of ROS can contribute to increased breast cancer risk (Gago-Dominguez, Jiang et al. 2007).

1.1.3 Classification of breast cancer

Different types of breast cancer can be named as ductal carcinoma *in situ* (DCIS), lobular carcinoma *in situ* (LCIS), infiltrating ductal carcinoma (IDC) and infiltrating lobular carcinoma (ILC) depending on the invasiveness of the tumors, of which IDC is the most common type accounting for more than 80% of breast cancer cases. Breast cancer can be classified by different schemes based on purpose and type of information used. They include histopathological features, hormone receptor status and more recently molecular and genomic features. It is important to note that not all tumors in a specific histological subtype belongs to the same molecular classification.

1.1.3.1 Histopathology

Traditionally, breast cancer classification is histological based on physical and anatomical properties of breast cancer. Histological grading and TNM staging schemes can evaluate the cancer aggressiveness (American Joint Committee on Cancer, 2010). In breast cancer grading, cancer cells are given a grade when checked under microscope. This grade is given by how cancer cells look like to normal breast cells. A grade number can usually correspond to whether the cells are slow growing which are less likely to spread (lower grade number) or fast growing which are more likely to spread (higher grade number). Grade 1 is given if the cells are well differentiated, grade 2 if the cells are moderately differentiated and grade 3 if the cells are poorly differentiated. The TNM staging system classifies breast cancer according to tumor size (T0-T4), involvement of lymph node (N0-N3) and if there is metastatic spread (M0-M1). Combining the T, N and M classification, breast cancers are given different five stages from 0-4 (American Joint Committee on Cancer, 2010). Stage 0 is used for non-invasive DCIS and stages 1-4 are used for invasive breast cancer.

1.1.3.2 Expression of hormone receptors

Breast cancer can be classified by expression of three hormone receptors, namely estrogen receptor (ER), erb-b2 receptor tyrosine kinase 2 (HER2) and progesterone receptor (PR). For example, studies from gene expression analysis revealed ER-positive tumors and ER-negative tumors to be molecularly different. These receptors expression are assessed by immunohistochemistry (IHC) and HER2 amplification can also be confirmed by fluorescence in situ hybridization (FISH) (Cornejo, Kandil et al. 2014). Expressions of hormone receptors provide information of selecting appropriate treatment. For example, endocrine therapy can be used for ER-positive patients while HER2-specific monoclonal antibodies are used against HER2-positive tumors as

targeted therapy. Triple negative (ER-negative, HER2-negative and PR-negative tumor) patients have relatively poor prognosis due to lack of treatment options.

1.1.3.3 Molecular and genomic features

As a more recent approach, breast cancer can be further classified into different molecular intrinsic subtypes using gene expression analysis on DNA microarrays: luminal-A, luminal-B, HER2-enriched, basal-like and normal-like (Sorlie, Perou et al. 2001). Most luminal tumors are ER-positive and PR-positive. Luminal tumors are subdivided into luminal-A type which are low grade tumors and luminal-B type which are higher grade tumors. HER2-enriched are high grade tumors showing amplification and overexpression of ERBB2 gene while basal-like tumors are triple negative and are the most clinically aggressive type. The use of cell markers are useful for dictating prognosis and treatment.

Luminal A and luminal B tumors both have similar expression of ER and they are distinguished from expression of proliferation/cell-cycled related pathways and luminal/hormone-regulated pathways. Expression of genes involved in proliferation/cell-cycle, such as *MKI67* and *AURKA*, are higher while genes in luminal pathways like *PR* and *FOXA1* have lower expression in luminal B tumors (Prat, Pineda et al. 2015). The HER2-enriched tumors are featured with high HER2-related and proliferation-related expression (*ERBB2* and *GRB7*), intermediate luminal-related expression (ER and PR) and low basal-related expression (*keratin 5* and *FOXC1*) (Prat, Pineda et al. 2015). As for basal-like subtype, it is related to high expression of proliferation-related genes and keratins, intermediate expression of HER2-related genes, and very low expression of luminal-related genes (Prat, Pineda et al. 2015). Employing additional technology platforms provided further characterization of molecular features

of breast cancer. The Cancer Genome Atlas Network (TCGA) has shown a more comprehensive way to characterize the above subtypes of breast cancer through analyzing primary breast cancers by genomic DNA copy number, DNA methylation, exon sequencing, mRNA arrays, microRNA sequencing and reverse-phase protein arrays (Cancer Genome Atlas 2012) (Figure 1.2). Clinically, there are already gene profiling assays for different subtypes of breast cancer to provide prognostic information, predict risk of recurrence and response to chemotherapeutics, such as Oncotype DX, MammaPrint and PAM 50 Breast Cancer Intrinsic Classifier (Taherian-Fard, Srihari et al. 2015).

Subtype	Luminal A	Luminal B	Basal-like	HER2E
ER$^+$/HER2$^-$ (%)	87	82	10	20
HER2$^+$ (%)	7	15	2	68
TNBCs (%)	2	1	80	9
TP53 pathway	*TP53* mut (12%); gain of *MDM2* (14%)	*TP53* mut (32%); gain of *MDM2* (31%)	*TP53* mut (84%); gain of *MDM2* (14%)	*TP53* mut (75%); gain of *MDM2* (30%)
PIK3CA/PTEN pathway	*PIK3CA* mut (49%); *PTEN* mut/loss (13%); *INPP4B* loss (9%)	PIK3CA mut (32%) PTEN mut/loss (24%) INPP4B loss (16%)	PIK3CA mut (7%); PTEN mut/loss (35%); INPP4B loss (30%)	PIK3CA mut (42%); PTEN mut/loss (19%); INPP4B loss (30%)
RB1 pathway	Cyclin D1 amp (29%); *CDK4* gain (14%); low expression of *CDKN2C*; high expression of *RB1*	Cyclin D1 amp (58%); *CDK4* gain (25%)	*RB1* mut/loss (20%); cyclin E1 amp (9%); high expression of *CDKN2A*; low expression of *RB1*	Cyclin D1 amp (38%); *CDK4* gain (24%)
mRNA expression	High ER cluster; low proliferation	Lower ER cluster; high proliferation	Basal signature; high proliferation	HER2 amplicon signature; high proliferation
Copy number	Most diploid; many with quiet genomes; 1q, 8q, 8p11 gain; 8p, 16q loss; 11q13.3 amp (24%)	Most aneuploid; many with focal amp; 1q, 8q, 8p11 gain; 8p, 16q loss; 11q13.3 amp (51%); 8p11.23 amp (28%)	Most aneuploid; high genomic instability; 1q, 10p gain; 8p, 5q loss; *MYC* focal gain (40%)	Most aneuploid; high genomic instability; 1q, 8q gain; 8p loss; 17q12 focal *ERRB2* amp (71%)
DNA mutations	*PIK3CA* (49%); *TP53* (12%); *GATA3* (14%); *MAP3K1* (14%)	*TP53* (32%); *PIK3CA* (32%); *MAP3K1* (5%)	*TP53* (84%); *PIK3CA* (7%)	*TP53* (75%); *PIK3CA* (42%); *PIK3R1* (8%)
DNA methylation	–	Hypermethylated phenotype for subset	Hypomethylated	–
Protein expression	High oestrogen signalling; high MYB; RPPA reactive subtypes	Less oestrogen signalling; high FOXM1 and MYC; RPPA reactive subtypes	High expression of DNA repair proteins, PTEN and INPP4B loss signature (pAKT)	High protein and phospho-protein expression of EGFR and HER2

Percentages are based on 466 tumour overlap list. Amp, amplification; mut, mutation.

Figure 1.3 Highlight of genomic, clinical and proteomic features of breast cancer subtypes from TCGA. (Cancer Genome Atlas 2012).

1.2 Tumorigenesis of breast cancer

Human breast tissues contain three major cell types: epithelial cells, myoepithelial cells and alveolar cells when in pregnancy and lactation. Certain epithelial cells of normal mammary glands of cells and from benign and neoplastic lesions have the properties of stem cells and these epithelial stem cells give rise to other cell types but malignant epithelial cells with stem cell properties did not have to ability to differentiate (Rudland,

Barraclough et al. 1997). Different groups have proposed a model in which breast tumorigenesis is driven by malignant stem cells (Reya, Morrison et al. 2001, Dontu, Al-Hajj et al. 2003, Pardal, Clarke et al. 2003). In this model, cancer cells are thought to be aroused via multiple mutations over time. Mammary stem cells are slow dividing and long lived cells with high proliferative capacity and thus able to accumulate series of mutation or alterations during tumorigenesis. Weinberg R.A. and his group have proposed six hallmarks of cancer, capabilities that enable tumor growth and metastasis, during cancer development. They includes sufficient proliferative signaling, insensitivity to growth suppressors, activation of tissue invasion and metastasis, replicative immortality, induction of angiogenesis and resistance to cell apoptosis (Hanahan and Weinberg 2000).

Different genetic and epigenetic changes have been implicated in the tumorigenesis of breast cancer. The results of these mutations/alterations in the progenitor cells and the origin of cell types determine the subtype of that breast cancer belongs to (Dontu, El-Ashry et al. 2004).

1.2.1 Genetic changes

As previous section has mentioned many genes have been implicated in development of breast cancer. There is extensive evidence which have shown that these well recognized genes are found to have somatic mutations in genomes of breast cancer tumors. Study by Stephens et al. identified somatic driver substitutions and small insertions/deletions in *BRCA1, TP53, PTEN, CDH1, RB1, AKT1, GATA3* and *PIK3CA* (Stephens, Tarpey et al. 2012), genes that are involved in apoptosis, regulation of cell cycle and transcription regulation. The same study also found somatic mutations in another group of cancer genes previously identified in other cancer types (*APC,*

ARID1A, ARID2, ASXL1, BAP1, KRAS, MAP2K4, MLL2, MLL3, NF1, SETD2, SF3B1, SMAD4, and *STK11*) and newly identified genes previously not implicated (*ARID1B, CASP8, MAP3K13, MAP2K13, SMARCD1, CDKN1B* and *NCOR1*) (Stephens, Tarpey et al. 2012). Genetic changes varies distinctly between individual tumors showed the heterogeneity of breast cancer. Consistently, analysis conducted by TCGA identified mutations in nearly all genes implicated in breast cancer (Cancer Genome Atlas 2012). Also, the study by TCGA gives evidence that different molecular subtypes of breast cancer have different genetic characteristics but at the same time cannot be defined by one type of genetic change or mutation. For example, luminal A and luminal B subtypes mainly have missense mutations in *TP53* while basal-like subtype contains mostly non-shift and frameshift mutations (Cancer Genome Atlas 2012). Moreover, levels of copy number changes are found correlated with different subtypes such as focal amplification in *PIK3CA, EGFR, FOXA1* and *HER2* gene regions, and focal deletion *MLL2, PTEN, RB1*,and *MAP2K4* gene regions (Cancer Genome Atlas 2012).

1.2.2 Epigenetic changes

Epigenetic changes are molecular alterations without any changes of DNA sequence. The changes include DNA methylation and histone modification. These alterations are reversible and heritable. These alterations can regulate the gene expression. They are critical in normal growth and development. In addition, epigenetic changes are also critical for the development and progression of many types of cancer, including breast cancer. DNA hypermethylation and abnormal histone modification are often associated with epigenetic silencing of tumor suppressor genes and genomic instability in breast cancer (Huang, Nayak et al. 2011). Compared to noncancerous tissues, multiple genes are methylated in breast cancer. DNA methylation is mediated by DNMTs (DNMT1, DNMT3a and DNMT3b) which add methyls groups to cytosines in targeted CpG

dinucleotides. DNA methylation accumulated over time would lead to silencing of genes involved in important biological processes leading to breast carcinogenesis, such as *ER, PR* and *RAR-β* for growth and proliferation, *HOXA5, DAPK* and *GPC3* for apoptosis, *E-cadherin* and *SYK* for tissue invasion and metastasis, and *cyclin D2, p16* and *GSTP1* for replicative immortality (Widschwendter and Jones 2002). Post translational modification of histones can determine whether genome are open and genes are transcriptional active or genome are close thus transcriptional inactive. One of the most studied histone modification are histone acetylation/deacetylation. Histone acetylation status is maintained by the balance between activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Hyperacetylated are associated with transcription activation while hypoacetlyated histones are correlated with transcription repression (Smith 2008). In general, hyperacetlyation may promote cell growth, thus inhibition of HDACs might leads to tumor growth inhibition (Damaskos, Garmpis et al. 2017). A study which characterized 880 human breast carcinoma show that changes in histone acetylation status and methylation pattern might be the evidence of early sign of breast cancer (Elsheikh, Green et al. 2009).

1.3 Treatment for breast cancer

There are several treatments to treat breast cancer which depends on its stage of cancer, size of the tumor, hormone receptor status and patient's health condition. Some are called local therapies which includes surgery and radiation therapy. Local therapies treat cancer without affecting the rest of body and more likely to be useful for less advanced cancers. Other treatments are called systemic treatments involving the use of drugs given orally as well as intravenously. Drugs administered can therefore reach cancer cells anywhere in the body. Depending on the type of breast cancer and different type of drugs, treatments can be categorized as chemotherapy, endocrine therapy and

targeted therapy. Breast cancer patients usually would receive more than one type of treatment. Adjuvant therapy is the method that is given to the patient after main treatment like surgery to enhance the prevention of cancer while neoadjuvant therapy is the method given to the patient before main treatment to reduce the size of tumors to facilitate surgery (Majeed, Aslam et al. 2014).

1.3.1 Surgery

Surgery has long been the primary option for the treatment of breast cancer. Most patients with early stage breast cancer such as DCIS would receive surgery treatment first (Matsen and Neumayer 2013). For the past decades, surgery treatment of breast cancer have been transitioned from more radical approaches such as mastectomy (removal of the whole breast) to less extreme approaches like nipple and areola sparing mastectomies, lumpectomy (breast conserving surgery). Removal of axilla lymph nodes are for staging and prognosis use though not providing any treatment benefits (Karam 2013). The choice of surgery approach depends on the size of tumor, if there is any sign of metastasis or relapse as well as patient's wish. As a better approach of breast conserving surgery is combined with radiation therapy for reducing risk of recurrence. There are certain conditions such as pregnancy, multicentric tumor and large tumor to breast ratio, lumpectomy should not be considered. Recurrences of breast cancer are deal with by mastectomy (Matsen and Neumayer 2013).

1.3.2 Radiation therapy

Radiation therapy is the therapy using high energy radiation to induce ionization to exposed tissue. Radiation can cause damages to DNA strands of cancer cells which inhibit their growth. Though radiation can also affect healthy tissues, it is more effective against cancer cells. They are fast-growing and therefore not able to repair the induced

DNA damage rapidly, ultimately causing cell death (Majeed, Aslam et al. 2014). Radiation therapy is usually implemented to remove the remaining cancer cells after lumpectomy for early stage breast cancer. Radiation therapy after breast conserving surgery has become a standard part of breast cancer treatment as many randomized trials have shown a significant benefit in breast cancer control and resulted in advantages of overall survival compared to surgery alone (Speers and Pierce 2016).

1.3.3 Hormonal therapy

Hormones are chemical messengers in the body and exert their effects to different organs when brought to the target organs by blood flow. Ovaries are responsible for producing the two main hormones estrogen and progesterone in premenopausal women. As in postmenopausal women, androgens released by adrenal glands are converted to estrogen by aromatase that found in adipose tissue and muscle. These female hormones are important for female secondary development and the maintenance of menstrual cycle. But exposure of hormones in hormone sensitive breast cancer triggers dimerization of ER and promotes estrogen related gene transcription; enhanced levels of these hormones can lead to development of cancer.

Hormonal therapy aims to reduce the development of hormone receptor positive cancer by blocking the effect of estrogen, inhibiting the production of estrogen and progesterone as well as inhibiting the actions of hormones. Different classes of endocrine therapy have been developed such as selective estrogen receptor modulators (SERM), selective estrogen receptor downregulator (SERD), aromatase inhibitors (AI) and luteinizing hormone releasing hormone agonists (LHRH agonist) (Reinbolt, Mangini et al. 2015). One of the well-known examples of SERM is tamoxifen which can partially inhibit ER dimerization. Its activity is tissue specific: acting as an estrogen

antagonist in breasts and vaginal muscosa but as an agonist in endometrium and bones (Lumachi, Luisetto et al. 2011). On the other hand, SERD is antagonist in all tissues, like fulvestrant that block ER dimerization completely and ultimately cause downregulation of ER expression (Reinbolt, Mangini et al. 2015). AIs inhibit the action of arimatase and thus block the production of estrogen. Examples of AI are anastrozole, letrozole and exemestane, in which the first two are non-steroidal reversible inhibitors while the later is steroidal irreversible inhibitors (Lumachi, Luisetto et al. 2011). LHRH agonist such as Goserelin are given to premenopausal women to suppress steroidogensis via binding and blocking luteinizing hormone releasing agonist receptors on the pituitary gland, eventually inhibit the release of estrogen from ovaries (Reinbolt, Mangini et al. 2015).

Hormonal therapy has been proven as an effective treatment for ER positive cancer. Adjuvant therapy given to patients after primary surgery for five years have shown to delay local and distant relapses and improves overall survivals (Early Breast Cancer Trialists' Collaborative 2005). However, it is not uncommon for ER positive breast cancer patients reported to be not responsive to first line endocrine treatment due to primary (*de novo*) resistance and other patients who initially respond but eventually become not responsive due to development of secondary (acquired) resistance (Ring and Dowsett 2004).

1.3.4 Chemotherapy

Chemotherapy refers to the treatment of cancer that utilizes cytotoxic drugs to stop cancer cell proliferation or causing cell death. It is common to administer to breast cancer patients for neoadjuvant therapy and/or adjuvant therapy, i.e. combining chemotherapy with surgery. Chemotherapy is often associated with severe side effects

and thus not suitable for all breast cancer patients though it could be the only available option for patients with advanced stage and metastasis to reduce as many cancer cells as possible. Anthracyclines, taxanes, platimnum compounds and alkylating agents and antimetabolites are the main types of chemotherapeutic drugs.

To achieve better treatment outcomes, breast cancer patients are usually given a combination of two to three drugs together. Examples of these combinations are, listed in CRUK, AC (Adriamycin/Doxorubicin, Cyclophosphamide), EC (Epirubicin,Cyclophosphamide), CMF (Cyclophosphamide, Methotrexate, 5-Flurouracil) and FEC (5-Flurouracil, Epirubicin, Cyclophosphamide).

Example of anthracyclines are doxorubicin and epirubicin, they exert their cytostatic and cytotoxic effect via several mechanisms: intercalation into DNA/RNA strands, induce DNA cross-linking, DNA binding and alkylation, interference with DNA unwinding or DNA strand separation and helicase activity; initiation of DNA damage via inhibition of DNA topoisomerase II; generation of free radicals, leading to DNA damage or lipid peroxidation and direct membrane effect (Minotti, Menna et al. 2004).

The taxanes group, such as paclitaxel, doctaxel and cabazitaxel, act by binding to and stabilizing microtubules. Microtubules are intracellular filaments forming part of the cytoskeleton and are important for chromosome separation during cell division and mitosis. Disruption of microtubules by taxanes prevents microtubules disassembly, eventually inhibit essential interphase cellular mechanisms (Komlodi-Pasztor, Sackett et al. 2011). Also taxanes can inhibit antiapoptotic function of the BCL2 family and upregulate cyclin dependent kinase inhibitor 1A and tumor suppressor protein p53,

leading to cell cycle arrest and cell apoptosis (Moos and Fitzpatrick 1998, Jordan and Wilson 2004).

Cisplatin and carboplatin are anticancer drugs that contain metal platinum. These platinum compound are crosslinking agents that induce DNA monoadduct, DNA inter-strand crosslinks, DNA intra-strand crosslinks and DNA-protein crosslinks, thereby blocking DNA repair and DNA synthesis in cancer cells (Dasari and Tchounwou 2014). Cyclophosphamide, an alkylating agent, works with similar mechanisms as platinum compounds. It interferes with DNA replication by inserting an alkyl moiety into the DNA structure which block cell division (O'Shaughnessy 1999).

Antimetabolites work by being incorporated into macromolecules such as DNA and RNA, or by essential biosynthesis inhibition. 5-flurouracil is an example of antimetabolites. It acts as an analog of uracil with a fluorine atom in place of hydrogen atom, therefore 5-flurouracil can enter cells as uracil and mis-incorporated into RNA and DNA. Also metabolic products of 5-flurouracil, FdUMP, inhibit the action of nucleotide synthetic enzyme thymidylate synthase to disrupt DNA replication and repair (Longley, Harkin et al. 2003).

1.3.5 Targeted therapy

Targeted therapy are treatments that targeting specific characteristics of cancer cells, such as a protein that allows abnormal growth of cancer cells, cell surface markers that are pro-proliferative. Currently, there are several targeted therapies physicians use to treat breast cancer including trastuzumab, trastuzumab-DM1 (T-DM1), pertuzumab, lapatinib and neratinib, everolimus, palbociclib and ribociclib. The HER2 target drugs are used to treat patients with HER2-overexpressed breast cancer, usually given along

with other chemotherapeutics. Trastuzumab, T-DM1 and pertuzumab are man-made version of monoclonal antibodies against HER2 to inhibit HER2 signaling pathways and tumor cell growth. Trastuzumab is usually used to treat early and late stage breast cancer while pertuzumab is given to patients with advanced stage breast cancer. T-DM1 is trastuzumab conjugated with potent cytotoxic agent DM1 and used to treated advanced breast cancer patients previously treated with trastuzumab and chemotherapy. Lapatinib and neratinib are reversible and irreversible tyrosine kinase inhibitors respectively and directly inhibits the kinase activity of HER2 as well as other HERs. They are used when trastuzumab is no longer working (Saini, Azim et al. 2011, Parakh, Gan et al. 2017). Everolimus is a mTOR inhibitor via binding to intracellular receptor FKBP12 to prevent mTOR downstream pathway which control cell cycle progression, cell growth and proliferation (Royce and Osman 2015). Everolimus is currently approved by FDA to treat advanced breast cancer that is hormone receptor positive, HER2 negative in postmaenopausal women together with aromatase inhibitors. Palbociclib and ribociclib slow cancer growth by blocking cyclin dependent kinases, particularly CDK4 and CDK6. These CDK4/6 inhibitors are also usually used in combination with hormonal therapy (Hamilton and Infante 2016).

1.3.6 General mechanisms of drug resistance

Current chemotherapeutic methods in single or in combination with other methods have significantly improved cancer patients' survival. Drug resistance, intrinsic or acquired one, has become the major cause of treatment failure in cancer chemotherapy. Intrinsic resistance refers to a tumor which fails to respond to therapy at the first interval imaging assessment clinically, possibly due to the existence of resistance mechanism prior to treatment at the cellular level. On the other hand, acquired resistance refers to an initial

clinically responsive tumor which subsequently becomes progressive on treatment, having developed resistance mechanism over time (Burrell and Swanton 2014).

To understand and overcome drug resistance, many mechanisms of drug resistance have been proposed. Drug resistance mechanisms can be typically characterized as affecting the pharmacodynamics or pharmacokinetics of anticancer agents. Pharmacodynamics resistance includes altered expression levels of the drug target, upregulated DNA repair, activated adaptive pro-survival responses and failed cell death induction due to deregulated apoptosis. Pharmacokinetic resistance usually includes altered stability, metabolism, excretion and distribution of anti-cancer drugs at the tumor site (Holohan, Van Schaeybroeck et al. 2013, Damia and Garattini 2014). Moreover, repeated exposure to single anti-cancer agents may result in cross resistance to other agents of the same classes or even different classes. This can be explained by sharing of common metabolic pathways, efflux transport systems, or cytotoxic targets (Li, Zha et al. 2015).

In breast cancer, mechanisms of resistance to endocrine therapy, tamoxifen resistance in particular, may involve loss of expression of ERα due to methylation of CpG islands or histone deacetylation at *ESR1* promoter. Also, changes in growth factor signaling such as HER2, IGFR1 and FGFR1 pathways induced activation of the mitogen-activated protein kinase (MAPK) signaling or phosphoinositide 3-kinase (PI3K) pathway and stimulate growth of tamoxifen resistance cells. Recently, numerous ncRNAs have also been implicated in acquired resistance to endocrine therapies (Hayes and Lewis-Wambi 2015).

Breast cancer resistance to chemotherapy such as anthracyclines may involve members of the ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp), breast

cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRPs). ABC transporters are a superfamily of energy dependent membrane transporters and they mediate drug resistance by controlling drug efflux and reducing intracellular drug concentrations. Overexpressions of these ABC transporters result in alteration of membrane transport and drug efflux (Mechetner, Kyshtoobayeva et al. 1998, Burger, Foekens et al. 2003). Resistance to anthracyclines can also be contributed by changes in topoisomerase II activity and apoptotic signaling pathways due to mutations in p53 (Riordan and Ling 1985, Aas, Borresen et al. 1996). On the other hand, mechanisms of taxanes resistance include altered expression of isotopes of β-tubulin, such as β-III and the expression of ABC transporters. P-gp expression was found to mediate paclitaxel resistance and in-vitro drug sensitivity assays in primary cancer cells (Mechetner, Kyshtoobayeva et al. 1998). As shown by microarray expression profiling analysis, low expression microtubule-associated protein tau is a significant predictor of paclitaxel sensitivity. This indicated that microtubule-associated proteins also involved in paclitaxel resistance (Rouzier, Rajan et al. 2005).

1.4 Reactive oxygen species and oxidative stress

1.4.1 Introduction of reactive oxygen species (ROS)

ROS are broadly defined as oxygen-containing chemical species with reactive characters. They are radicals, ions or molecules that have a single unpaired electron in their outermost electron shell. Oxygen molecules (O_2) are essential for all aerobic organisms. Aerobic energy metabolism depends on oxidative phosphorylation in which oxidoreduction energy is converted to high energy phosphate bond in ATP via mitochondrial enzymatic complex. O_2 serves as final electron acceptor for cytochrome-c oxidase which catalyzes the reduction of O_2 to H_2O. During these electron transfer reactions, O2 metabolites, such as superoxide anion ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2)

may form. They are partially reduced and highly reactive and a more reactive hydroxyl radical ($OH^{\cdot}$) can be formed in the presence of transition metal ion. These O_2 metabolites are named as "reactive oxygen species" as they are more reactive than O_2 (Thannickal and Fanburg 2000). ROS can be classified as two types: free oxygen radicals and non-radicals ROS. Free oxygen radicals includes $O_2^{-\cdot}$, $OH^{\cdot}$, nitric oxide, organic radicals, sulphur containing radicals. Examples of non-radicals ROS are H_2O_2, singlet oxygen, ozone, organic hydroperoxides, nitrogen containing anions/molecules and highly reactive lipid or carbohydrate derived carbonyl compounds. Related to cancer, $O_2^{-\cdot}$, H_2O_2, $OH^{\cdot}$ are the most well studied ROS types (Liou and Storz 2010)..

Cancer cells have been characterized by increased aerobic glycolysis and high level of oxidative stress contributed by ROS (Cairns, Harris et al. 2011). Altered signaling pathways of cell metabolism in cancer lead to increased ROS generation. Accumulation of ROS in cancer cells is the result of imbalance between ROS generation and ROS elimination. In breast cancer, ROS levels are found upregulated when compared to normal breast tissue, especially level of O_2^{-}, possibly because of change in redox state of breast tumors (Haklar, Sayin-Ozveri et al. 2001)

1.4.2 Generation of ROS in cells

In cancer cells, dysregulation of these ROS production mechanisms leads to the elevated levels of ROS. It can be result from mitochondrial dysfunction, increased metabolic activity, imbalance of peroxisome activity, increased cellular receptor signaling, activated oncogene and increased activity of various ROS enzymes (Liou and Storz 2010). ROS are mainly generated in mitochondria, endoplasmic reticulum (ER) and peroxisomes (Figure 1.4).

Mitochondria have been considered as main production site of ROS. In an experiment

published by Chance and colleagues, approximately 1-2% of oxygen molecules consumed by mitochondria forms superoxide anions under particular conditions (Chance, Sies et al. 1979).In mitochondria, ROS are produced as byproduct of oxidative phosphorylation. Superoxide anions are generated via electron transport chain complexes I and III at the inner membrane of mitochondria and then released to mitochondrial matrix or cytosol via mitochondrial permeability transition pore. Superoxide anions are converted to hydrogen peroxide by superoxide dismutases either in mitochondrial matrix (MnSOD) or in cytosol (Cu/ZnSOD) (Liou and Storz 2010).

Endoplasmic reticulum is the site for proper protein folding and bond formations depends on redox status of endoplasmic reticulum. ROS are generated as a byproduct of protein oxidation in the endoplasmic reticulum. The inner lumen of endoplasmic reticulum is an oxidizing enviornment. Endoplasmic reticulum oxidoreductase, including protein disulfide isomerase (PDI), catalyzes the PDI-mediated oxidation of substrate polypeptides to form disulphide bonds. The PDI are then recycled by endoplasmic reticulum oxidoreductin 1 (ERO1) reduction. ERO1 transfers electrons to oxygen molecules, leading to ROS production (Malhotra and Kaufman 2007). It has been estimated by a study that 25% of cellular ROS production is contributed by ERO1 mediated oxidation during protein folding in endoplasmic reticulum (Princiotta, Finzi et al. 2003).

Peroxisomes are multifunctional organelles with dual roles in generation and decomposition of ROS (Bonekamp, Volkl et al. 2009). Oxidases in peroxisomes, such as palmitoyl-CoA oxidase, pristanoyl-CoA oxidase, D-amino acid oxidase, L-α-hydroxyacid oxidases and xanthine oxidase, are responsible to catalyze fatty acid β- and α-oxidation, amino acid and glycoxylate metabolism, and synthesis of lipidic compounds. During these oxidative breakdown processes, hydrogen is extracted from

an appropriate substrate to oxygen molecules, forming hydrogen peroxide. Other ROS like superoxide anions and nitric oxide radicals can also be produced by peroxisomal oxidase (Antonenkov, Grunau et al. 2010, Di Meo, Reed et al. 2016).

1.4.3 Elimination of ROS in cell

The intracellular ROS levels are maintained to prevent cells from oxidative damage and to avoid redox imbalance under normal physiological condition. Cells have developed wide range of antioxidant defense to eliminate the excess ROS (Birben, Sahiner et al. 2012). Antioxidant defense is facilitated by antioxidant enzymes such as the glutathione peroxidase, superoxide dismutase, catalase and thioredoxin peroxidase as primary defense (Figure 1.4) . There are also non enzymatic compounds (vitamin A, vitamin C, vitamin E, glutathione, thioredoxin) served as secondary antioxidant defense.

The glutathione system contributes the breakdown of H_2O_2 and hydroperoxides using selenium as the cofactor. It comprises of glutathione and three enzymes: glutathione reductase, glutathione peroxidases and glutathione S-transferases. Through the oxidation of glutathione to glutathione disulfide, glutathione can protect cells from oxidative damage by reducing disulphide bonds of cytoplasmic proteins to cysteines (Brigelius-Flohe 1999). Glutathione reductase serves to reduce glutathione disulfide and recycle to fill glutathione pools (Carlberg and Mannervik 1975). Glutathione peroxidases can metabolize H_2O_2 and lipid hydroperoxides (Arthur 2000). Glutathione S-transferases catalyze the conjugation of glutathione with other compounds contain an electrophilic center and reduce organic hydroperoxides (Sherratt & Hayes, 2001). Comparing to normal human breast tissues, it was founds that activities of glutathione reductase, glutathione peroxidases and glutathione S-transferases are all higher in human breast tumor tissues (di Ilio, Sacchetta et al. 1985), suggesting that breast cancer

cells can tolerate higher levels of ROS by their upregulated ROS defense system.

The superoxide dismutase family is one of the first and most important line of antioxidant enzyme defense against ROS, especially against superoxide anions. Superoxide dismutases are metal containing enzymes that catalyze the reaction of superoxide anions to form oxygen and hydrogen peroxide molecules. There are three distinct isoforms of superoxide dismutases identified in mammals: Cu/Zn-SOD or SOD1, Mn-SOD or SOD2 and EC-SOD or SOD3. Both Cu/Zn-SOD and EC-SOD have Cu and Zn as their catalytic center while Mn-SOD has manganese as cofactor. The three different SOD enzymes are localized in different subcellular compartments: Cu/Zn-SOD in cytoplasm, Mn-SOD in mitochondria and EC-SOD in extracellular elements (Zelko, Mariani et al. 2002). Genetic polymorphism in MnSOD gene, such as Ala-9 to Val, was associated with higher risk of breast cancer among women with high levels of oxidative stress or low intake of antioxidants (Ambrosone, Freudenheim et al. 1999, Cai, Shu et al. 2004).

Both catalase and thioredoxin peroxidase are endogenous antioxidant enzymes that neutralize ROS by converting hydrogen peroxide to water and oxygen molecules. In addition thioredoxin peroxidase can metabolize other ROS such as organic hydrogen peroxides and peroxynitrite.

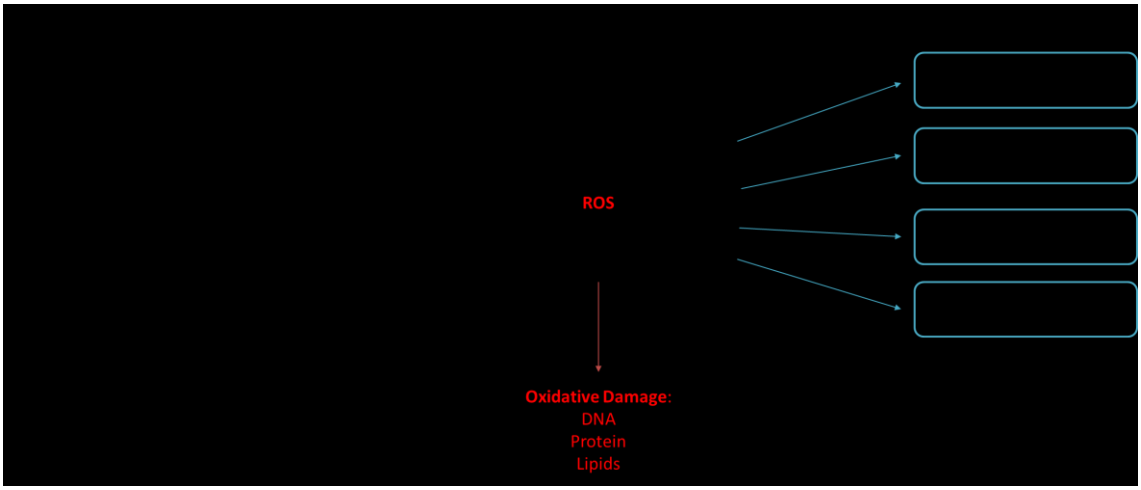


Figure 1.4 Major mechanisms of ROS generation and elimination. Mitochondria produce ROS during oxidative phosphorylation via electron transport chain. ROS are produced in the endoplasmic reticulum by protein oxidation during protein folding. Peroxisomes produce ROS during metabolism of fatty acids or detoxification activities. High levels of ROS have damaging functions for DNA, protein and lipids. Cells have developed a wide range of ROS scavenging systems to eliminate the excess ROS. The glutathione system comprises of glutathione and three enzymes to protect cells from oxidative damage. Superoxide dismutase serves to convert superoxide anions to oxygen and hydrogen peroxide. Other antioxidant enzymes include catalase and thioredoxin reductase, they convert hydrogen peroxide and other ROS to water and oxygen.

1.5 Roles of ROS in cancer

It is well established that ROS has destructive actions on DNA and proteins. There is also growing evidence that ROS can act as a chemical messenger, such as involvement in receptor-mediated pathways and transcription activation. Studies have revealed that ROS production can be triggered by growth factor such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), angiotensin II and estrogen, as well as by cytokine stimulations. These ligand stimulated ROS generation suggest that

intracellular ROS participates in different roles of complex signal transduction network (Finkel 2000).

ROS, particularly hydrogen peroxide, is postulated to act as second messengers to mediate various cell signaling pathways (Sundaresan, Yu et al. 1995, Colavitti, Pani et al. 2002). Hydrogen peroxide can mediate modification of specific cysteine residues in enzymes and transcription factors via reversible oxidation, thereby altering their activities (Lennicke, Rahn et al. 2015).

As mentioned earlier, cancer cells are characterized by production of high levels of ROS and an enhanced metabolic activity, resulting in changes of the cellular redox state. Several ROS sensitive signaling pathways are persistently upregulated in many cancer cells. They are involved in cell survival and cell proliferation. However, high levels of intracellular ROS can also induce cell cycle arrest or apoptosis. Due to these dual roles of ROS, cellular anti-oxidative systems are thought to be a key component for maintenance of cellular redox homeostasis.

1.5.1 Cell proliferation and cell survival

Several key ROS-dependent signaling pathways including mitogen-activated protein (MAP) kinase/ERK cascade, phosphoinositide-3-kinase (PI3K)/AKT-regulated signaling cascades, as well as the IκB kinase (IKK)/nuclear factor κ-B (NF-κB)-activating pathway mediates cell survival and proliferation (Figure 1.5).

An increase in ROS such as superoxide anions and hydrogen peroxide found in cancer cells has been associated with cell survival, increase cell growth and cell proliferation through the activation of mitogen-activated protein kinase (MAPK)/ extracellular regulated kinase 1/2 (ERK1/2) cascade, phosphoinositide-3-kinase (PI3K)/Akt-

regulated signaling cascades as well as IκB kinase (IKK)/nuclear factor κ-B (NF-κB)-activating pathway.

The MAPK/ERK1/2 pathway in cancer is activated by growth factors and K-Ras stimulated pathways (Khavari and Rinn 2007, Roberts and Der 2007). The upstream activator for ERK1/2, Ras, can be activated directly through oxidative modification at its cysteine 118 residue, leading to the inhibition of GDP/GTP exchange (Lander, Hajjar et al. 1997). Upstream kinases of ERK1/2 such as p90RSK can also be activated by ROS (McCubrey, Steelman et al. 2007, Chan, Liu et al. 2008). Estrogen metabolism in breast cancer generate hydrogen peroxide as a by-product and hydrogen peroxide is responsible for activation of ERK1/2, resulting in increased cell proliferation (Irani, Xia et al. 1997, Reddy and Glaros 2007). In addition, activation of ERK1/2 via ROS has other roles in cell survival, anchorage-independent growth and motility in multiple cancer types including breast, melanoma and ovarian cancer (McCubrey, Steelman et al. 2007, Roberts and Der 2007). The regulation of ROS activated ERK1/2 signaling in cell survival is cell type specific (Liou and Storz 2010). For example, inhibition of ERK1/2 or its upstream mitogen-activated protein kinase kinase (MEK), or treatment with ROS scavengers promote cell apoptosis and cell adhesion in breast cancer cell lines (Ostrakhovitch and Cherian 2005, Zhou, Chen et al. 2008). But treatment with exogenous ROS activates ERK1/2 and triggers cell death, probably due to the high basal level of ROS in these cancer cells (Liou and Storz 2010).

ROS generated from estrogen metabolism or other mammary carcinogens was shown to activate the PI3K/Akt signaling pathway in breast cancer (Burdick, Davis et al. 2003, Park, Na et al. 2009). Also, oxidative stress was shown to activate PI3K/Akt signaling pathway. The PI3K/Akt signaling pathway functions in cell survival through the

phosphorylation and inactivation of its target such as pro-apoptotic proteins Bad, Bax, Bim and FOXO transcription factors (Brunet, Bonni et al. 1999, Pastorino, Tafani et al. 1999, Xin and Deng 2005, Qi, Wildey et al. 2006). ROS such as hydrogen peroxide acts by reversibly oxidizing and by inactivation of the phosphatases PTEN and PTP1B, which are negative regulators of PI3K/Akt signaling pathway (Lee, Yang et al. 2002, Salmeen, Andersen et al. 2003). Moreover, PTEN deletion in mouse embryonic fibroblasts show increased levels of ROS due to deregulation of antioxidant enzymes expression such as peroxiredoxins and Cu/Zn SOD (Huo, Li et al. 2008).

Nuclear factor κ-B (NF-κB) is a redox-regulated transcription factor (Li and Karin 1999). Inactivated NF-κB is maintained in cytosol and tightly bound to its inhibitor IκB. Upon activation by cytokines such as TNFα or IL-1, NF-κB-inducing kinase (NIK) is phosphorylated and activates its downstream IKK complex. Activated IKK phosphorylate IκB, leading to subsequent IκB ubiqutination and proteasomal degradation. Degradation of IκB translocate NF-κB to nucleus and NF-κB can act as a transcription factor to drive expression of anti-apoptotic and anti-inflammatory genes (Beg, Finco et al. 1993). NF-κB act as a sensor for oxidative stress and can be activated by low doses of hydrogen peroxide (Schreck, Albermann et al. 1992). Oxidative stress activates NF-κB signaling via various pathways. For example, in breast cancer cell line MCF-7, induction by cytokines TNFα, IL-1β or carcinogen sodium arsenite generates hydrogen peroxide and superoxide, leading to activation of NF-κB and increased cell proliferation (Liou and Storz 2010). Activation of NF-κB in response to ROS can also be IKK independent via tyrosine phosphorylation of IκBα which leads to dissociation of NF-κB from IKK complex without degradation of IκB (Imbert, Rupec et al. 1996).

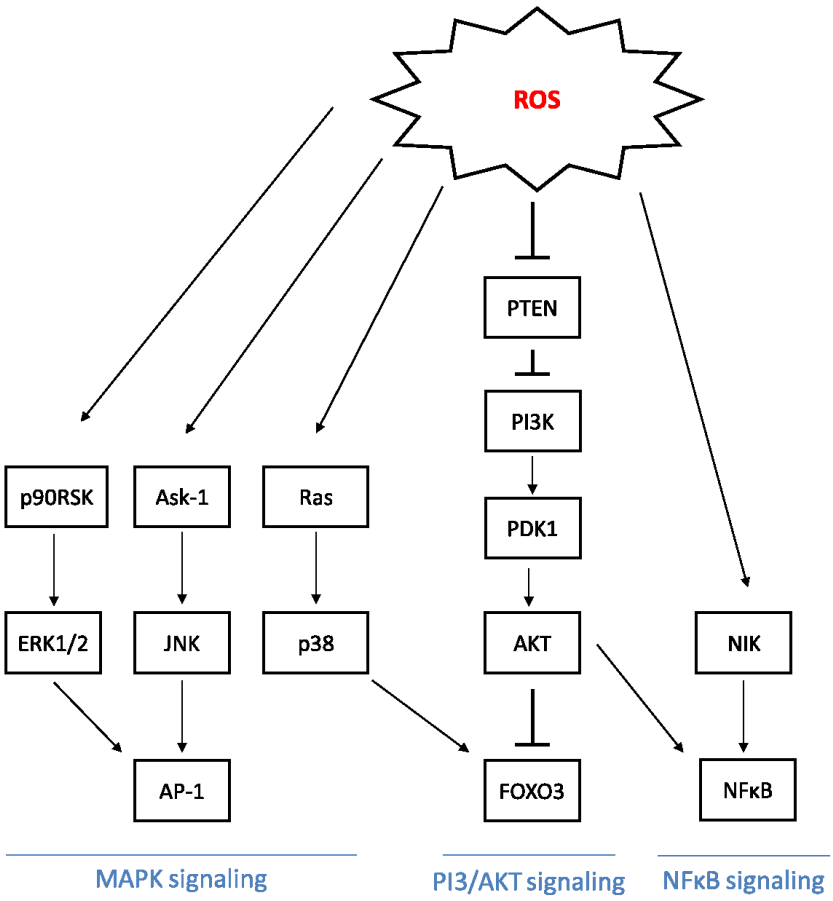


Figure 1.5 Major signaling pathways regulated by ROS in cancer. Imbalance of ROS leads to activation of different signaling cascades, such as MAPK (ERK1/2, JNK and p38) signaling cascades, the PI3K/AKT signaling pathway and NFκB signaling pathway. Redox-regulated transcription factors such as AP-1 or FOXO3 can also activated by ROS. Activations of these signaling cascades mediate cell survival and proliferation as well as cell apoptosis.

1.5.2 DNA damage and genome instability

ROS accumulation is common in different type of cancers. Such accumulation can cause direct DNA damage, mainly by oxidation of nucleotides and strand cleavage (Avendaño and Menéndez 2008) . ROS mediated DNA damage has been suggested to play a role in the pathogenesis of human carcinogenesis, as a driving force of initiation of carcinogenesis as well as malignant transformation (Herath, Leggett et al. 2006).

Cancer initiation and progression are associated with accumulation of mutated genes, leading to altered cell growth, cell survival and cell differentiation. Genome instability accounted for genetic diversity of human cancers. Tumor cells need certain genome instability to allow cells to evolve and drive tumorigenesis (Sieber, Heinimann et al. 2003). ROS are genotoxic in different cellular processes. For example, hydroxyl radicals can attack pyrimidines, purines of DNA and chromatin proteins, causing base modifications and DNA damage (Fruehauf and Meyskens 2007). DNA damage can cause DNA oxidation, resulting different DNA alteration such as deletions, insertions and base pair modifications and double strand breaks. These deleterious lesions can cause deletions and translocations. ROS mediated damage induce genomic instability and eventually alter gene expression if these damage are insufficiently repaired (Cooke, Evans et al. 2003).

ROS-mediated DNA damage, ineffective DNA repair mechanisms are found commonly in breast cancer cell lines and human breast tumor tissue samples. Compared to nonmalignant human breast cells, increased levels for most types of DNA damage were detected in breast cancer cells while the processing of double strand breaks and oxidatively induced clustered DNA lesions was deficient (Francisco, Peddi et al. 2008).

1.5.3 Apoptosis and cell death

A disproportional increase in ROS in cancer cells can be achieved by depleting antioxidant proteins within cells with induced ROS generation by immune cells or treatment of chemotherapy. Toxic levels of intracellular ROS can induce cell cycle arrest, senescence and apoptosis (Liou and Storz 2010). Cell death can be initiated by intrinsic apoptotic signaling in the mitochondria and extrinsic apoptotic signaling by death receptors. Caspase activation is one of the important mechanism of apoptosis,

triggering cleavage of different substrate such as poly ADP ribose (PARP), DNA fragmentation and cell death (Groeger, Quiney et al. 2009).

In intrinsic apoptotic signaling, increased ROS production from mitochondrial electron transport chains causes damage to the mitochondrial membrane resulting in the release and translocation of cytochrome c to the cytoplasm. Released cytochrome c can complex with Apaf-1 and pro-caspase 9, inducing the cleavage of caspase-3 and 7 and eventually result in apoptosis (Simon, Haj-Yehia et al. 2000). Also, loss of cytochrome c have been shown to further elevate ROS production following damages to mitochondrial electron transport chain (Chen and Lesnefsky 2006).

In extrinsic apoptotic signaling, binding of corresponding ligands to death receptors such as Fas, TNF receptor 1 (TNFR1), TNF-related apoptosis inducing ligand receptor 1 (TRAIL- R1) and TRAIL receptor 2 (TRAIL-R2) triggers initiator caspase 8 activation, resulting in cleavage of caspase 3. Caspase 8 activation also induces cleavage of Bcl-2 protein Bid to tBid which translocates back to mitochondria and also trigger the release of cytochrome c in the intrinsic apoptotic signaling. Evidences have shown a role of ROS in death receptors activation. Antioxidants, such as N-acetylcysteine, were found to be able to block TNF induced apoptosis and FAS receptor mediated apoptosis, the result suggested a functional role of ROS in this process (Simon, Haj-Yehia et al. 2000).

In addition, elevated ROS production can activate the Jun N-terminal kinases (JNK) pathway, resulting in apoptosis initiated by both intrinsic apoptotic signaling in the mitochondria or extrinsic apoptotic signaling by death receptor pathways (Dhanasekaran and Reddy 2008) Another MAPK family member p38 is also involved in apoptotic pathways in response to elevated ROS production. Activation of JNK and

p38 via ASK1 (apoptosis signal regulating kinase 1) is regulated by thioredoxin, as reduced form of thioredoxin can bind and inhibit ASK1 (Saitoh, Nishitoh et al. 1998). Other signaling molecules such as the forkhead transcription factors and p53 have also been implicated in apoptosis regulation in response to ROS (Liou and Storz 2010).

1.5.4 Adaptation to ROS

As mentioned above, ROS can modulate various signaling pathways that are involved in stress response and cell survival. Cancer cells are well adapted to persistent intrinsic oxidative stress. They develop an enhanced, endogenous antioxidant system to respond to such stress and make the malignant cells resistant to exogenous stress, survive under stress and ROS-mediated DNA damage. Cells that survived from intrinsic oxidative stress may have a set of adaptive mechanisms which not only activate ROS scacvenging system but also inhibit apoptosis (Pervaiz and Clement 2004). Increased activity of antioxidant enzymes such as MnSOD, peroxiredoxins and thioredoxin peroxidase were found upregulated in tumor cells than their normal counterparts (Janssen, Bosman et al. 2000, Young, Mei et al. 2004). The enhanced antioxidant systems may also serve as a key mechanism to evade ROS induced apoptosis, as oncogenic Ras-transformed cells have shown higher resistance to hydrogen peroxide induced cell death (Young, Mei et al. 2004)

Mechanisms of ROS adaptation may involve multiple pathways to activate stress induced transcription factors to increase expression of ROS scavenging enzymes and antioxidant molecules such as SODs, catalase, thioredoxin and the glutathione system, as well as the expression of cell survival molecules such as BCL2 family and Akt pathway. Also, alteration of cell death factors such as caspases may also involve in adaptation to ROS. These transcription factors include NF-κB, NRF2, c-JUN and HIF-

1 (Chen, Hewel et al. 2007, Sullivan and Graham 2008). They also regulate gene expression that are involved in cell proliferation, immortalization, angiogenesis and metastasis, providing additional survival advantage for adapted cancer cells (Chen, Hewel et al. 2007)

In addition, tumor cells acquire adaptations via hypoxia, contributing a more aggressive phenotype. ROS have been shown to play a role. Hypoxia arises as a result of insufficient supply of oxygen in tumor tissues. Normal cells result in cell death under prolonged exposure to hypoxia while cancer cells are adapted to hypoxia through a metabolic switch activating glucose metabolism utilizing glycolysis regardless of oxygen supply, known as the Warburg effect (Harris 2002). Previous studies have showed that increased glycolysis in tumor cells can prevent hydrogen peroxide induced cell death (Aykin-Burns, Ahmad et al. 2009). HIF-1 is one of the transcription factor activated by hypoxia and it comprises two subunit HIF-1α and HIF-1β. ROS have been shown to mediate HIF-1α stabilization under hypoxic conditions. A study in breast cancer cell line MCF-7 showed that increased MnSOD activity induce suppression of accumulation and activity of HIF1. This finding suggested a role of superoxide anions and hydrogen peroxide molecules in HIF-1α regulation (Wang, Kirk et al. 2005).

1.5.5 Oxidative stress and chemotherapy

ROS has been shown to interfere with many cellular functions, including cell survival, cell cycle progression, DNA damage and apoptotic pathways. Many drugs used for cancer chemotherapy can induce oxidative stress. Elevated lipid peroxidation products; reduction in total radical-trapping capacity of blood plasma; lowered plasma levels of antioxidants such as vitamin E, vitamin C, and β-carotene; and decreased tissue glutathione levels are found in patients who received chemotherapy (Conklin 2004).

Most anti-cancer drugs can induce the formation of ROS which can trigger cell apoptosis. One of the drug induced apoptotic pathways involves the release of cytochrome c from mitochondria. Release of cytochrome c triggers diversion of electrons to electron transport chains to oxygen by NADH dehydrogenase and reduction of coenzyme Q10, eventually producing superoxide radicals (Kaufmann and Earnshaw 2000).

Among various types of chemotherapy, anthracyclines such as doxorubicin and epirubicin generate the highest levels of oxidative stress. In addition to generating ROS at other cellular sites, anthracyclines can divert electrons from electron transport chains of mitochondria, resulting the production of superoxide radicals (Gille and Nohl 1997). Platinum compounds including cisplatin may also elicit their genotoxic stress response through enhanced ROS production via JNK and p38 MAPK activation (Benhar, Dalyot et al. 2001). Other types of anti-cancer drugs such as taxanes and antimetabolites induce relatively lower levels of oxidative stress (Conklin 2004).

1.6 NRF2: Master regulator of oxidative stress response

Several defense systems against oxidative stress have been shown to be upregulated in cancer cells via the transcription factor nuclear factor erythroid-derived 2-like 2, also known as NFE2L2 or NRF2. These include the thioredoxin/thioredoxin reductase (Trx/TrxR) system, peroxiredoxins (Prxs) and several glutathione S-transferases (GSTs) (Ganan-Gomez, Wei et al. 2013). They are responsible for mediating the cellular redox homeostasis and at the same time allow redox modifications of specific redox-sensitive factor, triggering redox signaling events.

1.6.1 Introduction of nuclear factor erythroid-derived 2-like 2 (NRF2)

Studying β-globin gene expression and characterization of locus control regions as having regulatory properties leads to the discovery of NRF2 (Tuan, Solomon et al. 1985, Forrester, Thompson et al. 1986). One of the regulatory regions termed hypersensitive site 2 of β-globin contains a tandem activating protein 1 and nuclear factor erythroid 2 (AP1-NFE2) motif with strong enhancer activity. Subsequent work by several groups lead to the cloning of transcription factors that bind to this AP1-NFE2 site. They are identified as members of the cap 'n' collar (CNC) subfamily of the basic leucine zipper (bZIP) transcription factors, including NFE2, NRF1 (Chan, Han et al. 1993), NRF2 (Moi, Chan et al. 1994), NRF3 (Kobayashi, Ito et al. 1999) and two distantly related proteins, i.e., Bach1 (Oyake, Itoh et al. 1996) and Bach2 (Muto, Hoshino et al. 1998), as well as other CNC family proteins discovered and characterized later.

NRF2 was first cloned by Moi et al in 1994. It is encoded within a 2.2-kb transcript and with predicted protein size of 66 kDa. NRF2 protein contains a bZIP DNA binding domain which is highly homologus to NF-E2. NRF2 is expressed ubiquitously and contains a strong acidic activation domain. It first thought to be involved in mediation of enhancer activity of hypersensitive site 2 in erythroid cells and participation in the transcriptional stimulation of beta-globin genes (Moi, Chan et al. 1994). However, early studies have indicated NRF2 is not essential for erythropoiesis, growth and development of murines (Chan, Lu et al. 1996). Recent studies have shown a well-established role of NRF2 as a master regulator of oxidative stress response in multiple tissues and cell types.

Analysis on NRF2 activity and structure revealed that NRF2 contains six highly conserved domains, named Neh (Nrf2-ECH<chicken Nrf2> homologus domain (Itoh,

Chiba et al. 1997). Detailed structure of NRF2 describing roles and functions of these domains are shown in figure 1.6. The N-terminus domain Neh2 was found to harbor a negative regulatory role on NRF2 transcriptional activity. Neh2 deletion significantly increases transcriptional activity of Nrf2, suggesting that Neh2 contain interaction site for binding of negative regulator. Cytoskeletal anchoring protein Kelch-like ECH-associated protein 1 (KEAP1) was identified as the major Neh2 domain interactor using a yeast two hybridization system and Neh2 as bait (Itoh, Wakabayashi et al. 1999).

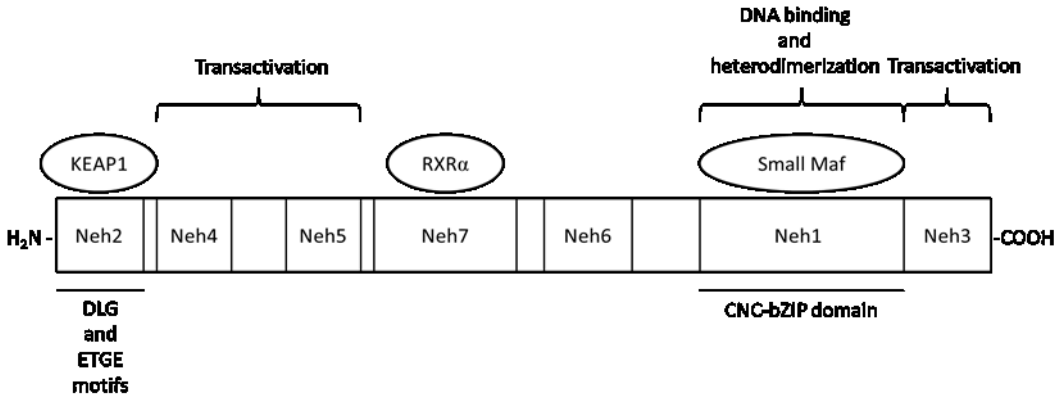


Figure 1.6. Structure of NRF2 and functions of its conserved domain. There are seven highly conserved domains known as Nrf2-ECH homology (Neh) domains in human NRF2 protein. Neh2, the most highly conserved domain among species, is located in the N-terminus and found to interact with Keap1 via the DLG and ETGE motifs. The Neh3–5 domains function in transactivation by binding to different components of the NRF2 transcriptional complex. Neh7 interacts with retinoic X receptor α (RXRα) to mediate the repression of NRF2. Two redox-independent motifs are found in Neh6 which are not recognized by KEAP1 but as target region by E3 ubiquitin ligase β-TrCP. Neh1 contains the CNC–bZIP domain, which mediates DNA binding and heterodimerization with small Maf (Canning, Sorrell et al. 2015).

Under basal conditions, NRF2 binds and forms a complex with NRF2 through extensive hydrogen bonds in the cytoplasm. This leads to polyubiquitination and constitutive protein degradation of NRF2 by proteasome pathway. Under conditions of oxidative stress, KEAP1 is oxidized and modified such that KEAP1 is not able to bind to NRF2, resulting the release from KEAP1 and stabilization of NRF2. Increased cytoplasmic level of NRF2 induces NRF2 translocation into the nucleus (Itoh, Chiba et al. 1997, No, Kim et al. 2014). NRF2 in nucleus heterodimerizes with various transcriptional regulatory proteins such as small Maf family of transcription factors (MafK and MafG). The exact role of small Maf in NRF2 transcriptional activity on ARE is controversial, some studies suggested their participation as positive (Itoh, Chiba et al. 1997) or negative regulators (Venugopal and Jaiswal 1996). Stress-responsive proteins such as members of the activator protein-1 family including Jun and Fos, ATF1 and PMF-1 were also suggested to be interacting partner with NRF2 to control ARE-regulated genes expression (Itoh, Chiba et al. 1997, Jeyapaul and Jaiswal 2000, Jaiswal 2004). Similar to the case of small Maf, these regulatory proteins roles as coactivators or corepressors are controversial.

The NRF2 complexes bind to antioxidant response element (ARE) at the upstream promoter regions to drive a battery of target genes transcription (Rushmore, Morton et al. 1991, Itoh, Chiba et al. 1997). ARE core sequence is suggested as 5'-puGTGACNNNGC-3' 3'-pyCACTGNNNCG-5', where N is any nucleotide. ARE is required for transcription activation of antioxidants and is responsive to oxidative stress (Rushmore, Morton et al. 1991).

1.6.2 NRF2 target genes

High throughput genomic analysis using oligonucleotide microarray has been used to

analyze the effects of oxidant (sulforaphane) treatments into mouse models with functional or disrupted Nrf2. A number of genes directly or indirectly related to oxidative defense are reported to be upregulated and dependent on Nrf2 (Thimmulappa, Mai et al. 2002, Lee, Calkins et al. 2003, Rangasamy, Cho et al. 2004). These upregulated genes can be functionally categorized into different classes, including antioxidant enzymes; regulation of glutathione synthesis and regeneration; phase I, II and III xenobiotic detoxification systems; multidrug response transporters and enhancer of chemical export; NADPH-generating enzymes. Several typical Nrf2 target genes are listed in table 1.1. Regulation of NRF2 target genes usually include both constitutive levels and stress inducible levels, still, not all genes are completely dependent on Nrf2 for their expression.

Gene	Function	Reference
Antioxidant enzymes and proteins		
HO-1	Catalysis of pro-oxidant heme to bilirubin	(Alam and Cook 2003)
NQO1	Reduction of highly reactive quinones	(Venugopal and Jaiswal 1996)
GI-GPx	Reduction of hydrogen peroxide	(Banning, Deubel et al. 2005)
Ferritin H	Sequestion of ions	(Pietsch, Chan et al. 2003)
Prx1	Thiol specific peroxide detoxification	(Kim, Ahn et al. 2007)
Txn1	Reduction of oxidaixed protein resideus to restore enzyme activity	(Kim, Masutani et al. 2001)
Glutathione homeostasis		
GCLC	GSH synthesis	(Mulcahy, Wartman et al. 1997)
GCLM	GSH synthesis	(Erickson, Nevarea et al. 2002)
Conjugation		
GSTA3	Conjugation of GSH to electrophiles for detoxification	(Jowsey, Smith et al. 2003)
GSTP1	Conjugation of GSH to electrophiles for detoxification	(Ikeda, Serria et al. 2002)
UGT1A1	Conjugation of glucuronic acid to xenobiotics to increase excretion	(Yueh and Tukey 2007)
Transporters		
MRP2	Energy dependent membrane transporters to mediate drug efflux	(Vollrath, Wielandt et al. 2006)
ABCG2	Energy dependent membrane transporters to mediate drug efflux	(Singh, Wu et al. 2010)

Table 1.1. List of typical NRF2 targets with identified AREs reported in literature.

1.6.3 The oncogenic roles of NRF2

NRF2 has been implicated to play important roles in chemoprevention of diseases because of its roles to protect the cell from cytotoxicity associated with oxidative and electrophilic stresses. NRF2 has been traditionally considered as a cytoprotective gene and a tumor suppressor. Nrf2 deficiency in mouse appeared to have increased sensitivity to carcinogenesis, lost anticarcinogenic efficacy of chemopreventive agents (Ramos-Gomez, Kwak et al. 2001) and increased cell migration and metastasis (Rachakonda, Sekhar et al. 2010, Satoh, Moriguchi et al. 2010). The beneficial effects of activating NRF2 signaling in cancer prevention have been studied by different groups. Many small molecules that modulate KEAP1-NRF2-ARE transcription machinery have been described as potential therapeutic agents (Hayes, McMahon et al. 2010). However, the roles of NRF2 signaling in cancer have become controversial (Sporn and Liby 2012). There is growing evidence which suggest that activating NRF2 pathway might not be beneficial in all cancer types and stages. Several mutations and aberrant signaling of the NRF2 pathway has been found in cancer and NRF2 activation are associated with the development of cancer as well as detrimental for the outcomes of cancer treatment. The following section will discuss the role of NRF2 in cancer focusing on its role as a potential oncogene.

1.6.3.1 NRF2 induces antioxidant genes in cancer

Many target genes of NRF2 have been described to promote cell growth and tumorigenesis.

One of the well-studied NRF2 targets is heme oxygenase 1 (HO-1). HO-1 is an enzyme that catalyzes breakdown of heme group, generating free iron, carbon monoxide and bilirubin. HO-1 serves as a strong antioxidant based on the combined effects of its products (Otterbein, Soares et al. 2003). HO-1 overexpression is found in many cancer

types such as breast (Noh, Bae et al. 2013), prostate (Maines and Abrahamsson 1996), renal (Goodman, Choudhury et al. 1997), gastric (Yin, Liu et al. 2012), colorectal (Yin, Fang et al. 2014)and lung (Degese, Mendizabal et al. 2012). Overexpression of HO-1 has been found to promote breast cancer cell proliferation (Deng, Wang et al. 2013), confers resistance to doxorubicin in breast cancer cells by promoting autophagy (Tan, Wang et al. 2015) as well as mediates tumor escape from immune system by inhibiting T cell-mediated cytoxicity (Di Biase, Lee et al. 2016).

Similarly, NQO1 has been found to be overexpressed in a number of solid tumors including breast cancer, colon cancer, hepatoblastoma and non-small-cell lung cancer (Schlager and Powis 1990). NQO1 catalyzes reductions of quinones, quinoneimines, nitroaromatics, and azo dyes. NQO1 is a strong antioxidant as these reductions lower quinone levels and thereby depress the generation of ROS by redox cycling (Dinkova-Kostova and Talalay 2010). Studies have uncovered the roles of NQO1 in breast cancer development. Antiestrogens such as tamoxifen can activate NQO1, protecting cells from toxic and tumor-promoting effects of carcinogens. NQO1 activation can also inhibit the genotoxic effects induced by the oxidative metabolism of estrogen (Montano, Bianco et al. 2005). NQO1 enhances TNF-induced apoptosis in human breast tumors and a homozygous missense variant NQO1 was identified, which predicts poor survival among breast cancer patients, in particular those after anthracyclines adjuvant chemotherapy (Fagerholm, Hofstetter et al. 2008).

1.6.3.2 NRF2 roles in cancer cell proliferation

Functions of NRF2 are not limited to control antioxidant genes expression. There is growing evidence showing NRF2 particiaption in regulating other transcription factors and genes involved in cell growth and survival. A global mapping the NRF2 binding

sites by ChIP-Seq and gene-expression profiling analyses using wildtype, Keap1 knockout and Nrf2 knockout mouse embryonic fibroblast cell lines have identified new targets of Nrf2. These targets are involved in cell proliferation and survival pathways as well as cell-cycle regulation, which includes cell proliferation regulator *Als2, Npnt, Bmpr1a, Igf1, Itgb2, Jag1* and *Pdgfc*; cell cycle genes *Cdkn1a (p21)* and *Cdkn2b (p15)* (Malhotra, Portales-Casamar et al. 2010).

In addition to enhancing cellular stress response, NRF2 can modulate cellular metabolism. Nrf2 redirects glucose metabolites to pentose phosphate pathway and induces GSH and NADPH synthesis to increase metabolism (Mitsuishi, Taguchi et al. 2012). Nrf2 also regulates genes involved in lipid metabolism (Kitteringham, Abdullah et al. 2010). It is found that active PI3K-Akt pathway promotes accumulation of nuclear Nrf2 and enables Nrf2 to promote metabolic activities (Mitsuishi, Taguchi et al. 2012). Increased metabolite availability facilitates cell proliferation, which can account in part for tumor growth induced by NRF2.

Cadherins are the proteins responsible for cell-cell adhesion. Their expressions control cell morphogenesis, rearrangements, migrations, and epithelial-mesenchymal transition (EMT). Switching from E-cadherin to N-cadherin is the main feature of the EMT process of cancer development and is associated with cancer progression and metastasis as well as chemoresistance (Lamouille, Xu et al. 2014). E-cadherin interacts with the C terminus of Nrf2 and inhibits accumulation of nuclear Nrf2. Overexpression of E-cadherin decreased the Nrf2 transcriptional activity. Knockdown of E-cadherin could enhance Nrf2 activity and protect cancer cells from doxorubicin. This finding suggests that, upon E-cadherin loss, tumor growth and chemoresistance of cancer cells are attributable to Nrf2 hyperactivation (Kim, Kim et al. 2012).

1.6.3.3 NRF2 roles in cancer cell apoptosis

During cancer development, cells not only increase their proliferative potential but also need to develop mechanisms that allow them to escape their own tumor suppressor systems. Developing resistance to cell apoptosis is one of hallmark of cancer. Several studies reported a crosslink between NRF2 pathway and certain effectors of the apoptotic pathways

Bcl-2 family proteins contain six antiapoptotic members including Bcl-2 and regulate cell death and survival. Altered expression of Bcl-2 family genes are usually shown to be prognostic significant in many types of cancer treated by chemotherapy including breast cancer (Yip and Reed 2008). A study conducted by Niture & Jaiswal found that Keap1 triggered proteasomal degradation of Bcl-2 by directly interacting with BH2 domain of Bcl-2 and induced its ubiquitination by Cul3 (Niture and Jaiswal 2011). The BH2 domain is required for heterodomerization with the proapoptotic protein Bax (Yin, Oltvai et al. 1994). Therefore, Keap1 induced Bcl-2 turnover by competing with Bax for its Bcl-2 binding site, resulting in stabilization of Bax and facilitating apoptosis (Niture and Jaiswal 2011). The same group have also identified an ARE on the Bcl-2 gene promoter that was essential for activation of Bcl-2 gene expression and altering Nrf2 expression leads to altered Bcl-2 induction. Taken together these findings support the role of NRF2 pathway to regulate apoptotic cell death via the control of Bcl-2 expression.

Tumor suppressor p53 induction of apoptosis upon DNA damage is partially dependent on oxidative stress (Liu and Xu 2011). It has been shown that p53 suppresses the NRF2 dependent transcription of antioxidant genes to prevent a strong intracellular levels of antioxidants that could hinder apoptosis (Faraonio, Vergara et al. 2006).However,

another study showed NRF2 is mediated by p53 transcriptional target p21. p21 can directly interact with NRF2 to compete with KEAP1 for NRF2 binding and inhibit ubiquitination of NRF2. These findings complicate how NRF2 contribute to p53-meidated cell survival or apoptosis. A two phase model was proposed by Chen et al (2012). In the induction phase: subtoxic doses of ROS inducer activate NRF2 and maintain low p53 expression levels; while in the repression phase: higher doses of these agents induce a stronger activation of p53, resulting inhibition of NRF2 (Chen, Jiang et al. 2012).

1.6.3.4 NRF2 roles in drug resistance

The functions of NRF2 in cytoprotection, promoting cell survival and proliferation and counteracting proapoptotic signal making this transcription factor not only promoting cancer development but also an important antagonist of the effects of chemotherapy. Genetic aberrations of the KEAP1/NRF2 pathway in cancer cells have been found to be correlated with the resistance to chemotherapy. Resistance to chemotherapeutic drugs such as platinum compounds, etoposide and doxorubicin (Shibata, Kokubu et al. 2008, Wang, Sun et al. 2008) are reported to be associated with alterations of KEAP1/NRF2 pathway. In general, these drugs can induce ROS generation and production of free radicals that interact with DNA, causing DNA damages and triggering cell death event. Direct relationship between NRF2 signaling and oxidative stress response showed that Nrf2 overexpression or hyperactivation is one of the mechanisms of action in resistance to chemotherapy.

The constitutive NRF2 activation could protect cancer cells from chemotherapy. It was found that endogenous expression of NRF2 in doxorubicin resistant MCF-7 cells was higher than that in ordinary MCF-7 cells (Zhong, Zhang et al. 2013).. Antioxidant

enzymes HO-1 and NQO1 which are NRF2 target genes were also expressed at higher levels in the resistant cells. Knockdown of NRF2 reversed doxorubicin resistance of the resistant cells and NRF2 activator treatment to ordinary MCF-7 cells could enhance the resistance to doxorubicin. These findings suggested that activation of NRF2 was associated with the drug resistance in breast cancer cells (Zhong, Zhang et al. 2013).

Proapoptotic effects of compounds not only targeting redox balance can also hinder NRF2 signaling. One of the proposed mechanisms is the elevation of protein exprerssion of the drug efflux pumps, preventing intracellular drug accumulation. In fact, members of ABC transporter family have been found to be overexpressed in cancer cells with mutations of KEAP1 and/or NRF2 (Sasaki, Shitara et al. 2012). The ABC transporter family, which can mediate efflux of anticancer drugs, has found to be transcription targets of NRF2. For example, expression of MRP1 is regulated by NRF2 in lung cancer cell lines as well as other cancer tissues including breast, colon and gastric cancer. NRF2 upregulates MRP1 through binding to the defined ARE sequence and NRF2-ARE-MRP1 level contribute to difference in sensitivity to chemo drugs between ordinary cancer cell line and drug resistant cell line (Ji, Li et al. 2013). Similar findings have also been observed for MRP3 (Sasaki, Shitara et al. 2012)and ABCG2 (Singh, Wu et al. 2010).

1.6.4 Mechanism of NRF2 regulation

Under normal physiological conditions, formation of KEAP1-NRF2 complex entrapped NRF2 in cytoplasmic compartments and renders low basal expression of genes driven by ARE. Under oxidative or electrophilic stressed conditions, there is increased accumulation of nuclear NRF2 which represents a significant pre-requisite event of NRF2 transcription activation on ARE (Kensler, Wakabayashi et al. 2007).

Regulation mechanisms of NRF2 transcription system involve collaboration with multiple interacting proteins during basal state and during stressed conditions. These mechanisms are continually being discovered.

1.6.4.1 NRF2 protein degradation

KEAP1 is the major repressor of NRF2 through multiple regulatory mechanisms. From studies using ARE reporter assays, KEAP1 overexpression significantly reduced Nrf2 transactivation activity (Itoh, Wakabayashi et al. 1999). Antioxidant treatments reversed the repression of NRF2 by KEAP1 suggesting that the KEAP1-NRF2 complex can be destabilized by alteration of cell redox states. *KEAP1* knockout mouse models showed high constitutive expressions of genes driven by ARE with persistent nuclear accumulation of NRF2 but such characteristics were not observed in *KEAP1* and *NRF2* double knockout mouse models (Wakabayashi, Itoh et al. 2003). Recent analysis on human cancer tissues from various origins including lung, breast, colon, stomach, liver, larynx and prostate, and leukaemias revealed NRF2 hyperactivity, shown by upregulated expressions of NRF2-activated cytoprotective proteins NQO1 and GCLC, were attributed by somatic mutations of *KEAP1*. However, no *NRF2* mutations were detected in the same study. This finding suggested *KEAP1* mutations and inactivations might protect cancer cells from oxidative stress and play roles in cancer development (Yoo, Kim et al. 2012). Taken together, both *in vitro* and *in vivo* studies have shown KEAP1 as a specific negative regulator of NRF2.

The role of KEAP1 is well recognized for its participation in regulating NRF2 homeostasis via protein degradation. NRF2 proteins were expressed at low level and had a limited half-life (18.5 min) although there was abundant mRNA expression of NRF2 at unstressed basal conditions. This suggested that a rapid degradation exists for

maintaining NRF2 homeostasis (Itoh, Wakabayashi et al. 2003). The fast turnover of NRF2 protein involves a facilitated ubiquitin-proteaosomal degradation system. Neh2 domain of NRF2, which is redox sensitive, was shown to participate in the proteasome dependent degradation of NRF2 in an engineered *NRF2-LacZ* knock in mouse model (Itoh, Wakabayashi et al. 2003) and KEAP1 interacts with Neh2 to enhance proteasome dependent degradation (McMahon, Itoh et al. 2003). Under stressed conditions, interactions between KEAP1 and Neh2 domain of NRF2 were disrupted, resulting in prolonged half-life and increased protein stability of NRF2 (Nguyen, Sherratt et al. 2003). The BTB and IVR domains of KEAP1 have been shown to be essential in recruitment of ubiquitin-proteasome factors in NRF2 degradation (Kobayashi, Kang et al. 2004). The BTB/POZ domain is required for homodimerization of KEAP1 to sequester NRF2 in cytoplasm (Zipper and Mulcahy 2002). The IVR domain of KEAP1 associates with the N terminal region of a subunit of the E3 ligase, Cul3, to promote ubiquitination of NRF2. KEAP1 functions as an adaptor and NRF2 as a substrate, for Cul3-based E3 ligase system to regulate proteasomal degradation of Nrf2 (Kobayashi, Kang et al. 2004).

In addition to KEAP1 dependent protein degradation, KEAP1 independent proteasomal degradation has also been suggested to regulate NRF2 degradation. This degradation pathway was thought to be functioned through specific recruitment of nuclear NRF2 participating in the transcription complex. However, it was not known whether this pathway is crucial in NRF2 degradation under stress conditions (Itoh, Tong et al. 2004). Taken together, studies have indicated the important roles of KEAP1 in proteasomal degradation of NRF2, especially under unstressed basal conditions.

1.6.4.2 Stress mediated cell signaling kinases cascades

Several stress mediated cell signaling kinases cascades has been suggested to participate in regulation of NRF2. These additional regulatory mechanisms may collaborate with KEAP1 to regulate NRF2 and downstream NRF2 target genes. It is hypothesized that inhibition or inactivation of Keap1-dependent ubiquitination and protein degradation may stabilize NRF2 protein, and additional post-translational modification of KEAP1-NRF2 complex is needed for NRF2 to disassociate from the complex completely (Shen, Jeong et al. 2005, Kensler, Wakabayashi et al. 2007). These stress mediated cell signaling kinases cascades includes MAPK, PI3K-AKT, PKC and PERK regulated signaling cascades. They have been shown to mediate NRF2 activation and expressions of ARE-driven genes. The involvements of these signaling cascades in activating the KEAP1/NRF2 transcription system are detailed below. However, it is worthy to note that there were inconsistency among different studies, possibly due to differences in species, cell types and/or NRF2 inducers used in experiments, which might have distinct modulatory effects in this interaction.

Extracellular signaling-related kinase 1/2 (ERK1/2) is a downstream multi target kinase effector of MAPK. ERK1/2 functions in promoting cell survival during cellular stress by phosphorylating a number of transcription factor (Dhillon, Hagan et al. 2007). In an *in vitro* kinase assays by Zipper and Mulcahy in 2000, they showed that purified recombinant NRF2 is a substrate of ERK1/2. But site directed mutagenesis analysis of HepG2 cells reported by the same group later that *NRF2* mutations at all conserved phosphorylation sites did not reduce NRF2 capability to response to pyrrolidine dithiocarbamate (PDTC) treatment and to execute ARE-mediated transactivation (Zipper and Mulcahy 2003). These results suggested that NRF2 is a phosphorylation target of ERK1/2 but such phosphorylation of NRF2 may not be required for NRF2

activation and subsequent ARE-driven genes transcription activation. On the other hand, a co-activator of NRF2, CBP/300, can be directly phosphorylated by MAPK cascade and induce NRF2-ARE-driven genes transcription activation (Shen, Hebbar et al. 2004)

Activation of p38 MAPK signaling cascade is common in different cell systems exposed to oxidative stress including breast cancer (Koul, Pal et al. 2013). The role of p38 MAPK in regulating NRF2 activity has also inconsistent results. There are reports which suggest a positive regulation between p38 MAPK and NRF2/ARE activation using selective inhibitors of p38 (Alam, Wicks et al. 2000, Balogun, Hoque et al. 2003). But studies using ectopic expression of dominant positive p38 MAPK isoforms showed a negative regulation of p38 MAPK and NRF2/ARE activation. It was found that NRF2 is a substrate for p38 MAPK and p38 MAPK activation promote NRF2 association with KEAP1. The different and opposing result might be due to possible non-specific effects of the pharmacological inhibitors being used that might interfere other signaling pathways (Keum, Yu et al. 2006). It is known that p38 MAPK might counterbalance the effects of ERK1/2 activation, so it is possible that p38 MAPK is a mediator of positive regulation of ERK1/2 MAPK (Park, Choi et al. 2003). Taken together, the precise mechanisms through which the MAPK pathways activate NRF2 transcription machinery has yet to be elucidated.

Another cell survival related signaling kinase cascade, PI3K and its downstream AKT (PI3K-AKT) activation has been associated with NRF2 activation and upregulation of ARE driven genes by various NRF2 activators including chemotherapeutic drugs (Martin, Rojo et al. 2004, Kang, Woo et al. 2007). Similar to MAPK, mechanisms of how PI3K-AKT regulates NRF2 is not completely known. It has been shown that PI3K regulates NRF2 nuclear translocation via actin rearrangement of the cytoskeleton,

thereby destabilizing KEAP1-NRF2 complex in rat-derived cells. However, whether such translocation of NRF2-actin complex would directly result in activation of NRF2 transcription and induced expression of ARE-regulated genes was not shown (Kang, Lee et al. 2002). Hyperoxia stimulated an NRF2 ARE transcriptional response in pulmonary epithelial cells which involves the activation of both PI3K-AKT and ERK1/2 MAPK pathways. The crosstalk between PI3K-AKT and ERK1/2 MAPK pathways suggested that ERK1/2 MAPK can also be downstream target effectors of PI3K-AKT (Papaiahgari, Zhang et al. 2006). This might explain inhibition of either pathway can attenuate NRF2/ARE activation.

Protein kinase C (PKC) has been found to directly phosphorylate NRF2 both *in vitro* and *in vivo*. The phosphorylation site of NRF2 by PKC is at Serine 40 (Ser40). This phosphorylation site is located in the Neh2 domain of NRF2 and phylogenetically conserved. Modifying Ser40 could disrupt NRF2 association with KEAP1 and correspond to increased ARE mediated activation. But PKC mediated NRF2 phosphorylation did not alter affinity of NRF2/MafK complex binding to the ARE of *NQO1* in vitro (Huang, Nguyen et al. 2002). These findings were agreed by another studies, they found that NRF2 phosphorylation is dispensable for nuclear accumulation and stabilization of NRF2 as well as subsequent ARE-mediated gene expression (Bloom and Jaiswal 2003). Similar to ERK1/2 MAPK, studies revealed that NRF2 phosphorylation may be a mechanism to disrupt interaction between NRF2 and KEAP1 in the cytoplasm. But NRF2 phosphorylation is not required for stabilization of nuclear NRF2 and subsequent target genes transcription activation.

Accumulations of unfolded proteins in the endoplasmic reticulum (ER) induce ER stress, promoting translation inhibition and cell cycle arrest. Protein kinase R (PKR)-

like endoplasmic reticulum kinase (PERK) function is essential for cell survival under ER stress. NRF2 has been identified as a phosphorylation target of PERK. PERK dependent phosphorylation of NRF2 triggered dissociation of NRF2 from NRF2/KEAP1 complex and inhibit reasscoiation of NRF2 to NRF2/KEAP1 complex *in vitro*. PERK activators triggered unfolded protein response is required for disscoication of the NRF2/KEAP1 complex in the cytoplasm (Cullinan, Zhang et al. 2003). NRF2 phosphorylation by PERK contribute to disruption of NRF2/KEAP1 complex, followed by accumulation of nuclear NRF2, enhanced ARE-driven genes transcription, supporting cell survival (Cullinan, Zhang et al. 2003, Salaroglio, Panada et al. 2017)

1.6.4.3 Other modes of regulation of NRF2

Recent studies suggest that there are more complex regulatory mechanisms involved in regulation of NRF2 transcription machinery. Different proteins with activating roles have positive regulation towards NRF2/ARE transcription axis while there are other mechanisms have taken part in negative regulation of NRF2 transcription machinery.

BRCA1 had been implicated in this signaling cascade as BRCA1 can lower cellular levels of ROS (Saha, Rih et al. 2009)and *BRCA1* mutation induce oxidative stress (Martinez-Outschoorn, Balliet et al. 2012). Wild type expression of BRCA1 induced transcription of a number of antioxidant genes expressions and conferred relative oxidative stress resistance, implicating increased NRF2 activity (Bae, Fan et al. 2004). It is found that BRCA1 physically associated with NRF2; preventing NRF2 binding to KEAP1 and KEAP1 mediated ubiquitination and degradation. This leads to subsequent NRF2 stabilization, downstream target genes activation and anti-stress response. Knockdown of KEAP1 in BRCA1 deficient mammary cells activated NRF2, rescued

survival defect associated with BRCA1 loss and restored ROS levels. These findings indicated that this mechanism is physiologically relevant (Gorrini, Baniasadi et al. 2013). Partner and localizer of BRCA2 (PALB2), also appeared to regulate this pathway. PALB2 is found commonly mutated in hereditary breast cancer and pancreatic cancer (Tischkowitz and Xia 2010). Wild type PALB2 could bind to KEAP1, thus preventing NRF2 KEAP1 mediated ubiquitination and degradation of NRF2 (Ma, Cai et al. 2012). This resulted in a similar effect as wild type BRCA1 in regulating oxidative stress. Although it is not known whether or how BRCA2 involves in NRF2/KEAP1 complex or how PALB2 activity may be regulated in response to oxidative stress, both BRCA1 and BRCA2 (via PALB2) may participate in regulating NRF2 activity, implicating functions of these hereditary cancer genes.

From a genome-wide screening analysis that was used to identifying ARE activators, sequestosome 1 (SQSTM1) or known as p62 and dipeptidylpeptidase 3 (DPP3) were reported to promote NRF2/ARE transcription activity via activation of PI3K, PKC and MAPK signaling cascades, showing there are intermediate proteins acting as mediator of stress mediated cell signaling kinases to regulate NRF2 activity (Liu, Kern et al. 2007). DJ-1 or PARK7, a protein associated with cancer and Parkinson's disease, had been found to protect cells from toxic stresses. DJ-1 knockdown lowered expression of *NQO1* which is an ARE mediated gene. DJ-1 stabilizes and activates NRF2 by interfering NRF2 association to KEAP1 complex, inhibiting subsequent NRF2 ubiquitination and degradation in both primary cell lines and transformed cells of both human and mouse origins(Clements, McNally et al. 2006). Monocytic leukemia zinc-finger protein (MOZ or MYST3) is a histone acetyltransferase, were reported to act as a coactivator for Nrf2/MafK complex and induce expression of Nrf2 target genes such as glutathione transferase P (Gstp) in rat cells. Upregulation of Moz were found during

hepatocarcinogenesis, inducing expression tumor marker gene Gstp suggesting that increased Nrf2-mediated pathway contribute to development of some cancers (Ohta, Ohigashi et al. 2007).

The bZip proteins including BACH1, NRF3 and p65 isoform of NRF1 act as negative regulators of NRF2 (Sankaranarayanan and Jaiswal 2004, Dhakshinamoorthy, Jain et al. 2005, Shan, Lambrecht et al. 2006, Wang, Kwok et al. 2007). They function to compete with NRF2 for small Maf transcription factors and interfere with the binding of NRF2 to ARE, repressing NRF2-mediated transcription and NRF2 target genes induction. For example, BACH1 was found to be recruited by NRF2 activators and involved in a dynamic exchange for small Maf with NRF2 (Shan, Lambrecht et al. 2006). The finding suggested that these bZip proteins may participate in a negative feedback regulation in controlling Nrf2 mediated transcription on ARE. Similarly activating transcription factor 3 (ATF3) has shown to be an interacting protein of the NRF2 transcription complex partner CBP at ARE in the promoter region of target genes (Brown, Sekhar et al. 2008). ATF3 represses NRF2 activity via direct ATF3 NRF2 protein-protein interactions to displace of CBP from the ARE, thereby suppress gene transcription.

Studies have also shown nuclear receptors can inhibit NRF2 transcription and ARE-mediated gene transactivation. Inhibition of NRF2 pathway by nuclear receptors such as estrogen receptors, retinoic acid receptors and retinoid X receptor have been shown in breast cancer cells (Namani, Li, Wang, & Tang, 2014; Wang, Hayes, Henderson, & Wolf, 2007; Yao, Brodie, Davidson, Kensler, & Zhou, 2010) . For example, the retinoic acid receptor (RARs) is a type of nuclear receptor which can also act as transcription factors. RARs are activated by retinoic acids (a major metabolite of vitamin A). The

retinoic acid activity is mediated mainly by three members of the RAR subfamily, namely RARα, RARβ and RARγ (di Masi, Leboffe et al. 2015). In MCF-7 breast cancer cell line, retinoic acid treatment lowered NRF2 activity to mediate ARE-driven genes induced by chemopreventive agents. Retinoic acid did not inhibit accumulation of nuclear NRF2 but inhibited the binding of NRF2 to ARE as RARα physically associate and form complex with NRF2 (Wang, Hayes et al. 2007). Besides nuclear receptor, transcription co-regulator has been implicated in modulating NRF2 activity. In addition to the well-known role of nuclear receptor co-repressor 2 (NCOR2) as a corepressor of nuclear receptors including RARα. NCOR2 has been shown to inhibit NRF2 transcription via protein-protein interaction. NCOR2 is recruited to the Neh4/5 transctivation domains of NRF2 upon activation of glucocorticoids receptor, suppressing the gene expression of the glutathione S-transferase (Ki, Cho et al. 2005). NCOR2 will be introduced in a later section.

1.7 Forkhead Box M1 (FOXM1) transcription factor

There are more than 50 forkheadbox (FOX) genes identified in the human genome (Jackson, Carpenter et al. 2010). The FOX proteins are characterized by the Forkhead box or winged helix DNA binding domain. The Forkhead box M1 (FOXM1) was previously named as Trident, HFH-11, FKHK16, WIN or INS-1 (Laoukili, Stahl et al. 2007, Jackson, Carpenter et al. 2010). It is a transcription factor that plays central roles in normal cell growth and cell proliferation through the regulation of cell cycle transition points and mitotic spindle. FOXM1 is also implicated in carcinogenesis which FOXM1 is activated by various mechanisms.

FOXM1 transcriptionally regulate target genes through binding to core consensus sequence, TAAACA (Koo, Muir et al. 2012). Human FOXM1 gene is located at chromosomal band 12p13 (Korver, Roose et al. 1997). It has ten exons with two alternatively expressed exons (exon Va and exon VIIa) which give rise to three different isoforms: FOXM1a, FOXM1b, and FOXM1c. Exon Va was shown play as a transcriptional repressor, while exon VIIa does not have any apparent transcriptional significance (Ye, Kelly et al. 1997).FOXM1a contains both alternatively expressed exons and is thought to be transcriptionally inactive; FOXM1b contains none of the exons and FOXM1c contains exon Va only, they are transcriptionally active and thought to be mediate the effect of FOXM1 (Laoukili, Stahl et al. 2007, Koo, Muir et al. 2012). It is found that FOXM1b has higher level in cancer cells and greater transforming potential, compared to FOXM1c (Lam, Ngan et al. 2013).

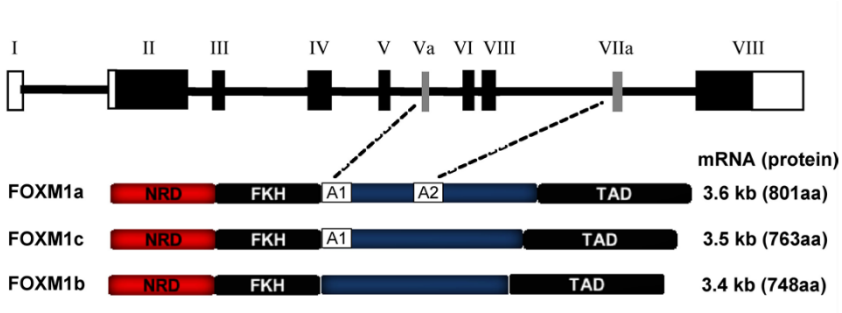


Figure 1.7 Structure of FOXM1 and FOXM1 isoforms. FOXM1 protein has three isoforms due alternative splicing of exons Va and VIIa. FOXM1b and FOXM1c are transcriptional activators but FOXM1a is transcriptionally inactive. NRD: N-terminal Repressor Domian; FKH: Forkhead/Winged-helix domain; TAD: Transactivation domain. (Schwenen et al, 2009)

1.7.1 Clinical significance of FOXM1

Elevated expression of FOXM1 is observed in different cancer types, including breast cancer, suggesting that FOXM1 is important for cancer cell proliferation (Laoukili, Stahl et al. 2007, Koo, Muir et al. 2012). High expression of FOXM1 is associated with advanced tumor stage, metastasis and poor prognosis (Liu, Dai et al. 2006, Degese, Mendizabal et al. 2012, Xu, Jia et al. 2013). The FOXM1 regulatory network is identified as a major predictor of adverse outcomes in 39 types of malignancies from a recent meta-analysis of expression signatures from 18000 human tumors (Gentles, Newman et al. 2015). An independent study of 688 breast cancer cases showed that FOXM1 is a poor prognostic factor for survival in hormone receptor-positive/HER2-negative subtype but not in the HER2-positive subtype or triple negative subtype (Lee, Lee et al. 2016). Other studies on breast cancer patients also revealed that there was no significant difference in all patient survival but high FOXM1 expression is correlated with poorer survival in ER positive tumors (Bergamaschi, Madak-Erdogan et al. 2014,

Ahn, Sim et al. 2015), suggesting the prognostic significance of FOXM1 in hormone receptor positive subtype breast cancer.

1.7.2 FOXM1 as an oncogenic driver

Chromosomal amplification of FOXM1 12p13 locus as well as other genomic regions are observed in different cancer including breast cancer, cervical cancer, head and neck cancer and nasopharyngeal cancer (Heselmeyer, Macville et al. 1997, Singh, Gogineni et al. 2001, Rodriguez, Khabir et al. 2005, Hu, Mao et al. 2016). Enhanced transcription of FOXM1 can be driven by promoter binding of oncogenic proteins such as c-Myc and E2F (Blanco-Bose, Murphy et al. 2008, Millour, de Olano et al. 2011); and reduced transcription repression due to inactivation of tumor suppressors such as p53 (Pandit, Halasi et al. 2009). Various oncogenic signaling pathways such as hedgehog, PI3/AKT, EGF and Raf/MEK/MAPK signaling have also been shown to mediate phosphorylation and activation of FOXM1 (Teh, Wong et al. 2002, Koo, Muir et al. 2012).

FOXM1 plays critical roles in different aspects of cancer development. Recent studies have shown that it is involved in all major hallmarks of cancer: sustaining tumor cell proliferation, evasion of growth/tumor suppressor activities, enhanced resistance against apoptosis, enabling of replicative immortality, inducing angiogenesis, activation of invasion and metastasis as well as promoting genomic instability (Halasi and Gartel 2013). Two mechanisms have been proposed to explain FOXM1 role as an oncogenic driver in human cells. FOXM1 act as an oncogene by transcriptional activation of downstream targets that serve different pro-tumorigenic effect. For example, stimulation of ERα transcription is mediated by FOXM1, leading to enhanced cell proliferation (Madureira, Varshochi et al. 2006); FOXM1 upregulates antiapoptotic genes *XIAP* and *surivivin* expressions (Nestal de Moraes, Delbue et al. 2015); VEGF

regulates angiogenesis and its expression can be dependent on FOXM1 (Karadedou, Gomes et al. 2012). The oncogenic role of FOXM1 can also be mediated by the interaction of FOXM1 with other proteins, hence regulating other oncogenic pathways. Xu et al have shown that FOXM1 interacts with SMAD3 to sustain activation and nuclear retention of SMAD3/SMAD4 complex, thereby promoting TGF-β signaling pathway and metastasis in breast cancer cells (Xue, Lin et al. 2014)

1.7.3 FOXM1 and anti-oxidative stress response

Recent studies have shown that FOXM1 can be induced by various stresses including ionic radiation, ultraviolet radiation, heat-shock and oxidative stress, suggesting that FOXM1 could be a stress response protein (Dai, Gong et al. 2013). FOXM1 overexpression is found to be ROS dependent via TNF-α/ROS/HIF-1 pathway in hepatocellular carcinoma, which control cell proliferation and resistance to apoptosis, showing that FOXM1 can be regulated by oxidative stress (Xia, Mo et al. 2012). Moreover, depletion of FOXM1 in different cell types have shown to induce cellular senescence, increased ROS production and inhibition of antioxidant enzymes. Depletion of FOXM1 also sensitizes cells to ROS-induced apoptosis (Li, Smith et al. 2008, Park, Carr et al. 2009, Kwok, Leung et al. 2016, Smirnov, Panatta et al. 2016). These findings suggested that FOXM1 is a key regulator of oxidative stress and ROS homeostasis. Cancer cells have been shown to upregulate ROS scavenging pathways in order to survive, proliferate, and induce resistance to radiotherapy and chemotherapy. A bioinformatics analysis have identified 116 anti-oxidative stress genes that are overexpressed in multiple cancers. These anti-oxidative stress genes are mostly downstream transcriptional targets of FOXM1 as well as NRF2 and predicts poor patient outcomes in breast and lung cancer (Rotblat, Grunewald et al. 2013). A recent study have showed that FoxM1 might transcriptionally activate Nrf2 in hepatocellular

carcinoma. A Ras/Erk/FoxM1/Nrf2 pathway is proposed which mediate Prx1 expression and suppression of ROS-induced hepatic cancer-cell death (Han, Shin et al. 2016). Taken together, these findings support that FOXM1 is functionally important to drive cancer progression and chemoresistance by mediating anti oxidative stress response.

1.7.4 FOXM1 and chemoresistance

A growing body of evidence has revealed the significance of FOXM1 in chemoresistance. Studies have shown that FOXM1 expression level is a critical determinant of sensitivity of breast cancer chemotherapeutic drugs including epirubicin, doxorubicin, paclitaxel and cisplatin (Kwok, Peck et al. 2010, Millour, de Olano et al. 2011, Park, Jung et al. 2012, Khongkow, Gomes et al. 2016). It is found that FOXM1 expression is downregulated by chemotherapeutic drugs treatment in the sensitive breast cancer cell line MCF-7 and upregulated in the established chemoresistant cell lines.

Chemoresistance conferred by FOXM1 is contributed by enhancing DNA damage repair, promoting cell cycle progression, reducing the stringency of cell cycle checkpoints and inhibiting cell apoptosis. FOXM1 is found to regulate genes involved in DNA damage repair, such as *EXO1 , RFC4, PLK4, BRIP, RAD51* and *NBS1* (Park, Jung et al. 2012, Zhang, Wu et al. 2012, Monteiro, Khongkow et al. 2013, Khongkow, Karunarathna et al. 2014). It has also been shown that activation of mitogen-activated protein kinase-activated protein kinase 2 leads to increase FOXM1 expression, mediating epirubicin resistance (de Olano, Koo et al. 2012). Microtubulin-associated kinesin KIF20A are critical for normal formation of mitotic spindle. It has been shown that FOXM1 regulate KIF20A expression, mediating paclitaxel resistance (Khongkow,

Gomes et al. 2016). FOXM1 can also directly regulate the expression of antiapoptotic genes such as *XIAP* and *Survivin*. Upregulation of these antiapoptotic genes could enhance resistance to apoptosis. Therefore, this could confer resistance to epirubicin, docetaxel, and paclitaxel as they employ apoptosis to eliminate cancer cells (Nestal de Moraes, Delbue et al. 2015).

1.8 Nuclear receptor co-repressor 2 (NCOR2) and its spice variant BQ323636.1

1.8.1 Transcriptional regulators

Nuclear receptors (NRs), such as glucocorticoid receptors and ER, and other transcriptional factors are essential for gene transcriptional regulation, however, they do not possess the enzymatic activities needed to modulate chromatin structure (Mangelsdorf, Thummel et al. 1995, Gronemeyer, Gustafsson et al. 2004). Transcriptional co-regulators are recruited in response to different signals including ligand–receptor binding signaling cascades and interact with transcription factors that bind to regulatory regions of genes in a sequence specific manner. Transcriptional co-regulators play main roles in epigenetic regulation as they recruit different chromatin modifying enzymes to form functional co-regulator complexes (Giguere 1999, Battaglia, Maguire et al. 2010). These co-regulators can be further categorized to co-activators and co-repressors.

Co-activators are involved in chromatin relaxation as they possess histone acetyltransferase activity or serve as a docking partner of enzyme with such activities. Chromatin relaxation is a prerequisite for recruitment and association of basic transcription machinery. Examples of co-activators includes SRC-1, TIF2, and GRIP. On the other hand, co-reprerssors such as NCOR1 and NCOR2 (also known as SMRT)

are involved in chromatin condensation and repression of gene expression. They can recruit various forms of histone deacetlylases and preferentially binds to unactivated receptors or transcriptional factors. Both co-activators and co-repressors appear to be an integral part of cell signaling pathways (Pascussi, Gerbal-Chaloin et al. 2008).

1.8.2 Nuclear receptor co-repressor 2 (NCOR2)

The nuclear receptor co-repressor 2 (NCOR2), also known as silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), is a proto-typical co-repressor that maintain transcriptional silencing of certain target genes. NCOR2 is mainly localized in the nucleus and it operates as a central platform recruiting other co-repressors components to assemble a larger, functional co-repressor complex, tethering to its transcription factors partners (Privalsky 2004).

NCOR2 contains 4 repression domains (RD-1 to RD-4) located at the N-terminal and central region. The RDs are responsible for recruitment of other corepressor subunits, such as GPS2, TBL-1, histone deacetylases (HDACs) and a number of other modulatory and effector proteins (Ordentlich, Downes et al. 2001, Privalsky 2004). The two SANT domains located in RD2 are responsible for interaction with HDACs. SANT2 is essential for interacting with HDAC3. (Battaglia, Maguire et al. 2010). NCOR2 binds to NRs via “CoRNR box” motifs/ nuclear receptor interaction domains at the C-terminus. Interaction sites of other transcription factors are also mainly at C-terminus side (Goodson, Jonas et al. 2005) (Figure 1.8). By forming a functional co-repressor complex, NCOR2 can suppress the transcriptional activation of genes of nuclear receptors or transcription factors such as NR, RARα and PLZF.

It is found that NCOR2 activity can be altered by post translational modification. Growth factor receptor/MAP kinase signaling cascades can phosphorylate NCOR2. NCOR2 phosphorylation releases NCOR2 from its transcription factor partners, resulting de-repression of target genes (Goodson, Jonas et al. 2005). Also, AKT signaling can phosphorylate NCOR2 and lead to its nuclear export (Privalsky 2004).

1.8.3 NCOR2 in breast cancer

NCOR2 can interact with ER-α in the absence of ligand as well as in the presence of estrogen or tamoxifen in breast cancer. It is found that expression levels of BCL-2, cyclin D1 and PR are decreased while pS2 mRNA level is increased upon NCOR2 depletion by specific siRNAs (Peterson, Karmakar et al. 2007). The different response of the expression level of these ER-α target genes suggested that NCOR2 can function as both co-activator and co-repressor for ER-α in a gene specific manner in breast cancer (Blackmore, Karmakar et al. 2014)

The dual function of NCOR2 on ER-α makes it hard to determine whether NCOR2 exerts a pro-tumorigenic or anti-tumorigenic role in breast cancer because immunohistochemical analysis of tissue microarrays indicated moderate or high levels NCOR2 expression are found in 16.7 % of breast tumors, which are associated with poor prognosis of the patients (Green et al., 2008). In another study, high NCOR2 mRNA level can predict better outcomes in untreated ER positive patients, suggesting a protective role of NCOR2 in breast cancer (van Agthoven, Sieuwerts et al. 2009). Still, these reports showed that SMRT expression and functions are altered in breast cancer.

Recent studies have implicated NCOR2 role in anti-estrogen resistance and progression in breast cancer. NCOR2 level appears unchanged in tamoxifen responsive tumor

samples while it is downregulated in patients samples with tamoxifen resistance. NCOR2 is one of the transcription regulators that is deregulated in tamoxifen resistant cell lines and its gene expression signature is correlated with ER positive subtype (van Agthoven, Sieuwerts et al. 2009). Tamoxifen is a selective estrogen receptor modulator and competes with estrogen for ER (Reinbolt, Mangini et al. 2015). Recruitment of NCOR2 and other co-repressor complex subunits to ER can be triggered by tamoxifen binding to ER. This in turn suppress transcriptional activation of ER target genes and the ER pro-proliferative pathway.

1.8.4 NCOR2 splice variant BQ323636.1

NCOR2 can give rise to a series of additional corepressor isoforms by alternative mRNA splicing. Two N-terminal truncated variants of NCOR2 ,s-SMRT and TRAC-1, were previously identified, which lack N-terminal repression domains found in wildtype NCOR2 (Goodson, Jonas et al. 2005). SMRTτ is another isoform of NCOR2, it lacks 47 amino acids following the ND1 domain. Loss of these amino acids significantly reduces NCOR2 isoform affinity to certain receptors (Privalsky 2004). It has also been suggested the NCOR2 splice variants could mediate a subset of the repression functions of wildtype NCOR2, or even act as "anti-corepressors" (Goodson, Jonas et al. 2005).Alternative splicing of NCOR2 generate different isoforms which alters NCOR2 affinities to different transcription factors, thus creates functional diversity and customizing corepressor function in different cells and contexts (Privalsky 2004).

Our group has profiled a splice variant of NCOR2 from SpliceArray profiling of breast cancer, termed as BQ323636.1 (BQ). It is formed from exon 11 skipping during mRNA processing, leading to early termination of the protein product. BQ is predicted to be

362 amino acids in length with molecular mass of 42.6 kDa. Amino acid sequence of BQ shared the same sequence of N-terminus of NCOR2, the only difference is the last 10 amino acids before the stop codon are translated from exon 12 out of frame (Zhang, Gong et al. 2013). Compared to its wildtype form, BQ only retains the RD1 domain, thus predicted to be unable to interact with nuclear receptors and most transcription factors (Zhang, Gong et al. 2013) (Figure 1.8). Protein level of BQ is expressed at a comparable level as NCOR2 though mRNA level of BQ is greatly lower than that of NCOR2, this suggests that there are some post transcriptional modification promoting BQ translation or BQ is not actively degraded (Zhang, Gong et al. 2013). Sustained protein level of BQ could make BQ functionally important in cells.

Overexpression of BQ is observed in tamoxifen -resistant breast cancer cell lines (Zhang, Gong et al. 2013). From the same study, BQ mRNA expression is significantly correlated with tamoxifen resistance in a set of 77 breast cancer patients' samples. High BQ expression is significantly associated with development of distant metastasis in patients who were ER positive and had received tamoxifen treatment. It is also observed that BQ showed opposite effect of wildtype NCOR2 in modulating ERα transcriptional activation activity (Zhang, Gong et al. 2013). These results suggest a role of BQ in mediating tamoxifen resistance and BQ can serve as a new biomarker in predicting breast cancer patients' response to tamoxifen. This study revealed the clinical significance and possible function of BQ in mediating tamoxifen resistance in breast cancer. Since NCOR2 can regulate multiple transcription factors, it is possible that BQ could modulate other functions played by NCOR2.

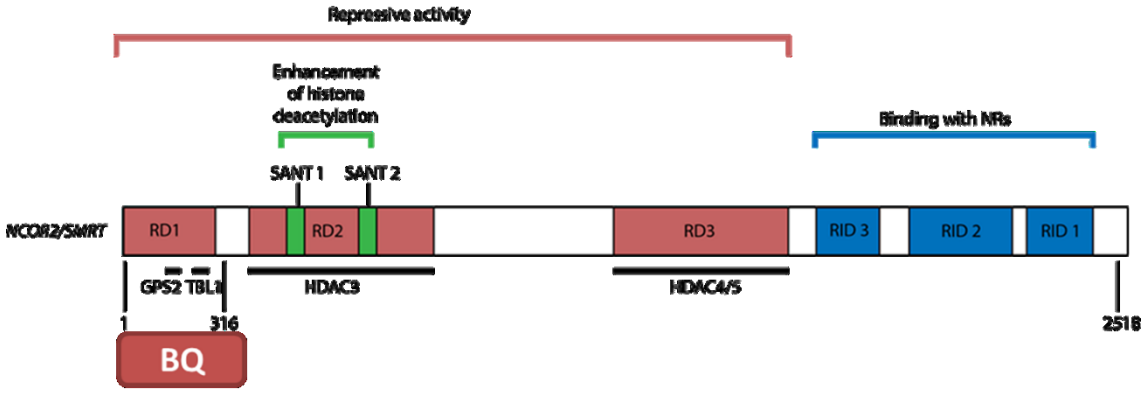


Figure 1.8 Structure of NCOR2 and its splice variant BQ323636.1**.** NCOR2 serves as a Central platform recruiting other co-repressors components (GPS2, TBL-1, HDACs) to assemble a larger, functional co-repressor complex, thereby attenuates activities of nuclear receptors (NRs) and other transcription factors. The splice variant of NCOR2, BQ323636.1 (BQ), retains RD1 as the only functional domain from wild type NCOR2. It is predicted that BQ lose the ability of regulating transcription factor.

1.9 Research initiative and hypothesis

Breast cancer is the most common type of female cancer. Breast cancer tumorigenesis could be due to alteration of levels of reactive oxygen species, inducing oxidative stress in cells. Oxidative stress regulation plays an important role in regulating signaling pathways that control cell survival, cell proliferation, DNA damage response, and cell apoptosis. Aberrant cell signaling and metabolism contribute to differences in redox status and elevated ROS levels in cancer cells, compared to their normal counterpart.

Chemotherapy, in single or in combination with other methods, has long been integrated as a standard part of treatment to breast cancer patients and has been significantly improved cancer patients' survival. Drug resistance, however, has become the major cause of treatment failure in cancer chemotherapy. Development of drug resistance such as anthracyclines, taxanes or platinum compounds are not uncommon in breast cancer patients. It is estimated more than 30 % of metastatic breast cancer patients would not respond to chemotherapy (Yardley 2013). Chemoresistance in breast cancer have been intensively studied over the past years, yet not all mechanisms of resistance are defined.

The nuclear factor erythroid 2-related factor 2 (NRF2) is the main regulator of cytoprotective response to oxidative stress and electrophilic stress. Upregulation of NRF2 provides growth advantage for breast cancer cells. Deregulated NRF2 pathway is associated with resistance to chemotherapy. Although there has been large amount of work trying to address the relationship between NRF2 and chemoresistance, the underlying mechanisms on regulating NRF2 are not fully understood.

My PhD project aims to identifying molecular mechanisms that are involved in the development of drug resistance via modulation of oxidative stress in breast cancer. My

study would investigate novel molecular mechanism through which NRF2 activity would be enhanced to confer drug resistance in breast cancer cells.

Cell signaling pathways involved in anti-oxidative stress can protect cells against cytotoxic effects of chemotherapy. In this study, we hypothesize that the adaptive response to oxidative stress may eventually contribute to the development of resistance to chemotherapy. The NRF2/ARE pathway is the main pathway controlling anti-oxidative stress response of cells. The role of FOXM1 in mediating response to chemotherapy has been well studied. The relationship between FOXM1 expression and NRF2 expression in breast cancer cells would be elucidated. The results showed that FOXM1 regulates the transcription of NRF2 which mediates the response to epirubicin and eventual epirubicin resistance in breast cancer cells. My study therefore uncovered a novel mechanism through which FOXM1/NRF2 axis confer to the drug resistance in breast cancer. These findings may be eventually translatable to serve as predictive markers for response to chemotherapy.

In addition, the role of the novel splice variant of NCOR2, BQ323636.1, in contributing the regulation of oxidative stress and chemoresistance would be described, and the underlying mechanism would be explored. BQ was shown to have an opposite effect of wildtype NCOR2 in modulating ERα transcriptional activation activity. Given that NCOR2 can interact with NRF2 and suppress transcription of antioxidant genes, it was therefore hypothesized that BQ could compromise the suppressive effect of NCOR2 on repressing the transcriptional activity of NRF2, leading to the development of the drug resistance. The results suggested that this splice variant of NCOR2, BQ323636.1, could become a robust biomarker in predicting ROS-mediated chemoresistance.

Chapter 2 Materials and Methods

2.1 Cell lines and culture

2.1.1 Breast cancer cell lines used

Human mammary epithelial cell line MCF-10A, human breast cancer cell lines MCF-7 and ZR-75-1 were originated from the American Type Culture Collection (ATCC) and were re-authenticated previously. MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco) supplemented with 5% horse serum (Gibco), 20 ng/ml epidermal growth factor (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 0.01 mg/ml insulin (Sigma-Aldrich), and 500 ng/ml hydrocortisone (Sigma-Aldrich). MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 1% penicillin–streptomycin (Gibco) and 10 % fetal bovine serum (FBS) (Gibco). ZR-75-1 cells were cultured in Improved Minimum Essential Medium (IMEM) (Gibco) supplemented with 1% penicillin–streptomycin and 10 % FBS.

Human breast cancer cell lines BT-474, MDA-MB-453, HCC-70 and MDA-MB-231 were originated from ATCC and obtained from Cancer Research UK (London, UK). MCF-7, MDA-MB-453 and MDA-MB-231 cells were cultured in DMEM-low glucose (Sigma-Aldrich) supplemented with 4 mM L-glutamine, penicillin–streptomycin (Gibco) and 10 % fetal calf serum (FCS) (First Link Ltd). BT-474 and HCC-70 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco) supplemented with 4 mM L-glutamine, penicillin–streptomycin and 10 % FCS. All cell lines were cultured in an incubator maintained at 37 ℃ with 10 % CO_2.

The epirubicin resistant breast cancer cell line MCF-7 EpiR and the paclitaxel resistant

breast cancer cell line MCF-7 TaxR were derived from parental MCF-7 cells. These cell lines were established in our group previously (Khongkow, Olmos et al. 2013). MCF-7 EpiR and MCF-7 TaxR was established by continuous exposure of drug sensitive MCF-7 cells to increasing concentration of the drug until resistance to 5 μM epirubicin (Accord healthacare) and 1 μM paclitaxel (Hospira) respectively. The established cell lines MCF-7 EpiR and MCF-7 TaxR were cultured in DMEM-low glucose supplemented with 4 mM L-glutamine, penicillin–streptomycin (Sigma) and 10 % FCS, and maintained in 1 μM epirubicin and 50 nM paclitaxel respectively. The drugs were removed from the culture medium for at least 24 h.

Stable BQ323636.1 overexpression cell lines were previously generated in our group using lentiviral system. In brief, lentiviruses were produced using human embryonic kidney 293 fast transform cells (HEK293FT) by co-transfecting lentivirus expression plasmids (pCDH-CMV-MCS-EF1-Puro-GFP-6xHis-BQ323636.1 or its empty vector as control) and pPACK Packaging Plasmid Mix (System Biosciences). At post-transfection 48 h, the packaged lentivirus were harvested and used to transduce ZR-75-1 cells. The infected cells expressed green fluorescence protein (GFP) which can be examined under fluorescent microscope. The successfully infected cells were selected by continuous exposure of increasing concentration of puromycin (Gibco) from 0.5 μg/ml until a pure population was formed, which all cells expressing GFP was obtained. The stable cell lines were cultured in IMEM supplemented with 1% penicillin–streptomycin, 10 % FBS and 5 μg/ml puromycin.

2.1.2 Maintenance of cells in culture

Breast cancer cells were grown and split when cell confluency was around 70 – 80 %. Cell culture medium was aspirated and cell monolayer were rinsed with 1 x phosphate

buffered saline (PBS) to remove any residual FCS. The cells were then trypsinized by 1 x trypsin in EDTA (Sigma-Aldrich) incubated at 37 °C. After cell detachment, the cell were resuspended in appropriate cell culture medium and seeded on to appropriate cell culture flasks or plates.

2.1.3 Treatment of cells

Tert-Butyl hydroperoxide (tBHP) solution [70 % weight in H_2O; 7.78 M] (Sigma-Aldrich) is an exogenous ROS inducer. A 50 mM intermediate dilution of tBHP was prepared with the stock and 1 x PBS. Different dilutions of tBHP were then freshly prepared with cell culture medium.

Brusatol (Carbosynth) is a NRF2 inhibitor. Brusatol powder was solubilized in dimethyl sulfoxide (DMSO) (Thermo-Scientific) and a stock solution of 1 mM was made. During experiments, the cells were treated with brusatol at nanomolar concentrations.

Tert-butylhydroquinone (tBHQ) (Sigma-Aldrich) is a compound that stabilize NRF2 protein and enhance NRF2-mediated transcriptional activation. tBHQ powder was solubilized in DMSO as a 10 mM stock. The cells were treated with tBHQ to achieve a final concentration of 100 µM.

N-acetyl-L-cysteine (NAC) (Sigma-Aldrich) is a synthetic precursor of intracellular cysteine and glutathione and known to inhibit ROS. NAC powder was solubilized in DMSO and a 250 mM stock was made. The cells were pre-treated with NAC of final concentration of 5 mM for 1 h before other treaments.

Chemotherapeutic drugs epirubicin [2 mg/ml] (Accord healthacare) and paclitaxel [6 mg/ml] (Hospira) were obtained from Imperial College Healthcare (UK). Intermediate stock solutions were prepared with appropriate cell culture medium at 100 µM and ? 50 µM for epirubicin and paclitaxel respectively. These drugs were freshly diluted to designated concentrations to treat cells.

2.2 Plasmid constructs

NRF2 expression plasmid pFN21A-human full length NFE2L2 (Halo-NRF2; Flexi Halo Tag clone, FHC06144) was purchased from Promega and Kazusa DNA Research Institute. FOXM1 expression plasmid pcDNA3-FOXM1 was generated in Prof. Eric Lam's lab and has been described (Ma, Tong et al. 2005, Kwok, Peck et al. 2010). NCOR2 expression plasmid pCMV6-Entry-NCOR2 and its empty vector were purchased from Origene. BQ Lentivirus expression plasmid pCDH-CMV-MCS-EF1-Puro-GFP-6xHis-BQ323636.1 was constructed and cloned in Prof. U.S. Khoo's lab previously. BQ expression plasmid pcDNA3.1-6xHisBQ was generated by Prof. U.S. Khoo's lab and has been described (Zhang, Gong et al. 2013). Luciferase reporter vectors pGL3-basic and pRL-SV40 were purchased from Promega.

2.3 DNA cloning

2.3.1 Cloning of antioxidant response element (ARE) into an pGL3-basic vector

Antioxidant response element (ARE) of human NQO1 gene has been identified with nucleotide sequence: 5'-CAGTCACAGTGACTCAGCAGAATCT-3' (Dhakshinamoorthy and Jaiswal 2000). The sequence of human NQO1 gene and promoter were identified from Ensembl (Transcript: NQO1-201, ENST00000320623.9). Primers were designed using Primer3Plus online software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primers were aligned with the NQO1 promoter containing the described ARE sequence and cutting sites for NheI and XhoI restriction enzymes were added to 5' ends of forward and reverse primers. The sequences of forward primer and reverse primer were: 5' - CTAGCTAGCTAGCCGAGTAGCTGGGACTTACAGG – 3' and 5' - CCGCTCGAGCGG GCACGAAATGGAGCAGAAAAAGAGC – 3' respectively. PCR was carried out with human genomic DNA (Promega) and the 503 bp PCR

products (inserts) were extracted by agarose gel electrophoresis.

Restriction enzyme NheI and XhoI (New England Biolabs) digestions were performed overnight with appropriate buffers at 37°C according to the manufacturer's protocol for cutting both inserts and vector (pGL3-basic). DNA fragments (both insert and vector) with compatible overhangs were covalently joined by T4 DNA ligase (Roche) overnight at 16 °C. The molar ratio of vector and insert was 1:3 in a 30 µl final reaction volume. 5 µl of the ligation reaction was used for transformation. Only transformed cells would survive on the agar plates with 100 µg/ml ampicillin as they contained the pGL3 with insert (pGL3-ARE).

These positive colonies were expanded in larger culture and pGL3-ARE plasmid was obtained by plasmid miniprep or maxiprep. The extracted plasmids from positive clones were re-digested with NheI and XhoI enzymes and gel electrophoresis was performed to confirm the presence of ARE insert. DNA sequencing of the plasmids were performed by Centre for Genomic Sciences, HKU to further validate the plasmids sequence.

2.3.2 DNA quantification

DNA concentration was measured using ND-1000 NanoDrop spectrophotometer (Thermo-Scientific) and the nucleic acid purity was assessed by ratio of absorbance at 260 nm and 280 nm for as well as the ratio of absorbance at 260 nm and 230 nm.

2.3.3 Bacterial transformation and culture

OneShot TOP10 Chemically Competent E. coli cells (Invitrogen) were used for propagating the ligated DNA transformed cells or available glycerol stock of plasmids.

DNA and cells mixture were heat shocked at 42 ℃ for 1 min and then returned to ice bath for 10 min. The transformed cells were allowed to recover in 500 µl of super optimal broth with catabolite repression (SOC) medium (Invitrogen) at 37 °C in a shaker before plating the transformed cells on LB agar plate with appropriate selection antibiotics (100 µg/ml ampicillin or 50 µg/ml kanamycin). Single colony was then picked and inoculated in 5 ml or 250 ml LB broth with appropriate selection antibiotics for culture at 37 ℃ overnight.

2.3.4 Plasmid Miniprep and Maxiprep

DNA was purified from the overnight-grown culture (5 ml or 250 ml) using a PureLink HiPure Plasmid Miniprep Kit (Invitrogen) or PureLink HiPure Plasmid Maxiprep Kit (invitrogen), the manufacturer's protocols were followed.

2.4 Transient transfections

2.4.1 Plasmid transfection using FUGENE 6

Transient transfection was performed using FUGENE 6 transfection reagent (Promega) according to manufacturer's protocol. Cells were seeded onto appropriate culture dish to reach approximately 60 -70 % confluency at the time of transfection. Desired amount of plasmid DNA was diluted in Opti-MEM medium (Gibco) and incubated at room temperature for 5 min. Then it was mixed with FUGENE 6 reagent at a ratio of 1:3 (1 µg of DNA to 3 µL of FUGENE 6), and incubated at room temperature for 15 min. The DNA-FUGENE6 complex was then added to cells in a drop-wise manner. Cells were returned to incubator for 24-48 h before being harvested or used for further experiments

2.4.2 Plasmid transfection using Lipofectamine 2000

Transient transfection for luciferase reporter assays was performed using

Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells were seeded onto 6-well plates to reach approximately 60 -70 % confluency at the time of transfection. Desired amount of luciferase reporter plasmids (pGL3 vectors and pRL-SV40) and expression plasmids; as well as Lipofectamine 2000 reagent were diluted in 250 µl Opti-MEM medium separately and incubated at room temperature for 10 min. Then they were mixed together and incubated at room temperature for additional 25 min. The DNA-Lipofectamine 2000 complex was then added to cells in a drop-wise manner. Cells were returned to incubator for 48 h before being harvested for subsequent luciferase assays.

2.4.3 Plasmid transfection using XtremeGENE HP

To increase the transfection efficiency of Halo-NRF2 expression plasmid, XtremeGENE HP reagent (Roche) was used. The cells were seeded in an appropriate culture dish to reach a confluency of 50 - 60 % on the day of transfection. Following manufacturer's protocol, a ratio of 5:1 of XtremeGENE HP to plasmid DNA was used. The plasmid DNA was diluted in Opti-MEM and vortexed for 10 sec. The appropriate amount of XtremeGENE HP was then added and incubated at room temperature for 15 min The mixture was then added to cells in a drop wise manner. Cells were returned to incubator for 48 h before being harvested or used for further experiments.

2.4.4 Small interfering RNAs (siRNAs) transfection

RNA interference (RNAi) was used to repress the expression of specific genes of interest and was performed using small-interfering RNA (siRNA). All siRNAs for this study were ON-TARGETplus SMARTPool siRNAs (Dharmacon) and this siRNAs product contains a mixture of 4 distinct siRNAs against the same gene, thereby reducing the potential off target effects. The SMARTPool siRNAs employed in this study were:

siFOXM1 (L-009762-00-0010) and siNRF2 (L-003755-00-0010); ON-TARGETplus Non-Targeting Pool (NSC) (D-001810-10-50) was served as negative control in all siRNA experiments. . All siRNAs were resuspended to a 20 μM stock, in 1 X siRNA buffer (Thermo Scientific).

Cells were seeded onto an appropriate culture dish or plate to reach cell confluency of 70-80 % on the day of transfection. SiRNAs were transfected into cells using Oligofectamine (Invitrogen) according to manufacturer's protocol. For a well of a 6 well plate scale, 5 μl Oligofectamine reagent was diluted in 60 μL of Opti-MEM and 7.5 μL of 20 μM siRNA stock was diluted in 250 μL of Opti-MEM. The oligofectamine dilution and the siRNAs dilution were incubated at room temperature for 5 min, followed by mixing these dilutions together. After incubation at room temperature for additional 20 min, Opti-MEM was toped up to a final volume of 500 μL. The cell culture medium was aspirated and the monolayer of cells was rinsed by 1 x PBS. The siRNA-Oligofectamine transfection complex was added onto the cells in a drop wise manner. Cells were returned to incubator for 48 h before being harvested or used for further experiments.

2.5 RNA analysis

2.5.1 Total RNA extraction and determination of RNA concentration

Total RNA was extracted from frozen cell pellets which were kept at -80 ℃ using RNeasy Kit (QIAGEN) according to manufacturer's protocol. RNA was eluted in DEPC-treated water (Ambion). RNA concentration and nucleic acid purity were measured by ND-1000 NanoDrop spectrophotometer.

2.5.2 Reverse transcription

The purified total RNA was reverse transcribed into first strand cDNA using Superscript III First-Strand system (Invitrogen). Briefly, 2000 ng of total RNAs were mixed with 1 µl of 10 mM dNTPs mix (NewEngland Biolabs) and 1 µl of 50 µM Oligo dT (Invitrogen), was toped up to a final volume of 13 µL using DEPC-treated water. Samples were denatured at 65 ℃ for 5 min. 1 µl of 0.1 M DTT, 1 µl of RNaseOUT recombinant inhibitor, 1 µL of 200U/µL superscript III reverse transcriptase and 4 µL of 5 x first-strand buffer were added to the mixture and incubated as follow: 25℃ for 10 min, 50 ℃ for 50 min, 70 ℃ for 15 min and hold at 4 ℃.The cDNA sample was diluted in 1:4 with DEPC-treated water, either used immediately for RT-qPCR or stored at -20 ℃.

2.5.3 Reverse transcription quantitative PCR (RT-qPCR)

Reverse Transcriptase quantitative PCR (RT-qPCR) was carried out using 7900HT Fast Real-time PCR System (Applied BioSystems). Eacg RT-qPCR reaction contains 2 µl of cDNA sample, 1 µl of 10 µM forward and reverse primer, 10 µl of 2 x Power SYBR Green PCR Master Mix (Applied Biosystems) and 7 µl DEPC-treated water. Housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or ribosomal L19 were used for internal control and normalization. Relative transcript levels were quantified using ΔΔCt method or relative standard curve method.

2.5.4 Primers used

The primers used for RT-qPCR were designed using Primer3Plus online software and the specificity of the primer pair was assessed by Primer-Blast tool (National Center for Biotechnology Information, NCBI; https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were purchased from Invitrogen. The primers used were presented in table 2.1.

Gene of interest	Sequence of the primer (5' to 3')	
ABCG2	Forward:	AGTGGCTTTCTACCTTGTCGA
	Reverse:	ATGGCGTTGAGACCAGGTTT
BQ323636.1	Forward:	AAGGTGGAGCGCATCGAGAAC
	Reverse:	GCATCTGCTTCTCCAGGTTCTCTG
CCNB1	Forward:	CAGTTATGCAGCACCTGGCTAAG
	Reverse:	TGTGGTAGAGTGCTGATCTTAGCAT
FOXM1	Forward:	TGCAGCTAGGGATGTGAATCTTC
	Reverse:	GGAGCCCAGTCCATCAGAACT
GAPDH	Forward:	GAAGGCTGGGGCTCATTT
	Reverse:	CAGGAGGCATTGCTGATGAT
GCLC	Forward:	GGATCCTCCAGTTCCTGCAC
	Reverse:	TTCTCCCCAGACAGGACCAA
GCLM	Forward:	GGAACCTGCTGAACTGGGG
	Reverse:	TGGGTTGATTTGGGAACTCCA
GSTP1	Forward:	CCCTACACCGTGGTCTATTTCC
	Reverse:	CAGGAGGCTTTGAGTGAGC
HMOX1	Forward:	AAGACTGCGTTCCTGCTCAAC
	Reverse:	AAAGCCCTACAGCAACTGTCG
L19	Forward:	GCGGAAGGGTACAGCCAAT
	Reverse:	GCAGCCGGCGCAAA
NCOR2	Forward:	ACGAGGTGTCAGAGATCATCGA
	Reverse:	TGATGAACTTGATGCGCTGCT
NQO1	Forward:	GGCAGAAGAGCACTGATCGTA
	Reverse:	TGATGGGATTGAAGTTCATGGC
NRF2	Forward:	ATGATGGACTTGGAGCTGCC
	Reverse:	ACTCTTTCCGTCGCTGACTG

Table 2.1 List of RT-qPCR primers used in this study.

2.6 Protein Analysis

2.6.1 Protein extraction and determination of protein concentration

Cells cultured in flasks or on plates were trypsinised as described above. The cell suspension was centrifuged for 5 min at 1200 rpm. The supernatant was discarded and centrifuged again for 3 min at 1200 rpm, any residual supernatant was removed. The

cell pellets were the lysed in cell lysis buffer [1 % NP-40, 50 mM Tris-HCL (pH7.4), 0.5 mM EDTA, 150 mM NaCL, 50 mM HEPES] supplemented with 1 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4), 2 mM phenylmethysulfonyl fluoride (PMSF) and 1 x complete protease inhibitors (Roche), and incubated on ice for 10 min. Supernatants, which contained the cell proteins, were transferred to a new 1.5 ml tube after centrifugation at 13000 x g at 4 ℃ for 10 min. Protein concentration was measured by BCA protein assay (Bio-Rad) according manufacturer's protocol. The absorbance at 595 nm was determined by microplate reader (Tecan) and protein concentration of sample was calculated according to the standard curve obtained from serial dilutions of BSA. Protein sample were mixed with 6 x SDS loading dye [0.375 M Tris pH 6.8, 12 % SDS, 60 % glycerol, 0.6 M DTT, 0.06 % bromophenol blue] and heated at 100 ℃ for 10 min. Samples were either immediately proceeded for SDS-PAGE or stored at -20 ℃.

2.6.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and electro-transfer

To detect protein expression, western blot analysis was employed. Protein samples of cells were separated according to their molecular weight using sodium dodecyl dulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring separated proteins to nitrocellulose membrane. Protein expression would be visualized after immunoblotting.

SDS-polyacrylamide gels were made up of two layers: stacking gel and resolving gel. The percentage of resolving gel was choosen according to the molecular size of protein of interests that need to be detected, ranging from 5 % to 14 %. Low percentage gels allowed better separation of proteins of large molecular size and high percentage gels

allowed the visualisation of proteins with small molecular weight. Ammonium persulphate (APS) and tetraethylethyleneduanine (TEMED) were used to catalyze gel polymerisation. SDS-polyacrylamide gels were made according the recipe (Table 2.2). 10 to 20 µg of proteins of each sample per well were loaded to gels, alongside with Novex Sharp Pre-Stained protein standard (Invitrogen) and ran in a running buffer [25 mM Tris, 109 mM Glycine and 0.1 % SDS] under 90 V. After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Bio-Rad) using the wet transfer method in a transfer buffer [25 M Tris, 190 mM glycine and 20 % (v/v) ethanol

2.6.3 Immunoblotting

The membranes were stained with Ponceau S (Sigma) to check the quality of the electro-transfer and de-stained by washing with 1x Tris-buffered saline Tween-20 (TBST). Membranes were then blocked in 5 % bovine serum albumin (BSA) (Sigma) at room temperature for 1 h, and then incubated with desired primary antibodies at 4 ℃ overnight. After primary antibodies incubation, membranes were washed five times with TBST, followed by secondary antibodies incubation at room temperature for 45 min. Antibodies used are listed in table 2.3. Subsequently, membranes were washed for three times with TBST to remove any unbound antibodies. The Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer) was applied to the membranes according to manufacturer's protocol and signals were detected by Hyperperformance Chemiluminescence films (Amersham) on an automatic x-ray film processor.

Reagents	Resolving gel				Stacking gel
	5%	**7%**	**10%**	**12%**	**5%**
1.5 M Tris, pH 8.8 (ml)	2.5	2.5	2.5	2.5	/
1.5 M Tris, pH 6.8 (ml)	/	/	/	/	0.42
10 % SDS (µl)	100	100	100	100	50
30% Acrylamide/Bis Solution, 37.5:1 (ml)	1.16	2.33	3.33	4	0.83
dH20 (ml)	5.68	5.02	4.02	3.35	3.67
25 % APS (µl)	10	10	10	10	10
TEMED (µl)	10	10	10	10	5

Table 2.2 Recipe for SDS-PAGE gels of different percentage

Antibody	Company	Species	Catalog number
FOXM1	Santa Cruz	Rabbit	sc-502
FOXO3	Cell Signaling	Rabbit	2497
NRF2	Novus Biologicals	Rabbit	NBP1-32822
β-Tubulin	Santa Cruz	Rabbit	sc-9104
HO-1	Cell Signaling	Rabbit	5853
Halo Tag	Promega	Mouse	G9211
Cyclin B1	Santa Cruz	Rabbit	sc-752
BQ323636.1	Homemade	Mouse	/
β-Actin	Santa Cruz	Mouse	sc-4778
6 x His Tag	Wako	Mouse	010-21861
NQO1	Santa Cruz	Mouse	sc-32793
NCOR2	Abcam	Rabbit	ab24551
Rabbit HRP	Dako	Goat	P0448
Mouse HRP	Dako	Goat	P0447

Table 2.3 Lists of antibodies used in this study.

2.7 Cell viability assay

In order to measure the cell survival after treatments, either sulforhodamine B (SRB) assay or MTT assay were used. For assessing cells long-term survival and renewal, clonogenic asaay was employed. These cell viability asaays examined the effectiveness

of the treatment to cells being tested.

2.7.1 Sulforhodamine B (SRB) assay

Cells were seeded on 96-wells plate, 2000 cells per well, prior to the assay. 24 h after seeding, the cells were treated with different concentration of the desired reagents or drugs for the designed period of time. On the day of harvest, cells in each well were first fixed with 100 μL of 40 % trichloroacetic acid (TCA) (Sigma-Aldrich) and incubated at 4 °C for 1 h. After the incubation, the plates were rinsed with water for 3 times, followed by stained with SRB solution [0.4 % SRB (Sigma-Aldrich) in 1 % acetic acid] at room temperature for 1 h. Excess dye were washed away with 1% acetic acid and air-dried overnight. Protein-bound dye was dissolved in 10 mM Tris solution at room temperature over a period of 1 h. The absorbance at 492 nm was measured by a microplate reader (Tecan). Result was presented as cell viability relative to the reading on day 0 or untreated control.

2.7.2 MTT assay

Cells were seeded on 96-wells plate, 2000 cells per well, prior to the assay. 24 h after seeding, the cells were treated with different concentration of the desired reagents or drugs for the designed period of time. On the day of harvest, cells in each well were incubated with 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) solution [10% MTT (Affymetrix) in standard culture medium] at 37 °C for 4 h. After the incubation, the solution in the wells were discarded. The purple precipitated formed in wells was dissolved in 100 μL isopropanol with 4 mM hydrochloric acid and 0.1% NP-40. The absorbance at 570 nm was measured by a microplate reader (Tecan). Sample reading was subtracted by culture medium background. The corrected absorbance data were presented as cell viability relative to the reading on day 0 or

untreated control.

2.7.3 Clonogenic assay

Cells were seeded on 6 well plates at a density of 1000 cells/well. Cells were treated with different concentrations of desired reagents or drugs on the following day. The medium was replaced with fresh cell culture medium 48 h after treatment. The surviving cells were allowed to grow and form colonies for 10 to 14 days. After inucation, medium were aspirated and the wells were rinsed with 1 x PBS. The cell colonies were fixed with 4 % paraformaldehyde (PFA) for 15 min at room temperature, and washed with 1 x PBS for 3 times. Fixed cells were stained with 0.5 % crystal violet in PBS for 30 min. Excess crystal violet washed off with water and left air-dried overnight. The images of colonies were taken by camera, followed by dissolving the stained colonies using 33 % acetic acid. The dye was quantified by measuring the absorbance at 592 nm using a microplate reader (Tecan).

2.8 Luciferase reporter assay

Cells were seeded onto a 6-well plate at a density of 100000 cells/well. On the following day, cells were co-transfected with firefly luciferase reporter plasmid (pGL3-basic or pGL3-ARE), the expression plasmid (pCMV6-NCOR2 or pCMV6-Entry; pCDNA3.1-BQ or pCDNA3.1-empty) and reniila luciferase plasmid (pRL-SV40) as transfection efficiency control, using lipofectamine 2000. The dual luciferase reporter assay system from Promega was used in this study and the manufacturer's instructions were followed. Briefly, 48 h after transfection, cells were treated with desired drugs for a desired period or left untreated. After treatment, cells were rinsed with 1 x PBS and then lysed in 1 x Passive Lysis buffer at room temperature for 15 min. The supernatant was obtained after centrifugation at 13000 x g for 30 s. 20 µl/well of the cleared lysate was added to a 96-

well luciferase plate (Perkin Elmer) and mixed with 100 µl/well of Luciferase Assay Reagent II. The intensity of firefly luminescence was recorded by a VICTOR3 multilabel plate reader (Perkin Elmer). Following which, 100 µl/well of Stop & Glo Reagent was added to the well and the intensity of renilla luminescence was recorded by the plate reader. The ratio of luminescence of luciferase Reporter/Renilla reporter for each samples were calculated and the value represented the normalized luciferase activity.

2.9 Detection of reactive oxygen species (ROS)

Detection of ROS in cells were done by CellROX Deep Red Flow Cytometry Assay Kit (Molecular probes, Life technologies). The CellROX Deep Red reagent is a cell permeable dye that does not fluoresce in a reduced state while becomes fluorescent upon oxidation by ROS. The emission maxima of CellROX Deep Red is approximately 665 nm and is measurable by flow cytometry. Breifly, cells were treated with desired reagents or drugs. After treatment, cells were trypsinized and counted by hemocytometer. 1 x 10^6 cells/ml were resuspended in cell culture medium (without phenol red). Cells were stained with CellROX Deep Red reagent in the 37 ℃ incubator for 1 h. The samples were immediately analyzed by flow cytometry on a LSR Fortessa analyzer (BD Biosciences). The data were analyzed by the FlowJo analysis software (FlowJo,LLC) and the values for mean fluorescence intensity ,which represent the relative ROS levels, were obtained.

2.10 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed to study binding of protein to DNA. The magnet beads were first incubated with antibodies.For each sample, 20 µl of Dynabead Protein A was washed with 200 µl of TSE I buffer for three times and

resuspended in 40 µl of TSE I buffer. 4 µg of antibodies of anti-FOXM1 (Santa Cruz) were diluted with 200 µl of Buffer D before adding to the pre-washed beads. The bead-antibodies mixture was incubated at 4 ℃ overnight with gentle shaking.

On the second day, cells cultured in 150 mm dish (around 90 % confluency), treated with desired drugs, and were incubated with 1 % paraformaldehyde (in PBS) for 10 min to allow crosslinking. Cells were rinsed with 1x PBS (ice-cold) and incubated with 0.25 M glycine (in PBS) at room temperature for 5 min to stop crosslinking. The cells were then harvested by scrapping in 1.4 ml scrapping buffer. The cells were centrifuged at 4 ℃ at 5000 rpm for 5 min and the cell pellet was sequentially washed in 1 x PBS (ice-cold), Buffer I and Buffer II, and finally resuspended in 300 µl ChIP lysis buffer, incubate on ice for 10 min. The cell resuspension was sonicated in ice-cold water bath for 16 cycles, each cycle with 30 s on and 30 s off, in a sonicator (Diagenode). Sonicated sample was then centrifuged at 4 ℃ at 13000 x g for 10 min and the supernatant was transferred to a new 1.5 ml tube. The sample was then mixed with 300 µl of Buffer D and 100 µl was taken as Input. The remaining sample was divded into two equal parts, one part was mixed with antibody anti-FOXM1 and the prepared Dynabead Protein A while the other part was mixed with prepared Dynabead Protein A only. The samples were incubated at 4 ℃ with gentle rotation.

On the third day, the incubated beads were obtained by magnetic stand from the samples. The beads were sequentially washed with TSE I, TSE II and TE buffer. 100 µl ChIP elution buffer was added to the beads and the mix was rotated at room temperature for 1 h. Eluted samples were transferred to a new 1.5 ml tube and the beads were eluted again in 100 µl ChIP elution buffer. A total of 200 µl of eluted samples were collected and incubated at 65 ℃ overnight to reverse crosslinking.

On the fourth day, DNA in the eluted samples was extracted and purified using SPRI select reagent (Beckman Coulter). The left side size selection method was used as the proper size of sonicated DNA would be around 100-300 bp, according to the manufacturer's protocol. In brief, the sample was mixed with equal volume of SPRI beads and incubated at room temperature for 1 min. The beads were allowed to settle on magnetic stand for 10 min. The beads were washed with 85 % ethanol and left air-dried for 5 min. 50 µl of DEPC-treated water were used to elute the DNA and the eluted DNA was transferred to a new tube. The gene of interest were quantified by RT-qPCR using primers flanking the predicted FOXM1 binding region. The forward primer used was: 5' - TTGCGCAACAGATCAACAGC – 3'. The reverse primer used was: 5' - CTTCTAGTTCGGACGCGGTG – 3'. Results were calculated by ΔΔCt method and expressed as fold-change relative to the bead control.

The recipes of all buffers used in ChIP are listed in table 2.4. It is noted that all the buffers (except ChIP elution buffer) were supplemented with 1 x protease inhibitor and were kept on ice before use.

Buffer	Recipes
Scraping Buffer	100 mM Tris-HCL, pH 9.4; 0.1 % SDS; H_2O
ChIP Lysis Buffer	1 % SDS; 10 mM EDTA; 500 mM Tris-HCL, pH 8.1; H_2O
Buffer I	0.25 % Triton X-100; 100 mM EDTA; 0.5 mM EGTA; 10 mM HEPES, pH 6.5; H_2O
Buffer II	200 mM NaCl; 10 mM EDTA; 0.5 mM EGTA; 10 mM HEPES, pH 6.5; H_2O
Buffer D	1 % Triton X-100; 2 mM EDTA; 20 mM Tris-HCL, pH 8.1; 150 mM NaCl; H_2O
TSE I	0.1 % SDS, 1 % Triton X-100, 2 mM EDTA; 20 mM Tris-HCL, pH 8.1; 150 mM NaCl; H_2O
TSE II	0.1 % SDS, 1 % Triton X-100, 2 mM EDTA; 20 mM Tris-HCL, pH 8.1; 500 mM NaCl; H_2O
TE Buffer	100 mM Tris-HCL, pH 8.0; 1 mM EDTA; H_2O
ChIP Elution Buffer	0.1 mM $NaHCO_3$; 1 % SDS; H_2O

Table 2.4 Recipes of buffers used in ChIP.

2.11 Co-immunoprecipitation (Co-IP)

Co-immunoprecipitation was performed for identifying protein-protein interaction. Cells were lysed in cell lysis buffer supplemented with 1 x complete protease inhibitors (Roche) and incubated on ice for 10 min.Th supernatant was transferred to a new 1.5 ml tube after centrifugation at 13000 x g at 4 ℃ for 10 min. Protein concentration of the lysate was determined and then the lysate was pre-cleared with Dynabeads Protein A (Invitrogen) (pre-washed with 1 x PBS). Dynabeads were removed from the lysates using DynaMag magnetic stand (Invitrogen). After preclearing, lysate containing 300 µg of proteins was incubated with specific primary antibodies including anti-NCOR2 (Abcam) and anti-NRF2 (Cell Signaling) as well as pre-washed Dynabeads Protein A at 4 ℃ overnight with gentle rotating. Lysate containing 300 µg of proteins was also incubated with rabbit immunoglobin (IgG) (Dako) and pre-washed beads as negative

control. 30 μg of proteins were mixed with 6 x SDS loading dye under denaturing condition as input. On the following day, the beads that had bound with antibody and target proteins were obtained using DynaMag magnetic stand. These samples were then washed with 1 x PBS for 3 times, to wash off the unbound antibodies and proteins. Samples were mixed with 2 x SDS loading dye and boiled at 100 ℃ for 5 min to elute the bound proteins from Dynabeads. The prepared samples were then proceeded to SDS-PAGE or stored at -20 ℃.

2.12 Immunohistochemical staining of patient samples

2.12.1 Tissue microarray (TMA)

With the approval by the Institutional Review Board of The University of Hong Kong, the tissue microarray for analysis were constructed. Histological sections of all cases were reviewed by pathologist. The representative paraffin tumor blocks were selected as donor block for each case and the selected areas marked for TMA blocks construction. With clinical follow up data retrieved from the records of the Department of Pathology (Queen Mary Hospital, Hong Kong), a total of 99 cases could be used scoring of NRF2 and FOXM1 staining in one set of TMA while a total of 124 cases could be used scoring of BQ323636.1 and NQO1 staining in another set of TMA. Each case in the TMA blocks was constructed in duplicates and average score was taken as the score for that case.

2.12.2 Immunohistochemistry

TMA sections were deparaffinized and rehydrated with repeated incubation of ethanol and xylene. 0.01 M citrate buffer (pH 6) was used for antigen retrieval and the slides were then immersed in 3 % H_2O_2 (in methanol) at room temperature for quenching endogenous peroxidase. The slides were then rinsed in 1 x PBS with 0.05 % Tween-20

(PBST) for two times, followed by incubation with primary antibodies at 4°C overnight. Antibodies used were anti-NRF2 (1:200), anti-FOXM1(1:250), anti-BQ323636.1 (1:50) and anti-NQO1 (1:200). On the next day, the slides were washed with 1 x PBST and incubated with EnVision+ System- HRP labelled Polymer Anti-Rabbit (Dako) at room temperature for 30 min in dark. The excess reagent was washed off with 1 x PBST and Chromogen DAB / substrate reagent (Dako) was added to the slides and incubated for 6 min. The slides were dehydrated and mounted. To visualize and assess protein expressions, Aperio ScanScope system (Aperio technology) was used. TMA slides were scanned and individual TMA spots were assessed using Aperio's image viewer, ImageScope.

2.12.3 Staining and scoring

Since NRF2, FOXM1 and NQO1 was detectable in both the cytoplasm and the nucleus, expressions in cytoplasm and nucleus were scored separately as previously described (Khongkow, Karunarathna et al. 2014). Scoring was done by two independent individuals (myself and Ms Ellen Man) to avoid subjectivity in evaluation. The intensity of staining was scored as follows: 1 = low, 2 = moderate, 3 = high. The percentage of cells positively stained was scored as follows: 1 ≤ 25%, 2 ≤ 50%, 3 ≤ 75%, 4 > 75%. The final score, cytoplasm or nucleus, was obtained by multiplying the score of intensity with the score of percentage. The total score was calculated as adding score of cytoplasm to score of nucleus.

Cytoplasm expressions of BQ were similar in most samples but nuclear expressions of BQ were found to be varying across all samples, the H-score system (Detre, Saclani Jotti et al. 1995) was employed in scoring of BQ expression. The H-score system is a semi-quantitative method of scoring nuclear staining intensity and percentage of nuclei

of a particular intensity. The BQ's H-score was (1 × % of cells stained at "low" intensity) + (2 × % of cells stained at "moderate" intensity) + (3 × % of cells stained at "high" intensity).The median of the scores, which was 130, was set as the cutoff value.

2.13 Statistical analysis

Statistical analysis were conducted using Excel (Microsoft) or GraphPad Prism version 6 (GraphPad Software Inc). Statistical differences between two groups' means were evaluated by two-tailed unpaired or paired Student's t-test. Statistical significant difference was considered when P value: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.005$. Results were in graphs drawn using GraphPad Prism version 6. Results from tissue microarray were analyzed in SPSS (IBM). The correlation between NRF2 and FOXM1 expression or BQ323636.1 and NQO1 expression was analyzed by Mann-Whiney U Rank test analysis. P values, * $P \leq 0.05$, were considered statistically significant.

Chapter 3 FOXM1/NRF2 in regulation of oxidative stress and epirubicin response of breast cancer

3.1 Introduction

Reactive oxygen species (ROS) are generated as a result of normal cellular metabolism. At low to moderate levels of ROS, they function as secondary messenger in cell signaling cascades and in various physiological processes (Valko, Rhodes et al. 2006). However, at high levels of ROS, they could damage cellular components such as DNA, proteins and lipids. To counterbalance the harmful effect of these oxidants, cells have developed a wide range of antioxidants: enzymatic and nonenzymatic (Birben, Sahiner et al. 2012). 'Oxidative stress' refers to when the balance between ROS and antioxidants are disrupted due to accumulation of ROS or depletion of antioxidants. Oxidative stress is involved in many pathological conditions and disease, including cancer (Birben, Sahiner et al. 2012).

Development of cancer in humans is a complex process. It requires both cellular and molecular alterations triggered by intracellularly or by exogenous sources. Oxidative stress to be one of the stimuli accounted for development of cancer. Cancer can be driven by chromosomal abnormalities and oncogene activation triggered by oxidative stress. Cancer cells are characterized by accumulation of ROS in cells due to imbalance between ROS generation and ROS elimination (Cairns, Harris et al. 2011). Dysregulation of ROS in cancer is contributed by altered activities in mitochondria, endoplasmic reticulum and peroxisomes, which are the main sites for ROS production. To deal with the increased levels of ROS, cancer cells have strong antioxidant systems

such that the levels of ROS are compatible to the cellular biological functions (Pervaiz and Clement 2004).

Despite an enhanced antioxidant system, cancer cells maintain higher ROS levels than normal cells. It was found that ROS levels is higher in breast tumors than normal breast tissue (Haklar, Sayin-Ozveri et al. 2001). It has been suggested that cancer cells are more sensitive to normal cells to additional oxidative stress, thus induction of oxidative stress could lead to preferential killing of cancer cells (Martin and Barrett 2002). Many drugs used for cancer chemotherapy can induce oxidative stress. Patients who received chemotherapy displayed signs of oxidative stress such as elevated levels of peroxidation products, lowered plasma levels of antioxidants and decreased tissue glutathione levels (Conklin 2004). Drugs such as anthraycyclines and platinum containing compounds generate extremely high ROS levels (Conklin 2004).

Chemoresistance has become the major cause of treatment failure in cancer chemotherapy. It is proposed that modulation of oxidative stress is one of the mechanisms for drug resistance. Resistance to ROS-inducing drugs should involve adaptive mechanisms that activate ROS scavenging system. Drug resistant cells should have lower ROS levels than drug sensitive cells such that additional oxidative stress induced by the drug would not trigger cell deaths of resistant cells. It is found that drug resistant cells have increased activities of antioxidant enzymes such as catalase, peroxiredoxins and thioredoxin peroxidase (Janssen, Bosman et al. 2000, Young, Mei et al. 2004). In this study, the involvement of modulation of oxidative stress in the development of chemoresistance is explored.

NRF2 is an essential transcription factor for relieving oxidative stress. It controls the expression of antioxidant genes and genes involved in glutathione homeostasis, conjugation and membrane transporters for drug efflux. Overexpression of NRF2 is found in many cancer types, including breast cancer and is associated with resistance to chemotherapy (Shibata, Kokubu et al. 2008, Wang, Sun et al. 2008). The forkhead box transcription factor FOXM1 is a well-known oncogene shown to play important roles in all hallmarks of cancer (Halasi and Gartel 2013). Also, different studies have shown that overexpression of FOXM1 mediates resistance to chemotherapeutic drugs including anthracyclines, platinum containing compounds and taxanes in breast cancer (Kwok, Peck et al. 2010, Millour, de Olano et al. 2011, Park, Jung et al. 2012, Khongkow, Gomes et al. 2016). Previous studies have suggested that the forkhead box transcription factors might take part in the regulation of NRF2 pathway (Guan, Zhang et al. 2016, Han, Shin et al. 2016).

In this study, we hypothesized that FOXM1 and NRF2 are involved together in contributing chemoresistance via modulation of oxidative stress in breast cancer. The relationship between FOXM1 expression and NRF2 expression in breast cancer cells will be studied and the underlying mechanisms are uncovered. It is expected that combinations of biomarkers will be more informative to predict response than any single target. Result from this study will provide support for FOXM1 and NRF2 to serve as markers together for predicting response to oxidative stress-inducing chemotherapy.

3.2 Results

3.2.1 Chemoresistant breast cancer cells have higher protection against oxidative stress than sensitive breast cancer cells

Since chemotherapy drug would induce oxidative stress to trigger cell death to kill cancer cells, the resistance cells should have different response towards the induction of oxidative stress, and thus resistant to cell death. The cell viability of drug sensitive MCF-7 cells and chemoresistant cells under oxidative stress were first determined. Two chemoresistant cell lines, namely MCF-7 EpiR (resistant to epirubicin) and MCF-7 TaxR (resistant to paclitaxel), would be employed. To induce oxidative stress, a chemical inducer, tert-butyl hydroperoxide (tBHP), was used. tBHP has been shown to induce oxidative stress in MCF-7 cells (Acharya, Das et al. 2010). MCF-7, MCF-7 EpiR and MCF-7 TaxR cells were treated with different concentrations of tBHP and their response were studied using sulforhodamine B (SRB) assay that could determine the cell viability. As shown in figure 3.1A, it was observed that both MCF-7 EpiR cells (Red lines) and MCF-7 TaxR cells (Green lines) showed significantly more resistant to tBHP induced cell death, marked by higher cell viability under the treatment of a series of concentration of tBHP (from 0 to 100 μM) compared to the ordinary drug sensitive MCF-7 cells (Blue lines). This result suggested that chemoresistant breast cancer cells are more resistant to oxidative stress.

The long-term cytostatic effects of oxidative stress were also studied by clonogenic assay. Chemoresistant MCF-7 cell lines and sensitive MCF-7 cell line were exposed to various concentrations of tBHP, grown for a longer time (2 weeks) than SRB assays (Figure 3.1B). Cell colonies produced are fixed, stained with crystal violet. The staining was quantified by absorbance at 592 nm when dissolved by acetic acid. In agreement with data of the SRB assays, both MCF-7 EpiR and MCF-7 TaxR had higher clonogenicity than sensitive MCF-7 cells when treated with tBHP. Results from both SRB assays and clonogenic assays evident that resistant cells had higher protection against oxidative stress. This finding demonstrated a correlation between resistance to

chemotherapy and protection against oxidative stress, which confirmed our hypothesis. It further suggested that oxidative stress signalling might contribute to the development of chemoresistance of breast cancer.

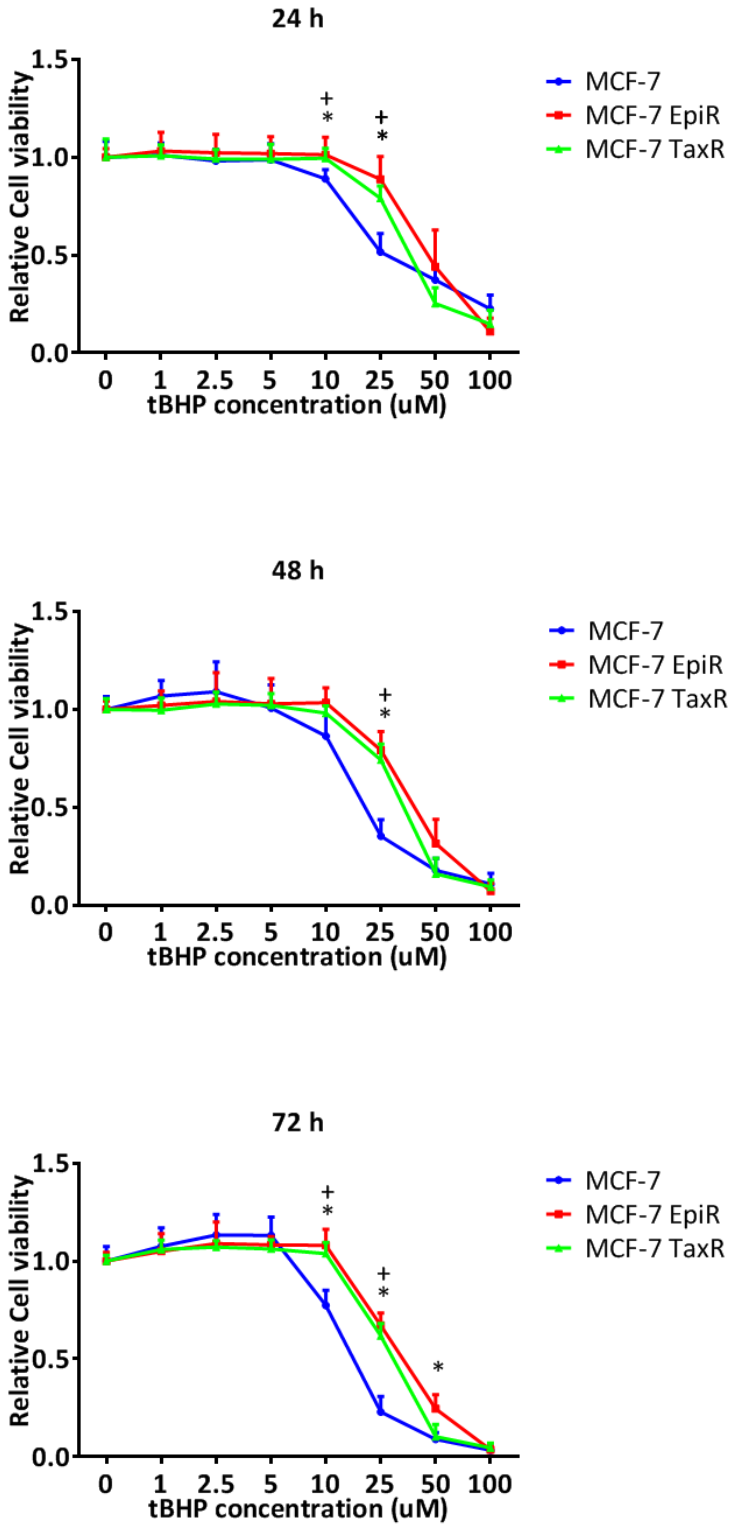
The viability assays' results suggested that the MCF-7 EpiR cell line demonstrated slightly higher resistance to oxidative stress than that of MCF-7 TaxR. As in keeping what is known in literature (reviewed in chapter 1), anthracyclines such as epirubicin generates higher levels of oxidative stress than paclitaxel in cells. We choose to use MCF-7 EpiR cell line and epirubicin treatment to illustrate how oxidative stress can mediate chemoresistance of breast cancer.

To investigate whether ROS is involved in development of drug resistance in breast cancer, the intracellular levels of ROS in sensitive cells and chemoresistant cells were evaluated. MCF-7 cells and MCF-7 EpiR cells were stained with CellROX Deep Red reagent to evaluate the basal levels of intracellular ROS. Flow cytometry was employed to detect and measure fluorescence signal of the stained cells (Figure 3.2A). Data from flow cytometry was analyzed by FlowJo software and the mean CellROX Deep Red reagent fluorescence values were obtained. The fluorescence signal represented the general ROS levels in cells. It was found that MCF-7 EpiR cells naturally had lower levels of ROS than MCF-7 cells. This result suggested that resistant cells might have more effective anti-oxidative stress mechanism, leading to constitutive low basal levels of intracellular ROS. As shown in Figure 3.2B, treating these cells with oxidative stress inducer tBHP, there was a relative significant increase of ROS levels upon treatment compared to untreated cells in MCF-7 indicating that drug sensitive MCF-7 cells were responsive to oxidative stress. Also, there was no significant difference between

untreated and tBHP treated condition in MCF-7 EpiR cells, showing that drug resistant MCF-7 EpiR cells were not responsive to tBHP in this experiment.

Collectively, our data suggested that effective oxidative stress defense mechanisms existed in chemoresistant cells, leading to a low basal level of ROS and higher tolerance to change of ROS levels under stressed conditions.

(A)

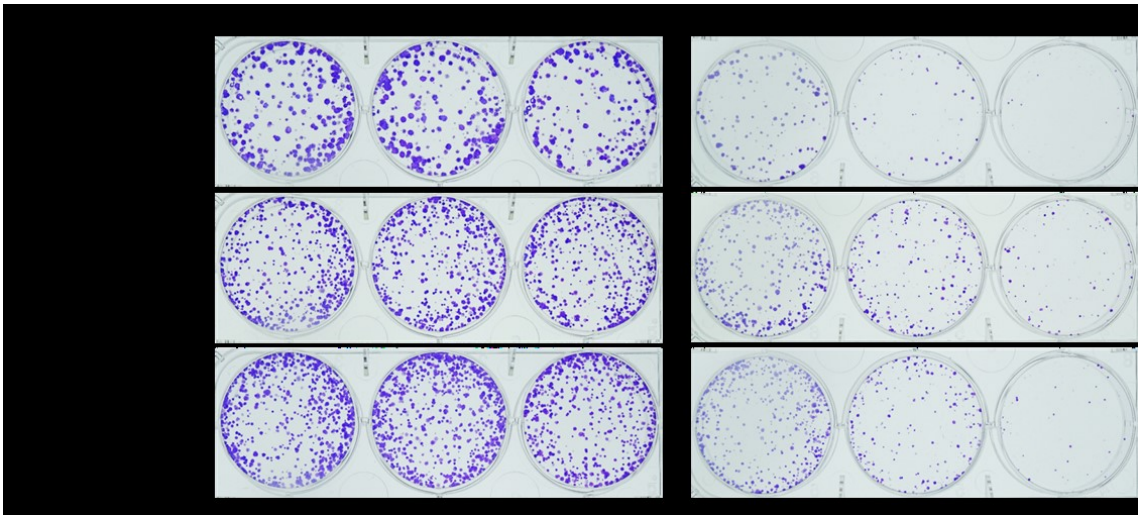


Note: * P<0.05 for MCF-7 EpiR compared to MCF-7;

$^{+}$ P<0.05for MCF-7 TaxR compared to MCF-7

(B)

(i)



(ii)

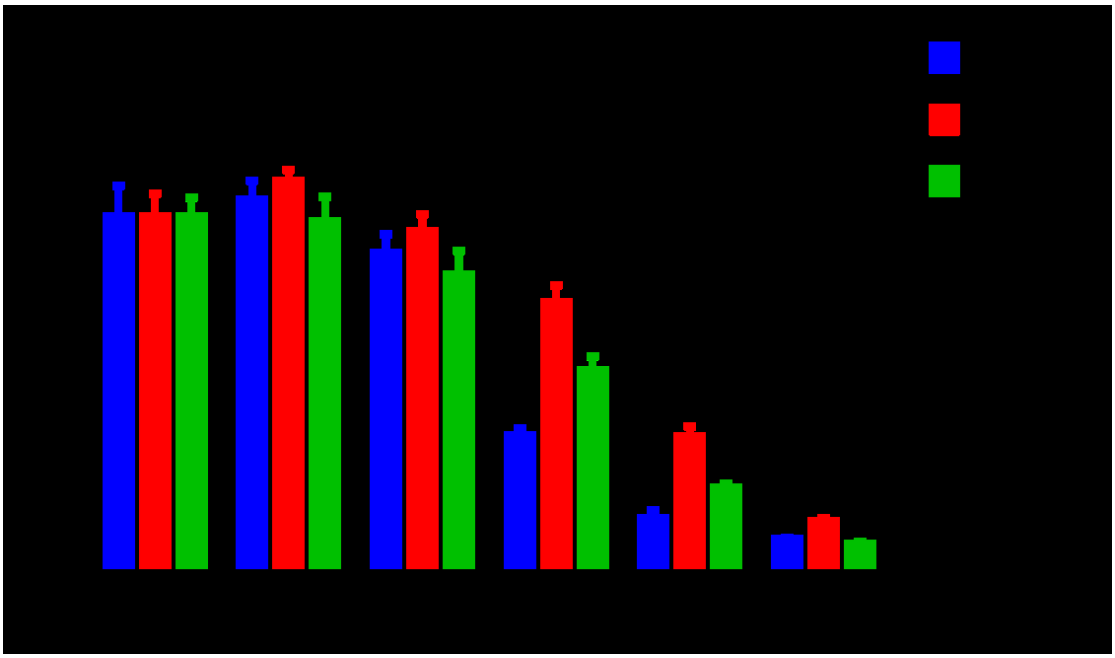
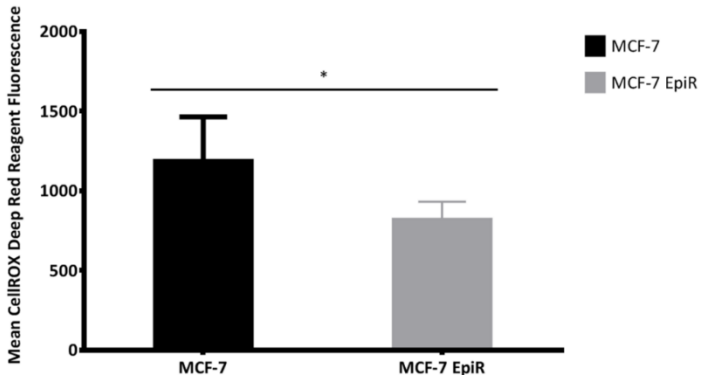


Figure 3.1 Chemoresistant breast cancer cells showed higher resistance to tBHP. Drug sensitive cells MCF7, chemoresistant cells MCF-7 EpiR and MCF-7 TaxR were treated with different concentrations of an oxidative stress inducer tBHP**. (A)** Cells were treated with tBHP (0, 1, 2.5, 5, 10, 25, 50 and 100 μM) for 24 h, 48 h and 72 h, followed by sulforhodamine B (SRB) assays. **(B)** (i) Cells were treated with tBHP (0, 1, 5, 10, 15 and 25 μM)for 48 h and then cultured with normal culture medium for 14 days. The cells were then stained with crystal violet. (ii) The quantitative analysis was measuring the intensity of crystal violet staining after dissolving the stain in acetic acid. Relative colony formation was expressed with reference to untreated cells (0 μM as 1). The experiments have been repeated for four times independently and data are shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001, when compared with MCF-7.

(A)



(B)

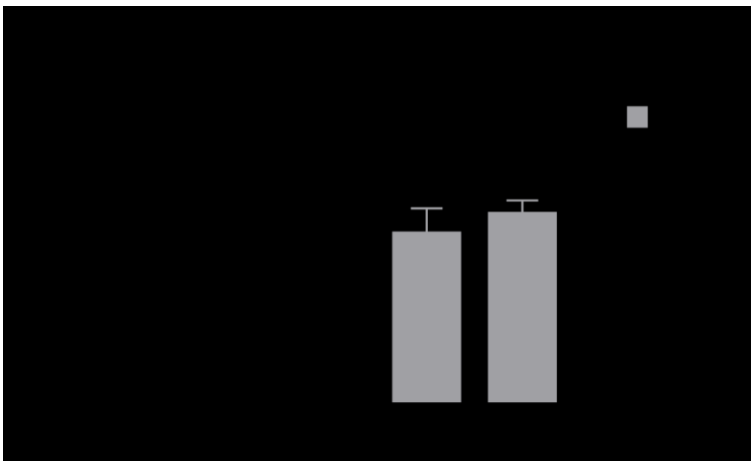


Figure 3.2 Chemoresistant cells have lower basal levels of ROS and were more resistant to change in levels of ROS after tBHP treatment. **(A)** The intracellular ROS of sensitive cells MCF-7 and epirubicin-resistant cells MCF-7 EpiR were detected by CellROX Deep Red reagents, followed by flow cytometry analysis. The mean fluorescence signal represent basal levels of ROS in these cells. **(B)** MCF7 and MCF-7 EpiR cells were treated with 100 μ M tBHP for 1 h. Cells were analyzed for relative ROS levels as compared with that obtained for control untreated cells (Untreated MCF-7 or MCF-7 EpiR as 1). The experiments have been repeated for three times independently and data are shown as mean ± SD;*P<0.05 when compared with MCF-7 cells (A) or controls (B).

3.2.2 NRF2 mediates epirubicin-resistance in breast cancer

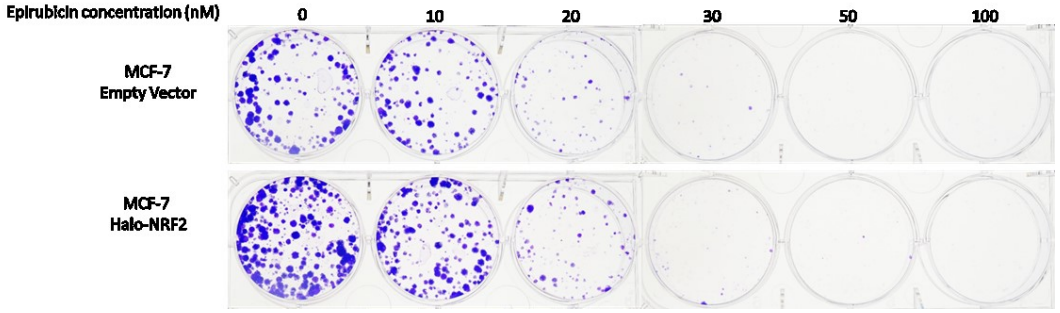
We hypothesized that chemoresistant breast cancer cells have higher protection against oxidative stress and that more effective oxidative stress defense mechanisms might exist in chemoresistant cells. Transcription factor NRF2 is the master regulator of oxidative stress. It has been reported that NRF2 regulate chemoresistance (Zhong, Zhang et al. 2013). The roles of NRF2 in controlling anthracycline resistance in breast cancer was first reconfirmed. Overexpression of NRF2 and knockdown of NRF2 in MCF-7 cell lines were performed to study their effects on cell viability in epirubicin treatment. Western blot results confirmed the overexpression of NRF2 and knockdown of NRF2 in MCF-7 cell lines (Figure 3.12) Clonogenic assays were performed as previously described (Khongkow, Karunarathna et al. 2014).

NRF2 was overexpressed by transfecting NRF2 expression plasmid (Halo-NRF2) in MCF-7 cells. Clonogenic assays were performed to study the effects of NRF2 overexpression on drug sensitvity to epirubicin of MCF-7 (Figure 3.3A). When compared to empty vector transfected MCF-7 as a control, NRF2 overexpressed cells had significant higher cell viability at various concentrations of epirubicin, showing that NRF2 overexpression could induce epirubicin resistance in MCF-7 cells. It was also observed that overexpression of NRF2 increased cells clonogenicity in MCF-7 without any epirubicin treatment, showing NRF2 had roles in promoting cell proliferation. Similarly, clonogenic assays were also performed to study the effects of NRF2-silencing on drug sensitivity of epirubicin of MCF-7 cells. NRF2 was knocked-down by NRF2-specific siRNA pool in MCF-7, followed by epirubicin treatment (Figure 3.3B). Loss of NRF2 further sensitized MCF-7 cells to epirubicin, marked by significant decrease in colony formation.

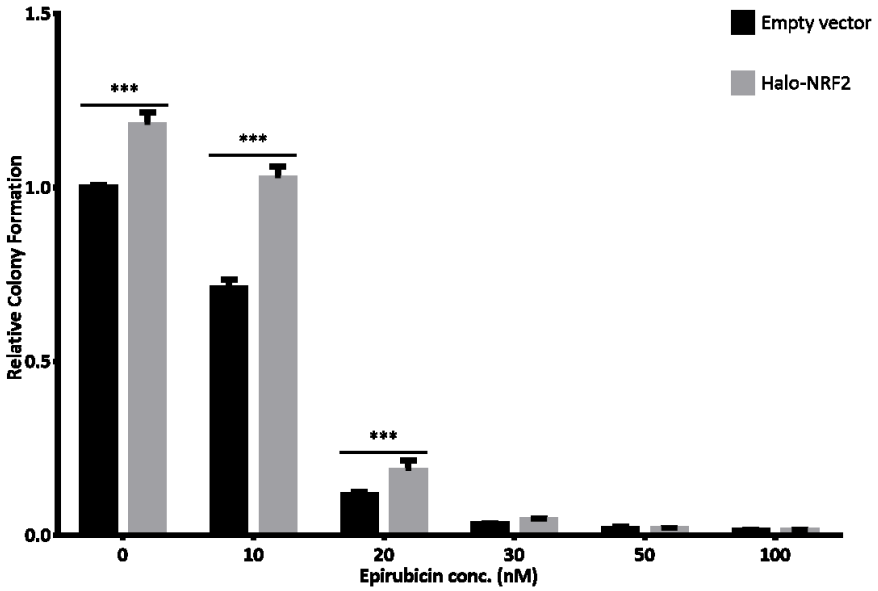
The effect of NRF2-silencing on MCF-7 EpiR were also studied (Figure 3.4). In agreement with the effect of NRF2 overexpression on promoting epirubicin resistance in MCF-7 cells, NRF2 silencing by specific siRNAs caused significant decrease in MCF-7 EpiR total colony formation. In addition, depletion of NRF2 in MCF-7 EpiR resensitized these cells to epirubicin, as shown by significantly reduction in relative colony formation (normalized) when compared to non-treated control.

Taken together, the findings showed NRF2 could regulate cell proliferation and confirmed the functional role of NRF2 in conferring epirubicin resistance in breast cancer.

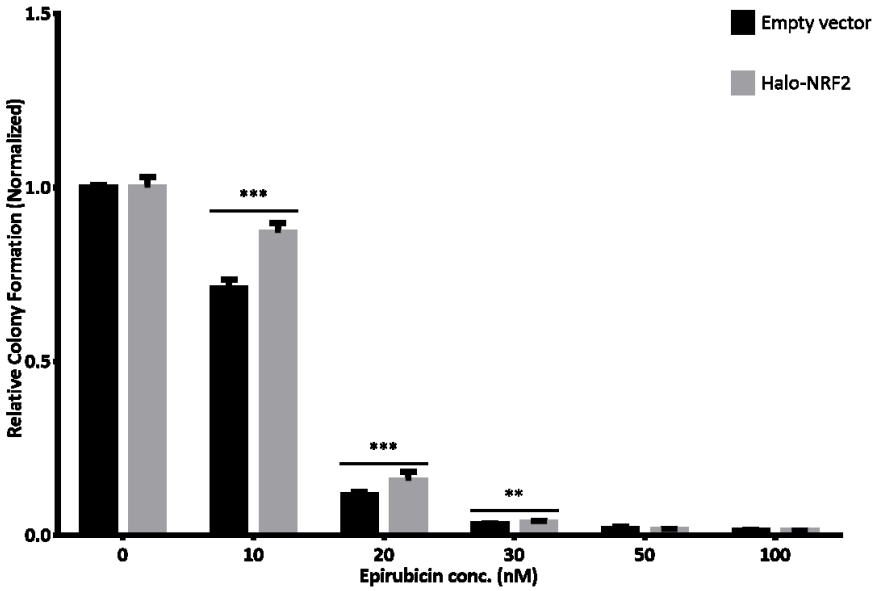
(A)


Epirubicin concentration (nM)
0
10
20
30
50
100
MCF-7
Empty Vector
MCF-7
Halo-NRF2

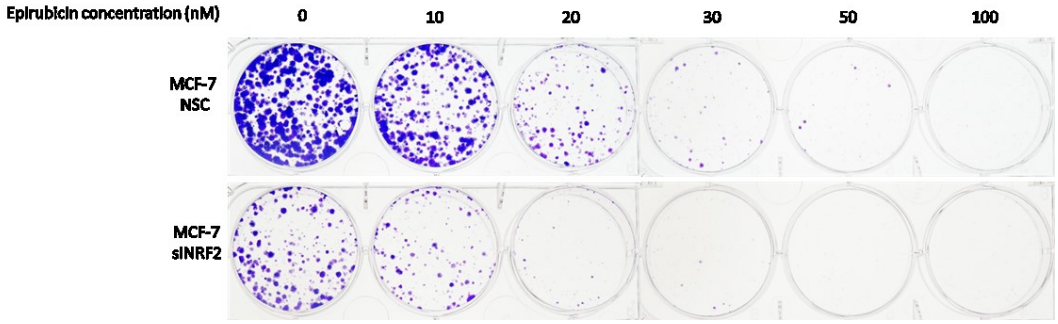
(i)


Empty vector
Halo-NRF2
Relative Colony Formation
1.5
1.0
0.5
0.0
***
***
***
0
10
20
30
50
100
Epirubicin conc. (nM)

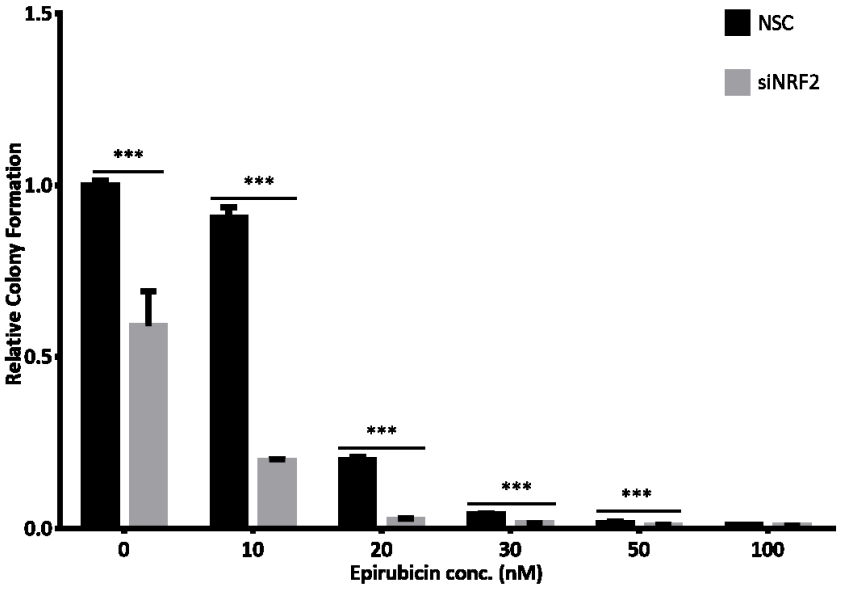
(ii)


Empty vector
Halo-NRF2
Relative Colony Formation (Normalized)
1.5
1.0
0.5
0.0
***
***
**
0
10
20
30
50
100
Epirubicin conc. (nM)

(B)


Epirubicin concentration (nM)
0
10
20
30
50
100
MCF-7
NSC
MCF-7
siNRF2

(i)


NSC
siNRF2
Relative Colony Formation
1.5
1.0
0.5
0.0
***
0
10
20
30
50
100
Epirubicin conc. (nM)

(ii)

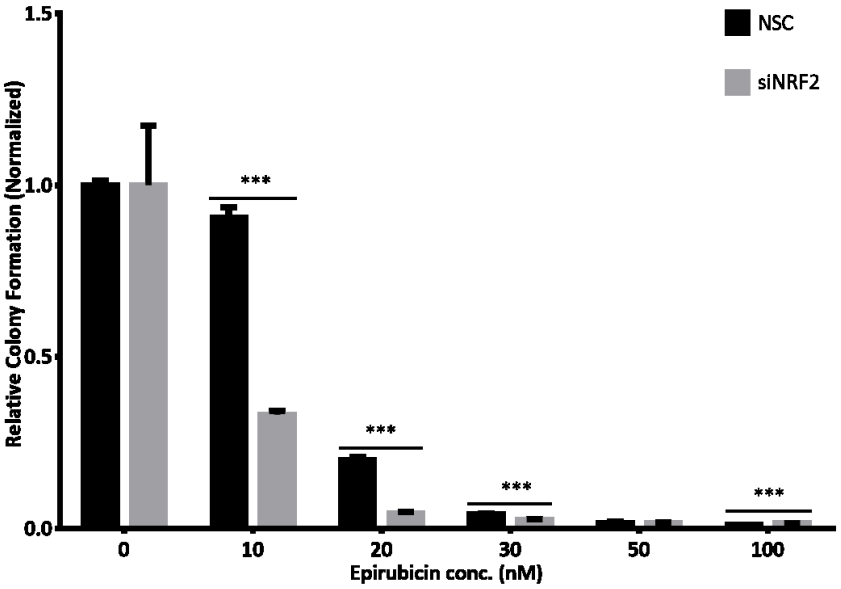
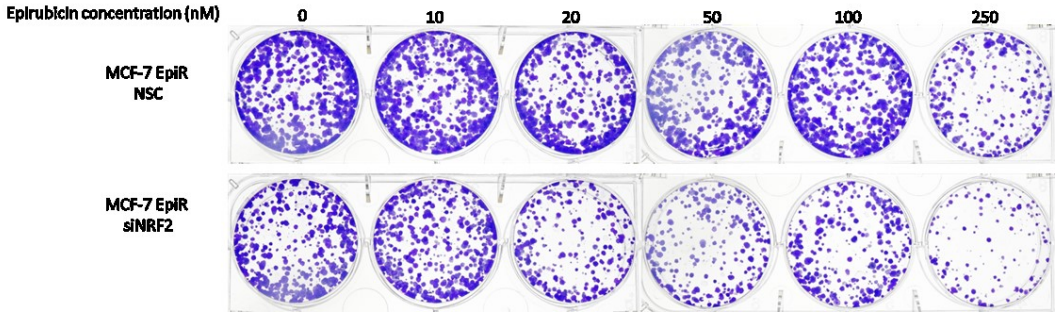
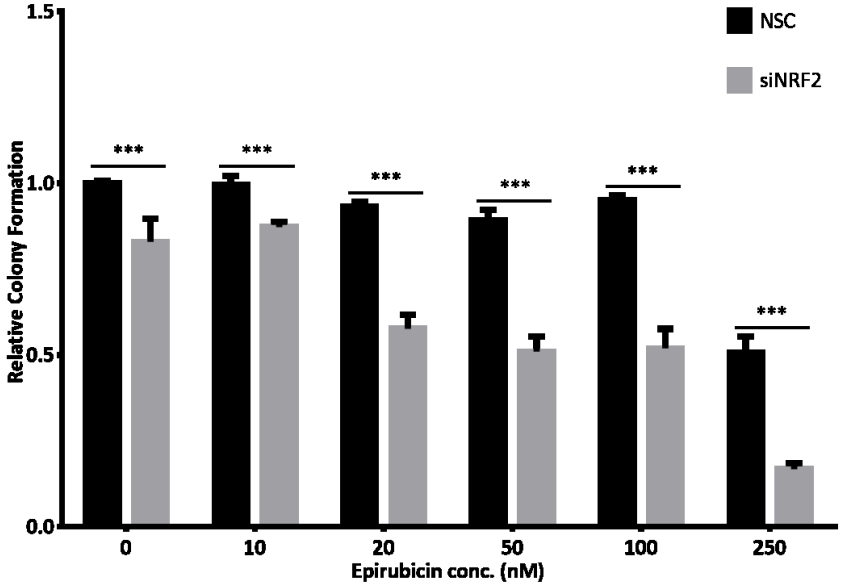

NSC
siNRF2
Relative Colony Formation (Normalized)
1.5
1.0
0.5
0.0
***
0
10
20
30
50
100
Epirubicin conc. (nM)

Figure 3.3 NRF2 expression control epirubicin resistance in breast cancer. **(A)** MCF-7 cells were first transfected with NRF2 expression plasmid (Halo-NRF2) or empty vector (as control) and then seeded for clonogenic assay. **(B)** MCF-7 cells were first transfected with specific siRNAs pool against NRF2 (siNRF2) or non-targeting siRNAs control (NSC) and then seeded for clonogenic assay. The cells were then treated with epirubicin for 48 hours at various concentrations (0, 10, 20, 30, 50 and 100 nM). After drug wash out, colonies were allowed to form for 14 days, followed by staining with crystal violet. The staining were quantified after dissolving in acetic acid and measured as absorbance (592 nm) by plate reader. Data were represented (i) as relative colony formation with reference to non-treated MCF-7 cells or (ii) as relative colony formation (normalized) at each epirubicin concentration were normalized to the control (non-treated condition 0 nM) as plotted in the graphs. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.


Epirubicin concentration (nM)
0
10
20
50
100
250
MCF-7 EpiR NSC
MCF-7 EpiR siNRF2

(i)


NSC
siNRF2
Relative Colony Formation
1.5
1.0
0.5
0.0
***
0
10
20
50
100
250
Epirubicin conc. (nM)

(ii)

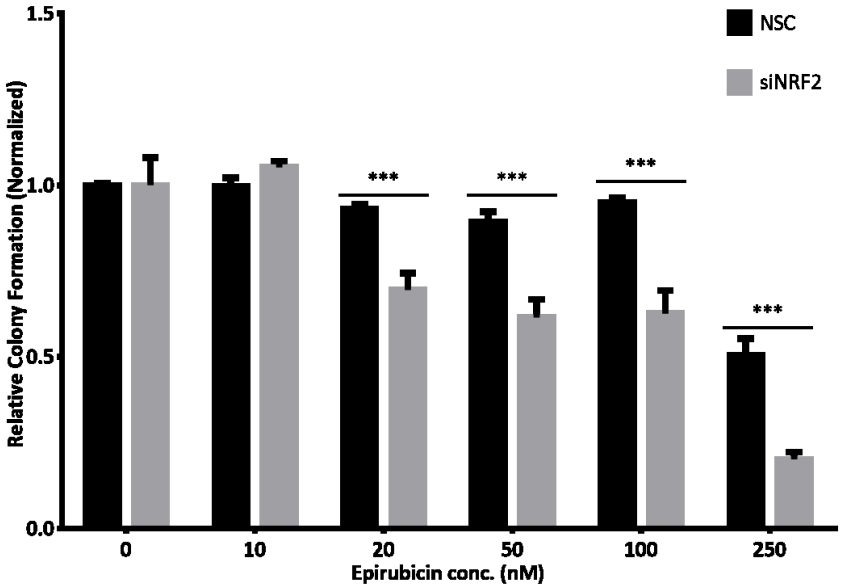

NSC
siNRF2
Relative Colony Formation (Normalized)
1.5
1.0
0.5
0.0
***
0
10
20
50
100
250
Epirubicin conc. (nM)

Figure 3.4 NRF2 depletion resensitize epirubicin resistant breast cancer cells to epirubicin treatment. MCF-7 EpiR cells were first transfected with specific siRNAs pool against NRF2 (siNRF2) or non-targeting siRNAs control (NSC) and then seeded for clonogenic assay. The cells were then treated with epirubicin for 48 hours at various concentrations (0, 10, 20, 50, 100 and 250 nM). After drug wash out, colonies were allowed to form for 14 days, followed by staining with crystal violet. The staining were quantified after dissolving in acetic acid and measured as absorbance (592 nm) by plate reader. Data were represented (i) as relative colony formation with reference to non-treated MCF-7 cells or (ii) as relative colony formation (normalized) at each epirubicin concentration were normalized to the control (non-treated condition 0 nM) as plotted in the graphs. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

3.2.3 NRF2 expression correlates with FOXM1 expression in breast cancer cells

Previous publications have reported that the roles of forkhead box transcription factors in drug resistance and they might take part in the regulation of NRF2 pathway (Guan, Zhang et al. 2016, Han, Shin et al. 2016). We hypothesized the forkhead box transcription factors were also involved in development of drug resistance in breast cancer via the NRF2 pathway.

To investigate the involvement of forkhead box transcription factors (FOXM1 and FOXO3) expression in the development of drug resistance via the NRF2 pathway, western blot analysis was first performed to compare the basal protein expression of FOXM1, FOXO3, NRF2 in MCF-7, MCF-7 EpiR and MCF-7 TaxR (Figue 3.5). The protein expressions of both FOXM1 and NRF2 were higher in MCF-7 EpiR and MCF-7 TaxR than MCF-7 while FOXO3 expressed at lower level in these resistant cells. It appeared that over-activation of FOXM1 in chemoresistant cells positively correlated with NRF2 upregulation while FOXO3 correlated negatively.

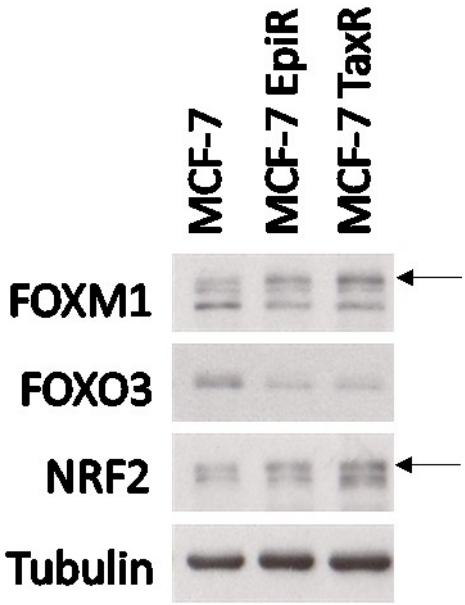
To further investigate the potential correlation between FOXM1 and the NRF2 pathway. MCF-7 and MCF-7 EpiR cells were treated with tBHP at 10 μM. The expression levels of FOXM1 and NRF2 were assessed by both Quantitative real-time PCR (qRT-PCR) and Western blot (Figure 3.6A). In MCF-7, although mRNA levels of FOXM1 and NRF2 did not show obvious changes within 24 hours of the treatment, both protein levels of FOXM1 and NRF2 were decreased at 16 h and 24 h. HO-1, an antioxidant enzyme and downstream target of NRF2 was induced, possibly as a response of tBHP mediated increase of NRF2. In contrast, for the MCF-7 EpiR cells, there were no significant changes in the levels of FOXM1, NRF2 or HO-1 during the 24 h treatment period, suggesting that MCF-7 EpiR cells were not responsive to the 10 μM tBHP

treatment in this experiment. This could be reasoned by the fact that 10 μM tBHP was not able to induce a significant reduction in cell proliferation in MCF-7 EpiR according to the SRB assay (Figure 3.1)

Similarly, MCF-7 and MCF-7 EpiR cells were treated with 1μM epirubicin for 0, 4, 8 16, 24 and 48 h (Figure 3.6B). Western blot analysis showed FOXM1 protein expression was decreased within 16 h and was completely abolished at 48 h in MCF-7 cells; NRF2 protein expression was also significantly lowered at 24 h and 48 h. Consistently, RT-qPCR showed epirubicin induced a strong reduction of FOXM1 and NRF2 mRNA levels in MCF-7 cells. Both FOXM1 and NRF2 expressions were downregulated in MCF-7 cells in the treatment of eprubicin. However, there was no obvious changes in the levels of FOXM1 and NRF2 following 48 h treatment in MCF-7 EpiR cells. FOXM1 mRNA levels at 48 h in MCF-7 EpiR was decreased while NRF2 levels in MCF-7 EpiR cells were not decreased. The decline of FOXM1 expression could more likely due to contact inhibition when cells were grown at high cell density (Coller, Sang et al. 2006) rather than as a response to epirubicin, as the cells were resistant epirubicin treatment.

Taken together, expressions of FOXM1 and NRF2 were down-regulated in response to oxidative stress and epirubicin in the sensitive MCF-7 cells, while FOXM1 and NRF2 expression might be deregulated in epirubicin resistant cells. These results again indicated the roles of FOXM1 and NRF2 in epirubicin sensitivity and resistance and is supportive that FOXM1 was positively related to NRF2 in MCF-7.

(A)


MCF-7
MCF-7 EpiR
MCF-7 TaxR
FOXM1
FOXO3
NRF2
Tubulin

(B)

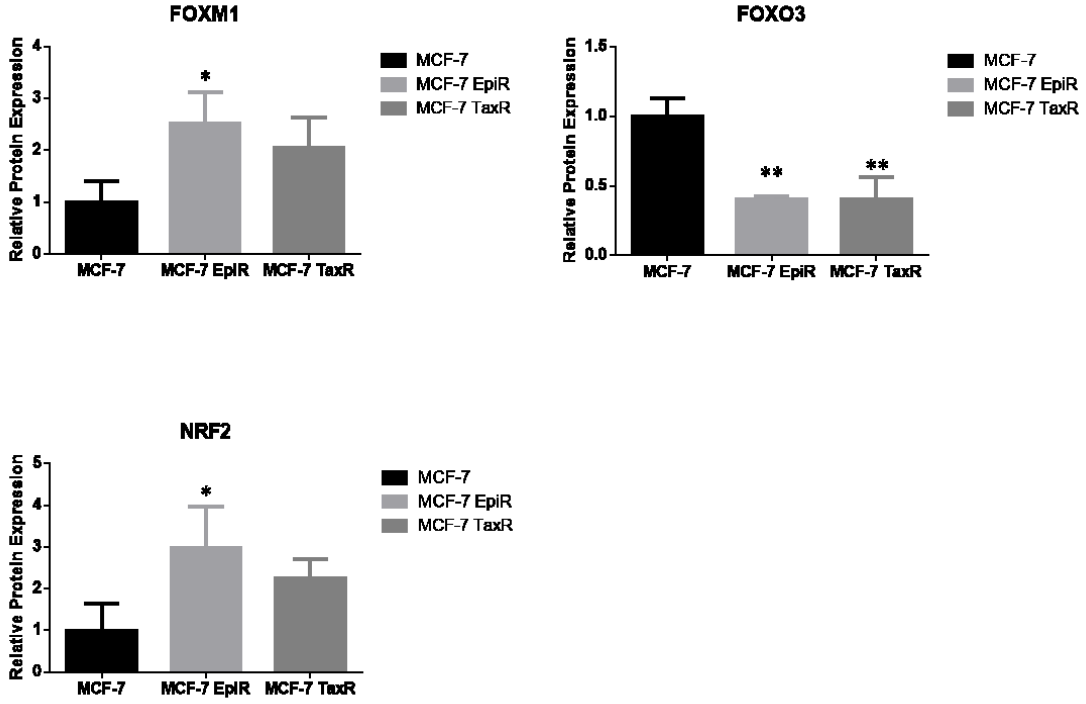
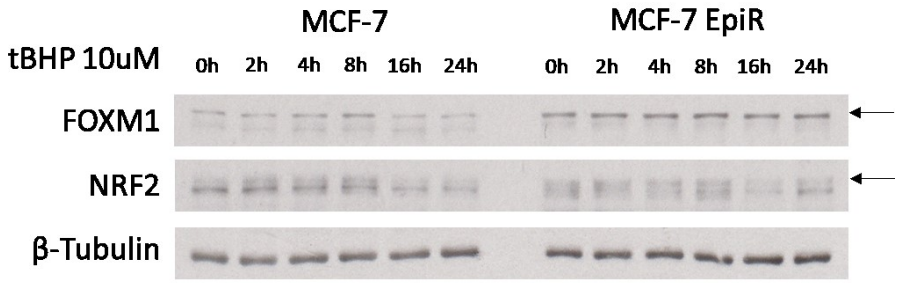

FOXM1
Relative Protein Expression
MCF-7
MCF-7 EpiR
MCF-7 TaxR
*
FOXO3
**
**
NRF2
*

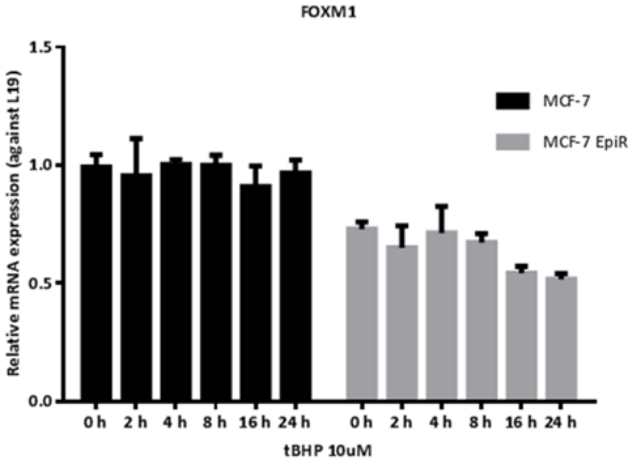
Figure 3.5 NRF2 correlates with FOXM1 expression but not FOXO3 expression at protein level in chemoresistant cells. Cell lysates of MCF-7, MCF-7 EpiR and MCF-7 TaxR cells were obtained and 10 μg of each cell line were loaded for SDS-PAGE followed by Western blotting. **(A)** Protein expression for FOXM1, FOXO3, NRF2 were shown and β-Tubulin served as internal control. Blot shown was representative image of three biological replicates. Arrows indicated the bands for the corresponding targets. **(B)** The band intensities were quantified by imageJ and normalized against the intensities of MCF-7. Results were represented as mean ± SD;*P<0.05; **P<0.01.

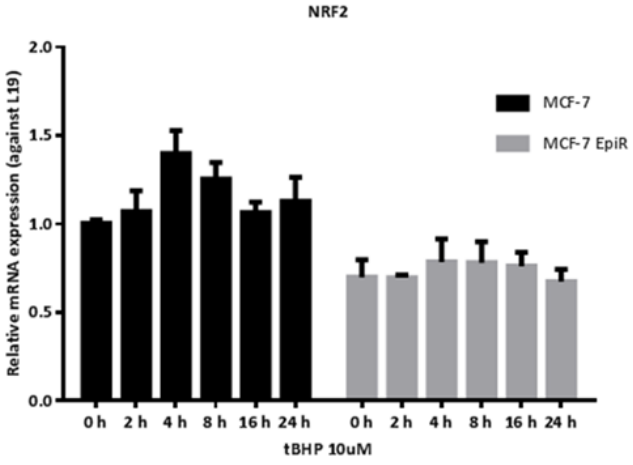
(A)

(i)


MCF-7
MCF-7 EpiR
tBHP 10uM
0h 2h 4h 8h 16h 24h
0h 2h 4h 8h 16h 24h
FOXM1
NRF2
β-Tubulin

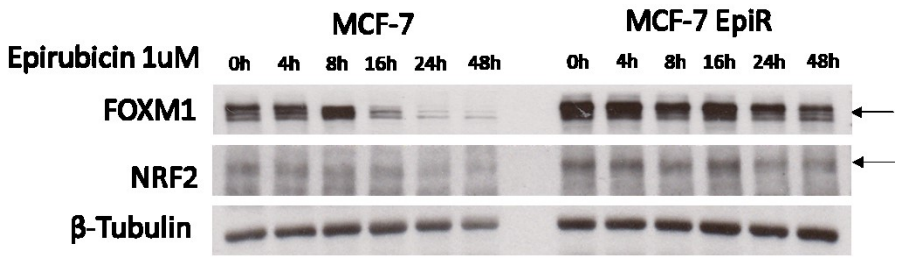
(ii)


FOXM1
Relative mRNA expression (against L19)
1.5
1.0
0.5
0.0
MCF-7
MCF-7 EpiR
0 h 2 h 4 h 8 h 16 h 24 h 0 h 2 h 4 h 8 h 16 h 24 h
tBHP 10uM

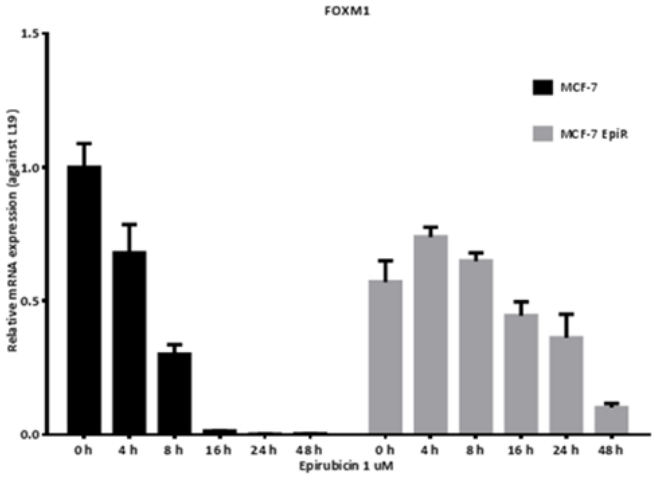

NRF2
Relative mRNA expression (against L19)
2.0
1.5
1.0
0.5
0.0
MCF-7
MCF-7 EpiR
0 h 2 h 4 h 8 h 16 h 24 h 0 h 2 h 4 h 8 h 16 h 24 h
tBHP 10uM

(B)

(i)


MCF-7
MCF-7 EpiR
Epirubicin 1uM
0h 4h 8h 16h 24h 48h
0h 4h 8h 16h 24h 48h
FOXM1
NRF2
β-Tubulin

(ii)


FOXM1
Relative mRNA expression (against L19)
1.5
1.0
0.5
0.0
MCF-7
MCF-7 EpiR
0 h 4 h 8 h 16 h 24 h 48 h 0 h 4 h 8 h 16 h 24 h 48 h
Epirubicin 1 uM

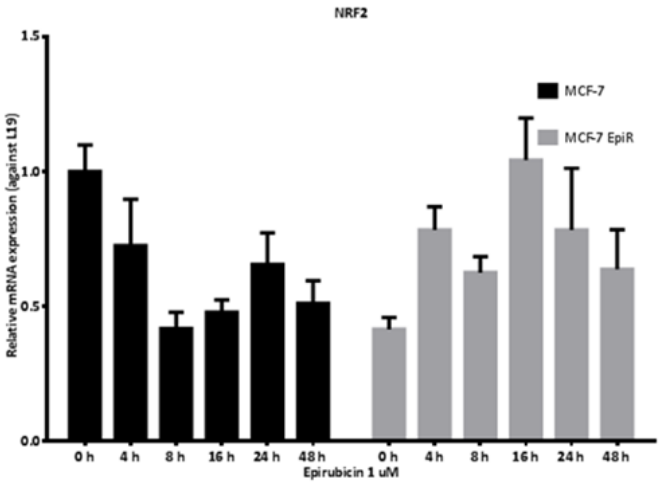

NRF2
Relative mRNA expression (against L19)
1.5
1.0
0.5
0.0
MCF-7
MCF-7 EpiR
0 h 4 h 8 h 16 h 24 h 48 h 0 h 4 h 8 h 16 h 24 h 48 h
Epirubicin 1 uM

Figure 3.6 Changes in FOXM1 and NRF2 expression in breast cancer cells in response to oxidative stress and epirubicin treatment. **(A)** MCF-7 cells and MCF-7 EpiR cells were treated with 10 μM tBHP for a duration of 24 h. Cells were harvested at 0, 2, 4, 8, 16 and 24 h. **(B)** MCF-7 cells and MCF-7 EpiR cells were treated with 1 μM epirubicin for a duration of 48 h. Cells were harvested at 0,4,8,16, 24 and 48 h. Untreated cells (0 h) were served as controls. (i) Western blot showed the changes in protein levels of FOXM1, NRF2 and HO-1; β-Tubulin served as loading control. Blot shown was representative image of three biological replicates. Arrows indicated the bands for the corresponding targets. (ii) RT-qPCR analysis showed changes in expression levels of FOXM1 and NRF2. The experiments were repeated three times.

3.2.4 FOXM1 regulates NRF2

Having demonstrated that a direct correlation between FOXM1 and NRF2, we hypothesized that FOXM1 could regulate NRF2 expression in human breast cancer. To this end, FOXM1 was overexpressed in MCF-7 cells to examine its effect on NRF2 expression by qRT-PCR and Western blotting. The result showed overexpression of FOXM1 caused a significant induction of NRF2 mRNA and protein levels (Figure 3.7A).

Similarly, FOXM1 was knocked down by FOXM1 specific siRNAs pool in MCF-7 and MCF-7 EpiR. It was found that NRF2 mRNA and protein levels were significantly reduced by depletion of FOXM1 in MCF-7 (Figure 3.7B). However, depletion of FOXM1 did not affect NRF2 expression in MCF-7 EpiR. These data indicated that FOXM1 is involved in the regulation of NRF2 expression in sensitive MCF-7 cells but not in the resistant cells. The control of FOXM1 on NRF2 expression might be deregulated in chemoresistant breast cancer.

Since it was shown that FOXM1 could regulate NRF2 expression in MCF-7, the effect of FOXM1 overexpression on NRF2 transcriptional activity was studied. Since antioxidant response element (ARE) is the main transcription binding site recognized by NRF2, a dual luciferase assay was employed to study the effect of FOXM1 overexpression on transcription of ARE-containing luciferase reporter (Figure 3.8). An ARE-containing luciferase reporter plasmid (pGL3-ARE) was constructed. MCF-7 cells were transfected with the FOXM1 expression plasmid (pCDNA3-FOXM1) or its empty vector control (pCDNA3-empty vector), ARE-dependent firefly luciferase reporter gene plasmid (pGL3-ARE) or its control vector (pGL3-basic) and renilla luciferase control reporter vector (pRL-SV40). The result were expressed as fold

activation normalized against vectors control (Empty vector + pGL3-basic was 1). Consistent to FOXM1 role in regulating NRF2 expression, ectopic overexpression of FOXM1 was found to significantly induce luciferase reporter activities in MCF-7 cells in all three conditions including non-treated (0 h), treated with 1μM epirubicin for 8 h and 24 h. This showed FOXM1 could mediate transcriptional activity on ARE, possibly via FOXM1 regulation on NRF2 expression.

Previously, it was noted that FOXM1 was found induced at protein level when treated with 1μM epirubicin for 8 h in MCF-7 (Figure 3.6). In this experiment, it was also found that transcription of ARE-containing luciferase reporter was induced by epirubicin treatment by comparing fold activation of empty vector + pGL3-ARE at untreated and 1μM epirubicin for 8 h conditions. Similar induction was also observed for FOXM1 expression plasmid + pGL3-ARE. This result indicated that transcriptional activity on ARE and hence NRF2 activity could be in response to epirubicin in sensitive MCF-7 cells.

As FOXM1 was found to regulate NRF2 expression and transcriptional activity on ARE, the effects of FOXM1 depletion on downstream NRF2 transcription target expression were studied. The mRNA expression of several previously reported NRF2 transcription targets, including GCLC, GCLM, ABCG2 and HMOX1 (HO-1), were evaluated by qRT-PCR (Figure 3.9). Compared to non-silencing siRNAs control (NSC), mRNA expression of these NRF2 target genes were significantly lowered by FOXM1 silencing in MCF-7 cells. The results showed FOXM1 could regulate NRF2 target genes expression and further supported that FOXM1 could control NRF2 activity in driving these target genes expression. It was found that depletion of FOXM1 in MCF-7 EpiR

cell line did not affect NRF2 expression. Therefore, it was not surprising that the NRF2 target genes did not show obvious change in mRNA levels upon depletion of FOXM1.

The data presented above indicated the overexpression of FOXM1 induced upregulation of NRF2 expression and ARE mediated transcription activity while knockdown of FOXM1 led to downregulation of NRF2 expression as well as downregulation of NRF2 target genes. Next, whether FOXM1 controls NRF2 at the transcriptional level in MCF-7 was investigated. Chromatin immunoprecipitation (ChIP) analysis was employed to investigate the putative binding region of FOXM1 at human NFE2L2 (NRF2) gene in MCF-7 cells.

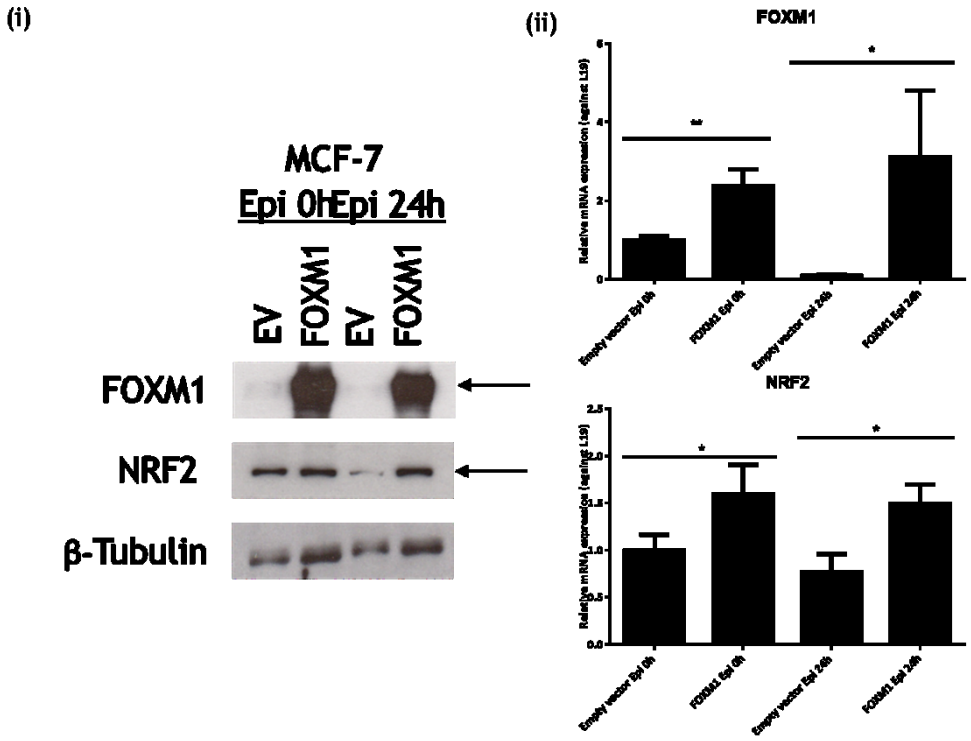
In silico analysis of the transcription factor ChIP-seq datasets was available in the Encyclopedia of DNA Elements (ENCODE) consortium. By analyzing the ChIP-seq data sets for FOXM1 in MCF-7 cells (ENCODE Experiment: ENCSR000BUJ), putative FOXM1 binding region for NFE2L2 was identified, located at NFE2L2 region with peak identified around chr2:178128000 to 178128500, which is near the transcription promoter region (Figure 3.10A). Recruitment of FOXM1 to this predicted binding site on NFE2L2 region was examined by ChIP followed by qRT-PCR (ChIP-qPCR) and primers for qPCR were designed to amplify across the same site. FOXM1 was either overexpressed or silenced in MCF-7 cells, followed by ChIP analysis (Figure 3.10B and Figure 3.10C). The result showed that FOXM1 binding site on NFE2L2 region was enriched for FOXM1 in MCF-7, denoted by the significant enrichment of protein binding comparing to the bead control. Overexpression of FOXM1 in MCF-7 further increased FOXM1 binding on NFE2L2 site significantly while silencing of FOXM1 significantly reduced FOXM1 binding on NFE2L2 site. This ChIP-qPCR result supported that NRF2 is a transcription target of FOXM1.

The effect of epirubicin treatment on FOXM1 binding on NFE2L2 region was then studied (Figure 3.11). MCF-7 cells and MCF-7 EpiR cells were treated with 1μM epirubicin for 0 and 24 h, followed by ChIP-qPCR analysis. In MCF-7, enrichment of FOXM1 binding to NRF2 region was significantly reduced upon treatment of epirubicin. This could be explained by the fact that FOXM1 expression was significantly reduced after 24 h treatment of epirubicin, therefore there were less FOXM1 available to bind to FOXM1 binding site. On the other hand, treatment of epirubicin to MCF-7 EpiR did not cause any significant change of enrichment in FOXM1 binding to NFE2L2 region as these resistant cells were not responsive to epirubicin treatment.

Comparing the FOXM1 binding to NRF2 region of MCF-7 and MCF-7 EpiR at basal condition (Epirubicin 0 h), it was found that enrichment in MCF-7 EpiR was significantly lower than that of MCF-7. This result suggested that there was intrinsically less FOXM1 binding on NFE2L2 region to drive the transcription of NRF2 in MCF-7 EpiR. The control of FOXM1 on NRF2 expression and NRF2 activity existed in MCF-7 but such control was possibly deregulated when MCF-7 developed resistance to epirubicin.

Collectively, the above results showed FOXM1 could regulate the expression of NRF2 by direct transcriptional control on the identified FOXM1 binding site on NFE2L2 region. FOXM1 regulation of NRF2 could alter transcriptional activity on ARE and the expression of downstream NRF2 target genes which can be driven by ARE. Also, epirubicin could mediate FOXM1 expression and hence NRF2 expression and NRF2 activity in sensitive MCF-7 cells. However, the regulation by FOXM1 on NRF2 was possibly deregulated when cells become resistant to epirubicin.

(A)


(i)
MCF-7
Epi 0h Epi 24h
EV
FOXM1
EV
FOXM1
FOXM1
NRF2
β-Tubulin
(ii)
FOXM1
NRF2

(B)

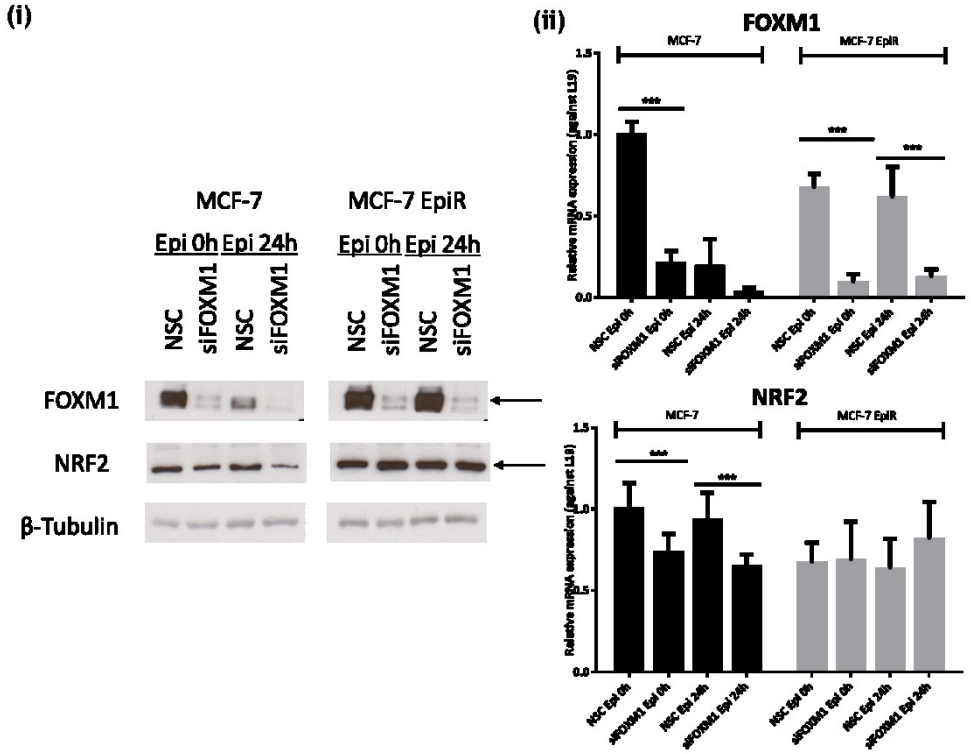

(i)
MCF-7
MCF-7 EpiR
Epi 0h Epi 24h
Epi 0h Epi 24h
NSC
siFOXM1
NSC
siFOXM1
NSC
siFOXM1
NSC
siFOXM1
FOXM1
NRF2
β-Tubulin
(ii)
FOXM1
NRF2
MCF-7
MCF-7 EpiR

Figure 3.7 FOXM1 regulates NRF2 expression in breast cancer. **(A)** MCF-7 cells were first transfected with FOXM1 expression plasmid (pCDNA3-FOXM1) or empty vector as control (pcDNA3-empty). **(B)** MCF-7 cells and MCF-7 EpiR cells were first transfected with specific siRNAs pool against FOXM1 (siFOXM1) or non-targeting siRNAs control (NSC). At post-transfection 48 h, the cells were either treated with 1 μM epirubicin (Epi) for 24 h or left untreated (as 0 h). (A i) and (B i) Western blot showed the changes in protein levels of FOXM1 and NRF2; β-Tubulin served as loading control. Blot shown was representative image of three biological replicates. Arrows indicated the bands for the corresponding targets. (A ii) and (B ii) RT-qPCR analysis showed changes in expression levels of FOXM1 and NRF2. The experiments were repeated three times. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

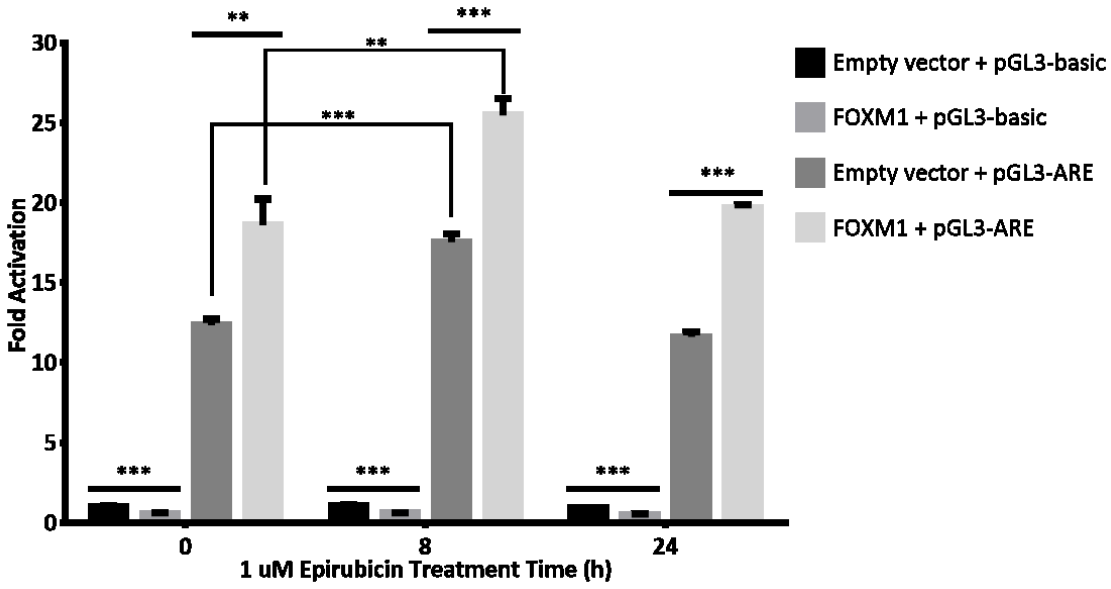


Figure 3.8 FOXM1 regulates transcription on antioxidant response element (ARE). MCF-7 cells were first transfected with FOXM1 expression plasmid (pCDNA3-FOXM1) or empty vector as control and ARE-firefly luciferase reporter plasmid (pGL3-ARE) or basic firefly luciferase reporter plasmid (pGL3-basic) together with renilla luciferase control reporter plasmid (pRL-SV40). At post transfection 48 h, transfected cells were either treated with 1 μM epirubicin (Epi) for 8h, 24 h or left untreated (as 0 h). At the designed time points, cells were harvested and the luciferase activities were measured by luminometer. The results were represented as fold activation normalized to renilla luciferase activity with reference to control cells (Empty vector + pGL3 basic as 1). The experiments were repeated three times. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

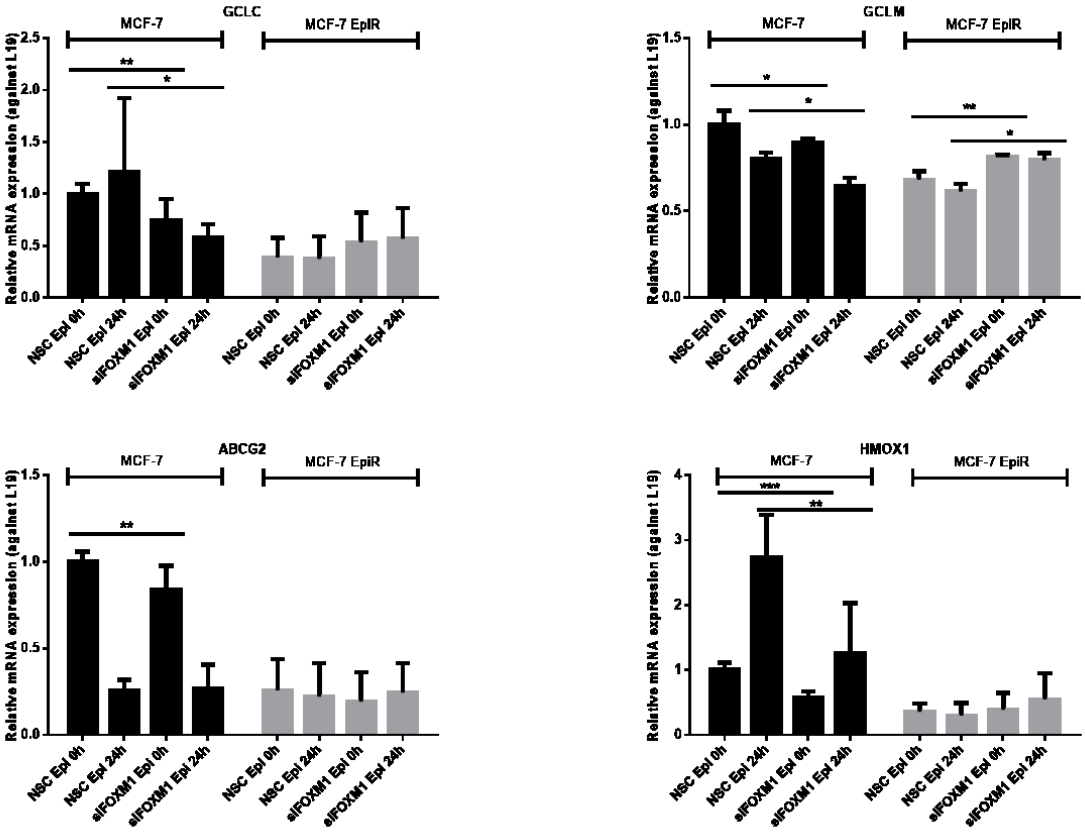
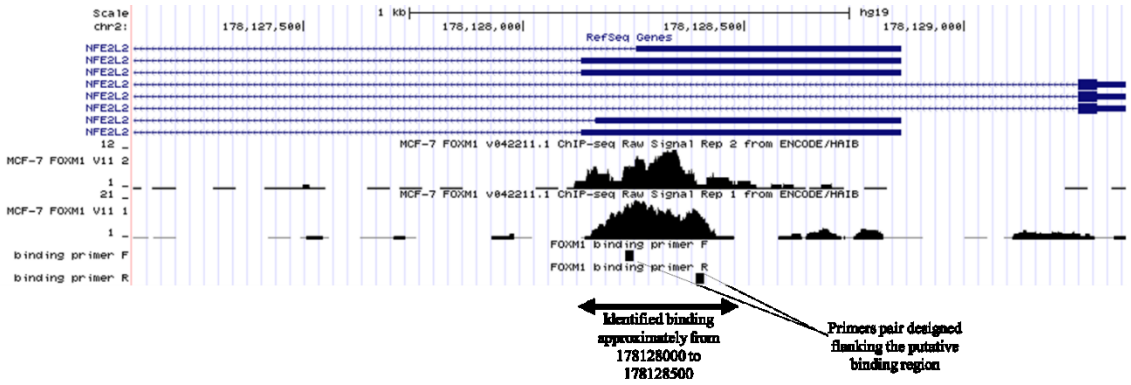
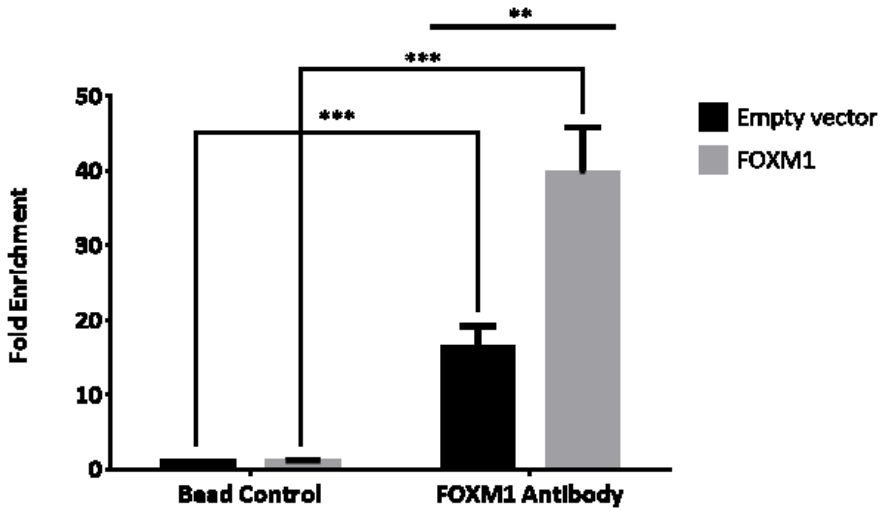


Figure 3.9 FOXM1 regulates mRNA expressions of NRF2 target genes in breast cancer. MCF-7 cells and MCF-7 EpiR cells were first transfected with specific siRNAs pool against FOXM1 (siFOXM1) or non-targeting siRNAs control (NSC). At post-transfection 48 h, the cells were either treated with 1 μM epirubicin (Epi) for 24 h or left untreated (as 0 h). RT-qPCR analysis showed changes in expression levels of GCLC, GCLM, ABCG2 and HMOX1. The experiments were repeated three times. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

(A)


Scale
chr2:
178,127,500
1 kb
178,128,000
178,128,500
hg19
178,129,000
RefSeq Genes
NFE2L2
MCF-7 FOXM1 V11 2
MCF-7 FOXM1 V11 1
binding primer F
binding primer R
MCF-7 FOXM1 v042211.1 ChIP-seq Raw Signal Rep 2 from ENCODE/HAIB
MCF-7 FOXM1 v042211.1 ChIP-seq Raw Signal Rep 1 from ENCODE/HAIB
FOXM1 binding primer F
FOXM1 binding primer R
Identified binding approximately from 178128000 to 178128500
Primers pair designed flanking the putative binding region

(B)


**
***
***
Fold Enrichment
50
40
30
20
10
0
Bead Control
FOXM1 Antibody
Empty vector
FOXM1

(C)

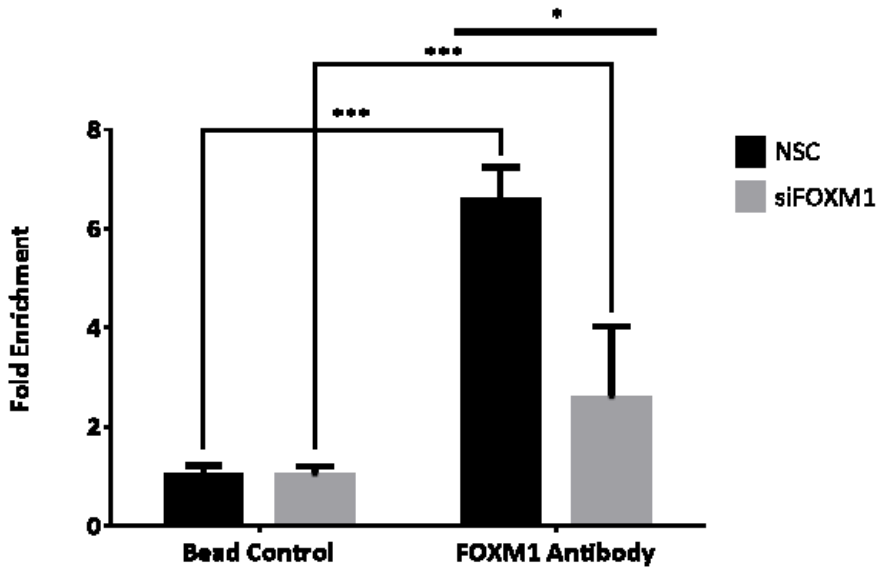

*
***
***
Fold Enrichment
8
6
4
2
0
Bead Control
FOXM1 Antibody
NSC
siFOXM1

Figure 3.10 FOXM1 binding to NRF2 region is detected in breast cancer. **(A)** The predicted binding site of FOXM1 to NRF2 region in MCF-7 cells. Image was adapted from UCSC Genome Browser and ENCODE annotation DATA. The putative binding region was indicated by arrows and positions of designed primers pair flanking the putative site were annotated in the image. **(B)** MCF-7 cells were either transfected with FOXM1 expression plasmid (pcDNA3-FOXM1) or empty vector as control; or **(C)** specific siRNAs pool against FOXM1 (siFOXM1) or non-targeting siRNAs control (NSC). Cells were harvested and fixed at post transfection 48 h, followed by CHIP assay. The results of CHIP-qPCR analysis were shown as fold enrichment with reference to non-binding control (bead control). The experiments were repeated three times. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

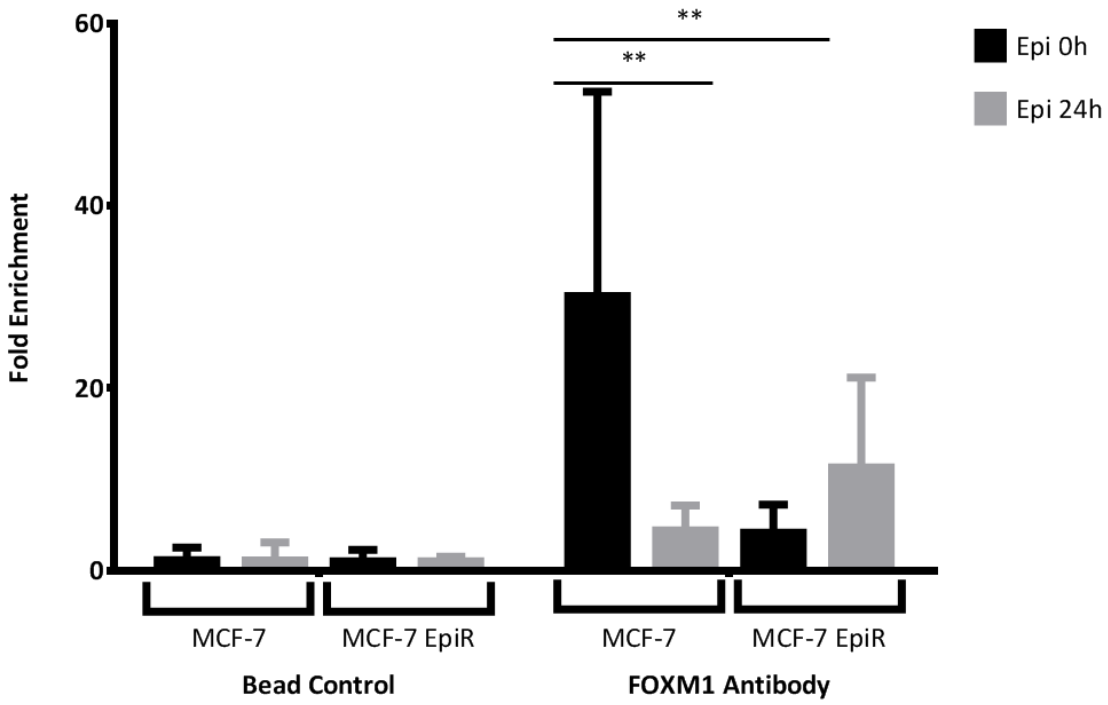


Figure 3.11 FOXM1 binding to NRF2 region in drug sensitive cells and chemoresistant cells in response to epirubicin treatment. MCF-7 cells and MCF-7 EpiR cells were treated with 1 μM epirubicin (Epi) for 24 h or left untreated (as 0 h). Cells were then harvested and fixed at post transfection 48 h, followed by CHIP assay. The results of CHIP-qPCR analysis were shown as fold enrichment with reference to non-binding control (bead control). The experiments were repeated three times. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

3.2.5 NRF2 can mediate the expression of FOXM1

It has been reported that NRF2 can promote cancer cell proliferation and regulate transcription factors and genes involved in cell growth and survival as well as cell cycle progression (Malhotra, Portales-Casamar et al. 2010). FOXM1 is a transcription factor important in cancer development and drug resistance (Kwok, Peck et al. 2010, Millour, de Olano et al. 2011, Park, Jung et al. 2012, Khongkow, Gomes et al. 2016), which also has function as transcriptional regulator of cell cycle progression (Chen, Muller et al. 2013). Although previous study from NRF2 pathway knockout mouse embryonic fibroblast cell lines was not able to identify FOXM1 as one of the NRF2 targets, it is possible that NRF2 could also regulate FOXM1 expression based on its role in promoting cell cycle progression.

To test this idea, NRF2 was overexpressed in MCF-7 cells and MCF-7 EpiR cells to examine its effect on FOXM1 expression with or without epirubicin treatment. As shown in figure 3.12A, both qRT-PCR and Western blot results showed NRF2 were successfully overexpressed in MCF-7 and MCF-7 EpiR though it appeared the extent of overexpression was higher in MCF-7. For MCF-7, although qRT-PCR result showed FOXM1 mRNA level was slight lowered upon overexpression of NRF2 without epirubicin treatment compared to that of empty vector control, Western blot results showed FOXM1 protein expression was increased. Overexpression of NRF2 in MCF-7 also lead to increased expression of FOXM1 upon epirubicin treatment at mRNA level albeit slightly at protein levels. CyclinB1 is a known transcription target of FOXM1 involved in cell cycle progression. In agreement with FOXM1 expression pattern, CCNB1 mRNA level was slight lowered in NRF2 overexpressing cells without epirubicin treatment compared to that of empty vector control. In MCF-7 EpiR,

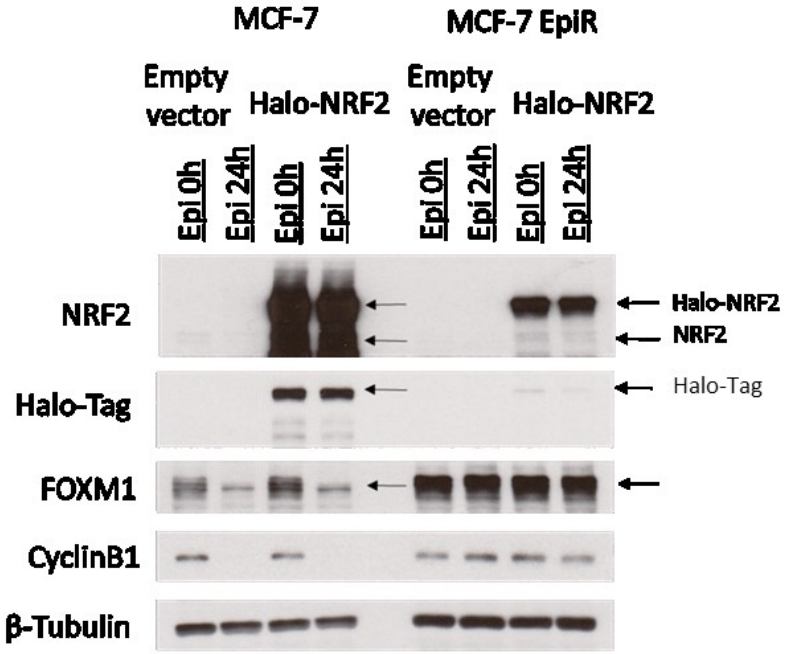
however, overexpression of NRF2 did not cause significant changes of FOXM1 mRNA and protein levels with or without epirubicin treatment.

Similarly, NRF2 was knocked-down by NRF2 specific siRNAs pool in MCF-7 and MCF-7 EpiR with or without epirubicin treatment (Figure 3.12B). For MCF-7, it was found that FOXM1 mRNA and protein levels was significantly reduced in untreated cells. However, there was no significant change in FOXM1 expression with or without NRF2 knockdown when under epirubicin treatment. It could be reasoned by that after 24 h epirubicin treatment, expression of FOXM1 was so drastically reduced that any possible difference of FOXM1 would be relatively negligible. Similar to FOXM1, expression of CyclinB1 was significantly lowered by 24 h treatment of epirubicin or by NRF2 silencing alone. At protein level, CyclinB1 expression was completely abolished by NRF2 silencing together with epirubicin treatment. Taken together, the above results showed that NRF2 regulates FOXM1 expression as well as FOXM1 downstream target CyclinB1 in sensitive MCF-7 cells. However, depletion of NRF2 did not affect FOXM1 or CyclinB1 expression significantly in MCF-7 EpiR.

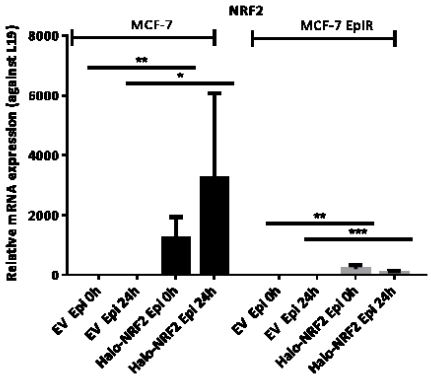
Taken together, knockdown of NRF2 lowered expressions of FOXM1 and CyclinB1 suggested that NRF2 might control their expressions. However, NRF2 overexpression only induced FOXM1 protein expression but these was no increase for FOXM1 or cyclin B1 at mRNA level. The result implied that NRF2 might not transcriptionally regulate FOXM1 directly. As *FOXM1* is a cell cycle regulated gene, the control of FOXM1 expression by NRF2 could be an indirect effect of NRF2 role in promoting cell cycle progression.

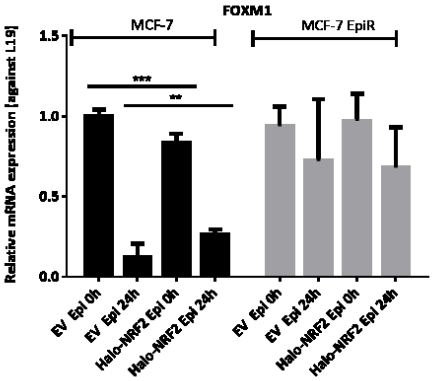
(A)

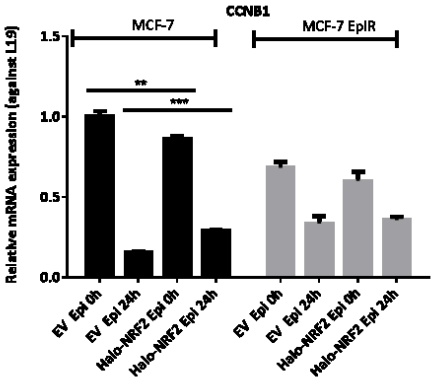
(i)


MCF-7
MCF-7 EpiR
Empty vector
Halo-NRF2
Empty vector
Halo-NRF2
Epi 0h
Epi 24h
Epi 0h
Epi 24h
Epi 0h
Epi 24h
Epi 0h
Epi 24h
NRF2
Halo-Tag
FOXM1
CyclinB1
β-Tubulin
Halo-NRF2
NRF2
Halo-Tag

(ii)

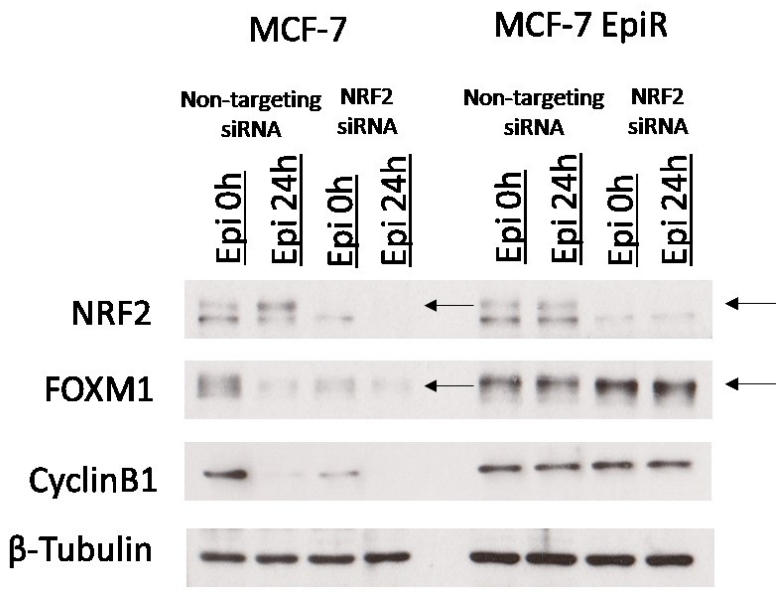

NRF2
MCF-7
MCF-7 EpiR
Relative mRNA expression (against L19)
8000
6000
4000
2000
0
**
*
**
***
EV Epi 0h
EV Epi 24h
Halo-NRF2 Epi 0h
Halo-NRF2 Epi 24h


FOXM1
MCF-7
MCF-7 EpiR
Relative mRNA expression (against L19)
1.5
1.0
0.5
0.0
***
**
EV Epi 0h
EV Epi 24h
Halo-NRF2 Epi 0h
Halo-NRF2 Epi 24h

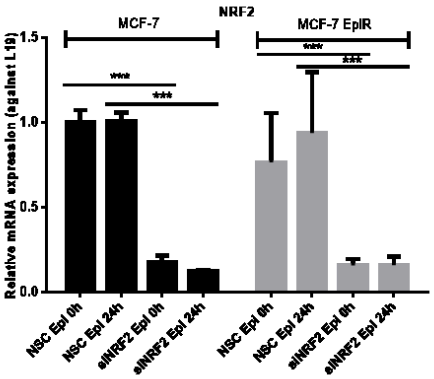

CCNB1
MCF-7
MCF-7 EpiR
Relative mRNA expression (against L19)
1.5
1.0
0.5
0.0
**
***
EV Epi 0h
EV Epi 24h
Halo-NRF2 Epi 0h
Halo-NRF2 Epi 24h

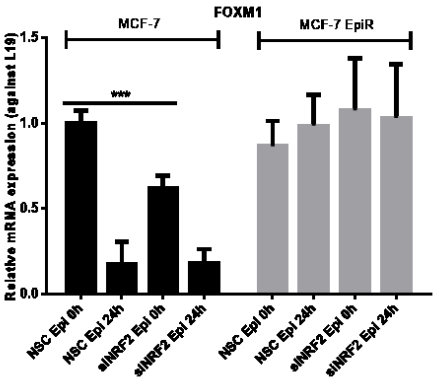
(B)

(i)


MCF-7
MCF-7 EpiR
Non-targeting siRNA
NRF2 siRNA
Non-targeting siRNA
NRF2 siRNA
Epi 0h
Epi 24h
Epi 0h
Epi 24h
Epi 0h
Epi 24h
Epi 0h
Epi 24h
NRF2
FOXM1
CyclinB1
β-Tubulin

(ii)


NRF2
MCF-7
MCF-7 EpiR
Relative mRNA expression (against L19)
NSC Epi 0h
NSC Epi 24h
siNRF2 Epi 0h
siNRF2 Epi 24h


FOXM1
MCF-7
MCF-7 EpiR
Relative mRNA expression (against L19)
NSC Epi 0h
NSC Epi 24h
siNRF2 Epi 0h
siNRF2 Epi 24h

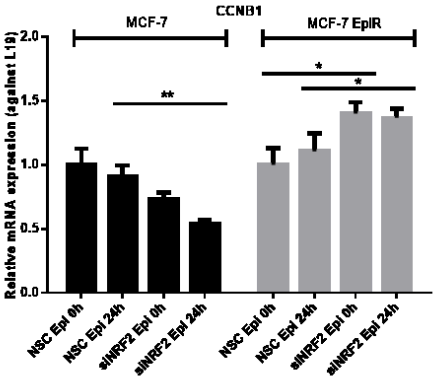

CCNB1
MCF-7
MCF-7 EpiR
Relative mRNA expression (against L19)
NSC Epi 0h
NSC Epi 24h
siNRF2 Epi 0h
siNRF2 Epi 24h

Figure 3.12 NRF2 mediates FOXM1 and Cyclin B1 expression in breast cancer. **(A)** MCF-7 cells and MCF-7 EpiR cells were first transfected with NRF2 expression plasmid (pFN21A-Halo-NRF2) or empty vector as control. **(B)** MCF-7 cells and MCF-7 EpiR cells were first transfected with specific siRNAs pool against NRF2 (siNRF2) or non-targeting siRNAs control (NSC). At post-transfection 48 h, the cells were either treated with 1 μM epirubicin (Epi) for 24 h or left untreated (as 0 h). (i) Western blot showed the changes in protein levels of FOXM1, NRF2 and CyclinB1; β-Tubulin served as loading control. Blot shown was representative image of three biological replicates. Arrows indicated the bands for the corresponding targets. (ii) RT-qPCR analysis showed changes in expression levels of FOXM1,NRF2 and CCNB1. The experiments were repeated three times. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

3.2.6 NRF2 inhibition enhanced drug sensitivity in breast cancer

NRF2 has shown roles in mediating drug response in breast cancer. Upregulation of NRF2 conferred epirubicin resistance in MCF-7 cells while depletion of NRF2 sensitized cells to epirubicin. An NRF2 inhibitor, brusatol (Olayanju, Copple et al. 2015), was used to study whether this small molecule has the potential to enhance response of breast cancer cells to epirubicin treatment. Brusatol is a naturally occurring small molecule found in *Brucea janica* plant. Its mechanism of action is suggested to be via inhibiting NRF2 mRNA translation at nanomolar concentrations (Zhu, Wang et al. 2016).

Different doses of brusatol were used to treat MCF-7 cells to determine the concentration needed to inhibit NRF2. MCF-7 cells were treated with 0, 0.1, 1, 5, 10, 20 nM of brusatol for 24 h and the cell samples were collected for Western Blot analysis (Figure 3.13A). The results showed that 5, 10 and 20 nM brusatol could significantly reduce the protein level of NRF2 while there were no obvious changes at 0.1 and 1 nM compared to the 0 nM control in MCF-7 cells. Protein levels of FOXM1 did not showed any obvious trend of changes with increasing concentration of brusatol. Its expression appeared to be slightly lower at 5 and 10 nM brusatol than the 0 nM control.

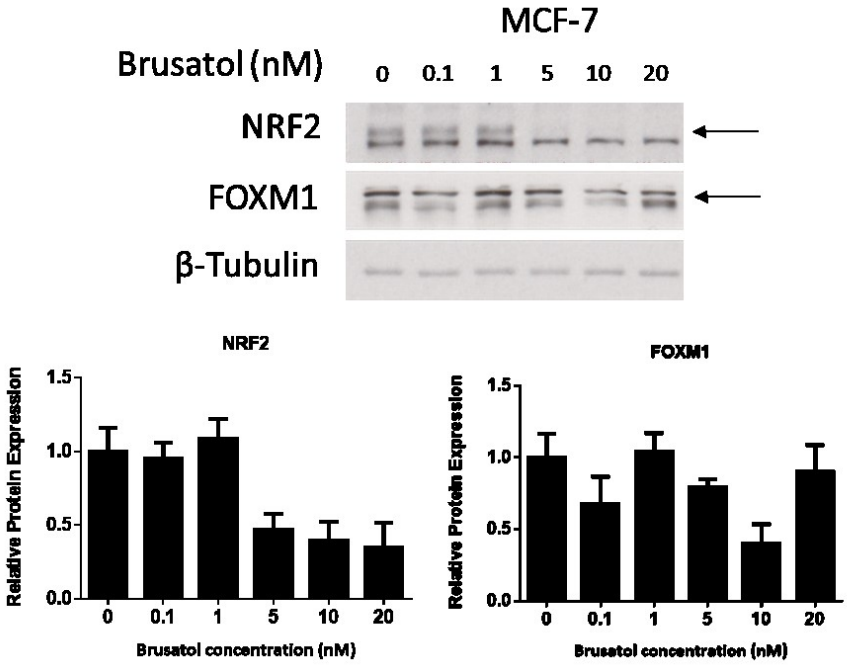
Next, the effect on cell viability of brusatol in MCF-7 cells was determined by clonogenic assay (Figure 3.13B). MCF-7 cells were treated with 0, 0.1, 1, 5, 10, 20 nM of brusatol to induce NRF2 inhibition and grown for 2 weeks. The clonogenic assay results showed that 20 nM brusatol could significantly decrease the clonogenicity of MCF-7 cells while other brusatol dosages either showed certain increase or no significant changes in clonogenicity of MCF-7 cells. The results indicated that all concentrations of brusatol were not toxic to MCF-7 cells in this experiment.

To test whether brusatol could enhance effect of epirubicin treatment to breast cancer cells, MCF-7 cells were co-treated with different concentrations of epirubicin (0, 1, 5, 10, 20 and 50 nM) and brusatol (0, 1, 5 and 10 nM), followed by clonogenic assays (Figure 3.14A). The result was expressed as normalized relative cell viability against 0 nM epirubicin control samples (Figure 3.14B), which can reflect the effect of different brusatol concentration on cell viability under the same epirubicin concentration. From data of treating epirubicin alone (Blue bars), 10, 20 and 50 nM epirubicin could significantly decrease the cell viability of MCF-7 cells while 5 nM epirubicin showed no significant changes and 1 nM epirubicin could significantly increase cell viability of MCF-7 cells. The increased cell viability at 1 nM could possibly due to the induction of cellular survival response at low dose of drug.

Under epirubicin of 1 nM, even though cell viability of MCF-7 were increased by epirubicin treatment, addition of brusatol of 5 (green bars) and 10 nM (purple bars) resulted in reduction of cell viability. Under 5 nM epirubicin treatment, MCF-7 cells showed no difference in cell viability whilst there was a slight reduction at epirubicin of 10 nM. Likewise at 5 nM and 10 nM epirubicin, cells treated with brusatol of 1 (red bars), 5 or 10 nM showed further reduction in cell viability of MCF-7 cells compared to 0 nM brusatol control.

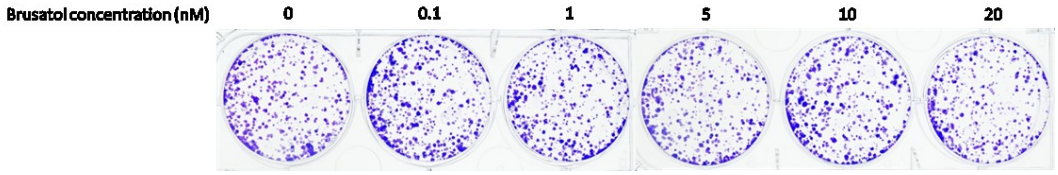
The results suggested that a sublethal dosage of brusatol could re-sensitize MCF-7 cells under a non- lethal dose of epirubicin. Epirubicin treatment could synergistically act together with brusatol in inducing cell death of breast cancer cells.

(A)


MCF-7
Brusatol (nM)
0 0.1 1 5 10 20
NRF2
FOXM1
β-Tubulin
NRF2
Relative Protein Expression
1.5
1.0
0.5
0.0
0 0.1 1 5 10 20
Brusatol concentration (nM)
FOXM1
Relative Protein Expression
1.5
1.0
0.5
0.0
0 0.1 1 5 10 20
Brusatol concentration (nM)

(B)

(i)


Brusatol concentration (nM)
0
0.1
1
5
10
20

(ii)

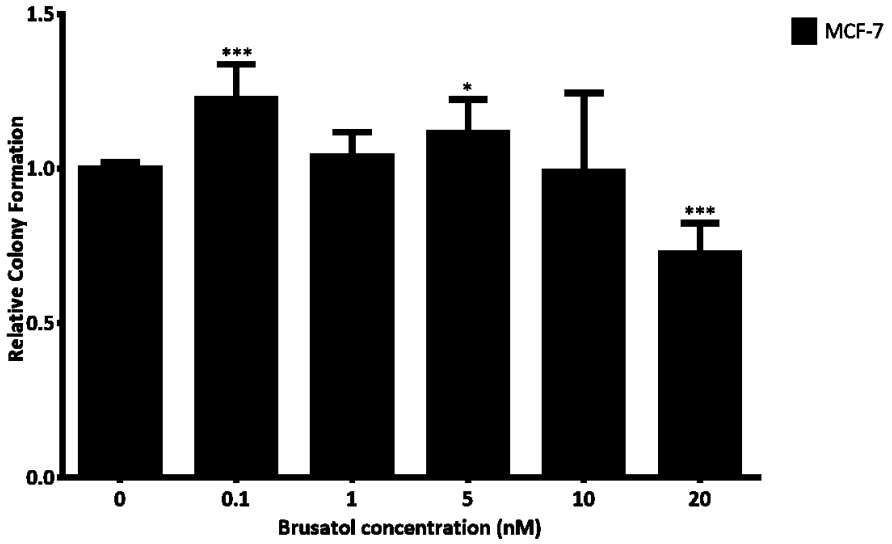
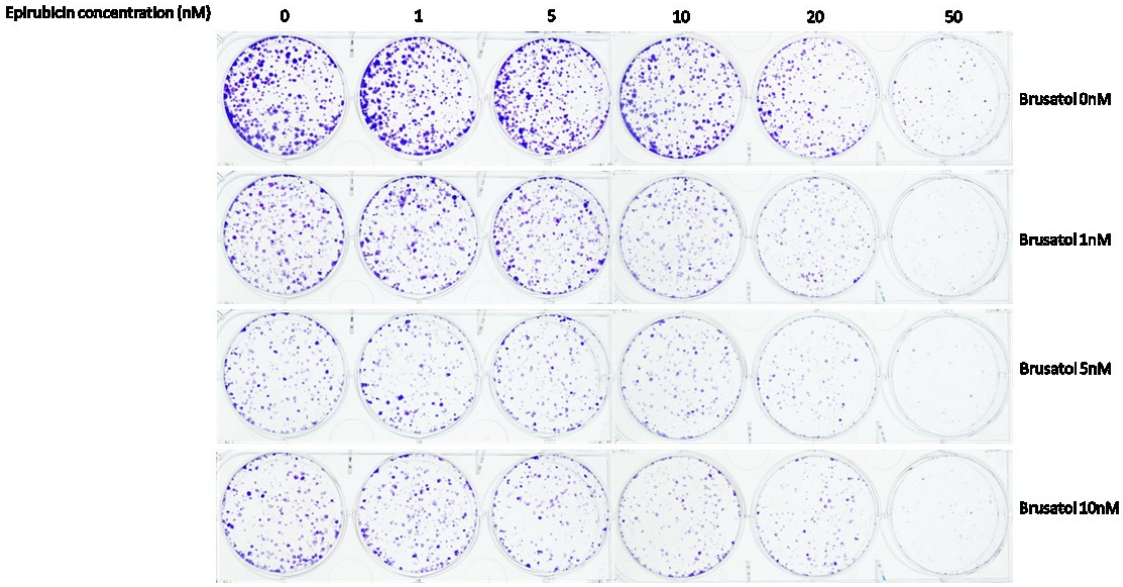

MCF-7
Relative Colony Formation
1.5
1.0
0.5
0.0
***
*
***
0 0.1 1 5 10 20
Brusatol concentration (nM)

Figure 3.13 Brusatol depletes NRF2 protein level in breast cancer cells. **(A)** MCF-7 cells were treated with brusatol of different concentrations (0, 0.1, 1, 5, 10, 20 nM) for 24 h. Protein lysates were obtained from treated cells, followed by SDSPAGE. Western blot showed the protein level of NRF2 and FOXM1; β-Tubulin served as loading control. Arrows indicated the bands for the corresponding targets. The band intensities were quantified by imageJ and normalized against the intensities of 0 nM. Results were represented as mean ± SD;*P<0.05; **P<0.01. **(B)** (i) Cells were treated with brusatol for 48 hours. After drug wash out, colonies were allowed to form for 14 days, followed by staining with crystal violet. The staining were quantified after dissolving in acetic acid and measured as absorbance (592 nm) by plate reader. (ii) Results are represented as relative colony formation with reference to non-treated MCF-7 cells; The experiments were repeated for three times and data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

(A)


Epirubicin concentration (nM)
0
1
5
10
20
50
Brusatol 0nM
Brusatol 1nM
Brusatol 5nM
Brusatol 10nM

(B)

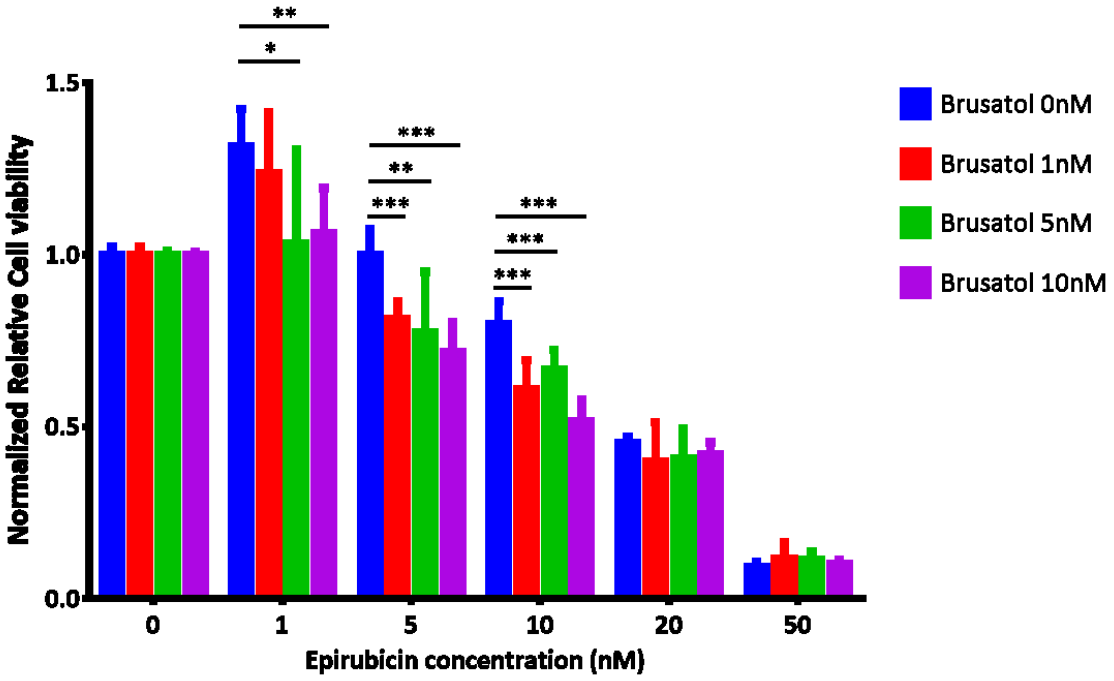

Normalized Relative Cell viability
1.5
1.0
0.5
0.0
0
1
5
10
20
50
Epirubicin concentration (nM)
**
*
***
**
***
***
***
***
Brusatol 0nM
Brusatol 1nM
Brusatol 5nM
Brusatol 10nM

Figure 3.14 Brusatol can act synergistically to sensitize breast cancer cells in response to epirubicin treatment. **(A)** MCF-7 cells were treated with various concentration of epirubicin (0, 1, 5, 10, 20 and 50 nM) alone or in combination with different concentrations of brusatol (0, 1, 5 and 10 nM) for 48 hours. After drug wash out, colonies were allowed to form for 14 days, followed by staining with crystal violet. The staining were quantified after dissolving in acetic acid and measured as absorbance (592 nm) by plate reader. **(B)** Results are represented as normalized relative colony formation with reference to non-epirubicin treated control; The experiments were repeated for three times and data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

3.2.7 FOXM1 and NRF2 expressions positively correlated in clinical samples of breast tumors

Having established that FOXM1 positively regulates NRF2 expression through direct transcriptional control and NRF2 might also regulate FOXM1 expressions thorough indirect effect in promoting of cell cycle progression in breast cancer cell line MCF-7, I next investigated the association between FOXM1 expression and NRF2 expression status.

To confirm the role of FOXM1 and NRF2 in the outcome of breast cancer patients, the relationship between these two factors and the survival of breast cancer patients were first analyzed. Kaplan-Meier analysis of mRNA levels of FOXM1 and NRF2 were performed using available public datasets from Kaplan-Meier Plotter (Gyorffy, Lanczky et al. 2010). Kaplan-Meier analysis of 3951 breast tumor samples demonstrated that higher mRNA expression of FOXM1 as well as higher mRNA expression of NRF2 were correlated with poorer disease-free survival (the time between primary treatment and the earliest of relapse or death) and poorer post progression survival (the time between disease progression and death) (Figure 3.15). These analyses might imply both FOXM1 and NRF2 behave as oncogenes.

We showed that FOXM1 expression correlated directly with NRF2 expression at both mRNA and protein levels in MCF-7 cell line. To further confirm the relationship at protein levels between FOXM1 and NRF2 in clinical breast tumor samples. Immunohistochemical (IHC) staining of FOXM1 and NRF2 and scoring of their expressions were performed in tissue microarray constructed with 99 cases of primary breast cancer samples. The representative images of the IHC staining of FOXM1 and NRF2 in breast cancer samples were shown in Figure 3.16. FOXM1 and NRF2

expressions were detected in both cytoplasm and nucleus. The expression levels of cytoplasmic and nuclear FOXM1 and NRF2 were scored respectively in a semi-quantitative manner as described in Chapter 2.12 (Khongkow, Karunarathna et al. 2014).

Since both FOXM1 and NRF2 are transcription factors and function in the nucleus. The TMA samples were divided into two groups: high nuclear FOXM1 expression and low nuclear FOXM1 expression, according to score of FOXM1 for each sample. Statistical analysis revealed that the median score of all samples analyzed for nuclear expression of FOXM1 is 6.25. The median score was set as the cutoff value, meaning that samples with score lower than or equal to 6.25 were regarded as low nuclear FOXM1 expression group while samples with score higher than 6.25 were regarded as high nuclear FOXM1 expression group. Nuclear score of NRF2 expression for each sample were plotted against these two group, as shown in Figure 3.17. It was found that the average NRF2 protein expression level were significant higher in high nuclear FOXM1 expression group than that of low nuclear FOXM1 expression group. This *in vivo* data provided strong evidence for the *in vitro* finding that FOXM1 positively regulates NRF2 expression and vice versa.

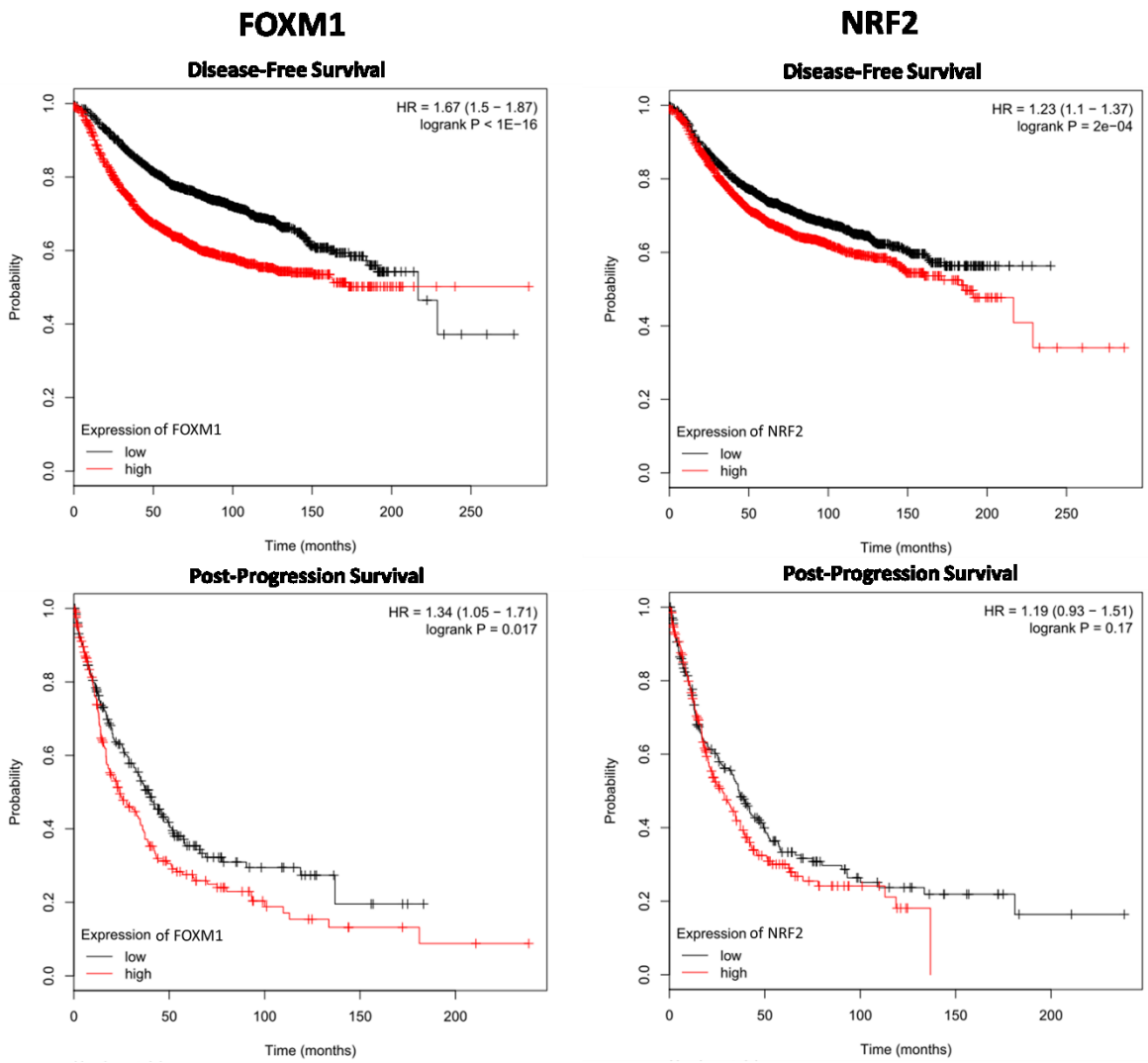


Figure 3.15 Prognostic significance of FOXM1 and NRF2 in breast cancer. Kaplan-Meier analysis of 3951 breast tumor samples using available public datasets from Kaplan-Meier Plotter. The mRNA expressions of FOXM1 and NRF2 were compared. (Gyorffy et al., 2010)

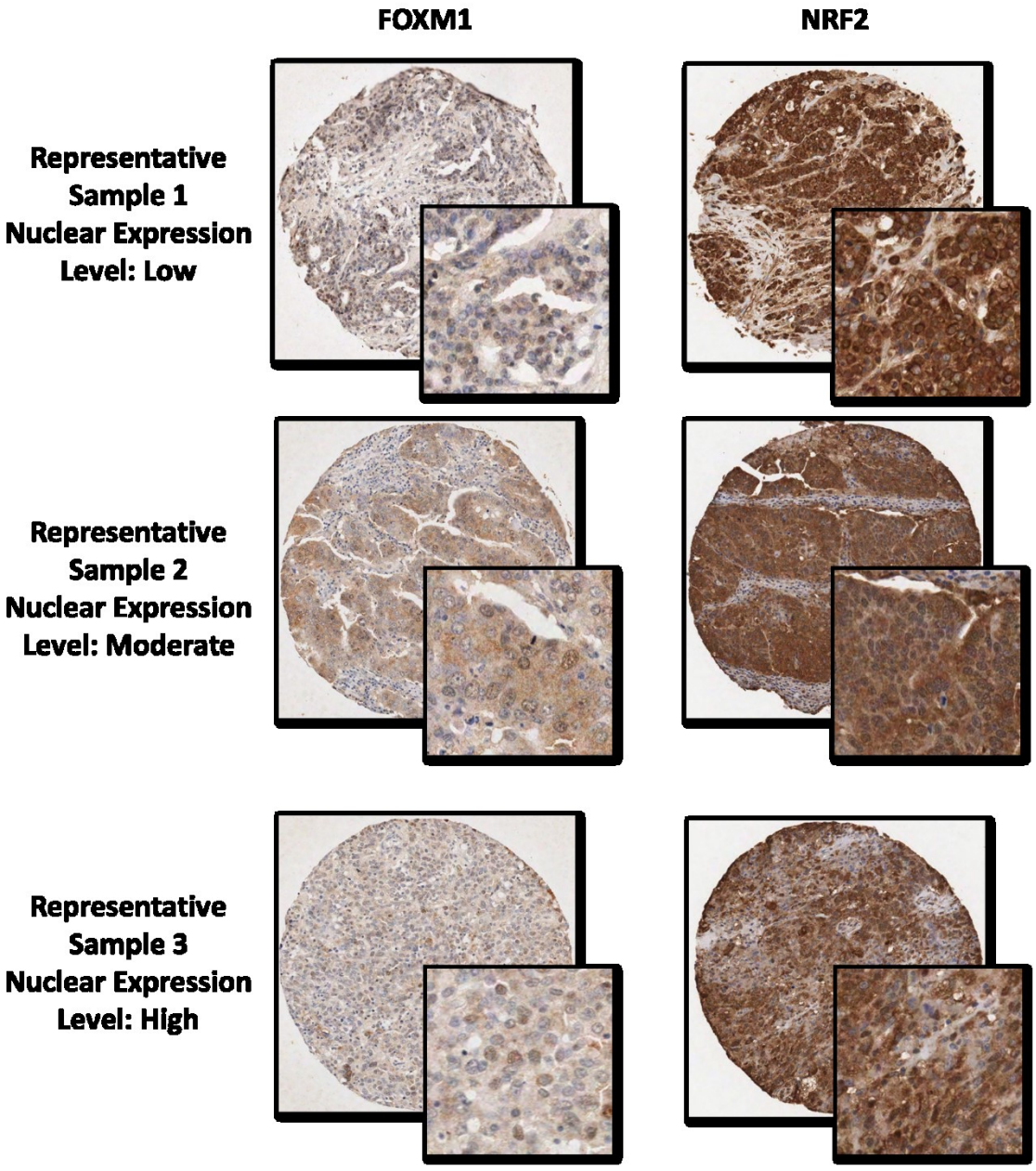


Figure 3.16 The representative images of the immunohistochemical (IHC) staining of FOXM1 and NRF2 in primary breast cancer samples. Images shown were selected corresponding to nuclear FOXM1 expression associated with nuclear NRF2 expression at low, moderate or high levels in the same clinical sample.

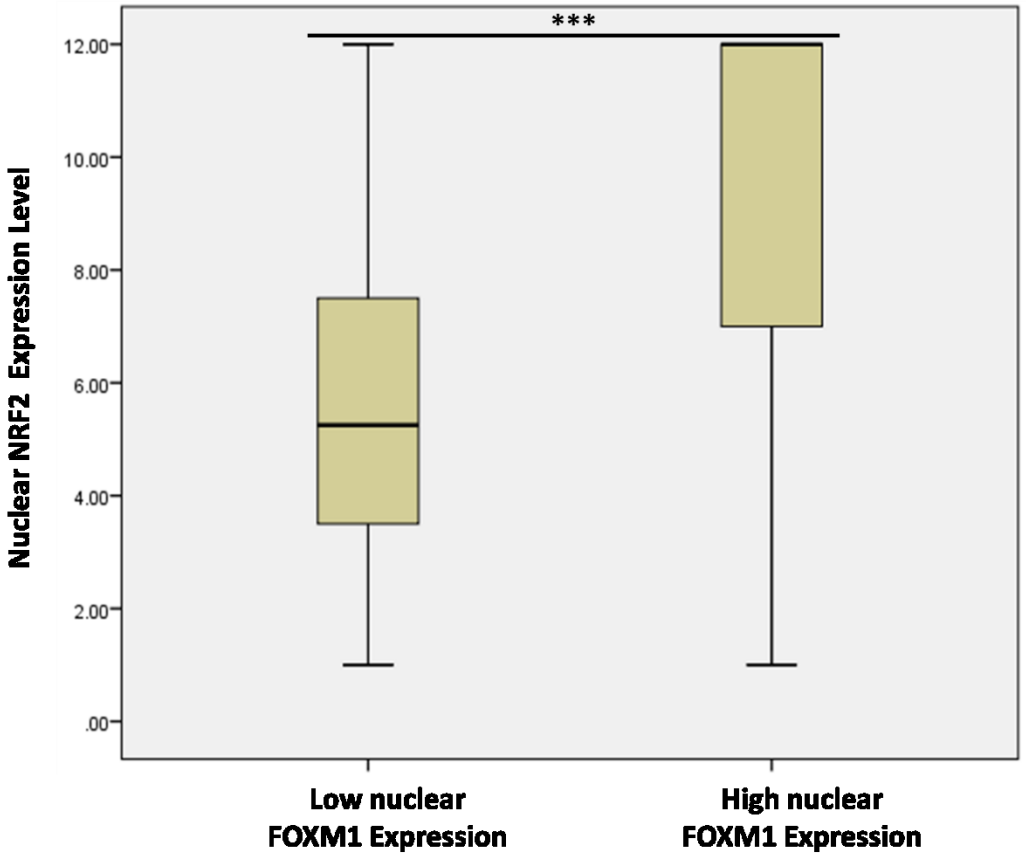


Figure 3.17 Comparisons of nuclear NRF2 expression levels with nuclear FOXM1 expression level by Mann Whitney U rank test using all primary breast cancer samples. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

3.3 Discussion

Chemoresistance poses difficulties to chemotherapy against cancer as chemoresistance cancer cells are harder to kill than chemosensitive cells using the same chemotherapeutic method. Even though patients might have high initial response rate, they might have relapse of cancer because of acquired resistance. Chemoresistance could be contributed by different molecular processes, including activation of ABC transporters, heterogeneity of cancer, deregulated DNA repair and apoptosis (Indran, Tufo et al. 2011). Redox signaling can mediate the cellular signaling of these molecular processes leading to chemoresistance. And reactive oxygen species (ROS) affect the redox homeostasis in cells, modifying proteins such as enzymes and transcription factors by oxidation (Lennicke, Rahn et al. 2015). Oxidation of these factors could modify their normal function, altering the cell signaling pathways and generate oxidative stress inside the cell, leading to altered molecular pathways. Therefore, understanding ROS and oxidative stress and their role in developing drug resistance in cancer cells are important to overcome the issue of chemoresistance.

In this study, it was shown that chemoresistant breast cancer cell lines (MCF-7 EpiR and MCF-7 TaxR) have higher resistance to oxidative stress-mediated cell death, indicated by both short-term cell viability SRB assays and long term clonogenic assays (Figure 3.1). This finding suggests having a higher protection against oxidative stress may be one of the mechanisms for the development of chemoresistance. Many drugs used for cancer chemotherapy can induce ROS generation, causing cancer cell death via apoptosis (Kaufmann and Earnshaw 2000). It has been reported that anthracyclines generate the highest levels of oxidative stress (Gille and Nohl 1997).

The intracellular ROS level of drug sensitive MCF-7 cells and epirubicin resistant (MCF-7 EpiR) cells as well as their response to epirubicin treatment were compared (Figure 3.2). It was found that epirubicin resistant cells have lower ROS content than the drug sensitive cells. This result revealed that ROS reduction could be a general characteristic for chemoresistance. It has been suggested that prolonged treatment with the same drug reduces the ROS level in cancer cells (Maiti 2010). Also, the result showed that treatment with epirubicin in resistant cells was no longer able to induce any ROS changes (Figure 3.2), implying that these cells could have adapted mechanisms to be more resistant to drug induced oxidative stress. Given that chemoresistant cells have lower intracellular ROS levels than sensitive cells, the same drug treatment might not able to generate enough ROS that could induce cell signaling for ROS mediated apoptosis as achieved in the case of sensitive cells. This implies that a ROS level threshold might exist to determine cell survival or cell death. ROS reduction is observed on prolonged treatment with anticancer drug as the resistant cell lines were established by prolonged treatment of the same drug. However, whether it is ROS reduction that makes the cancer cells resistant or that resistant cells reduce intracellular ROS levels is not known

NRF2 is a master regulator of oxidative stress and electrophilic stress. Because of its cytoprotective functions, NRF2 has been traditionally viewed as a tumour suppressor. Expression of the Nrf2-dependent proteins is important for elimination of toxic factors or carcinogens to maintain cellular redox homeostasis (Ramos-Gomez, Kwak et al. 2001). NRF2 activation has been shown to protect cells against carcinogenic exposure. However, there is growing evidence which reveals an oncogenic role for NRF2. NRF2 and its downstream targets are found to be overexpressed in many cancer types, including breast cancer (Sporn and Liby 2012). NRF2 overexpression provides cancer

cells an advantage for survival and growth. Recent studies have also indicated that upregulation of NRF2, that which in theory should lower ROS, can confers chemoresistance in various type of cancer, including breast cancer, cervical cancer and ovarian cancer (Konstantinopoulos, Spentzos et al. 2011, Ma, Cai et al. 2012, Zhong, Zhang et al. 2013). Here we show that overexpression of NRF2 in MCF-7 cells promoted cell proliferation and induced epirubicin resistance while knockdown of NRF2 reduced cancer growth and increased epirubicin sensitivity of MCF7-cells. Moreover, knockdown of NRF2 resensitized MCF7-EpiR cells to epirubicin (Figure 3.3 and Figure 3.4). These results confirm the role of NRF2 in favoring tumour growth and conferring chemoresistance in breast cancer.

The forkhead box transcription factor FOXM1 is a known oncogene. The oncogenic role of FOXM1 has been well studied; it plays prominent roles in promoting cell cycle progression, angiogenesis, cell migration and invasion, DNA damage response, epithelial–mesenchymal transition (EMT) stem cell renewal (Lam, Brosens et al. 2013). FOXO3 is another member of the forkhead box proteins. Unlike FOXM1, FOXO3 is traditionally viewed as a bona fide tumour suppressor and a key negative regulator of cell proliferation and survival. FOXO3 also regulates expression of specific cell cycle inhibitors, apoptosis, oxidative stress response (Lam, Brosens et al. 2013).

DeNicola et al. hypothesized that oncogenes increase NRF2 expression to activate the antioxidant system for ROS reduction and detoxification, promoting tumorigenesis (DeNicola, Karreth et al. 2011). Their study showed that oncogenes, K-Ras, B-Raf and c-Myc increased NRF2 transcription, thereby detoxifing cells from increased ROS level and induces tumorigenicity (DeNicola, Karreth et al. 2011). FOXM1 has been reported to be one of the targets shared by K-Ras, B-Raf and c-Myc as reported by different

studies (Blanco-Bose, Murphy et al. 2008, Park, Carr et al. 2009, Ma, Tong et al. 2010). It has also recently been reported FOXO3 could regulate the NRF2 pathway via transcription control of KEAP1 (Guan, Zhang et al. 2016). It is therefore reasonable to suspect that FOXM1 and FOXO3 can also mediate NRF2 expression in breast cancer. The current study investigated whether forkhead box transcription factors, and in particular FOXM1, are involved in NRF2 regulation and chemoresistance.

Protein levels of NRF2, FOXM1 and FOXO3 were first compared between sensitive cells and chemoresistant cells at basal level (Figure 3.5). In agreement with previous reports (Bao, Jaramillo et al. 2014), NRF2 protein level was higher in chemoresistant cells, MCF-7 EpiR and MCF-7 TaxR. FOXM1 was found to be overactivated while FOXO3 was downregulated in MCF-7 EpiR and MCF-7 TaxR cells, suggesting that FOXM1 is positively correlated while FOXO3 is negatively correlated with NRF2 expression. Moreover, changes of NRF2 expression were observed to follow a similar trend as the change of FOXM1 expression following the tBHP treatment and epirubicin treatment at both mRNA and protein level (Figure 3.6), further supporting the idea that the oncogene FOXM1 might regulate NRF2 expression in breast cancer.

FOXM1 overexpression was found to induce NRF2 expression while FOXM1 knockdown reduced NRF2 expression in MCF-7, confirming FOXM1 can regulate the expression of NRF2 (Figure 3.7). NRF2 binds antioxidant response element (ARE) to induce ARE-mediated transcriptional response, stimulating the expressions of antioxidant genes. Transcriptional activation of ARE plays a crucial role in modulating oxidative stress (Reddy 2008). It was found that FOXM1 overexpression significantly induced ARE-luciferase reporter activities in MCF-7 cells (Figure 3.8). In response to epirubicin, it was observed that FOXM1 protein was first activated at 8 h before

downregulated at 24 h and 48 h (Figure 3.6B). Similarly, ARE-luciferase activities was increased at 8 h and decreased at 24 h in response to epirubicin treatment (Figure 3.8). These results demonstrate that FOXM1 can modulate the transcriptional activation of ARE. Consistently, knockdown of FOXM1 downregulated the mRNA expression of antioxidant gens and NRF2 downstream targets. Treatment of epirubicin led to downregualton of FOXM1, therefore NRF2 and the antioxidant genes were also found to have lowered expression after epirubicin treatment at 24 h (Figure 3.9). To investigate whether FOXM1 controls NRF2 at transcriptional level, ChIP-seq data sets for FOXM1 binding in MCF-7 cells available in ENCODE was analyzed. A putative FOXM1 binding site was located at NRF2 region (Figure 3.10A). ChIP assays indicated FOXM1 could bind to this FOXM1 binding site of the NRF2 region, revealing that NRF2 is a transcription target of FOXM1 (Figure 3.10B).

In summary, from experiments conducted on sensitive MCF-7 cells found that FOXM1 controls NRF2 expression at transcriptional level, which in turn, regulates the expression of antioxidant genes via ARE transcription activation in breast cancer. Our results suggest that both FOXM1 and NRF2 have a role in epirubicin sensitivity and resistance. This is supported by a recent study in hepatocellular carcinoma and mice, Han B et al who have proposed the Ras/ERK/FoxM1/Nrf2 signaling pathway in controlling the expression an antioxidant enzyme peroxiredoxin I (Prx I) (Han, Shin et al. 2016). However, FOXM1/NRF2 axis function in chemoresistance was not investigated in this study.

Our study showed FOXM1 depletion in MCF-7 EpiR was not able to decrease the expression of NRF2, suggesting that FOXM1/NRF2 axis might no longer be functional in resistant cells (Figure 3.7B). As NRF2 expression level was not altered, mRNA

expression of antioxidant genes were also not significantly changed upon FOXM1 depletion by RNAi or epirubicin treatment (Figure 3.9). To elucidate the difference between drug sensitive cells and chemoresistant cells in FOXM1/NRF2 axis, a ChIP-qPCR assay was conducted to compare the response of MCF-7 cells and MCF-7 EpiR cells in response to epirubicin treatment (Figure 3.11). FOXM1 binding in MCF-7 cells was decreased when treated with epirubicin. This result agrees with a functional FOXM1/NRF2 axis in sensitive cells. However, it was found that there was less FOXM1 bound to the FOXM1 binding site at NRF2 region in MCF7-EpiR, compared to MCF-7 cells. These results imply that FOXM1 in chemoresistant cells are deregulated, so that FOXM1 was less efficient in binding to its original binding site at NRF2 region. The mechanism leading to deregulated FOXM1 binding activity remain to be elucidated.

Inhibiting either NRF2 or FOXM1 can induce cell cycle arrest at G2/M phase (Reddy, Kleeberger et al. 2008, Barsotti and Prives 2009), indicating that both NRF2 and FOXM1 share the role in promoting cell cycle progression. The effect of NRF2 on FOXM1 expression was also tested in this study (Figure 3.12). We found that knockdown of NRF2 lowered the expression of both FOXM1 and cyclinB1, a known FOXM1 downstream target involved in G2/M phase transition, suggesting that NRF2 might regulate FOXM1 expression. However, ectopic expression of NRF2 only induced FOXM1 protein expression but with no increase in FOXM1 or cyclin B1 at mRNA level. Moreover, neither depletion nor overexpression of NRF2 MCF-7 EpiR could affect FOXM1 or CyclinB1 expression significantly, indicating that FOXM1 is not regulated by NRF2 directly. It has been shown that oxidative stress conditions can induce cell cycle arrest and reduce FOXM1 expression (Chua, Yip et al. 2009). Therefore, induced oxidative stress and growth arrest by depletion of NRF2 (Reddy,

Kleeberger et al. 2008, DeNicola, Karreth et al. 2011) might explain the downregulation of FOXM1 observed in this study.

Compared to normal cells, cancer cells exhibit more active metabolism and generate higher levels of ROS. Increased levels of ROS in cancer cells also render them more vulnerable to oxidative stress and more dependent on ROS scavenging system. Several studies have attempted to selectively kill oncogenic transformed cells by targeting the abnormal levels of ROS via inducing oxidative stress (Trachootham, Zhou et al. 2006, Nogueira, Park et al. 2008). As previously described, NRF2 is the master regulator of antioxidant responses and FOXM1 has been shown to regulate oncogene-induced ROS. Also, upregulation of NRF2 and FOXM1 can confer chemoresistance. Therefore, it may be necessary to inhibit the FOXM1/NRF2 pathway during chemotherapy. A recent study demonstrated the use of FOXM1 inhibitors together with ROS inducers to induce cancer cell apoptosis. This approach could also inhibit the growth of breast tumor xenografts in mouse models (Halasi, Pandit et al. 2013). In this current study, epirubicin treatment could downregulate FOXM1 expression in epirubicin sensitive MCF-7 cells but not in chemoresistant cells (Figure 3.6B). The use of NRF2 inhibitor, brusatol, however could enhance the efficacy of epirubicin treatment to treat breast cancer (Figure 3.14).

In vitro results from this study are further supported by validating the correlation between FOXM1 and NRF2 expression in vivo. Both FOXM1 and NRF2 are poor prognostic markers which correlated with poorer disease-free survival and poorer post progression survival (Figure 3.15), implying that FOXM1 and NRF2 contribute to cancer progression and chemoresistance. From IHC stainings of a set of primary breast tumor samples in tissue microarray, we found that NRF2 protein expression was

positively correlated with FOXM1 protein expression (Figure 3.17). These results suggest that inhibiting FOXM1/NRF2 axis could be used as a therapeutic strategy to combat cancer cells, improving prognosis of breast cancer.

In summary, this chapter have showed the relationship between FOXM1 expression and NRF2 expression in breast cancer. The results showed that FOXM1 regulates the transcription of NRF2 which mediates the response to epirubicin in drug sensitive cells and uncovered a novel dysregulated FOXM1/NRF2 axis in drug resistance cells (Figure 3.18). In chemosensitive cells, anticancer drugs, such as epirubicin used in this study, could inhibit FOXM1 directly or indirectly. Inhibition of FOXM1 led to downregulation of NRF2 and NRF2 downstream targets which include a number of antioxidant enzymes, thereby ROS scavenging system in chemosensitive cells became inefficient and eventually leading to ROS induced cell death. In chemoresistant cells, however, the same drug could not target FOXM1 and there were higher expressions of FOXM1 and NRF2. This offered an efficient ROS scavenging system which could counteract the drug effects in inducing cell death.

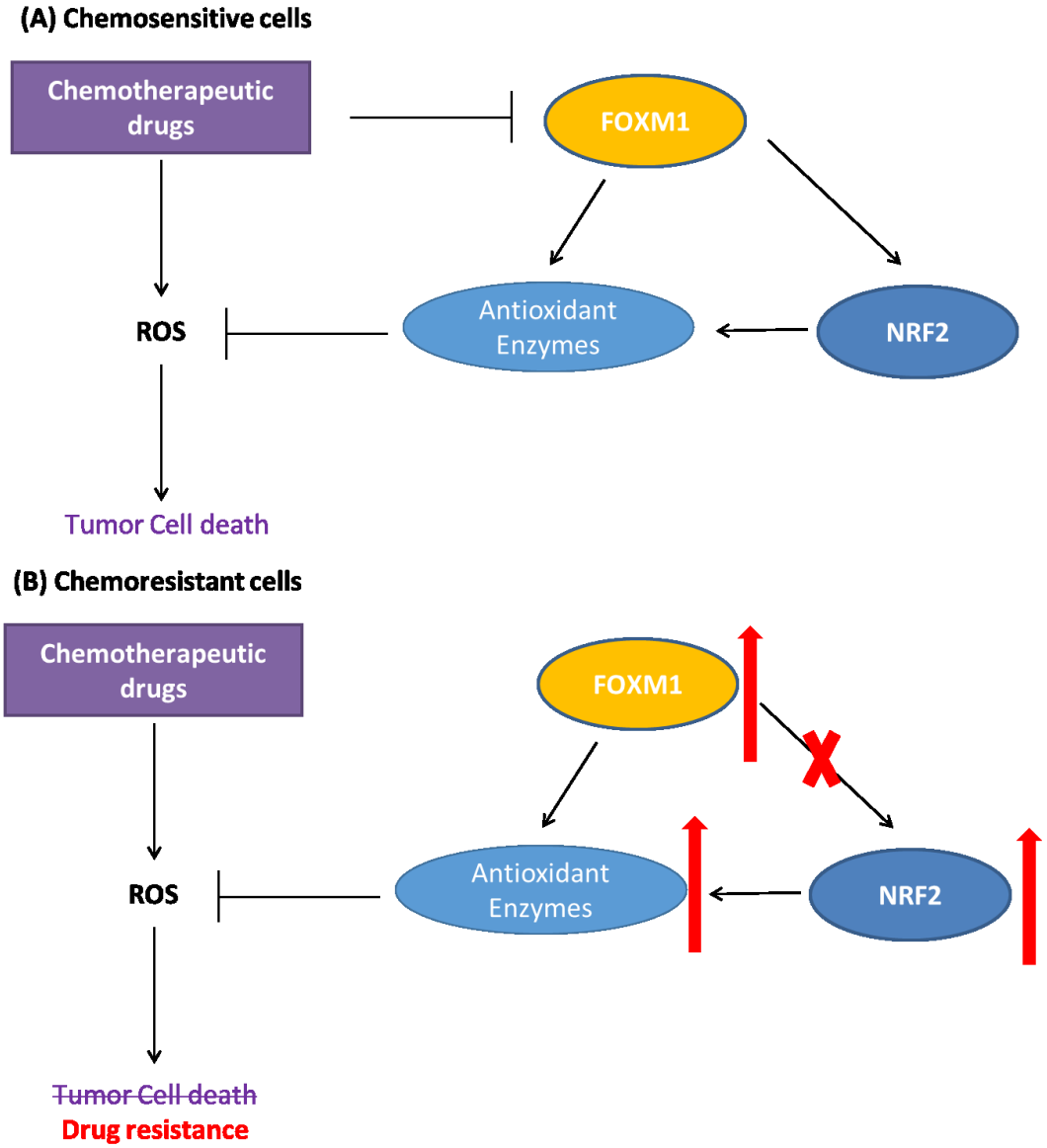


Figure 3.18. Proposed mechansim for FOXM1 and NRF2 regulation (i) chemosensitive cells and (ii) chemoresistant cells.

Chapter 4. The role of BQ323636.1, a splice variant of NCOR2, in modulation of oxidative stress in breast cancer

4.1 Introduction

As introduced in Chapter 1, our group previously identified BQ323636.1 (BQ) as a novel splice variant of which retains only the N-terminus repression domain 1 (RD1) of the NCOR2 wildtype protein. It was reported that overexpression of BQ at mRNA level was significantly associated with tamoxifen resistance and suggested that BQ could serve as a biomarker for predicting tamoxifen response (Zhang, Gong et al. 2013).

Overexpression of BQ conferred resistance to tamoxifen in both *in vitro* and in orthotropic mouse model. Mechanistically, co-immunoprecipitation showed BQ could bind to NCOR2 and inhibit the formation of co-repressor complex for suppression of the ER signalling. Consistently, BQ overexpression compromised the suppression role of NCOR2 in regulating estrogen-response element (ERE) activity and rescued the transcriptional suppression of tamoxifen on ER-target genes. Our group generated a monoclonal antibody specific for BQ used to predict patients' responses to tamoxifen treatment. Immunohistochemistry (IHC) performed on tissue microarray of 355 patients who had ER positive primary breast carcinoma and had received adjuvant tamoxifen treatment, showed highly significant association of nuclear BQ expression with tamoxifen resistance, cancer metastasis and disease relapse as well as with overall and disease-specific survival (Gong et al, under review)

Previously, our group had focused on understanding the role of BQ in tamoxifen

resistance. Little is known on whether BQ can mediate resistance to the other forms of drug in breast cancer. As mentioned in chapter 1, NCOR2 can bind to NRF2, suppressing the transcription of antioxidant genes. We hypothesized that BQ can interfere with the interaction between NCOR2 and NRF2 to modulate oxidative stress in breast cancer, thereby mediating the response of chemotherapy mediated by ROS.

4.2 Results

4.2.1 BQ overexpression protects breast cancer cells from oxidative stress

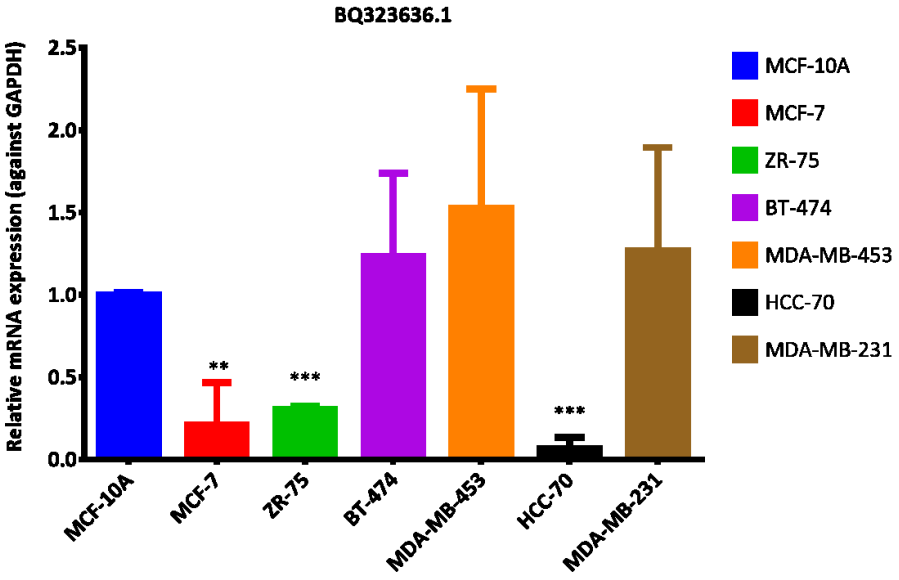
To investigate whether BQ can modulate oxidative stress, BQ overexpression was achieved in two selected breast cancer cell lines ZR-75 and MCF-7, based on their relatively low expression of BQ at both mRNA and protein levels among different breast cancer cell lines with reference to non-tumorigenic breast epithelial cell line MCF-10A (Figure 4.1A and Figure 4.1B). BQ was stably overexpressed using transduction of lentiviral expression vector (pCDH-CMV-MCS-EF1-Puro-GFP-6xHis BQ323636.1) in ZR-75. This stable cell line, ZR-75-BQ, was established previously by members in our group. Overexpression of BQ in MCF-7 was achieved by transient transfecting BQ expression plasmid (pCDNA3.1-6xHisBQ323636.1). Controls were generated by transducing the cells with empty vector alone in ZR-75 (ZR-75-GFP) or transfecting the cells with empty vector (pCDNA3.1-empty) in MCF-7. RT-qPCR was used to confirm the overexpression of BQ in these cell lines (Figure 4.2A). Since both expression plasmids used in ZR-75 and MCF-7 cell lines carried a 6xHistidine tag fused with BQ323636.1, the overexpression of BQ in these cells were also confirmed using anti-His Tag antibody (Figure 4.2B).

The response of BQ overexpression to oxidative stress treatment in both ZR-75 cells and MCF- cells were studied using MTT assay. It was found that, as shown in figure

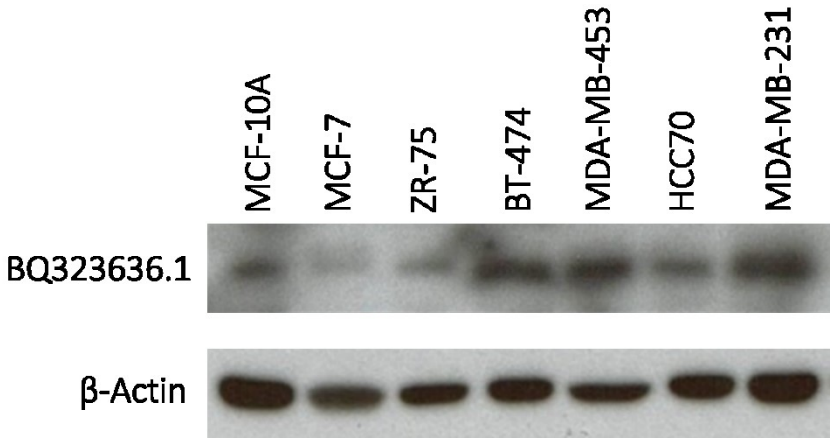
4.3A, BQ overexpression cells (Grey bars) have higher protection against oxidative stress, marked by higher cell viability under different concentrations of tBHP when compared with their vector controls (Black bars). Similarly, response of BQ overexpressing ZR-75 cells and MCF-7 cells to tBHP treatment were also compared by clonogenic assay (Figure 4.3B). The results also showed that BQ overexpressing cells had higher survival rate under different concentrations of tBHP when compared with their vector controls. Both short term MTT assays and long term clonogenic assay confirmed that BQ overexpression led to higher protection against oxidative stress, suggesting BQ could upregulate anti-oxidative stress mechanisms of breast cancer cells.

It should also be noted that, comparing BQ overexpressing cells and the control cells without tBHP treatment (i.e. tBHP 0uM) of both cell lines, the cell viability of BQ overexpressing cells were higher than that of vector controls in both MTT assays and clonogenic assay. This suggests that BQ overexpression can promote cell proliferation, supporting the possibility that BQ overepxression can favor tumor growth.

(A)


BQ323636.1
Relative mRNA expression (against GAPDH)
2.5
2.0
1.5
1.0
0.5
0.0
**
***
***
MCF-10A
MCF-7
ZR-75
BT-474
MDA-MB-453
HCC-70
MDA-MB-231

(B)


MCF-10A
MCF-7
ZR-75
BT-474
MDA-MB-453
HCC70
MDA-MB-231
BQ323636.1
β-Actin

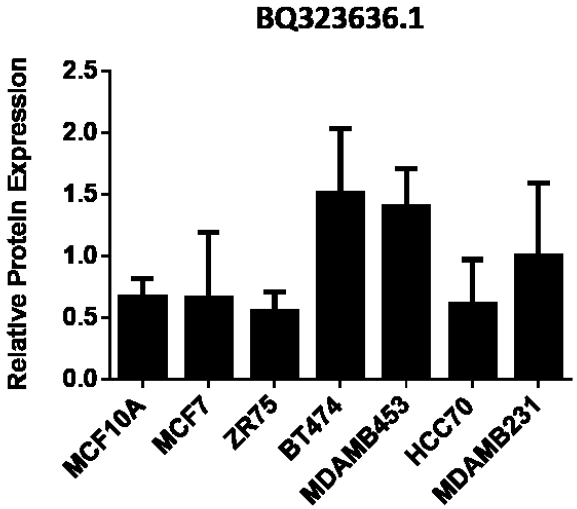
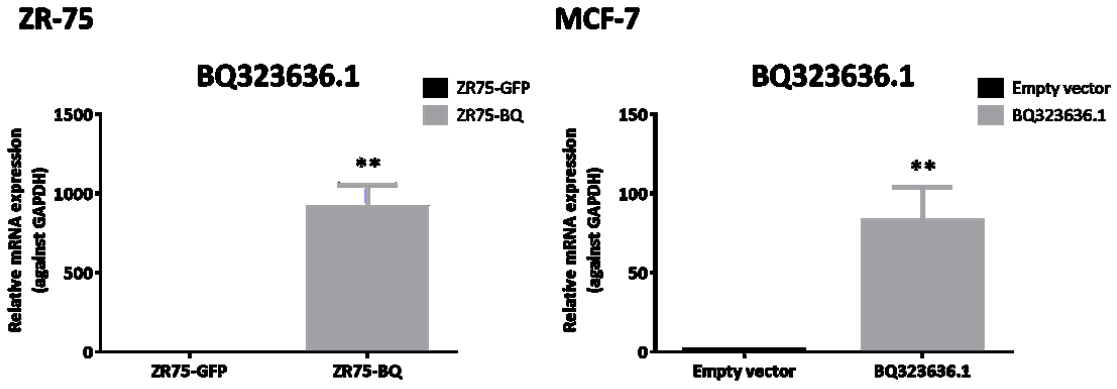

BQ323636.1
Relative Protein Expression
2.5
2.0
1.5
1.0
0.5
0.0
MCF10A
MCF7
ZR75
BT474
MDAMB453
HCC70
MDAMB231

Figure 4.1 BQ323636.1 expression at both mRNA and protein level in a panel of breast cancer cell lines. ER positive MCF-7 and ZR-75, HER2 positive BT-474, triple negative MDA-MB-453, HCC70 and MDA-MB-231 breast cancer cell lines mRNA and protein samples were analyzed by **(A)** RT-qPCR and **(B)** Western blot for expression levels of BQ323636.1. Expression of MCF-10A ,a non-tumorigenic breast epithelial cell line, was regarded as control (MCF-10A expression level as 1). Blot shown was representative image of three biological replicates. The band intensities were quantified by imageJ and normalized against the intensities of MCF-10A. The experiments were repeated three times. RT-qPCR data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

(A)

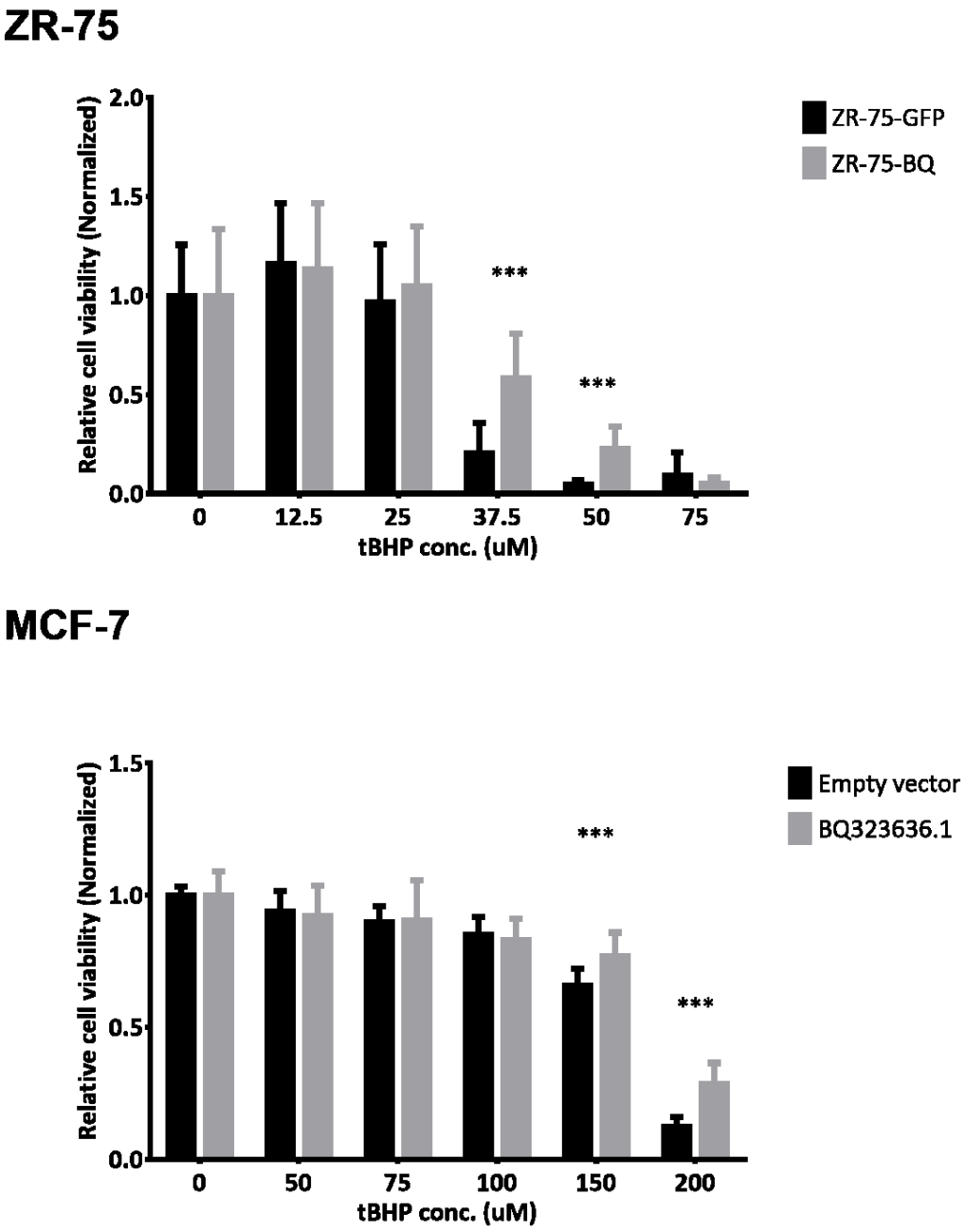


(B)

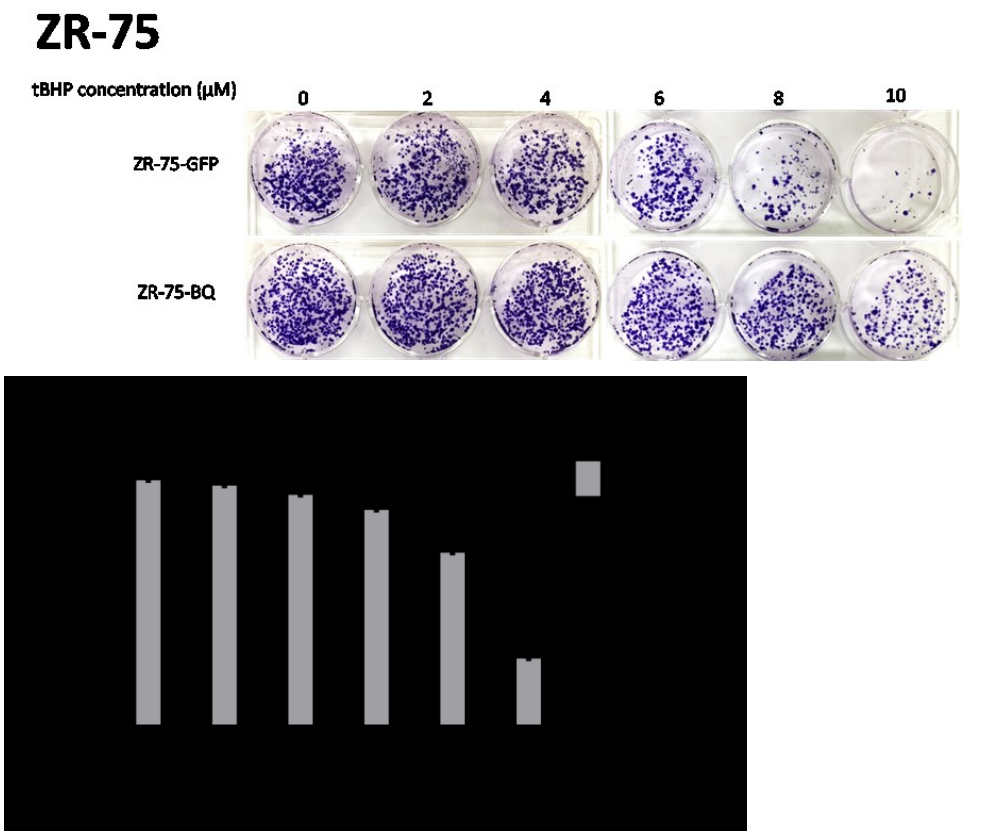


Figure 4.2 Overexpression of BQ in ZR-75 and MCF-7 cells. ZR-75-BQ and ZR-75-GFP was generated by stable transduction of lentiviral expression vectors (pCDH-CMV-MCS-EF1-Puro-GFP-6xHis BQ323636.1 or pCDH-CMV-MCS-EF1-Puro-GFP) in ZR-75 cells respectively. Transient transfection of BQ expression plasmid pCDNA3.1-6xHisBQ323636.1 or pCDNA3.1-Empty (annotated as BQ323636.1 or empty vector as control) in MCF-7 to achieve BQ overexpression. The overexpression of BQ were confirmed at **(A)** mRNA level and **(B)** protein level. The experiments were repeated three times. Blot shown was representative image of three biological replicates. RT-qPCR data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

(A)


ZR-75
Relative cell viability (Normalized)
2.0
1.5
1.0
0.5
0.0
***
***
0
12.5
25
37.5
50
75
tBHP conc. (uM)
ZR-75-GFP
ZR-75-BQ
MCF-7
Relative cell viability (Normalized)
1.5
1.0
0.5
0.0
***
***
0
50
75
100
150
200
tBHP conc. (uM)
Empty vector
BQ323636.1

(B)


ZR-75
tBHP concentration (μM)
0
2
4
6
8
10
ZR-75-GFP
ZR-75-BQ

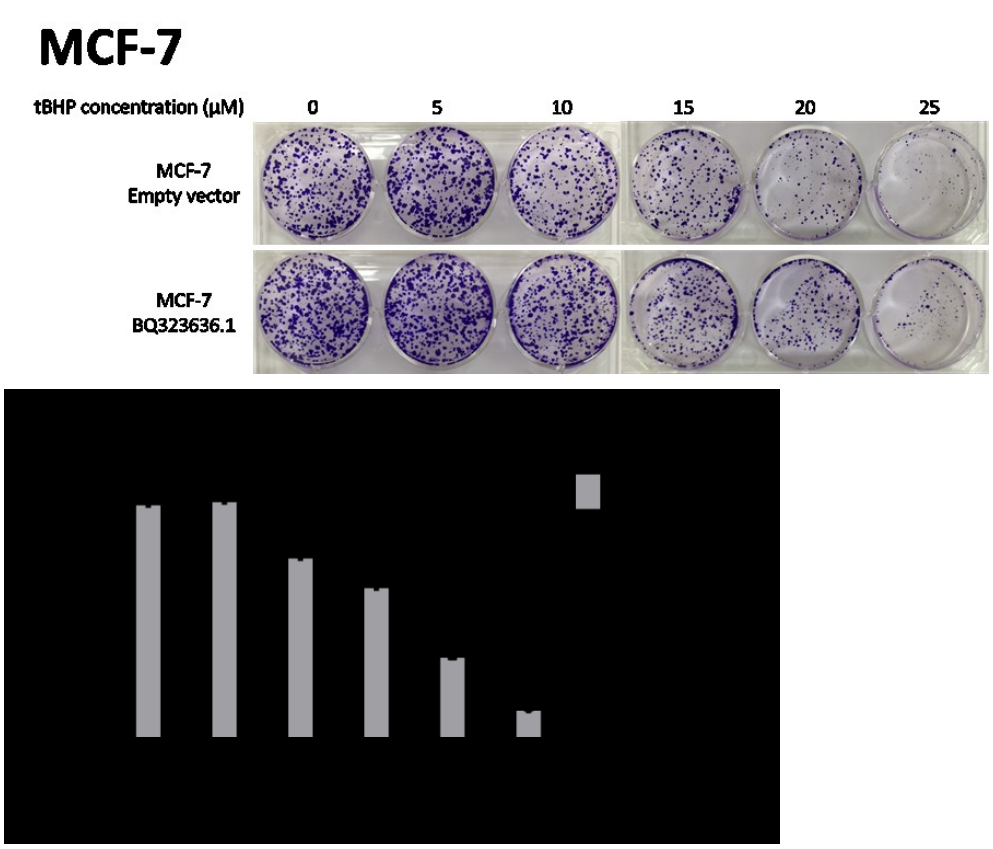

MCF-7
tBHP concentration (μM)
0
5
10
15
20
25
MCF-7
Empty vector
MCF-7
BQ323636.1

Figure 4.3 BQ overexpression promotes resistance to tBHP in breast cancer cells. BQ overexpressing ZR-75 and MCF-7 cells were treated with different concentration of an oxidative stress inducer tBHP. **(A)** Cells were treated with tBHP (0, 12.5, 25, 37.5, 50 and 75 μM for ZR-75; 0, 50, 75,100, 150 and 200 μM for MCF-7) for 72 h, followed by MTT assays. **(B)** Cells were treated with tBHP (0, 2, 4, 6, 8 and 10 μM for ZR-75; 0, 5, 10, 15, 20 and 25 μM for MCF-7) for 48 h and then cultured with normal culture medium for 14 days. The cells were then stained with crystal violet. The quantitative analysis was measuring the intensity of crystal violet staining after dissolving the stain in acetic acid. Relative colony formation was expressed with reference to untreated cells (0 μM as 1). The experiments have been repeated for four times independently and data are shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001, when compared with MCF-7.

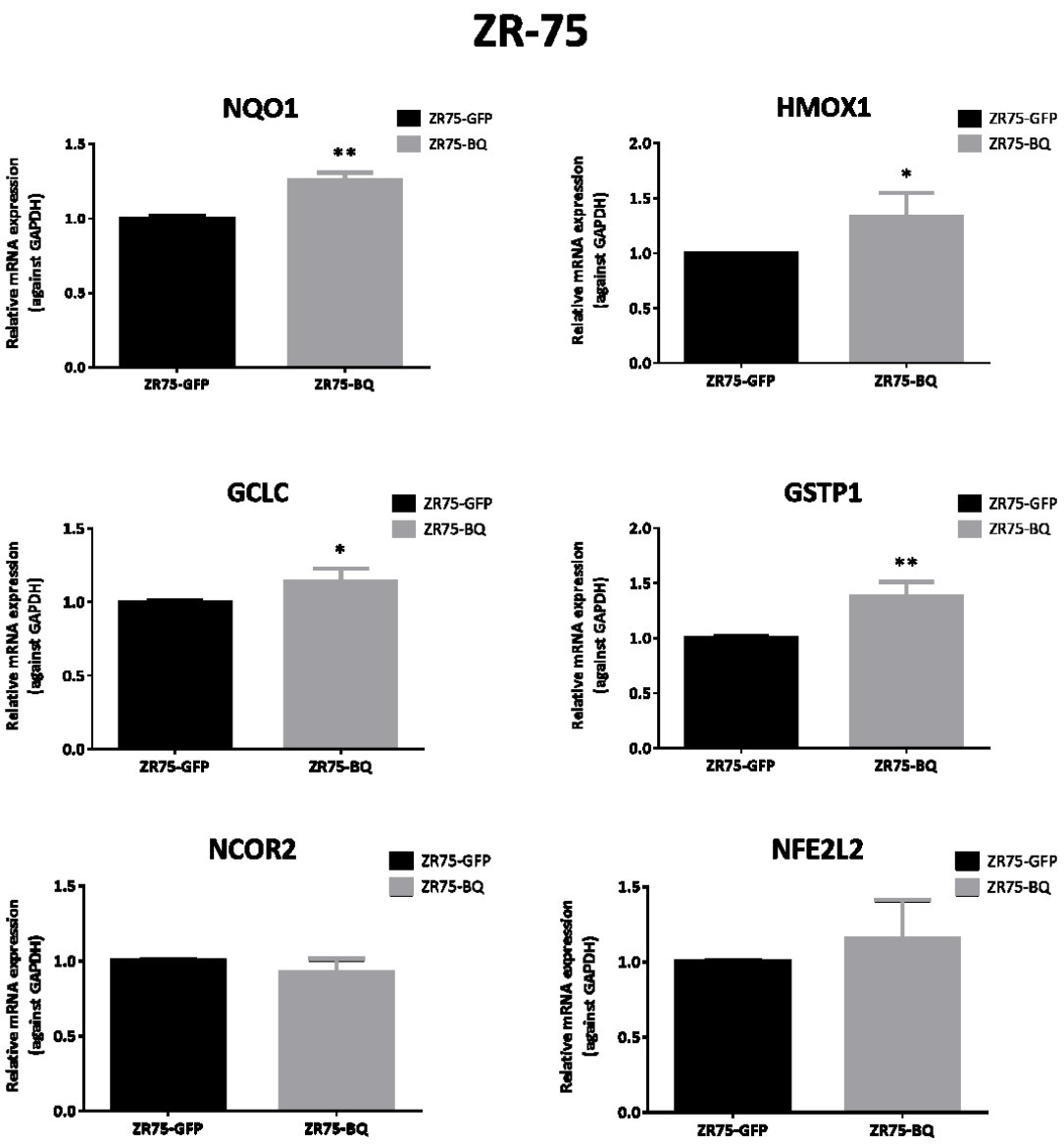
4.2.2 BQ overexpression upregulates antioxidant gene expression

It has been reported that NCOR2 binds to NRF2 and suppresses the transcription of antioxidant genes driven by the NRF2-ARE pathway (Ki, Cho et al. 2005). Since it was shown that BQ overexpression provided better defense against oxidative stress to breast cancer cells, we then hypothesized that BQ could mediate the expression of antioxidant genes regulated by NRF2, possibly through interference with the functions of NCOR2.

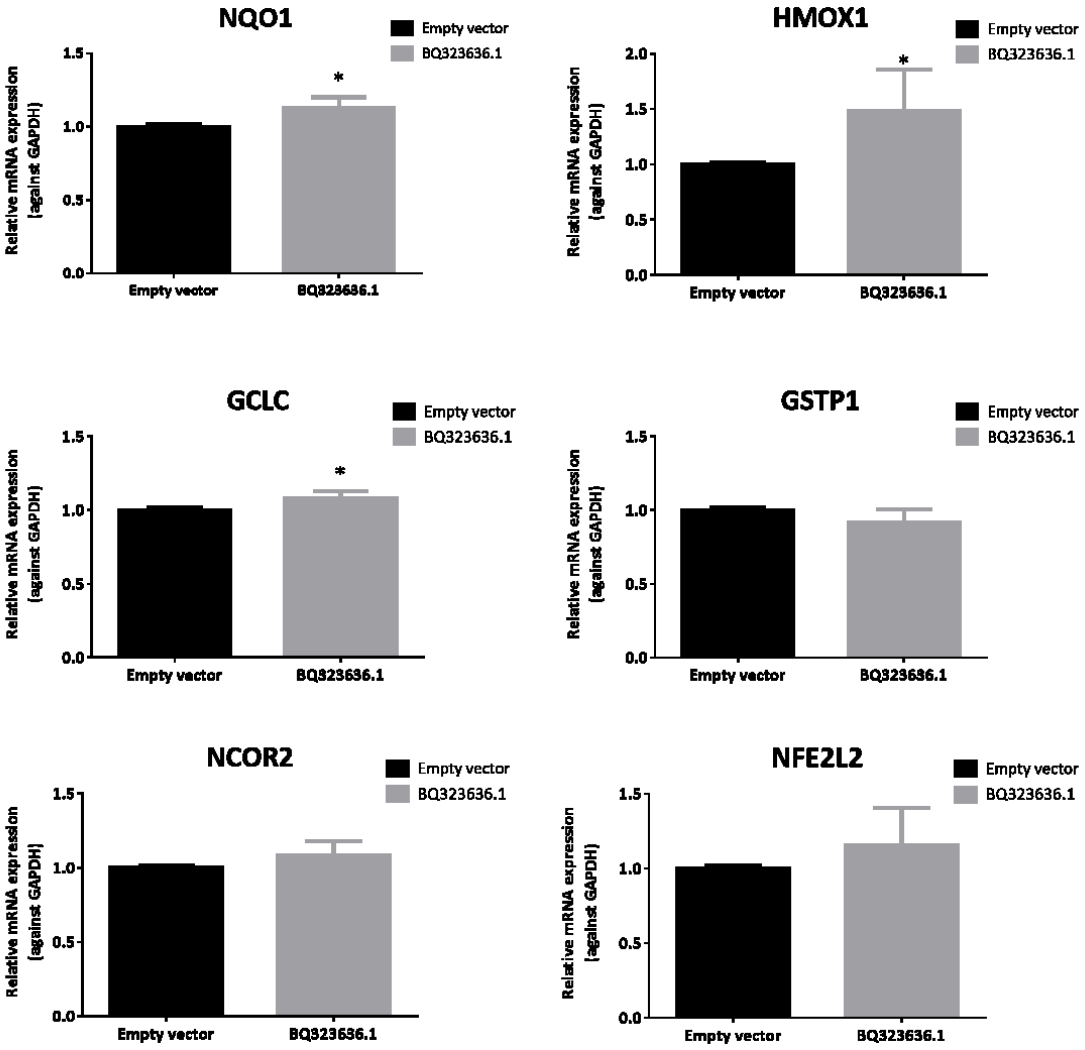
To test this hypothesis, the expression levels of NRF2 downstream target genes were examined, including antioxidant enzymes NQO1, HMOX1, GCLC and GSTP1, by RT-qPCR analysis comparing their expressions when BQ is overexpressed and with vector controls in both ZR-75 and MCF-7 cell lines (Figure 4.4A). The results showed that mRNA levels of NQO1, HMOX1, GCLC and GSTP1 were significantly higher in ZR-75 cells stably overexpressing BQ. Similarly mRNA levels of NQO1, HMOX1 and GCLC were also significantly higher in MCF-7 cells transfected with BQ expression plasmid. The higher expressions of NRF2 target genes in BQ overexpressing cells suggest that BQ can promote NRF2-ARE transcription. The mRNA expressions of NCOR2 and NRF2 (also know as NFE2L2) were also compared in these BQ overexpressing cells and their control cells. No significant difference in mRNA levels of NRF2 between BQ overexpressing cells and the control cells of both cell lines was observed (Figure 4.4A). To confirm the results shown by RT-qPCR analysis, protein expression of NCOR2, NRF2 and NQO1 as well as β-Actin as loading control are anaylzed by Western Blotting (Figure 4.4B). NQO1 was the most consistently and significantly increased NRF2 target gene in both cell lines. Consistent with RT-qPCR analysis, it was found that protein levels of NQO1 were higher in BQ overexpressing cells than their controls cell. Also, protein levels of NCOR2 and NRF2 were appeared not affected by BQ overexpression in both cell lines.

As expression level NRF2 were not significantly altered by BQ overexpression, this suggested that upregulation of NRF2 downstream targets was achieved by affecting the activity of NRF2 but not the expression of NRF2. Also, the mRNA level of NCOR2 was not affected, indicating that BQ overexpression did not alter the exprerssion of NCOR2. These results further indicate that alteration of transcription of NRF2 downstream target genes by BQ overexpression was not due to the change in expression of NCOR2. Taken together, evidences from RT-qPCR and Western blot analysis suggested BQ could modulate antioxidant genes exprerssions via a mechansim affecting the function or activity of NRF2 and NCOR2 rather than the expression of NRF2 or NCOR2.

(A)


ZR-75
NQO1
HMOX1
GCLC
GSTP1
NCOR2
NFE2L2
ZR75-GFP
ZR75-BQ
Relative mRNA expression (against GAPDH)
**
*
*
**
0.0
0.5
1.0
1.5
2.0

MCF-7


NQO1
Empty vector
BQ323636.1
Relative mRNA expression (against GAPDH)
1.5
1.0
0.5
0.0
*
Empty vector
BQ323636.1
HMOX1
Empty vector
BQ323636.1
Relative mRNA expression (against GAPDH)
2.0
1.5
1.0
0.5
0.0
*
Empty vector
BQ323636.1
GCLC
Empty vector
BQ323636.1
Relative mRNA expression (against GAPDH)
1.5
1.0
0.5
0.0
*
Empty vector
BQ323636.1
GSTP1
Empty vector
BQ323636.1
Relative mRNA expression (against GAPDH)
1.5
1.0
0.5
0.0
Empty vector
BQ323636.1
NCOR2
Empty vector
BQ323636.1
Relative mRNA expression (against GAPDH)
1.5
1.0
0.5
0.0
Empty vector
BQ323636.1
NFE2L2
Empty vector
BQ323636.1
Relative mRNA expression (against GAPDH)
1.5
1.0
0.5
0.0
Empty vector
BQ323636.1

(B)

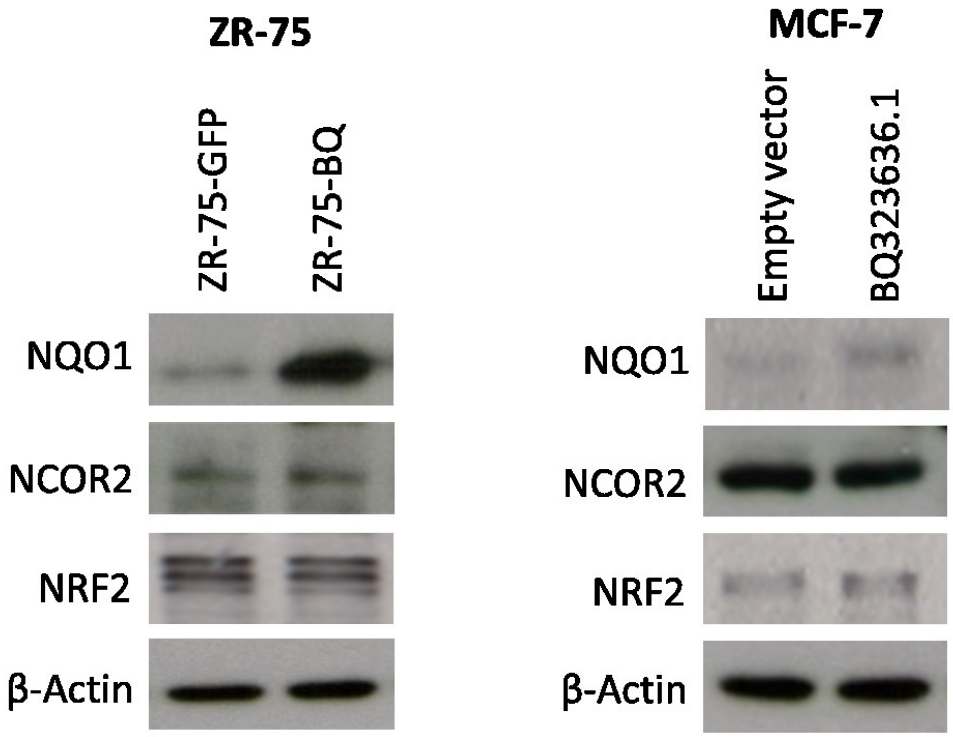


Figure 4.4 BQ overexpression induces antioxidant genes expression without altering expression of NRF2 and NCOR2 in breast cancer. The mRNA expressions and protein levels in BQ overexpressing ZR-75 and MCF-7 cells were analyzed, compared to cells transfected with empty vector. **(A)** RT-qPCR analysis showed expression levels of NQO1, HMOX1, GCLC, GSTP1, NCOR2 and NFE2L2 (NRF2). **(B)** Western blot showed the protein levels of NQO1, NCOR2, NRF2; β-Actin served as loading control. Blot shown was representative image of three biological replicates. The experiments were repeated three times. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

4.2.3 BQ overexpression upregulates NRF2 transcription activity

BQ overexpression induced higher protection against oxidative stress, suggesting that that BQ could upregulate anti-oxidative stress mechanims. It was found that BQ overexpression induced the expression of antioxidant genes without affecting the expression of NRF2 and NCOR2. Having ascertained the role of BQ in mediating oxidative stress, the next step was to understand the mechanism of how BQ may alter NRF2 transcription activity.

Previous study from our group showed that BQ can oppose the suppression effect of NCOR2 on transcription of estrogen response element (ERE)-containg luciferase reporter, suggesting a role of BQ in modulating ERα transcription activation activity (Zhang, Gong et al. 2013). It was suggested that BQ could compete with NCOR2 to interfere the suppression effect of NCOR2. As mentioned before, NCOR2 functions as a co-repressor and suppresses the transcription of antioxidant genes driven by NRF2 pathway. Also, NRF2 activates antioxidant gene expression via transcription on antioxidant response element (ARE). Therefore, we hypothesized that NCOR2 could suppress NRF2 transcription activation on ARE and BQ could compete with NCOR2 to interfere with the suppression effect of NCOR2, which in turn mediates the expression of NRF2 target genes.

To test this hypothesis, an ARE-containing luciferase reporter plasmid (pGL3-ARE) was constructed. The effects of overexpression of BQ and/or NCOR2 on transcription of ARE-containing luciferase reporter was studied. ZR-75 stably overexpressing BQ cells and the control cells were transiently transfected with NCOR2 or empty vector as control, followed by treatment of NRF2 activator tert-Butylhydroquinone (tBHQ) or untreated. tBHQ is a well-characterized NRF2 activator, by interfering with the ability

of Keap1 to repress Nrf2 via interaction with thiol groups of cysteine molecules on the KEAP1 protein (Li and Kong 2009). In this experiment, tBHQ treatment served to determine whether NRF2 functions can be affected by NCOR and/or BQ under NRF2 activated state. The results were expressed as relative luciferase activity normialized against empty vectors controls (empty vectors sample as 1) (Figure 4.5).

Consistent to its role as a co-repressor in breast cancer, ectopic overexpression of NCOR2 alone (ZR-75-GFP; NCOR2) significantly lowered the luciferase activity on ARE compared to empty vector control (ZR-75-GFP; Empty vector) in both non-treated and tBHQ treated conditions. (a) BQ overexpression alone (ZR-75-BQ; Empty vector) appeared to slightly induce ARE-luciferase activity under non-treated condition compared to the control cells (ZR-75-GFP; Empty vector) while (b) BQ overexpression alone significantly increased ARE-luciferase activity under tBHQ treated condition. tBHQ treatment amplified the difference in relative ARE-luciferase activity as tBHQ could activate NRF2, hence the ARE dependent transcription. The result indicated that NCOR2 and BQ overexpression altered ARE-dependent transcription and function at both basal and activated state of NRF2.

When NCOR2 was overexpressed in BQ overexpressing cells (ZR75-BQ; NCOR2), the ARE-luciferase activities were significantly higher when compared with (c) cells with overexpression of NCOR2 alone (ZR-75-GFP; NCOR2) under both non-treated and tBHQ treated conditions. This result suggested that NCOR2 suppression effect on ARE-luciferase activiy was, at least partially, rescued by BQ overexpression.

As mRNA and protein expression of NCOR2 upon BQ overexpression remained unchanged (Figure 4.4), it appears that BQ overexprerssion can oppose the effect on

NCOR2, not via altering the expression of NCOR2. From the ARE-luciferse reporter assay results, BQ overexpression could oppose the suppressive function of NCOR2 on ARE-mediated transcription. We therefore hypothesized that BQ exerts its negative effect by interfering with the binding of NCOR2 with NRF2, thereby inhibiting NRF2/ARE-medaited transcription.

To test this hypothesis, the interaction between NCOR2 and NRF2 was studied by co-immunoprecipitation in ZR-75 BQ overexpressing cells and the control cells. As shown in figure 4.5, the interaction of NCOR2 and NRF2 was confirmed in ZR-75 cells, consistent with the finding of previous published report (Ki, Cho et al. 2005). The effect of BQ overexpression on the interaction between NCOR2 and NRF2 was then compared to control cells. Co-IP revealed that BQ overexpression suppressed the interaction between NCOR2 and NRF2. This set of experiemnt was repeated for three times and IgG was used as a negative control. The band intensities of NRF2 were quantified using Image J software. Since it has been shown that BQ overexpression did not alter NRF2 protein level, the band intensities of NRF2 that had been pulled down by NCOR2 (IP:NCOR2) were divided by band intensities of NRF2 shown in the input (INPUT). The ratio of IP:NCOR2 to INPUT were represented as relative to control cells (ZR-75-GFP as 1) and this ratio reflects the relative amount of NRF2 bound by NCOR2. As shown in figure 4.5B, the results confirmed that there was significantly less NCOR2 binding to NRF2 when BQ was overexpresed. Taken togetherr, these data suggest that BQ could interfere with the binding of NCOR2 to NRF2, leading to inhibition of NCOR2 suppression effect on ARE mediated transcription, thus upregulating the expression of antioxidant genes which are NRF2 downsteam targets.

**

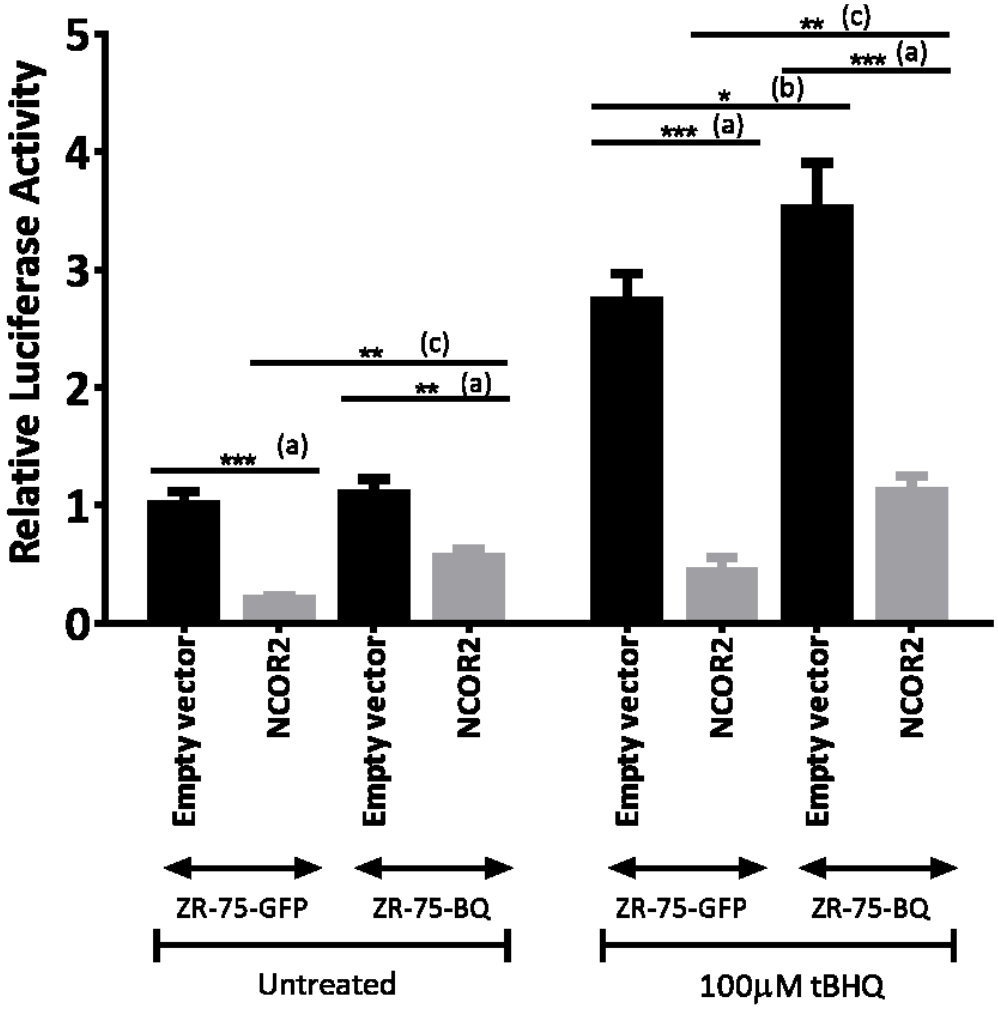
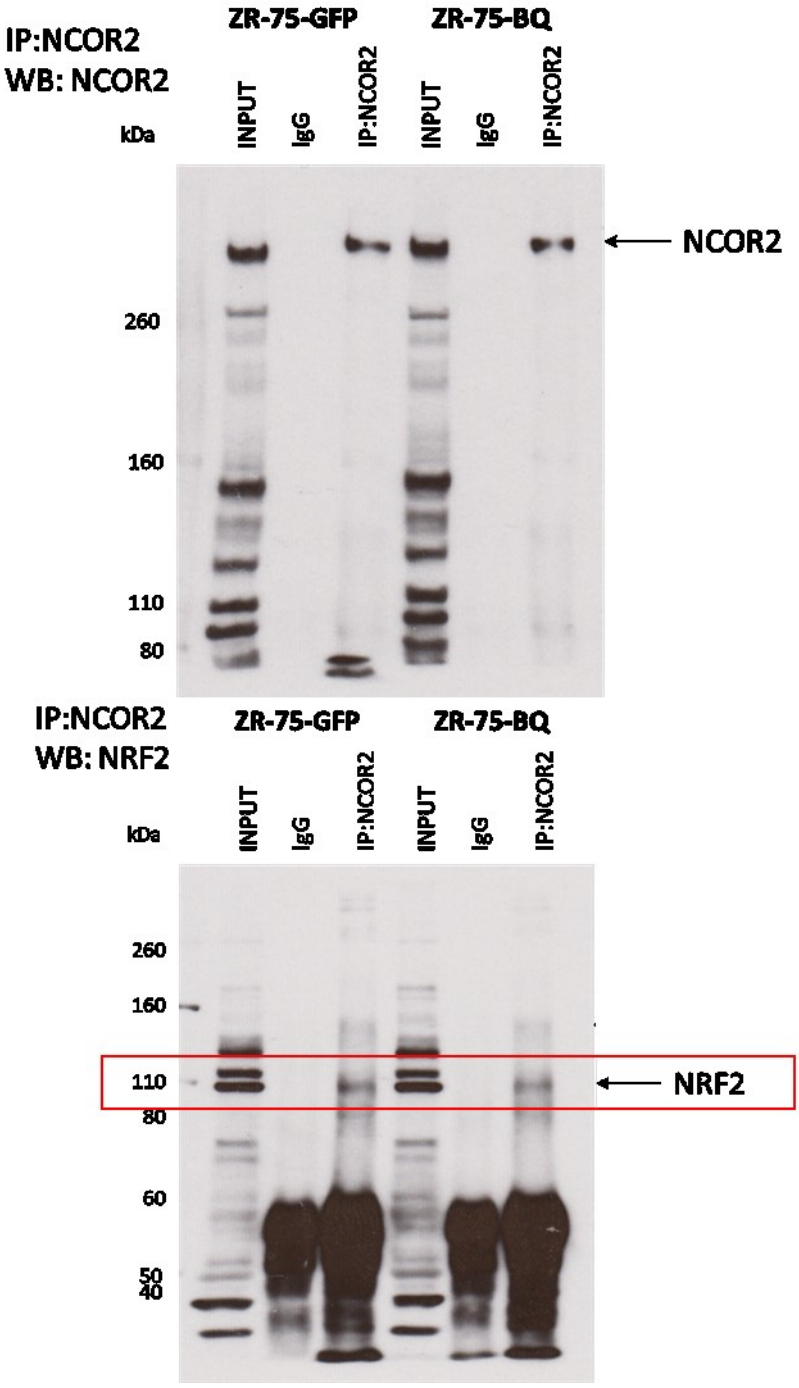

Relative Luciferase Activity
5
4
3
2
1
0
** (c)
*** (a)
* (b)
*** (a)
** (c)
** (a)
*** (a)
Empty vector
NCOR2
Empty vector
NCOR2
Empty vector
NCOR2
Empty vector
NCOR2
ZR-75-GFP
ZR-75-BQ
ZR-75-GFP
ZR-75-BQ
Untreated
100μM tBHQ

Figure 4.5 NCOR2 and BQ regulates transcription on antioxidant response element (ARE). ZR-75-BQ or ZR-75-GFP cells were transfected with NCOR2 expression plasmid (pCMV6-NCOR2) or empty vector as control and ARE-firefly luciferase reporter plasmid (pGL3-ARE) or basic firefly luciferase reporter plasmid (pGL3-basic) together with renilla luciferase control reporter plasmid (pRL-SV40). At post transfection 48 h, transfected cells were either treated with 100 μM tert-butylhydroquinone (tBHQ) for 24 h or left untreated. Cells were then harvested and the luciferase activities were measured by luminometer. Data were normalized to renilla luciferase activity and the resutls were represented as relative luciferase activity with reference to untreated empty vector control cells (ZR-75-GFP + Empty vector; untreated as 1). The experiments were repeated three times. Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

(A)


IP:NCOR2
WB: NCOR2
ZR-75-GFP
ZR-75-BQ
INPUT
IgG
IP:NCOR2
INPUT
IgG
IP:NCOR2
kDa
260
160
110
80
NCOR2
IP:NCOR2
WB: NRF2
ZR-75-GFP
ZR-75-BQ
INPUT
IgG
IP:NCOR2
INPUT
IgG
IP:NCOR2
kDa
260
160
110
80
60
50
40
NRF2

(B)

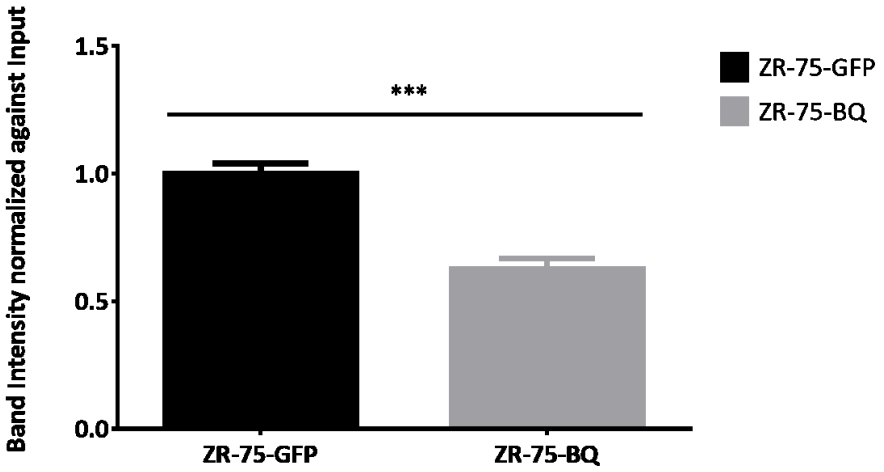


Figure 4.6 NCOR2 binds to NRF2 and BQ overexpression suppresses the interaction between NCOR2 and NRF2. ZR-75-BQ or ZR-75-GFP cells were harvested for immunoprecipitation by anti-NCOR2 antibody and normal rabbit IgG (IgG) as control. **(A)** Immunoblotting of the pulled down lysates as well as total cell lysate (as input control) by anti-NCOR2 antibody and anti-NRF2 antibody were shown. **(B)** Band intensities of NRF2 were quantified by Image J. Ratios of IP:NCOR2/Input in ZR-75-BQ and ZR-75-GFP were calculated as results were represented as mean ± SD;*P<0.05; **P<0.01; ***P<0.001. The experiments were repeated three times.

4.2.4 BQ overexpression mediates the response to epirubicin treatment in breast cancer cells

Transcription factor NRF2 is the master regulator of oxidative stress and modulation of oxidative stress. As illustrated in chapter 3, it has been shown that NRF2 could confer drug resistance in breast cancer cells. BQ overexpression was shown to upregulate the expression of NRF2 target genes and provide higher protection against oxidative stress. Therefore, we further hypothesized that BQ overexpression could contribute to chemoresistance in breast cancer.

To test this hypothesis, the response of ZR-75-BQ and ZR-75-GFP to treatment of two different chemotherapeutic drugs, epirubicin and paclitaxel, were studied using clonogenic assay (Figure 4.7). The results were shown as cell viability realtive to non-treated ZR-75-GFP (ZR-75-GFP at 0 nM as 1) which reflect the clonogenicity of cells under diffrerent conditions. As shown in figure 4.7A, BQ overexpression could significantly increase clonogencity in ZR-75 cells under all epirubicin concentrations tested (0, 1, 5, 10, 20 and 50 nM) in this experiment. On the other hand, BQ overexpression could significantly increase clonogencity in ZR-75 cells under paclitaxel at lower concentrations at 0, 0.5 and 1 nM (Figure 4.7B).

Since BQ overexpression favors tumor growth, BQ overexpressing cells (ZR-75-BQ) would have induced higher proliferation rate and hence higher clonogenicity at non-treated condition compared to control cells (ZR-75-GFP). Relative colony formation represented the total clonogencity, and this value can be affected by both drug treatment as well as cell proliferation. Therefore, the results were also presented as relative cell viability normalized agaisnt non-treated condition for ZR-75-GFP and ZR-75-BQ seperately (Figure 4.7Aiii and 4.7Biii). This ratio could opt out the effect of difference

in proliferation rate between these cells, reflecting the effect of drug treatment only. As shown in this plot, the results confirmed that BQ overexpression could significantly induce resistance to epirubicin in ZR-75 cells, marked by higher normalized colony formation under treatment of a series of epirubicin concentrations when compared with the vector controls. However, it was found that BQ overexpressing cells did not have higher normalized colony formation under treatment of different paclitaxel concentrations when compared with control cells. This indicated BQ overexpression might not contribute to resistance to paclitaxel in breast cancer cells. It was likely that the appeared higher total clonogencity under paclitaxel treatment would be due to the higher proliferation rate of BQ overexpressing cells.

As mentioned in Chapter 1, epirubicin generates higher levels of oxidative stress than paclitaxel. Deregulated control of oxidative stress might contribute to the development of chemoresistance in cancer cells. From the above results, it is evident that BQ overexpression could confer resistance to epirubicin but not to paclitaxel. Also, to further illustrate whether BQ overexpression could protect cells from drug induced oxidative stress, ROS levels in BQ overexpressing cells under epirubicin and paclitaxel treatment were compared to controls cells. ZR-75-BQ cells and ZR-75-GFP cells were treated with1 μM epirubicin and 10 nM paclitaxel alone or in combination with 5 μM N-acetyl-L-cysteine (NAC) for 8 h. Non-treated cells were used as control. NAC is a known ROS scavenger which is commonly used to identify and test ROS inducer. It can also be used to confirm the involvement of ROS in drug treatment (Halasi, Wang et al. 2013). Cells with different treatment were stained with CellROX Deep Red reagent to detect ROS, followed by flow cytometry analysis. The results were represented as CellROX Deep Red reagent fluorescence relative to non-treated control

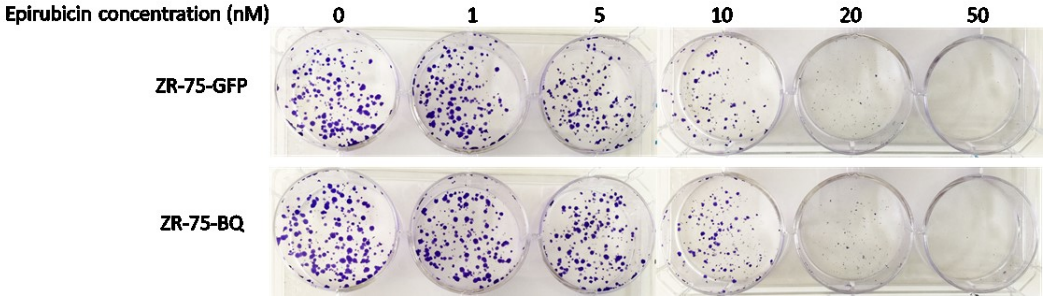
cells (non-treated ZR-75-GFP as 1), indicating the relative ROS levels of each cells sample (Figure 4.8).

Consistent with findings from literature, the results showed that epirubicin alone could significantly induce ROS in ZR-75 cells, marked by enhanced CellROX Deep Red reagent fluorescence under treatment with1 μM epirubicin when compared with non-treated control. Paclitaxel did not induce ROS in ZR-75 cells as there was no significant difference between paclitaxel treatment and non-treated control. Also, treatment with epirubicin in combination with NAC had significantly lower fluorescence signal than treatment with epirubicin alone in both ZR-75-GFP cells and ZR-75-BQ cells, indicating that 5 μM NAC in this experiment was sufficient to test the involvement of ROS in this experiment. This data showed that epirubicin, but less in paclitaxel, could contribute more to the generation of ROS in breast cancer cells.

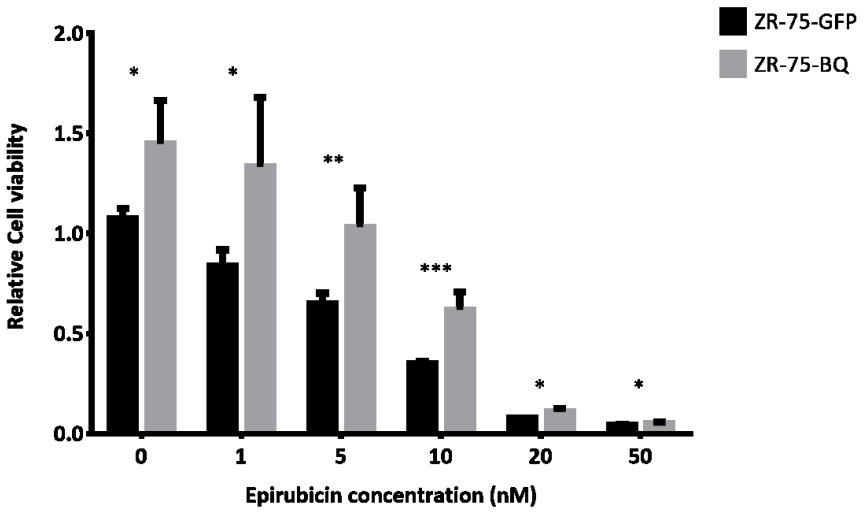
Comparing the fluorescence signal between ZR-75-GFP cells and ZR-75-BQ cells under different conditions, it was found that there was no significant difference between ZR-75-GFP cells and ZR-75-BQ cells, suggesting that the basal intracellular ROS levels were not affected by BQ overexpression. The induction of ROS by epirubicin treatment was significantly lower in ZR-75-BQ cells, suggesting that BQ overexpression had an effective anti-oxidative stress mechanism against epirubicin-induced oxidative stress. This result is consistent with our previous finding that BQ overexpression upregulates the expression of antioxidant genes.

(A)

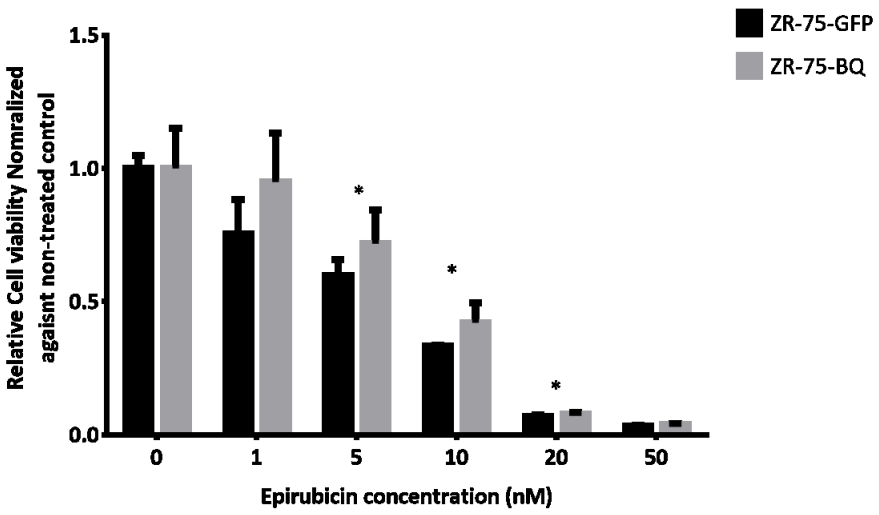
(i)


Epirubicin concentration (nM)
0
1
5
10
20
50
ZR-75-GFP
ZR-75-BQ

(ii)

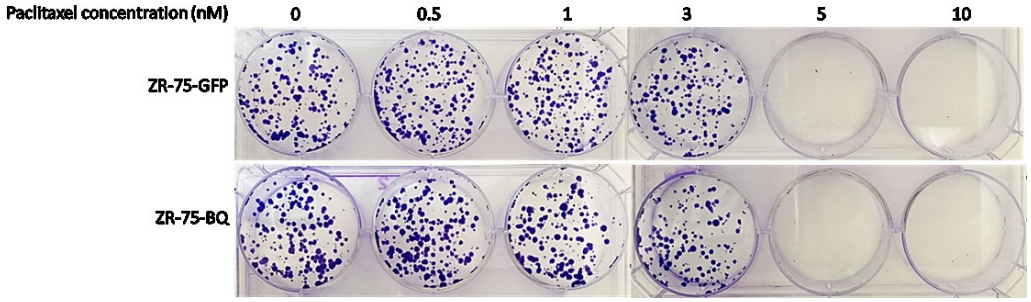

ZR-75-GFP
ZR-75-BQ
Relative Cell viability
2.0
1.5
1.0
0.5
0.0
*
*
**
***
*
*
0
1
5
10
20
50
Epirubicin concentration (nM)

(iii)

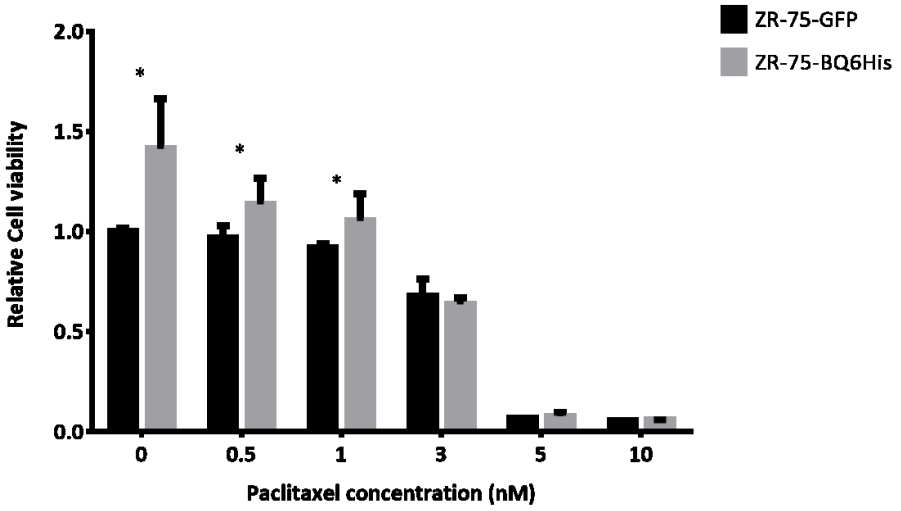

ZR-75-GFP
ZR-75-BQ
Relative Cell viability Nomralized against non-treated control
1.5
1.0
0.5
0.0
*
*
*
0
1
5
10
20
50
Epirubicin concentration (nM)

(B)

(i)


Paclitaxel concentration (nM)
0
0.5
1
3
5
10
ZR-75-GFP
ZR-75-BQ

(ii)


ZR-75-GFP
ZR-75-BQ6His
Relative Cell viability
2.0
1.5
1.0
0.5
0.0
*
*
*
0
0.5
1
3
5
10
Paclitaxel concentration (nM)

(iii)

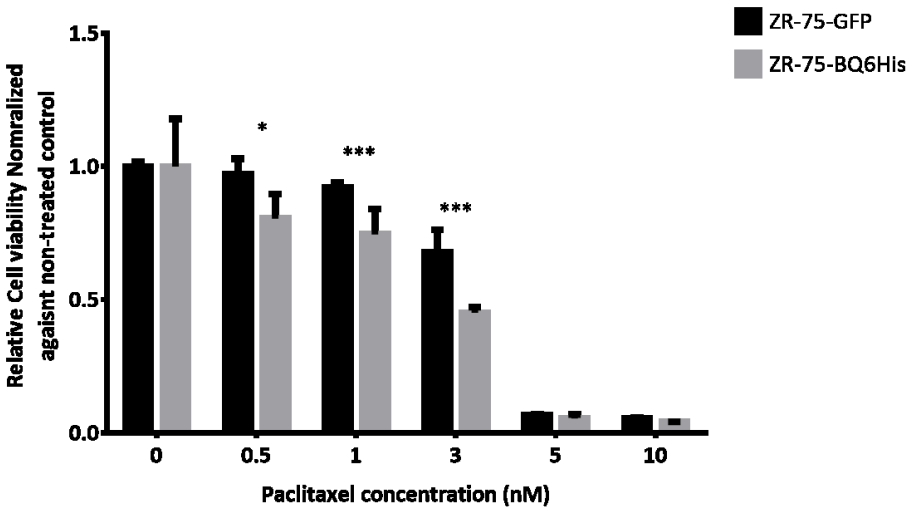

ZR-75-GFP
ZR-75-BQ6His
Relative Cell viability Nomralized against non-treated control
1.5
1.0
0.5
0.0
*
***
***
0
0.5
1
3
5
10
Paclitaxel concentration (nM)

Figure 4.7 BQ overexpression promotes chemoresistance in breast cancer cells. BQ overexpressing ZR-75 were treated with different concentration of **(A)** epirubicin or **(B)** paclitaxel. (i) Cells were treated with epirubicin (0, 1, 5, 10, 20 and 50 nM) or paclitaxel (0, 0.5, 1, 3, 5 and 10 nM) for 48 h. After drug wash out, colonies were allowed to grow for 10 days. The cells were then stained with crystal violet. The quantitative analysis was measuring the intensity of crystal violet staining after dissolving the stain in acetic acid. (ii) Relative colony formation was expressed with reference to untreated ZR-75-GFP cells. (iii) The result were also represented as relative colony formation (normalized) with regards to untreated condition (ZR-75-GFP or ZR-75-BQ at 0 nM as 1). The experiments have been repeated for three times independently and data are shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

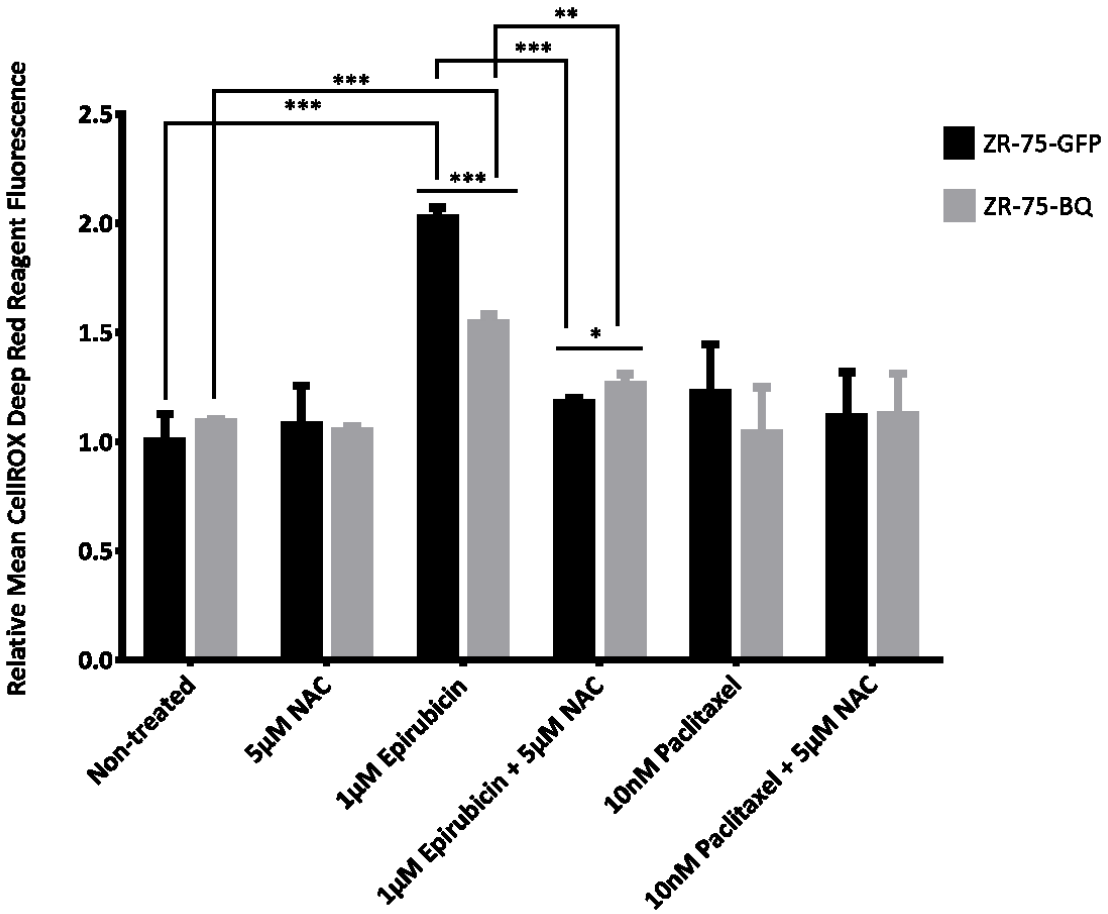


Figure 4.8 BQ overexpression mediates intracellular ROS levels in response to drug treatment in breast cancer. ZR-75-BQ and ZR-75-GFP cells were left untreated, treated with 1 μ M epirubicin or 10 nM paclitaxel alone for 8h. Cells were also pre-treated with 5 μ M NAC for 1 h. ROS levels were detected by CellROX Deep Red reagent and flow cytometry. Data were analyzed by FlowJo software and compared with non-treated control. The experiments have been repeated for three times independently and data are shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

4.2.5 BQ overexpression is associated with enhanced expressions of NRF2 target NQO1 in clinical samples of breast tumors

Having established the role of BQ in modulating NRF2 transcription activity and oxidative stress from *in vitro* experiments, immunohistochemical (IHC) staining was performed on tissue microarray constructed from 124 cases of primary breast tumors to validate the findings *in vivo*. It has been shown that BQ overexpression upregulates the expression of NRF2 targets, including antioxidant enzyme NQO1. The expression levels of BQ and NQO1 were scored as described in Chapter 2.12. Representative images of BQ and NQO1 IHC staining were shown in Figure 4.9. Total expression level of NQO1 was represented as the total sum of cytoplasmic score and nuclear score and were used for further statistical analysis. It was found that the BQ had similar expression levels in the cytoplasm in most samples while nuclear expression levels of BQ were found to be varying across all samples. The H-score system is a semi quantitative method of scoring nuclear staining intensity and percentage of nuclei stained of a particular intensity and was employed for assessing the expression levels of BQ, as described in Chapter 2.12.

According to the H-score determined of each sample, the cases were assigned as high nuclear BQ expression and low nuclear BQ expression group accordingly. The median score of BQ expression (equal to 130) was set as the cutoff value, meaning that samples with H-score lower than this value were regarded as low BQ expression group and vice versa. The NQO1 expression scores of each sample were plotted against these two groups, as shown in Figure 4.10. It was found that, although not statistically significant, the total NQO1 protein expression level were higher in high nuclear BQ expression group. However, the nuclear NQO1 protein expression was strongly correlated with nuclear BQ expression. This *in vivo* data provided strong evidence for the *in vitro*

finding that BQ overexpression upregulates NQO1 expression and nuclear level of NQO1.

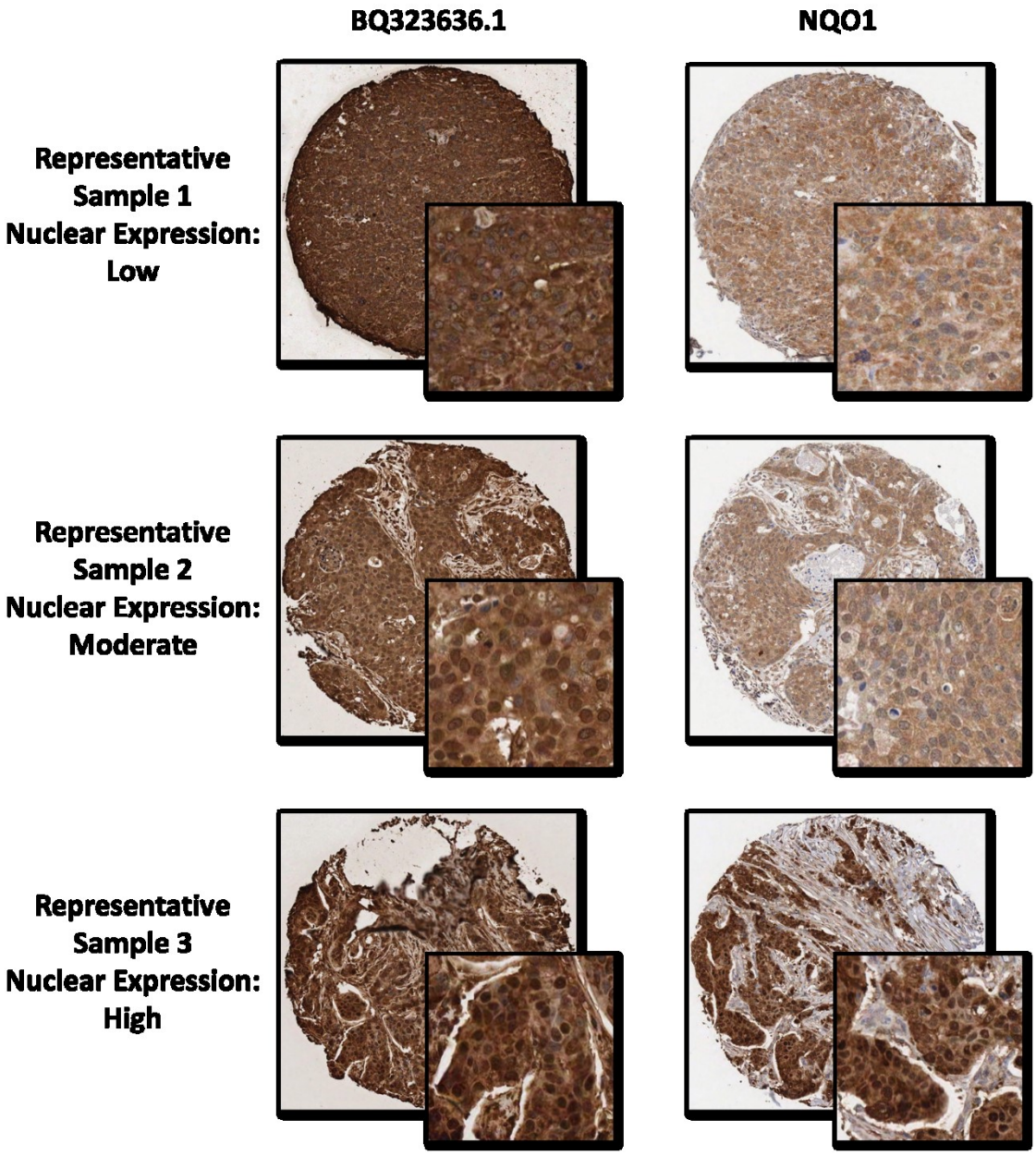
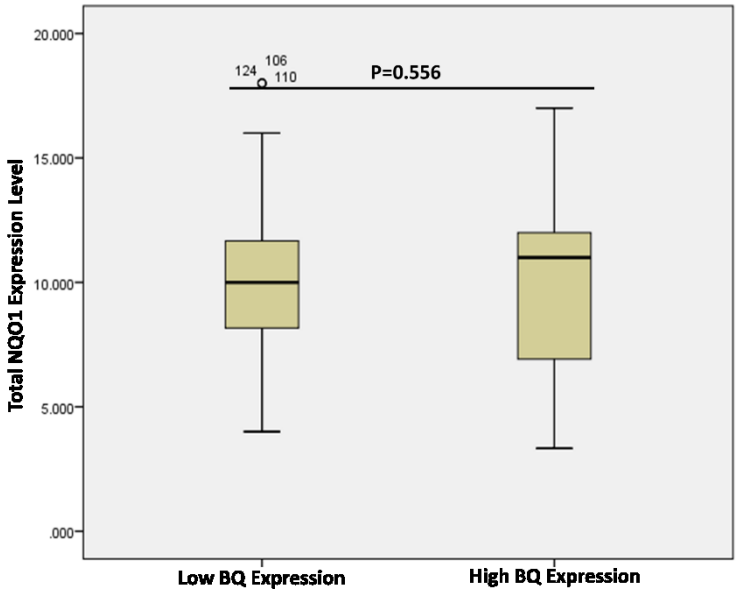


Figure 4.9 Representaive images of BQ323636.1 and NQO1 IHC staining in a set of tissue micrarray of primary breast tumor samples. Images shown with different nuclear expression level of BQ323636.1 (low, moderate or high) associated with NQO1 expression in the same clinical sample.

(A)



(B)

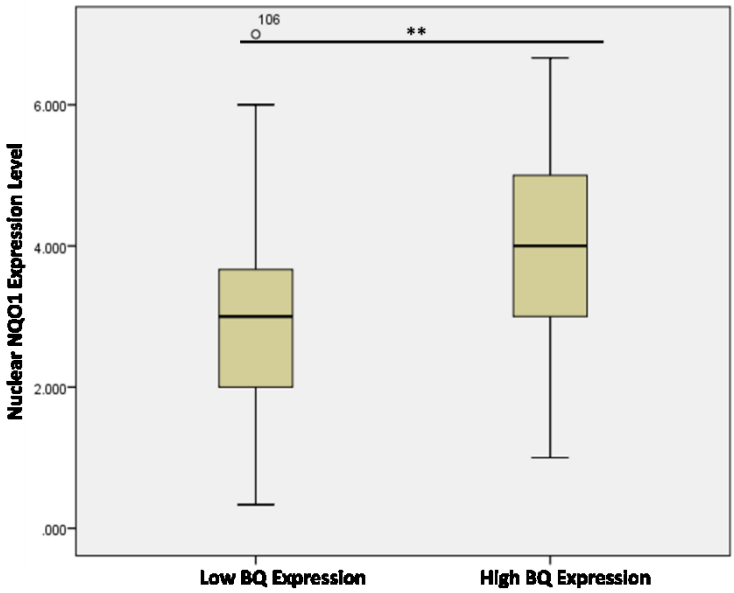


Figure 4.10 Comparisons of NQO1 expression levels with BQ expression level by Mann Whitney U rank test using all primary breast cancer samples. (A) Total NQO1 expression levels versus nuclear BQ expression (low or high). (B) Nuclear NQO1 expression levels versus nuclear BQ expression (low or high). Data were shown as mean ± SD;*P<0.05; **P<0.01; ***P<0.001.

4.3 Discussion

Upregulation of NRF2 has been reported in different types of cancer. A previous analysis of ER positive breast cancer patients showed that high NRF2 expression is significantly associated with poor patient outcome (Aritake, Tamaki et al. 2004). NRF2 is constantly degraded by KEAP1 via ubiquitination-proteasome pathway under basal condition. KEAP1 is inactivated under oxidative stress conditions, resulting in the release of NRF2 from KEAP1. NRF2 accumulation in the nucleus leads to induction of antioxidant genes (Itoh et al., 1997; No, Kim, & Song, 2014). As described in Chapter 1, stress mediated cell signaling kinases cascades can also regulate NRF2 transcriptional activity.

Besides this regulation induced by stress, a number of nuclear receptors are reported to interact with NRF2 to regulate NRF2 transcriptional activity. Inhibition of NRF2 pathway by nuclear receptors such as estrogen receptors, retinoic acid receptors and retinoid X receptor have been show in breast cancer cells (Wang, Hayes et al. 2007, Yao, Brodie et al. 2010, Namani, Li et al. 2014). The nuclear receptor co-repressor 2 (NCOR2) has been shown to inhibit NRF2 transcription upon activation of glucocorticoid receptor (Ki, Cho et al. 2005).

Our group has identified a spice variant of NCOR2, BQ323636.1 (called BQ in short) from splicearray profiling of breast cancer. BQ is formed from NCOR2 exon 11 skipping during mRNA splicing which result in an early stop codon. It is a truncated protein product retaining only the N-terminal side of the wild type NCOR2. It is found that BQ is associated with tamoxifen resistance and can mediate estrogen receptor ERα transcriptional activity (Zhang, Gong et al. 2013). Given the role of NCOR2 in inhibition of NRF2 activity and that BQ can control of ERα transcriptional activity, this

current study hypothesized that BQ could alter the suppressive role of NCOR2 on NRF2 transcription activity, favoring tumor growth and the development of chemoresistance in breast cancer.

In this study, BQ expression in a panel of breast cancer cell lines was compared. In general, It was found that BQ expression were lower in the ER positive breast cancer cell lines (MCF-7 and ZR-75) while higher in triple negative cell lines (MDA-MB-453 and MDA-MB-231 except HCC70) (Figure 4.1). Interestingly, BQ expression level in breast cancer cell lines shared similar pattern with BQ mRNA expression found in breast cancer samples and correlated to ER and PR status of the patients. BQ mRNA expression is significantly higher in ER negative patients and PR negative patients (Zhang, Gong et al. 2013). The endogenous low expression of BQ suggested that these ER positive cell lines are suitable to study the effect of ectopic BQ expression.

We found that BQ overexpression in ER positive cell lines resulted in higher resistance to tBHP induced cell death as shown by MTT assays and clonogenic assays (Figure 4.3). Also expression of antioxidant genes were found to be upregulated in BQ overexpressing cells. These genes includes NQO1, HMOX1, GCLC and GSTP1. High levels of these antioxidant enzymes has been found in many human cancer types, including breast cancer (Batist, Tulpule et al. 1986, Yang, Zhang et al. 2014, Chau 2015, Syu, Chi et al. 2016), suggesting their role in cancer progression and chemoresistance. In addition, NQO1 and GCLC has been suggested to drive tamoxifen resistance as they are functionally sufficient to confer a metabolic phenotype of tamoxifen-resistant breast cancer cells (Fiorillo, Sotgia et al. 2017). Consistently, BQ is also associated with tamoxifen resistance (Zhang, Gong et al. 2013). Given the roles of antioxidant enzymes in cancer progression and chemoresistance, it is postulated that BQ overexpression can

render cancer cells to survive in relatively high oxidative stress condition and thus protecting cancer cells from toxic action of chemotherapeutic agents.

The protein level of NQO1, as well as mRNA levels of antioxidant genes were found higher in BQ overexpressing cells (Figure 4.4), suggesting increased ARE-dependent transcription. Since NRF2 is the main transcription factor responsible for ARE-dependent transcription, this observation in BQ overexpressing cells could be explained either by increased expression of NRF2 or upregulated NRF2 transcription activity. It is of great interest to understand how BQ could upregulate the expression of antioxidant enzymes. From this current study, it appeared that NRF2 expression was not altered upon BQ overexpression. Also, expression of NCOR2 at both protein and mRNA levels showed no significant difference between BQ overexpressing cells and the control (Figure 4.4). The results indicated BQ did not exert a dominant effect on either NRF2 expression positively or NCOR2 expression positively. It has been shown that BQ exerts the opposite effect of NCOR2 as it can suppresses ERα transcriptional activity (Zhang, Gong et al. 2013), hence BQ might play a similar role in regulating NRF2 transcriptional activity rather than regulating the expression of NRF2.

We thus studied the function of BQ in regulating NRF2 signaling pathway by ARE-luciferase reporter assay. It has been shown that overexpression of NCOR2 also reduced the constitutive GSTA2 expression as well as reduced the induction of GSTA2 by tBHQ in H4IIE cells (Ki, Cho et al. 2005). In our study, an ARE-luciferase reporter was constructed based on ARE of NQO1 promoter. Consistently, overexpression of NCOR2 repressed ARE-luciferase activities as well as repressed the induction of ARE-luciferase activities by tBHQ, confirming the role of NCOR2 as a co-repressor in ARE mediated transcription. BQ overexpression rescued the repression of ARE-luciferase

activities, indicating the role of BQ in suppressing NCOR2 activity on ARE mediated transcription (Figure 4.5). The effect of BQ in opposing the NCOR2 suppressive activity on ARE-luciferase reporter could explain the upregulation of antioxidant genes upon BQ overexpression.

BQ323636.1 is a truncated protein product sharing amino acid sequence at the N-terminus of wildtype NCOR2. The only functional domain of wildtype NCOR2 retained in BQ is the repressor domain 1 (RD1), which is responsible for recruiting TBL1 and GPS2 of the core corepressor complex (Wong, Guo et al. 2014).In addition, it has been reported that the C-terminus of NCOR2 (amino acid 1788-1903) interacts with N-terminus of NCOR2 (amino acid 290-427) to form a protein dimer in anti-parrallel fashion serving as a central platform for recruiting other components in the co-repressor complex. As BQ shares the same structure only at the N-terminus of NCOR2, we believed that BQ would have lost the ability to recruit other factors in the core co-repressor complex or bind to transcription factors while retaining the ability to bind to the C-terminus of NCOR2. In other words, BQ could compete with NCOR2 for self-dimerization with the C-terminus of NCOR2. Based on this hypothesis, the mechanism of BQ in suppressing NCOR2 activity was studied by immunoprecipitation (Figure 4.6A). We confirmed that there was protein-protein interaction between NCOR2 and NRF2 in breast cancer cells, consistent with previous report that NCOR2 interacts with NRF2 upon glucocorticoid receptor activation (Ki, Cho et al. 2005). BQ overexpression significantly inhibited the interaction between NCOR2 and NRF2 (Figure 4.6B), indicating the suppressive effect of NCOR2 corepressor complex on NRF2 was inhibited by BQ. A mechanism of BQ is proposed, as shown in Figure 4.11.

As demonstrated in chapter 3, higher protection against oxidative stress is correlated with chemoresistance and upregulation of NRF2 confers epirubicin resistance in breast cancer cells. BQ overexpression also induces the expression of antioxidant genes and rescues the suppression of NRF2 on ARE transcriptional activity mediated by NCOR2. Due to its ability in modulating the NRF2/ARE pathway, we therefore studied whether BQ could also contribute to the development of chemoresistance. Results showed that BQ overexpression could favor cancer cell proliferation (Figure 4.7) and enhanced resistance of breast cancer cell against epirubicin (Figure 4.7A). However, BQ overexpression appeared not to confer paclitaxel resistance (Figure 4.7B).

The mechanisms of actions of epirubicin and paclitaxel are very different. Anthracyclines induces DNA damage by DNA/RNA intercalation, inhibition of DNA topoisomerase II and induction of DNA cross-linking. The cytotoxic activity of epirubicin is also contributed by the formation of ROS (Minotti, Menna et al. 2004). Paclitaxel, on the other hand, induces the dynamic instability of microtubules resulting in blocking of G2/M phase transition and consequent cell death (Komlodi-Pasztor, Sackett et al. 2011). Different mechanisms of drug resistance are also proposed for anthracyclines and taxanes. Anthracycline resistance can be mediated by drug transport proteins, drug metabolism alteration, topoisomerase modulation and enhanced antioxidant defense. Several potential taxane resistance mechanisms have been identified *in vitro*, they includes elevated expression of drug transporters, structural alterations in microtubule, apoptosis inhibition and activation of survival pathways (Chien and Moasser 2008, Edwardson, Chewchuk et al. 2013). In this study, it was also found that epirubicin, but not paclitaxel, could induce oxidative stress in breast cancer cells (Figure 4.8).

Enhanced antioxidant defense is one of the resistance mechanisms of anthracyclines. BQ overexpression could upregulate the expression of antioxidant genes (Figure 4.4) and reduce the effect of epirubicin-induced cell death (Figure 4.7A). Although BQ overexpression did not alter basal intracellular ROS levels, it was found that ROS induction by epirubicin treatment was significantly lower in BQ overexpressing cells (Figure 4.8). This result further supports the idea that BQ overexpression promotes epirubicin resistance via enhanced antioxidant defense.

These *in vitro* results are further supported by *in vivo* correlation between BQ nuclear expression and NQO1 expression. From IHC stainings of a set of primary breast tumor sample in tissue microarray, it was found that NQO1 protein expression was positively correlated with BQ protein expression (Figure 4.10A), confirming the i*n vitro* result that BQ overexpression upregulates antioxidant genes, including NQO1. More interestingly, in patients with high nuclear BQ protein expression, nuclear NQO1 expression was significantly higher (4.10B). It has previously been shown that NQO1 mainly demonstrates a cytoplasmic staining pattern in breast cancer (Yang, Zhang et al. 2014). In breast tumor samples analyzed in this set of TMA, it was also found that NQO1 staining was present in both the cytoplasm and the nucleus. Increased level of NQO1 in the nucleus is of significant importance. It has previously been suggested that nuclear NQO1 play roles in protection from chemotherapy and bioactivation of antitumor agents (Ross, Kepa et al. 2000, Winski, Koutalos et al. 2002). Therefore, BQ might not only upregulate NQO1 expression but also enhance the levels of NQO1 in the nucleus, which in turns, confer cancer growth and chemoresistance.

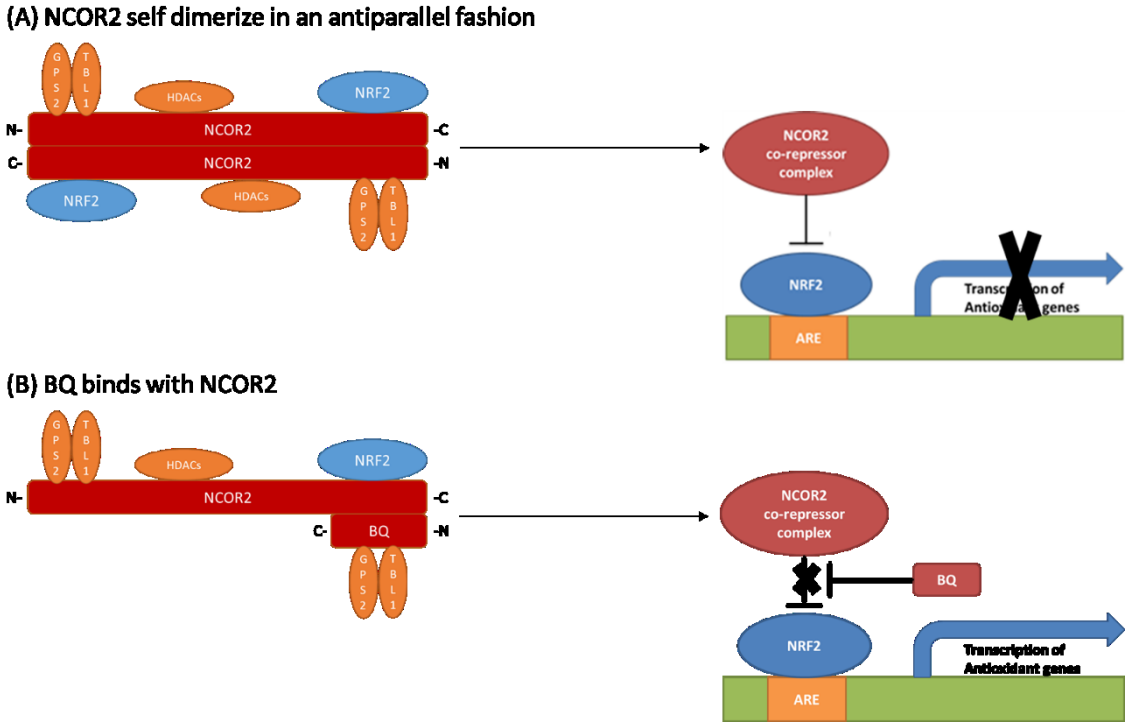


Figure 4.11 Proposed mechanisms of BQ in mediating NRF2 pathway via inhibition of NCOR2 suppressive effect. **(A)** NCOR2 self-dimerizes in anti-parallel fashion, forming a central platform to recruit other corepressor components such as GPS2, TBL1 and HDACs to form a functional NCOR2 corepressor complex. The NCOR2 corepressor complex binds with NRF2 via protein-protein interaction between NCOR2 and NRF2, suppressing the ARE mediated transcription of antioxidant genes. **(B)** In the presence of BQ, BQ binds with NCOR2 at the C-terminus, reducing the ability of NCOR2 to fully recruit other corepressor components such as HDACs. This gives rise to a non-fully functional NCOR2 corepressor complex, thus relieving the suppressive effect on NRF2, upregulating ARE mediated transcription of antioxidant genes.

Chapter 5 Final Discussion

Breast cancer is the most common malignancy in women. Breast cancer is ranked as the first leading cancer type and third leading causes of cancers deaths of female in Hong Kong (Hong Kong Cancer Registry, 2015). According to the WHO, there were an estimated 1.67 million new breast cancer cases diagnosed in 2012, which is 25% of all cancers. Breast cancer treatments are generally designed according to the stages of cancer and recently the molecular subtypes. Surgery, radiation therapy, hormonal therapy, chemotherapy and targeted therapy have achieved great improvement in breast cancer patient's survival rate. However, these treatment strategies appeared not to be effective in more aggressive and advanced stages, characterized by poor clinical outcomes and high incidence of recurrence (Videira, Reis et al. 2014). Due to failure of adjuvant therapy, development of distant metastasis over time is observed in about 15 % of all patients previously diagnosed with localized breast cancer. Although conventional chemotherapy and other treatments have improved survival outcomes, breast cancer is viewed as a chronic disease by some researchers as there is no cure available for metastatic breast cancer (Austreid, Lonning et al. 2014). Thus, there is a need for improved treatment methods for early as well as advanced breast cancer. A better understanding of the mechanisms of adjuvant therapy failure would be needed to improve current therapeutic methods.

Drug resistance is a major cause of treatment failure in cancer chemotherapy. Altered drug trafficking mediated by overexpression of ABC transporters has been proposed as a main cause of drug resistance. However, inhibitors targeting these drug efflux transporters did not show satisfactory clinical results (Palmeira, Sousa et al. 2012). Other mechanisms of chemoresistance includes enhanced DNA damage repair, drug

inactivation, deregulated apoptosis and induction of adaptive proliferative and survival signaling pathways. Besides, there are growing evidence to support that oxidative stress can contribute to resistance to chemotherapy. Oxidative stress results from an imbalance between ROS production and cell's antioxidant capacity. A complex intracellular redox homeostasis system has developed to adapt and protect cells against the harmful effects of oxidative stress. Cell signaling pathways involved in adaptive response to oxidative stress have also shown to be involved in protecting cells against cytotoxic effects of chemotherapy. Therefore, it is hypothesized that there is a correlation between adaptive response to oxidative stress and resistance to chemotherapy.

Understandings of the mechanisms causing resistance to chemotherapy are limited. Therefore, in this PhD study, my aim to understand molecular pathways that are involved in regulation of oxidative stress and chemoresistance in breast cancer. NRF2 is an essential transcription factor for antioxidant and detoxification genes. It has been shown that constitutive upregulation of NRF2 is common in many types of cancer. Due to its cytoprotective roles, NRF2 overexpression provides protection to cells against oxidative stress and anti-cancer drugs, providing growth advantage for cancer cells and thus contribute to chemoresistance. This PhD study is focused on novel molecular mechanisms that is implicated in the regulation of NRF2 and chemoresistance: 1) to investigate the role of FOXM1 in regulation of NRF2 expression and epirubicin response of breast cancer; 2) to characterize the role of a novel splice variant of NCOR2, BQ, in modulating oxidative stress and NRF2 transcriptional activity.

In chapter 3, the data suggested a distinct mechanism in breast cancer cells whereby transcriptional inactivation of NRF2 upon silencing of FOXM1 lead to downregulation of antioxidant enzymes in drug sensitive MCF-7 cell line. KEAP1 inhibition and cell

signaling kinases have been reported to control the level and activity of NRF2 protein. In contrast, little is known concerning the transcriptional control of the NRF2 gene. Our data revealed that FOXM1 is crucial for maintaining the basal levels of NRF2 by directing its transcription. Also, FOXM1 mediates the expression of NRF2 in response to epirubicin treatment. FOXM1 is a typical proliferation-associated transcription factor and overexpression of FOXM1 is commonly found in many type of human malignancies. It is implicated in different hallmarks of cancer (Halasi & Gartel, 2013). The identified FOXM1/NRF2 axis linking with tumor biology could serve as a useful target for breast cancer prognosis.

In chemoresistant MCF-7 EpiR cells, silencing of FOXM1 did not lower the expression of NRF2 and NRF2 downstream target genes, suggesting that the FOXM1/NRF2 axis is deregulated in epirubicin resistant cells (Figure 3.9). An FOXM1 binding site was identified in the NRF2 region by ChIP-seq analysis and confirmed by ChIP-qPCR in FOXM1 knockdown and overexpression assays. FOXM1 binding to the NRF2 region was less enriched in epirubicin resistant cells compared to sensitive cells (Figure 3.11). These results indicate deregulated FOXM1 binding on NRF2 region and transcriptional activity in chemoresistant cells. Transcriptional activity of FOXM1 can be regulated by expression of FOXM1 as well as post-translational modifications of FOXM1. It has been shown that ATM and p53 coordinately regulate FOXM1 expression through the E2F1 transcription factor in breast cancer cells, with ATM activating and p53 repressing FOXM1 transcription (Millour, de Olano et al. 2011). Recent studies have shown that FOXM1 activity could be mediated by phosphorylation, SUMOylation, ubiquitination and acetylation affecting FOXM1 functions positively or negatively (Song, Fiati Kenston et al. 2017). It is postulated these modifications can change the transcriptional activity of FOXM1 and may deregulate the FOXM1/NRF2 axis in the development of

chemoresistance. Additional studies would be required in order to elucidate which molecular mechanisms are altered in regulating FOXM1 activity during the development of chemoresistance.

Given that cancer cells harbor higher ROS levels than normal cells, inducing toxic levels of ROS in cancer cells represent a powerful approach to selectively kill cancer cells. This can be achieved by triggering ROS accumulation or inhibiting ROS scavenging systems. NRF2-ARE is a powerful antioxidant regulatory system which reduce ROS levels in cancer cells. In addition to regulation of NRF2 described in this study, previous reports have shown that FOXM1 could induce the expressions of antioxidant enzymes such as catalase, MnSOD and PRDX3 (Park, Carr et al. 2009). Inhibition of the NRF2-ARE pathway and the inhibition of FOXM1, which can reduce antioxidant genes expression and promote oxidative stress, could be a useful therapeutic method against cancer cells.

A number of small molecules could inhibit NRF2-ARE pathway, such as flavonoids, antitubercular drugs, vitamins and their derivatives. These small molecules inhibit NRF2-ARE pathway at multiple levels. The activity of NRF2 can be mediated by suppressing the cell signaling kinases cascades (such as AKT and ERK) phosphorylation on NRF2; blocking nuclear translocation of NRF2; promoting degradation of NRF2 mRNA or upregulating KEAP1 protein level and activity (Zhu, Wang et al. 2016). The small molecule brusatol was used in this study. Brusatol selectively inhibits the NRF2 pathway by altering NRF2 translation and post-translational regulation, and thus downregulate NRF2 protein levels (Ren, Villeneuve et al. 2011). It was shown that this NRF2 inhibitor could further sensitizes MCF-7 cells to epirubicin. Chemotherapy induces oxidative stress to cancer cells, and inhibition of

NF2 by brusatol enhances the efficacy of chemotherapeutic drugs. Hence, in the future, it might be useful to use NRF2 inhibitors combined with conventional chemotherapy in the treatment of breast cancer.

FOXM1 inhibition in combination with oxidative stress could also be useful to induce cancer cell death. Proteasome inhibitors such as bortezomib, MG115, MG132 and thiazole antiobiotics Siomycin A and thiostepton have been shown to inhibit FOXM1 expression and transcriptional activity (Bhat, Halasi et al. 2009, Gartel 2010). A study by Halasi *et al* have demonstrated the combinatorial treatment of FOXM1 inhibitor bortezomib and phenethyl isothiocyanate (PEITC) greatly inhibited proliferation of cancer cells and tumor growth, supporting that combination of ROS inducers and FOXM1 inhibitors could be used as new therapeutic approach to selectively kill cancer cells (Halasi, Pandit et al. 2013). However, PEITC and other ROS inducers such as sulforaphane (SFN), have been shown to induce phase II enzymes including GSTs, NQO1 and UGT. Induction of these enzymes is NRF2 dependent and coupled with activation of MAPK pathway (Cheung and Kong 2010). Overexpression of NRF2 could induce antioxidant enzymes expression and upregulate FOXM1 expression in MCF-7 cells. Taken together, the use of ROS inducers to trigger oxidative stress might minimize the effect of proteasome inhibitors in the treatment of cancer. The induction of oxidative stress might be better achieved by inhibiting ROS scavenging systems, such as the NRF2-ARE pathway. Therefore, combinational treatment of FOXM1 inhibitors and NRF2 inhibitors can be a feasible anticancer therapy to improve chemotherapeutic response in cancer treatment.

In chapter 4, it was shown that overexpressing the novel splice variant of NCOR2, BQ363232.1 (BQ), in breast cancer cell lines could promote cell proliferation, reduce

induction of ROS levels and protect cells from oxidative stress. This could be achieved through enhanced expression of antioxidant genes by BQ overexpression. NRF2 transcriptional activity, and thus expression of antioxidant genes, can be modulated by NCOR2. NCOR2 could attenuate transcription activation of ARE while BQ opposed the suppressive effect of NCOR2 since BQ could interfere the interaction between NCOR2 and NRF2. Furthermore, high BQ expression is significantly correlated with expression of an antioxidant enzyme NQO1, supporting its role in regulation of ROS scavenging system. It was also noted that BQ overexpression induced epirubicin resistance and mediated ROS induction by epirubicin treatment in breast cancer cells. Based on the role of BQ in modulation of oxidative stress, a new oncogenic role for BQ in contributing to epirubicin resistance is proposed. This current study strengthens the potential value of BQ not only as a biomarker for tamoxifen resistance in breast cancer but also could serve as a potential predictor of chemoresistance.

There are several molecular mechanisms which regulate gene expression such as epigenetic modulation, microRNAs and alternative splicing. Alternative splicing, whereby a precursor mRNA is processed to different mature mRNAs via splice site selection, provide a significant expansion of the proteome with limited gene numbers. (Dvinge, Kim et al. 2016). Recognition of alternative splice sites is usually cell type specific. Alternative splicing is involved in more than 90% of human gene expression, contributing to protein diversity in different cell context (Lee and Rio 2015). Although the functional roles of most isoforms generated by alternative splicing are yet to be determined, there is a growing body of evidence showing that specific isoforms of certain genes are involved in the development of cancer due to their ability to promote oncogenic transformation, cell progression/proliferation and resistance to anti-cancer drugs (David and Manley 2010). Alternative splicing have recently viewed as a new

hall mark of cancer (Ladomery 2013). Researchers in this field have discovered various alternative splicing isoforms implicated in breast cancer including BRCA1, DMTF1, FGFR, HER2, KLF6, Survivin and TP53 (Martinez-Montiel, Anaya-Ruiz et al. 2017). Different alternative isoforms of these genes are implicated in different aspects of breast cancer: tumor suppressors; resistance to HER2 targeted therapy; association with epithelial–mesenchymal transition, cell proliferation and survival pathways.

In this PhD study and previous works of our group, the roles of a novel alternative spliced isoform of NCOR2, BQ, in breast cancer were elucidated. It was found that BQ opposes the effect of wild type NCOR2, modulating the transcriptional activity of ERα (Zhang, Gong et al. 2013) and NRF2. High BQ expression was associated with poor clinical outcome of breast cancer patients and resistance to tamoxifen (Zhang, Gong et al. 2013), and our *in vitro* studies show it is potentially associated with resistance to chemotherapy that induces oxidative stress.

As BQ is formed from exon 11 skipping of NCOR2 mRNA during mRNA processing, future investigation can be focused on the mechanisms responsible for such alternative splicing of NCOR2 in breast cancer. Abnormal expression of splice factors in cancer changes the alternative splicing of pre-mRNAs. Deregulation of these splicing factors have been linked to breast cancer development (Silipo, Gautrey et al. 2015). More than 30 human splicing factors have been well defined in cells and tissue and some of them are characterized as either proto-oncogenes such as *SRSF1, SRFS3, DAM1, PELP1*, or tumour suppressors such as *RBM, PRMT6* in breast cancer (Silipo, Gautrey et al. 2015). Based on the oncogenic roles of BQ, alternative splicing of NCOR2 might possibly be mediated by these oncogenic splicing factors. Once these specific splicing factor(s) responsible for BQ generation is identified, targeting such factors could be a new

therapeutic approach for breast cancer. On the other hand, changes in alternative splicing might arise from mutations or single-nucleotide polymorphisms within the gene sequence, strongly affecting the splicing factor activities and influence the selection of splice sites. Future work might also need to define the specific mutations in NCOR2 genes that cause the alternative splicing of NCOR2 mRNA and generation of BQ.

The current study showed that FOXM1 regulates NRF2 expression and BQ could modulate NRF2 transcription activity. Another future research direction would be to investigate whether BQ could regulate FOXM1 expression and transcription activity. It has been shown that ERα activates FOXM1 transcription through an estrogen-response element (ERE) located within the proximal promoter region and ER-ligands (estrogen, tamoxifen and fulvestrant) regulate the expression FOXM1 (Millour, Constantinidou et al. 2010). Also, BQ could oppose the suppressive effect of NCOR2 in modulating the transcriptional activity of ERα on ERE (Zhang, Gong et al. 2013). It would be interesting to determine whether BQ could regulate FOXM1 expression and activity via modulation of ERα on ERE

NCOR2 has been reported to be associated with HDAC3 and FOXO3 to suppress the transcription of FOXO3 target gene IRF7 (Litvak, Ratushny et al. 2012). FOXO3 has been shown to antagonize FOXM1 activity via various mechanisms: (i) activated FOXO3 displaces FOXM1 from target gene promoters; (ii) FOXM1 is a negative transcriptional target of FOXO3; (iii) FOXO3 can recruit chromatin-remodelling proteins like HDACs, leading to condensed chromatin and limited binding of transcription factors, including FOXM1 (Koo, Muir et al. 2012).Given that NCOR2

has suppressive effect on transcription factors, NCOR2 may have suppressive effect on FOXO3. Thus BQ may oppose NCOR2 activity in regulating FOXM1 expression and/or transcriptional activity via FOXO3.

These findings will provide an insight into NCOR2/BQ in modulation of oxidative stress independent and/or dependent of FOXM1. Not all antioxidant genes possess ARE at their promoters, thus not all are regulated by the NRF2-ARE antioxidant system. There are other master genes in cancer that can regulate non-ARE genes to reduce ROS in cells. For instance, FOXM1 has been reported to regulate the expression of catalase, MnSOD and PRDX3 (Park, Carr et al. 2009). Defining BQ in the regulation of FOXM1 would extend our understanding of the role of BQ in modulation of oxidative stress and development of chemoresistance in the treatment of breast cancer.

To summarize, my results have characterized new molecular mechanisms that are involved in the regulation of NRF2 expression and transcriptional activity. And provided a better understanding of the molecular basis for the regulation of oxidative stress and the development of chemoresistance in breast cancer.

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