The Hong Kong Polytechnic University

Department of Health Technology & Informatics

Id-1 promotes cell proliferation through activation of EGFR, NF-κB p50 homodimer and Bcl-3 in MCF-7 breast cancer cell line

KO WAI TING

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

October 2009

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

_____ (Signed)

Ko Wai Ting (Name of student)

Abstract

Progression of breast cancer from hormone-dependent to hormone-independent causes a major problem in breast cancer therapy. The possible involvement of inhibitor of differentiation type 1 (Id-1) in this progression was suggested by the findings in our group that expression of Id-1 was higher in patients with a lower percentage of estrogen receptors (ER). Therefore, in this study, we over-expressed Id-1 in the hormone-dependent breast cancer cell line, MCF-7, to examine whether Id-1 confers growth advantage to these cells in the absence of estrogen. Our results showed that cell growth was increased in the Id-1 transfectants compared with the mock-transfected clone control over a 120 hour period. Cell growth of the transfectant was similar to the mock-transfected control after transient transfection of the Id-1 antisense oligonucleotide. Moreover, elevated levels of epidermal growth factor receptor (EGFR) which is associated with hormone-independent breast cancer, were found in Id-1 transfectants using Western blot and reverse-transcription (RT) - PCR analyses. The level of EGFR expression was decreased after transient transfection of Id-1 antisense oligonucleotide compared with the same Id-1 transfectant without Id-1 antisense oligonucleotide. The involvement of nuclear factor-kappa B (NF- κB) pathway which is one of the downstream pathways of EGFR, suggested to be

activated in ER negative breast cells, was investigated. After applying an inhibitor of NF- κB, parthenolide, cell growth and cells in the S phases of the cell cycle were significantly decreased by more than 50%. Results also showed that the percentage inhibitions were positively associated with the expression levels of Id-1, implying that Id-1 may be able to activate the NF-κB pathway. Interestingly, elevated expression of nuclear fragment p50 and Bcl-3, but not p65, were observed with increased level of Id-1 using Western blotting. By contrast, the expression of IκB-α, the corresponding inhibitor of the complex of NF-κB p50/p65 heterodimers, was not correlated with the expression of Id-1. Moreover, results of electrophoretic mobility shift assay (EMSA) showed that levels of NF-κB p50 were significantly increased in Id-1 transfectants and positively associated with levels of Id-1. Our results suggest that Id-1 may be able to modulate cell growth in the absence of estrogen, possibly through the activation of EGFR signaling pathways and through the activation of NF-κB p50/p50 homodimer and Bcl-3 but not NF-κB p50/p65 heterodimer. Although further study is needed, Id-1 may serve as a biomarker in the progression of breast cancer from hormone-dependent to hormone-independent. Inactivation of Id-1 may be a potential therapeutic target for breast cancer patients who have developed resistance to hormonal therapy.

List of Publication

Wai Ting, KO; Gina, LEUNG; Tony, TO (2008) The role of inhibitor of differentiation type 1 (Id-1) in the progression of breast cancer from hormone-dependent to hormone-independent. The latest advances in breast cancer research from basic science to therapeutics. 2nd JCA-AACR special joint conference. Japan. Abstract 20.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Dr. Gina Leung, who has supported me throughout my thesis with her patience, encouragement and knowledge. Without Dr. Gina Leung, the writing and completion of this thesis would not be possible. I have also learnt a lot of things other than laboratory techniques and knowledge from her during my MPhil study, which has enriched my life and also my view of working in clinical laboratory.

I also gratefully acknowledge my co-supervisor, Dr. Tony To, it is my pleasure to have guidance from him, he is always willing to share his ideas. His critical advice is really useful and helped me overcome difficulties.

It is my pleasure to study under the Department of Health Technology & Informatics of The Hong Kong Polytechnic University, I gratefully thank my department for giving me the chance to learn here, and for stipend during my study.

Many thanks also go to Ng Lui for his valuable technical advice and friendship, and to Michael Tsang, my research teammate.

I would also acknowledge Carina Luk, Sue Choi, Sindy Lai and Poon Ngork Wah for their support and encouragement in the office. I really thank those supporting me and providing me with useful information during discussions. I really treasure our friendship created in this period of time.

I would like to express my grateful thanks to my beloved, Ting Fung, for his company and support during the whole MPhil study.

Finally, I would like to thank my family and everybody giving me support and opinions throughout all my studies. I have to apologize that I cannot mention all of you one by one.

Table of Contents

List of Figures

List of Tables

Abbreviations

AF-1	Activation function -1
AJCC	American Joint Committee on Cancer
ANK	Ankyrin
BRCA	Breast cancer susceptibility protein
Cdk	Cyclin-dependent kinase
CI	Confidence interval
COX-2	Cyclooxygenase-2
CT	Computed tomography
DAPI	4'-6-diamidino-2-phenylindole
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptors
ERE	Estrogen response elements
ESCC	Esophageal squamous cell carcinoma
FBS	Fetal bovine serum
G-CSF	Granulocyte-colony-stimulating factor
HCC	Hepatocellular carcinoma
HLH	Helix-loop-helix

HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
Id-1	Inhibitor of differentiation type 1
IHC	Immunohistochemical staining
IKK	IκB kinase
IL-3	Interleukin-3
JNK	C-Jun NH(2)-terminal kinase
MAPK	Mitogen activated protein kinase
MDM2	Mouse double minute 2
MMP	Metalloproteinase
MRI	Magnetic resonance imaging
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF- κB	Nuclear factor-kappa B
PI	Propidium iodide
PI3K	Phosphoinositide-3 kinase
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homologue deleted on chromosome 10

Rb	Retinoblastoma protein
RHD	Rel homology domain
RIPA	Radioimmunoprecipitation assay
RR	Relative risk
RT	Reverse-transcription
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

Chapter 1: INTRODUCTION

In developed countries, cancer is the major cause of death after heart disease (American cancer society, 2009). Among these cancer deaths, breast cancer is the most common and the second most common cause of cancer-related mortality in women. Although in recent years a decreased trend of incidence was observed in breast cancer cases, the declined incidence was only observed in women with estrogen receptor (ER)-positive breast tumors, and seldom in ER negative breast tumors (Li & Brown, 2009).

Breast carcinogenesis is a complex, multi-factorial process with the inter-play of environmental, genetics and hormonal factors (Stewart & Pietenpol, 2001; Han et al., 2005; Corzo et al., 2006). Estrogen is a well-known hormonal mitogen in breast cells (Leung et al., 2002; Russo & Russo, 2006) and it has been established that it is able to initiate breast carcinogenesis through the binding between estrogen to ER which in turn stimulates the oncogenic pathways (Kuske et al., 2006). Prolonged exposure to estrogen may lead to an increased chance of getting breast cancer.

Surgery is the standard treatment for breast cancer patients. After surgery, additional therapy must be added to prevent micro-metastasis. Hormonal therapy is a useful adjuvant treatment for early breast cancer patients with ER-positive breast tumors (Winchester et al., 2006). It is mainly used to prevent the binding between estrogen to ER with a higher affinity or prevent the conversion of estrogen from androgen by inhibiting the enzymatic reaction (Bowcock, 1999; Macedo et al., 2009). Hormonal therapy is useful for patients with ER-positive breast tumors since it has fewer side effects and is more tolerable compared with chemotherapy and radiotherapy (Macedo, 2009). Unfortunately, hormonal therapy becomes ineffective to patients whose tumor has progressed to require hormone-independence after an initial period of hormone responsiveness (Wang & Cqsero, 2006). Hormone-independent cancer is often seen in patients with more aggressive tumors, poor survival rate and resistance to hormonal therapy. This causes a substantial problem in hormonal therapy, however the mechanisms of how breast cancer progress from hormone-dependent to hormone-independent are unclear.

Hormone-independent breast cancer is often associated with the loss of ER (Mclnerney et al., 2001) and increased expression of EGFR (Davidson et al.,

1987; Mclnerney et al., 2001; Hicks & Tubb, 2005; Nieto et al., 2007). Loss of ER implicated that cells can reach the stage of proliferation and bypass the stimulation of estrogen (Mclnerney et al., 2001). Overexpression of EGFR was found in 54%-72% of ER negative breast cancer (Livasy et al., 2006) and it has been suggested as a critical step in developing hormone-independent breast cancer (Mclnerney et al., 2001). Activation of EGFR was able to stimulate various signaling pathways including NF-κB pathway (Biswas et al., 2000; Biswas et al., 2001; Biswas and Iglehart, 2006). Transcription factor NF-κB is a well known regulator of inflammation, transformation, oncogenesis and cell survival (Cao & Karin, 2003). Elevated expression of NF-κB was often associated with ER negative breast cancer with overexpressed EGFR (Biswas & Iglehart, 2006). Results from studies suggested that activation of NF-κB is one of a downstream signaling pathway of EGFR especially in ER negative breast cancer cells (Biswas et al., 2000; Biswas et al., 2001; Biswas and Iglehart, 2006).

Inhibitor of differentiation (Id)-1 is a helix-loop-helix (HLH) transcription factor that lacks the basic domain. It functions as an inhibitor in cell differentiation (Ling et al., 2004; Di et al., 2006; Ling et al., 2006; Yuen et al., 2006). Id-1

was also believed to be an oncogene (Wang et al., 2002; Cheung et al., 2004; Zhang et al., 2007b). Up-regulation of Id-1 was found in many types of cancer such as prostate (Ling et al., 2004), ovarian (Zhang et al., 2004), cervical (Zhang et al., 2007b), endometrial (Wong et al., 2004), nasopharyngeal (Wang et al., 2002), pancreatic (Wong et al., 2004) and breast (Huh et al., 2006). It was suggested that ectopic expression of Id-1 was able to stimulate cell proliferation (Ling et al., 2002; Lin et al., 2005), enhance progression of cancer (Lin et al., 2000; Fong et al., 2003), and allow the development of hormone-independent cancer (Ling et al., 2004; Zhang et al., 2004).

We previously studied the estradiol (one type of estrogen) stimulated growth response in MCF-7, a hormone-dependent breast cancer cell line, three stable MCF-7-Id-1 transfectants and a mock-control MCF-7-puro transfectant (pBabe). Results showed that ectopic expression of Id-1 was able to reduce the estrogen stimulated growth response in the MCF-7 breast cancer cell line in terms of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and S phase of the cell cycle. Results of Western blotting showed that the reduced response to estrogen may be caused by the decreased expression of ER and progesterone receptor (PR) (unpublished results). Moreover, an *in vivo* study

using 128 breast cases to study the relationship between ER and Id-1 expression in breast tissues, results showed that the expression of Id-1 was inversely correlated to the expression of ER in breast tissues (unpublished results). These data provide evidence that Id-1 may be involved in the progression of breast cancer from hormone-dependent to hormone-independent, since the reduced hormone-responsiveness is an initial step in progression to hormone-independent breast cancer. Clarifying the progression of breast cancer from hormone-dependent to hormone-independent may assist the development of therapy for patients who are resistant to endocrine therapy. In this study, the possible involvement of Id-1 in the progression of breast cancer from hormone-dependent to hormone-independent are continued to be studied.

The previously used stable MCF-7-Id-1 transfectants, the mock-control MCF-7-puro transfectant and parental MCF-7 will also be used in this study. In addition, a hormone-independent breast cancer cell line, MDA-MB231 which does not express ER and therefore does not respond to estrogen, will be used as a hormone-independent positive control.

Firstly, the cell proliferation ability in the MCF-7-Id-1 transfectant in the absence of estrogen will be examined using MTT assay. Before breast cancer cells become resistant to hormonal therapy, they are expected to acquire the ability to bypass the stimulation from estrogen. Therefore in this study, whether ectopic expression of Id-1 gives ability to the MCF-7 cells to grow faster in the absence of estrogen will be examined. If ectopic expression of Id-1 provided advantage to the MCF-7 breast cancer to proliferate in the absence of estrogen, indicating increased level of Id-1 may give growth ability to breast cells to escape the stimulation from estrogen. In order to demonstrate the cell proliferation ability of the MCF-7-Id-1 transfectants in the absence of estrogen, a medium not containing any sex hormones will be used. Since the presence of phenol red strongly promoted cell proliferation and cell cycle progression in breast cancer cells (Wesierska-Gadek et al., 2007), therefore in this study phenol red free RPMI 1640 medium supplemented with dextran-coated charcoal-treated FBS (Hyclone, South Logan, UT) with all sex hormones pre-absorbed, will be used. The cell survival will be evaluated using the MTT assay. Results will be controlled by transient transfection of antisense oligonucleotide against the MCF-7-Id-1 transfectants.

Secondly, the relationship between Id-1 and EGFR will be investigated by Western blotting and reverse-transcription(RT)- PCR. As mentioned previously, increased expression of EGFR was highly associated with loss of ER expression (Mclnerney et al., 2001) and EGFR was overexpressed in more than 50% ER-negative breast cancer cases (Livasy et al., 2006). If we can show that ectopic expression of Id-1 is able to modulate the expression of EGFR, it would imply that Id-1 may be involved in the progression of breast cancer from hormone-dependent to hormone-independent and likely to be effective by suppressing the expression of ER and stimulating the expression of EGFR. This part of study, a hormone-independent breast cancer cell line (MDA-MB231) will also be examined for comparison. Cells will be cultured in an estrogen deprived condition, whole cell protein or RNA will then be extracted and evaluated using Western blotting or RT-PCR respectively. Results will be confirmed by transcient transfection of antisense oligonucleotide against Id-1.

Finally, the involvement of NF-κB pathway in the MCF-7-Id-1 transfectants will be studied. NF-κB pathway is one of the downstream pathways of EGFR (Biswas et al., 2000; Biswas et al., 2001; Biswas & Iglehart, 2006) and the activities of NF-κB overexpressed in ER-negative breast cancer cells (Biswas &

Iglehart, 2006). If we found that NF-κB pathway is involved in the MCF-7-Id-1 transfectants, it may provide evidence to support our hypothesis that Id-1 is involved in the progression of breast cancer from hormone-dependent to hormone-independent. The involvement of NF-κB pathway is studied by adding parthenolide to inhibit NF-κB pathway (Zhou et al., 2005. Cell survival is evaluated using the MTT assay. The shortest time at which more than 50% inhibition will be seen is chosen as experimental condition for the assay and cell cycle analysis. Cell cycle distribution and apoptosis will be examined by flow cytometric analysis. NF-κB dimmer involved in the MCF-7-Id-1 transfectant will be evaluated using Western blotting and confirmed using electrophoretic mobility shift assay (EMSA). A hormone-independent breast cancer cell line MDA-MB231 will be added to act as a reference.

Objectives of this study

1) To examine the growth ability of MCF-7-Id-1 transfectants in the absence of estrogen by MTT assay,
2) To investigate the relationship between Id-1 and EGFR by Western blotting in the MCF-7-Id-1 transfectants

3) To investigate the involvement of NF- κB pathway in Id-1 transfectants by adding a NF- κB inhibitor, parthenolide, and examined using MTT assay, Western blotting and EMSA.

Chapter 2: LITERATURE REVIEW

2.1 Overview of cancer

Cancer is a major cause of death in the United States, and is second only to heart disease (American cancer society, 2009). The American Cancer Society (2009) estimated that there will be 1,479,350 new cases and 562,340 deaths due to cancer in 2009, with approximately 1,500 deaths each day in America alone. Among these cases, about one-third are expected to be accounted for by cancer caused by overweight or obesity, and about 169,000 deaths are expected to be caused by cancer due to tobacco-smoking (American cancer society, 2009).

Cancer is a class of disease or disorder characterized by uncontrolled division of cells. The uncontrolled growth of cells can be caused by an imbalance between apoptosis (cell death) and cell proliferation (American Cancer Society, 2009). Cancer cells can metastases ethier by spreading through the bloodstream or lymphatic system to other organs; or by invasion, directly into adjacent tissue.

2.1.1 Apoptosis

Apoptosis is an important regulatory mechanism to initiate cells in the self-destruction cascade that is used to remove unnecessary or potentially

dangerous cells (Studzinski, 1999). Apoptosis occurs with little or no inflammatory response, it begins by shrinkage of the cell, thereby concentrating the cytoplasmic contents, making the cell looks darker (Potten & Wilson, 2004). The apoptotic cell becomes rounder and finally will be engulfed by neighboring cells, especially macrophages (Studzinski, 1999). Apoptosis occurs in a cascade sequence. Briefly, the apoptotic pathway can be divided into the extrinsic apoptotic pathway which mediates its action through receptor binding, whilst the other pathway is an intrinsic apoptotic pathway which mediates its action through the mitochondria (Studzinski, 1999). Activation of the intrinsic or extrinsic apoptotic pathway depends on the initiation of a family of proteases called caspases, or by inducing the signal of apoptosis (Chen et al., 2001). For example, a stimulus like the cytokine tumor necrosis factor alpha (TNF-α) activate apoptosis through an extrinsic apoptotic pathway. The extrinsic apoptotic pathway first initiates procaspase, mainly caspase-8, after a ligand binds to either the TNF receptor, Fas, OX40, CD40 or 4-1BB that contain a conserved protein-protein binding domain (Chen et al., 2001). The caspase-8 cleaves and activates the effecter caspase-3 and initiates the apoptotic process (Chen et al., 2001). On the other hand, the intrinsic apoptotic pathway is mediated through the mitochondria. After activation of the intrinsic apoptotic

pathway, mitochondria will release an adaptor protein, cytochrome c (Susin et al., 1999). After that, cytochrome c, Apaf-1, procaspase-9 and dATP will bind together to form the apoptosome death complex (Chen et al., 2001). The apoptosome death complex activates the procaspase-9, turning it into activated caspase-9 (Chen et al., 2001). The activated caspase-9 cleaves and activates the common effecter caspase of the extrinsic apoptotic pathway (De Laurenzi & Melino, 2000) (Figure 2.1).

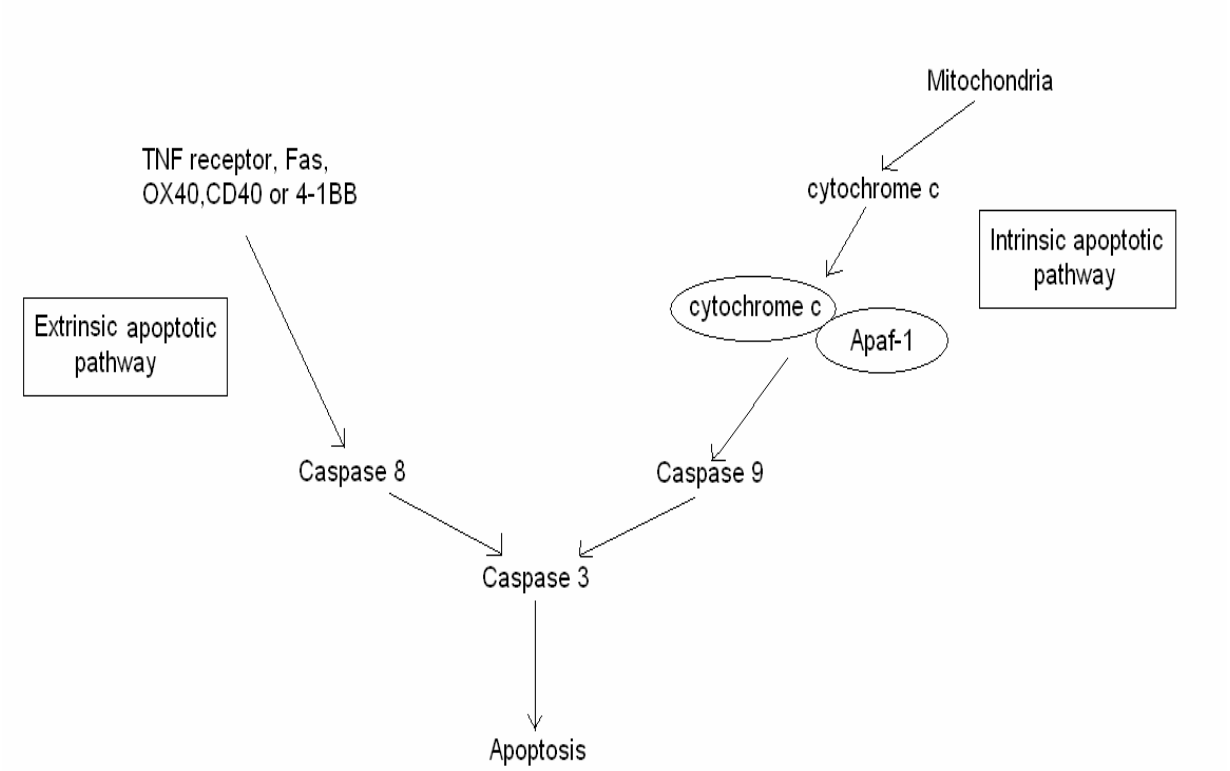


Figure 2.1 The intrinsic and extrinsic apoptotic pathways

The extrinsic apoptotic pathway is mediated through the binding between stimuli and receptor. After activation of the receptor, the procaspase 8 will be initiated to become activated caspase 8. The activated caspase 8 then activates caspase 3 and initiates the extrinsic apoptotic pathway. The intrinsic apoptotic pathway is mediated through mitochondria. If a cell needs to undergo apoptosis, the mitochondria will be stimulated to secret cytochrome c. Cytochrome c then forms an apoptosome death complex with other components such as Apaf-1. This apoptosome death complex then activates caspase 9, which in turn activates caspase 3 and initiation of the intrinsic apoptotic pathway occurs.

(Modified from Chen F, Castranova V, Shi X. (2001) New insights into the role of nuclear factor-kappaB in cell growth regulation. The American journal of pathology. 59(2):387-97.)

2.1.2 Cell cycle regulation

Regulation of the cell cycle is very important in cell replication, since aberration in this mechanism will trigger the initiation of tumorigenesis. Briefly, there are 4 major phases, G1, S, G2 and M phases, of a cell cycle in eukaryotic cells. These are DNA synthesis occurs in the S phase, while segregation of the newly synthesized chromatins occurs in the M phase (Kaldis, 2006) (Figure 2.2). In controlling the flow of the cell cycle from G1→S→G2→M phases, cyclin-dependent kinase (Cdk) is very important. Cdk becomes activated after heterodimerization with a regulatory subunit, cyclin, to form a complex cyclin/Cdk (Stein & Pardee, 2004). In mammalian cells, passage through G1 phase is controlled by ordered expression of cyclin D and cyclin E which associate with Cdk4/6 and Cdk2/3 respectively (Sherr, 1994). To prevent any defects occurring in the cells during replication, several checkpoints exist in the cell cycle. Before cells gain entry into the S phase from the G1 phase, they must be able to pass a checkpoint called the restriction point. The restriction point is used to ascertain if nutrients and growth factors present are sufficient for the cell to undergo DNA synthesis.

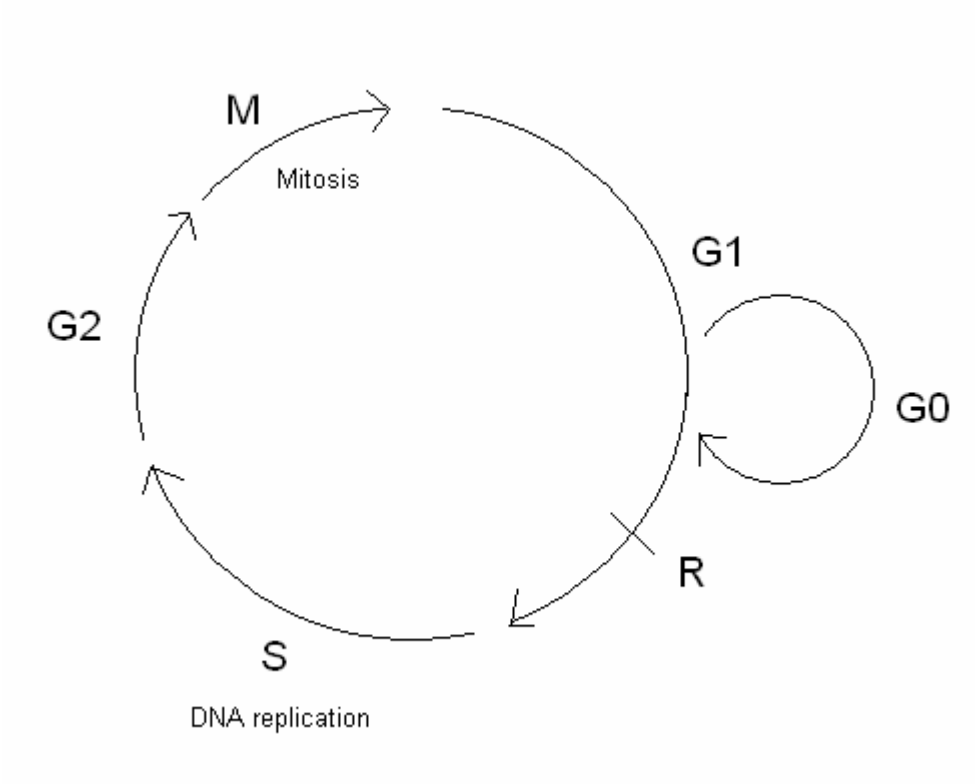


Figure 2.2 Cell cycle regulations

Cell division is tightly controlled by cell cycle regulation in which cell starts in G1 phase, if the cell can pass through the restriction checkpoint “R”, then goes to S phase and undergoes DNA replication. After DNA replication, cell goes through G2 phase and then to M phase that undergoes mitosis. G0 phase is a resting stage.

(Modified from Kaldis P. (2006) Cell cycle regulation. Springer. Berlin. New York.)

Retinoblastoma protein (Rb) is one of the key components of the restriction point since if functional Rb is absent, cells can pass through the restriction point even if the cells were subjected to a growth-limiting condition. Unphosphorylated Rb binds with the E2F transcription factor, and once binding occurs, it blocks the G1/S phase transition. The phosphorylation of Rb starts during the mid G1 phase, lasts until the end of the G1 phase (Planas-Silva & Weinberg, 1997) and is initiated by one of the complexes Cdk4/6-cyclin D. The phosphorylation of Rb causes the dissociation of E2F and the accumulation of E2F suppresses the Cdk activities in the G1 phase, thus allowing the cells to pass through G1 arrest (Blagosklonny & Pardee, 2002). Cyclin D1 is an important cyclin in the G1 phase, overexpression of cyclin D1 is able to increase the phosphorylation of Rb leading to a shortened G1 phase and overcoming the G1 phase arrest generated by DNA damage (Perry et al., 1998; Biswas et al., 2000; Kaldis, 2006). After DNA synthesis, DNA checking is very important to prevent mutation or the loss or gain of genetic materials. p53 is an essential regulator which comes into play after DNA synthesis. p53 is a tumor suppressor gene that acts as a “guard”, if any DNA damage has occurred, p53 stops the cell cycle progression through the inhibition of Cdk either at the G1/S or the G2/M phase. When DNA damage is detected, expression of p53

increases which leads to the increase of several effecter genes, including p16INK4a, p21Waf1 and p27Kip1. These effecter genes are Cdk inhibitors which can prevent the phosphorylation of Rb by inhibiting Cdk, (Ouyang et al., 2002). Once Cdk is inhibited, cell cycle arrest follows and DNA is allowed to repair.

2.2 Breast cancer statistics

Breast cancer is the most prevalent cancer and the second leading cause of cancer-related deaths in women (American Cancer Society, 2009). The American Cancer Society (2009) estimated that about 192,370 new cases of invasive breast cancer and about 62,280 new cases of carcinoma *in situ* will be diagnosed during 2009. Among all new cases of carcinoma *in situ* diagnosed, approximately 85% will be ductal carcinoma *in situ* which is more common than lobular carcinoma *in situ* (American Cancer Society, 2009). Breast cancer in females was also the top ranked cancer in incidence and the third most common cause of death in Hong Kong (Hong Kong Cancer Registry, 2007). Approximated 1 in 20 women have chance of getting breast cancer and 1 in 102 women died because of breast cancer (Hong Kong Cancer Registry, 2007).

Moreover, an increasing trend of incidence has been observed during the last two decades, before 1999, this increased incidence may be due to increased awareness of this disease, or more regulated examination of the breast or even a change in the reproductive pattern such as childlessness or having children at an older age (Hong Kong Cancer Registry, 2006; American Cancer Society, 2008). In recent years, the incidence rate of breast cancer slightly dropped to about 2.2% per year from 1999-2005, this improvement may be attributed to reduced utilization of hormone replacement therapy (HRT) (American Cancer Society, 2009). HRT was used in menopausal or postmenopausal women to reduce the severity of hot flushes and other menopausal symptoms (Poirier-Solomon, 2002). After the Women's Health Initiative (2002) reported that using HRT led to an increase in the risk of heart disease and breast cancer (Poirier-Solomon, 2002), there was reduction in the usage of HRT. Importantly, the declined incidence seen in breast cancer cases only applied to those aged 50 or older with estrogen receptor (ER) positive breast tumors, and less of a decline was seen in ER negative breast tumors (Li & Brown, 2009). The number of deaths caused by breast cancer in women has been stably declining since 1990, with about 3.2% decrease those younger than 50 and about 2% decrease in those 50 or older, per year. The decreased death rate seen may be due to earlier detection and better

treatment of early-stage breast cancer. Nevertheless, about 40,170 females are estimated to die from breast cancer during 2009 (American Cancer Society, 2009).

2.3 Risk Factors for breast cancer development

Risk factors can be categorized as environmental, genetic and hormonal factors (Stewart & Pietenpol, 2001; Han et al., 2005; Corzo et al., 2006).

2.3.1 Environmental factors

Environmental factors are modifiable factors including obesity, increased exposure to radiation and alcohol consumption, etc. Many case-control or cohort studies found that overweight or obese women have an increased risk of breast cancer, in addition to these groups, postmenopausal women are also at particular risk. A prospective cohort study of 337,819 women with 4,385 invasive breast cancer cases showed that the relative risk (RR) of women with BMI of 28kg/m^2 or above in developing postmenopausal breast cancer was 1.26 (95% confidence interval (CI): 1.09-1.46) (van den Brandt et al., 2000; Carmichael & Bates, 2004). A population-based case-control study consisted of 3,345 women, aged 50-74 years, with invasive breast cancer and 3,454

controls showed that the odd ratio of breast cancer in women who had gained 30kg or more since age 18 compared with women who had remained relatively unchanged in weight is 2.04 (95% CI: 1.20-3.48) (Carmichael & Bates, 2004). The positive association between obesity and postmenopausal breast cancer can be explained by the fact that adipose tissue is a source of estrogen after menopause since estrogen can be converted from adipocytes (Stoll, 2000; American Cancer Society, 2009). Thus, increased adipose tissues lead to an increase in the conversion of estrogen and in turn leads to an increased risk of breast cancer.

A prospective study of 1,484 cases of breast cancer among 38,454 women for 10 years found that alcohol consumption was associated with increased breast cancer risk with an RR of 1.32 (95% CI: 0.96-1.82) in women who consume 30g or more alcohol per day (Zhang et al., 2007a). Ingestion of alcohol may reduce hepatic estrogen metabolism (American Cancer Society, 2009), leading to an accumulation of estrogen.

2.3.2 Genetics factors

Approximately 10% of breast cancer cases are thought to be caused by genetic

factors (Venkitaraman, 2001). Genetic factors are nonmodifiable factors such as sex and inheritable gene mutation. Individuals who have inherited specific susceptibility gene mutations such as the breast cancer susceptibility protein (BRCA)1 and BRCA2 genes, or loss of tumor suppressor genes, such as p53, have a higher probability of developing breast cancer (Baum & Schipper, 1998; Stewart & Pietenpol, 2001; Steiner et al., 2008).

Among the 10% inherited breast cancer cases, about 20-60% are caused by loss-of-function mutation of BRCA1 or BRCA2 genes (Venkitaraman, 2001). Women inherited the mutated BRCA1 or BRCA2 have an 85% risk of developing breast cancer before the age of 70 and increased risk of developing ovarian, prostate and pancreatic cancers (Bertwistle & Ashworth, 1999). BRCA1 and BRCA2 genes are very similar (Zhang et al., 1998), are suggested to be the tumor suppressor genes that may be responsible for DNA damage in the cell cycle regulation before DNA synthesis (Davis et al., 1999). These genes are mainly responsible for the maintenance of genomic stability, rather than regulating cellular proliferation (Crook et al., 1998). Overexpression of BRCA1 was found to be able to inhibit activities of the estrogen receptor signaling pathway (Fan et al, 1999), implying that loss of BRCA1 function may

result in the increased capability of breast cells to proliferate in response to estrogen stimulation (Bertwistle & Ashworth, 1999). Interestingly, loss-of-function or mutation of p53 are often associated with BRCA-related breast cancer (Crook et al., 1997; Greenblatt et al., 2001). The role of p53 and BRCA proteins are suggested to be responsible for DNA damage, indicating that mutation of p53 or BRCA proteins cause a loss of DNA checkpoint control in tumor progression.

2.3.3 Hormonal factors

Breast cancer is widely accepted as being strongly associated with sex hormones, especially estrogen (Leung et al., 2002; Russo & Russo, 2006). Estrogen is primarily produced from developing follicles in the ovaries, although other tissues such as liver, adrenal glands and breasts, also produce small amounts of estrogen (Han et al., 2005). Although estrogen plays a role in breast carcinogenesis, estrogen also plays an important role in normal breast development. Any abnormalities occurring during the normal development of the breast may initiate neoplastic transformation.

There are three phases of breast development, lobule development which take

place between the ages of 10 and 25, glandular development which happens between the ages of 13 and 45, under the effect of menstrual hormones, and shrinkage of the milk duct which commences at about age 35 or above (Vander et al., 2001). It is believed that the breasts are not entirely mature until a woman has given birth and produced milk (Horseman, 1999). During pregnancy, some features of the breast may change such as breast swelling, prominence of the blood vessels in the breast and the enlargement and darkening of the areolas. All these changes are in preparation for breastfeeding the baby after birth (Vander et al., 2001). During menopause, the level of progesterone and estrogen become unsteady and the levels of estrogen dramatically decline by about 90% (Russo & Russo, 2006).

Epidemiological data showed that individuals who experienced nulliparity, late parity, early menarche (before age 12 years), late first pregnancy, childlessness or late menopause has a higher chance of developing breast cancer (Leung et al., 2002; Han et al., 2005; Steiner et al., 2008) as these result in prolonged exposure to estrogen. In addition, women who have taken long term (ten or more years) oral contraceptive and HRT, especially combined with estrogen and progestin therapy (American Cancer Society, 2009) will be at increased risk of breast

cancer (Phillips et al., 2009). All this data implies that prolonged exposure to estrogen increases the risk of developing breast cancer (Henderson & Feigelson, 2000; Leung et al., 2003; Yue et al., 2003; Gill et al., 2006).

2.4 Estrogen and breast carcinogenesis

Estrogen appears to be a well-known initiator of breast cancer (Russo & Russo, 2006), however, mechanisms of how estrogen initiates breast carcinogenesis have still not been fully elucidated. Two commonly proposed mechanisms are firstly, estrogen stimulates cell proliferation, thus increases the chance of error in cell proliferation (Yue et al., 2003; Russo & Russo, 2006). Two forms of ER have been established. These are ERα and ERβ. ERα is the major form in breast cancer and the major target in therapy (Kuske et al., 2006). Estrogen binds to ERα which initiates conformational change, activated ERs binds together and forms a dimer, then the complex translocates into the nucleus and binds to the target DNA which contains the site of conserved estrogen response elements (ERE) (Kumar & Chambon, 1988; Tsai et al., 1989; Biswas et al., 1998). ERα becomes functional after phosphorylation of the activation function -1 (AF-1) domain of ERα at both Ser^{118} and Ser^{167} (Kuske et al., 2006) and initiates transcription of genes responsible for cell proliferation. When cell proliferation

increases, the chance of error in DNA replication will also be increased. If any mutations had taken place in some essential steps in cell growth such as cellular proliferation, DNA repair or apoptosis, neoplastic transformation will occur (Preston-Martin et al., 1990; Liehr, 2000; Yue et al., 2003; Sasano et al., 2006).

Another commonly proposed mechanism of how estrogen is related to breast carcinogenesis is that estrogen can induce the formation of the genotoxic metabolite that directly reacts with DNA and causes depurination that in turn may cause a point mutation (Cavalieri et al., 2000; Liehr, 2000; Chakravarti et al., 2001; Yue et al., 2003; Russo & Russo, 2006). Depurination is the hydrolysis of a purine base (Adenine or Guanine) from the deoxyribose-phosphate backbone as illustrated in Figure 2.3. It occurs during estrogen stimulated cell proliferation. Cytochrome P-450 catalyzes the conversion of estradiol to 4-OH-estradiol, and then to estradiol- 3, 4- quinine. Estradiol- 3, 4- quinine binds to quinine or adenine to form 4- OH- estradiol- 1- N7- guanine or 4- OH- estradiol- 1- N3- adenine respectively. These complexes will be deleted from the DNA, causing depurination. When a purine base leaves the DNA without repair, a point mutation will occur. Point mutations are a potential initiator of neoplastic transformation (Chakravarti et al., 2001; Yue et al., 2003).

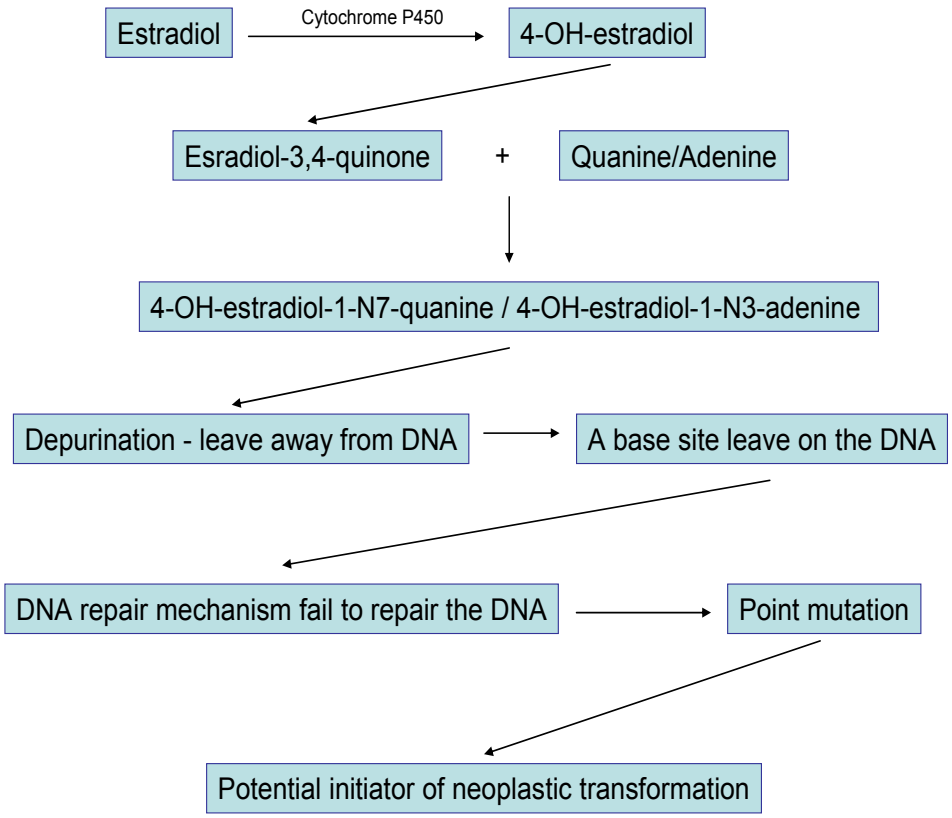


Figure 2.3 Mechanism of action of estrogen in breast carcinogenesis

Estrogen induces the formation of genotoxic metabolites through the reaction with cytochrome P450. The production of genotoxic metabolites directly reacts with DNA backbone bases and causes depurination leading to point mutation.

(Modified from Yue W, Santen RJ, Wang JP, Li Y, Verderame MF, Bocchinfuso WP, Korach KS, Devanesan P, Todorovic R, Rogan EG, Cavalieri EL. (2003) Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. The journal of steroid biochemistry and molecular biology. 86(3-5): 477-86).

2.5 Signs and symptoms of breast cancer

Patients with breast cancer are often pain-free and asymptomatic, only changes in breast appearance can be observed (Wayne, 1996). The common change of appearance are breast depression or lump, veins on the skin surface of the breast looking prominent, nipple becoming inverted, developing a rash or changes in skin texture. Apart from observable changes, a small number of patients will suffer from breast pain. Nipple discharge when unilateral, spontaneous or contains blood, is also another presentation of breast cancer (Wayne, 1996). Patients with Paget's disease which is a rare form of breast cancer will have eczematous changes of the nipple with itching and erythema (Lev-Schelouch et al., 2003). Symptoms appear in places other than the breast, and bone pain and neurological symptoms may also be due to metastasis of breast cancer cells (Wayne, 1996).

2.6 Breast cancer diagnosis

Mammogram is a useful screening test for women suspected of having breast cancer. It can provide a better image with a lower dose of radioactive material compared with traditional breast X-rays (American Cancer Society, 2009). In this test, the breast is compressed between an X-ray plate and a plastic plate,

then a low dose X-ray will pass through the breast to acquire a black and white X-ray picture. Any abnormal spot areas found in the mammography may due to the presence of breast lump. This suspected case will be carefully examined by physicians with questions such as family history of breast cancer and physical checking. After that, fine-needle biopsy will be used for further diagnosis (Breast cancer care, 2006). If breast cancer is diagnosed, the next step is to check the spreading of cancer cells. For instance, 1) using chest X-ray which provides a chest scanning picture, to examine whether cancer cells spread to the lung. 2) Bone scan to determine the possibility of cancer cell spread to bone. In this test, a small amount of low-level radioactive material will be injected into the patient's vein and location of the bone with an active turnover will show up as a hot-spot on the scanning picture. 3) Computed tomography (CT) scan that can produce detailed cross-sectional images to evaluate the metastasis of cancer cells to other organs. 4) Ultrasound or 5) Magnetic resonance imaging (MRI) scan to check the spread of cancer cells to other parts of the body.

2.7 Breast cancer staging

According to American Joint Committee on Cancer (AJCC), breast cancer can be categorized into five main stages (Stage 0, I, II, III, IV) based on the TNM

system, where is “T”, the tumor size and the spreading of tumor cells within the breast and to nearby organs, “N”, the involvement of lymph nodes, and “M”, status of metastasis (Figure 2.4). According to Hong Kong Cancer Registry (2006) about 27.4 % and 35% breast cancer patients were diagnosed as stage I and II breast cancer respectively.

Stage	Description
0	Non-invasive tumor such as DCIS or LCIS.
Ⅰ	Invasive tumor, the tumor measures up to 2 cm in size, but no lymph node involved.
ⅡA	Tumor cannot be found in the breast , but cancer cells can be found in the axillary lymph nodes, OR Tumor measures less than 2 cm and involves the spreading of cancer cells to axillary lymph nodes, OR Tumor size measures between 2 to 5 cm and do not involve the spreading of cancer cells to the axillary lymph nodes.
ⅡB	Tumor size measures between 2 to 5 cm and involves the spreading of cancer cells to the axillary lymph node, OR Tumor measures larger than 5 cm and do not involve the spreading of cancer cells to the axillary lymph node.
ⅢA	Tumor cannot be found in the breast, but cancer cells can be found in the axillary lymph node that are clumped together, OR sticking to other structure, or cancer cells spread to lymph node near the breastbone, OR Tumor measures less than 5 cm and involves the spreading of cancer cells to axillary lymph node that are clumped together or sticking to other structure, OR Tumor meausres larger than 5 cm and involves the spreading of cancer cells to axillary lymph node that are clumped together or sticking to other structure.
ⅢB	Tumor are present in any size, involves the spreading of cancer cells to the axillary lymph node that are clumped together or sticking to other structures, OR cancer cells spread to lymph node near the breastbone, and involves the spreading of cancer cells to the chest wall and/or skin of the breast.
ⅢC	Invasive cancer that cannot be found in the breast or any size of the tumor found in the breast that has spread to the chest wall and/or the skin of the breast, the cancer has spread to lymph nodes above or below the collarbone, and the cancer may have spread to axillary lymph nodes or to lymph nodes near the breastbone.
Ⅳ	Invasive breast cancer in which the cancer cells has spread to other organs of the body.

Figure 2.4 Five main stages of classifying breast cancer

Breast cancers are categorized into five main stages according to American Joint Committee on Cancer (AJCC) base on the TNM system.
(Modified from Breast cancer.org (2009) Stages of Breast Cancer)

2.8 Breast cancer treatment

Surgery is the standard treatment for early and hormone sensitive breast cancer patients (Macedo, 2009). Surgery includes breast-conserving surgery (the removal of the tumor with a clear margin, otherwise known as lumpectomy) and mastectomy (Hong Kong Hospital Authority, 2007). The decision of the type of surgery is based on two factors, the first being the tumor size, location and focality and the other factor being the physical characteristics of the patient such as breast size and age (Hong Kong Hospital Authority, 2007).

Breast cancer is considered a systemic disease. After surgery, adjuvant treatment is needed to prevent micro-metastasis and the recurrence of carcinogenesis (Hong Kong Hospital Authority, 2007). The choice of adjuvant treatment depends on the balance between the risk of relapse and survival, patient preference and the status of the hormonal receptor (Hong Kong Hospital Authority, 2007). Chemotherapy and radiotherapy are the usual adjuvant treatments after surgery. However, if possible, it is preferable to use hormonal therapy with/ without chemotherapy, since the use of hormonal therapy is better tolerated by the patient in comparison to radiotherapy and chemotherapy alone (Macedo, 2009). The use of hormonal therapy is based on the discovery of the

importance of estrogen in breast carcinogenesis, drugs targeting the ER were designed. Tamoxifen is an antiestrogen nonsteroidal hormonal therapeutic drug effective in premenopausal women was first used in 1971 (Bowcock, 1999). It prevents the action of estrogen against estrogen-mediated pathway by blocking the estrogen receptor (Bowcock, 1999). Use of this drug has been shown to be able to reduce recurrence of breast cancer after surgery by up to 33% (Morcel et al., 2008). Unfortunately, the reduction of recurrence was only observed in patients with an ER positive breast tumor but not in patients with an ER negative breast tumor (Li & Brown, 2009). The majority of breast cancer patients are post-menopausal (Macedo et al., 2009), and aromatase inhibitors are employed in these cases. Aromatase inhibitor is a common hormonal therapeutic drug effective against breast cancer in post-menopausal women (Hong Kong Hospital Authority, 2007). The major source of estrogen in post-menopausal women is not from the ovaries from androgen in the circulation. Androgen is catalyzed into estrogen through the enzymatic reaction of aromatase (Macedo et al., 2009). The use of aromatase inhibitor is to prevent the conversion from androgen to estrogen by inhibiting aromatase.

Hormonal therapy is a successful therapeutic approach in managing hormone-dependent ER positive breast cancer (Sarvilinna et al., 2006). Unfortunately, their progression to hormone-independent or hormone-resistant breast cancer causes a substantial problem in hormonal therapy. In the early stage of breast cancer, the tumor may progress from hormone-dependent to hormone-irresponsive and then to hormone-independent (Moudgil, 1994). The hormone-independent stage reflects a more aggressive tumor, which mostly occurs in premenopausal patients with ER negative breast tumors (Moudgil, 1994; Wang & Casero, 2006). Patients with ER negative breast tumors often have poor prognosis, poor survival rate and are resistant to hormonal therapy (Li & Brown, 2009). In addition, the use of hormonal therapy cannot prevent the conversion of ER positive breast tumors to ER negative breast tumors (Li & Brown, 2009). The mechanism of how breast cancer cells acquire resistance to hormonal therapeutic drugs is still not yet clear. The potential mechanisms could be explained using three broad areas (Newby et al., 1997): 1) the alteration of the pharmacodynamics of the drug, resulting in low level drug uptake within the tumor cell (Johnston et al., 1993) or the change of the balance between agonistic/ antagonistic metabolites (Wiebe et al., 1992). 2) The appearance of variant forms of the ER that are irresponsive to ligand binding or inhibited by

antagonists (Rose et al., 1985). 3) The alteration of the downstream effecter pathways of ER in cell proliferation, resulting in cell proliferation independent from estrogen stimulation (Fuqua et al., 1991; Newby et al., 1997).

2.9 Characteristics of hormone-independent breast cancer

2.9.1 Estrogen receptor

Patients who develop resistance to hormonal therapy almost all express low, or absent levels of ER (Johnston et al., 1995; Head & Johnston , 2003). Investigators have reported that reduced phosphorylation of ERα, reduced binding affinity to the ER modulator, loss of ER expression, enhanced ERβ expression or ER mutation is the cause of development toward hormone-independent breast cancer (Karnik et al., 1994; Moudgil, 1994; Mclnerney et al., 2001; Head & Johnston , 2003; Kuske et al., 2006; Sarvilinna et al., 2006). Loss of ER implies that cancer cells can proliferate through stimulation pathways other than estrogenic stimulation (Mclnerney et al., 2001) and ER may be able to cross-talk between different pathways (Johnston et al., 2003). Pathways other than estrogenic stimulation in breast cancer cells are complex and as yet still unknown.

Many studies revealed an inverse relationship between epidermal growth factor receptor (EGFR) and ER in breast cancer cells (Davidson et al., 1987; Klijn et al., 1992; Biswas et al., 2000; Mclnerney et al., 2001; Hicks & Tubb, 2005; Nieto et al., 2007), a relationship that is highly consistent across many studies (28/31 studies) (Klijn et al., 1992) and was associated with poor clinical outcome in breast cancer patients (Sainsbury et al., 1987; Lo & Hung, 2006). *In vitro* studies used the MCF-7 breast cancer cell line to demonstrate the relationship between ER and EGFR by using estrogen and antiestrogen, hydroxytamoxifen (Berthois et al., 1989). Results showed that estrogen and antiestrogen were able to modulate the expression of EGFR, indicating an inverse relationship between the activation of ER and EGFR. After short term treatment with estradiol (a type of estrogen), the expression of EGFR was decreased. In contrast, after treatment with antiestrogen, the expression of EGFR was increased (Berthois et al., 1989). Moreover many *in vivo* studies, for example, have used competitive binding and monoclonal antibody staining techniques (Sainsbury et al., 1985), multiple-point EGFR assay (Koenders et al., 1991), and immunohistochemical staining (Klijn et al., 1992) to show the significant inverse relationship between expression of ER and EGFR in breast cancer patients. It was also found that the expression of EGFR was greater in patients with

metastases than in patients with primary tumors only (Sainsbury et al., 1985; Koenders et al., 1991; Klijn et al., 1992).

The negative correlation of EGFR and ER implies that EGF stimulation factor may be a major growth stimulator in ER negative breast cancer cells (Biswas et al., 2000). Also studies revealed that overexpression of EGFR was a strong prognostic indicator and an important step in progressing to hormone-independent breast cancer (Mclnerney et al., 2001). Studies showed that there were several DNase I hypersensitive sites within the first intron of the EGFR gene that are potential regulatory regions, especially in hormone-independent breast cancer cells (Mclnerney et al., 2001). Researchers used the chloramphenicol acetyltransferase (CAT) assay to investigate the transcriptional activity of the first intron of EGFR. Results showed that a 140 bp region has enhancer capability exclusively in these hormone-independent breast cancer cells (Mclnerney et al., 2001). Therefore EGFR may be one of the pathways after bypassing the stimulation from estrogen in ER negative breast cancer cells (Fitzpatrick et al., 1984; Sainsbury et al., 1985; Davidson et al., 1987; Koenders et al., 1991; Newby et al., 1997; Mclnerney et al., 2001).

2.9.2 Epidermal growth factor receptor

The EGFR family of receptor tyrosine kinase is a 170kDa transmembrane glycoprotein (Rajkumar & Gullick, 1994) that contains intrinsic tyrosine kinase activity (Weber et al., 1984). It consists of four family members: EGFR (Erb-1), HER-2/neu (Erb-2), HER-3 (Erb-3) and HER-4 (Erb-4) (Ciardiello & Tortora, 2001). EGFR consists of three functional domains, an external EGF binding domain, a transmembrane domain and a cytoplasmic tyrosine kinase domain (Weber et al., 1984). In the resting state, EGFR exists as an inactive monomer. Once it undergoes ligand binding activation, the receptor becomes activated and causes homodimerization or heterodimerization with other family members of EGFR. After dimerization, the cytoplasmic tyrosine kinase domain is activated and initiates the activation of the intracellular cascade (Wells, 1999).

2.9.2.1 Role of EGFR in carcinogenesis

Functions of EGFR, for example, cell proliferation, apoptosis, angiogenesis and metastatic spreading are important for cancer development and progression (Ciardiello & Tortora, 2001). Overexpression, gene amplification or mutation of EGFR are associated with many types of cancer, for example, pancreatic

cancer (Korc, 1998), oral squamous cell carcinoma (Kiyota et al., 2000), bladder cancer (Ding et al., 2006), renal cancer (Li et al., 2007) and breast cancer (Mclnerney et al., 2001; Hicks & Tubb, 2005; Nieto et al., 2007). EGFR can activate gene expression involved in multiple pathways, since EGFR can activate the proto-oncogene Ras (Yu & Feig, 2002). Ras signaling is complex and involves multiple effecter pathways (Carpenter, 2004). Activation of Ras can initiate signaling cascades such as the activation of phosphoinositide-3 kinase (PI3K) (Yu & Feig, 2002), mitogen activated protein kinase (MAPK) (Ras -> Raf-1 -> Mek1/2 -> Erk1/2) (Leicht et al., 2007), NF-κB (Zhou et al., 2005) and c-Jun NH(2)-terminal kinase (JNK) (Leicht et al., 2007) pathways. All these pathways can eventually cause neoplastic transformation. The consequence of EGFR expression depends on pathways activated. Overexpressed EGFR also correlated with increased expression of cyclin D1 (Perry et al., 1998; Poch e al., 2001; Lo & Hung, 2006) when cyclin D1 accumulation occurs, it leads to an increase in the progression of the cell from G1/S phase in the cell cycle (Perry et al., 1998). Moreover, EGF has also been shown to induce activation of NF-κB in rat aortic smooth muscle cells (Obata et al., 1996), human epidermal carcinoma cell line A431 (Sun & Carpenter, 1998), osteoblastic MC3T3-E1 cells (Matsumoto et al., 1998), and ER negative breast

cancer cells (Biswas et al., 2000). Like EGFR, elevated expression of NF-κB is also suggested to be associated with ER negative breast cancer (Nakshatri et al., 1997; Cao & Karin, 2003; Zhou et al., 2005; Zhou et al., 2007) and is overexpressed in many types of cancer, which is discussed in detail below.

NF-κB is another common pathway related to tumorigenesis. It can be activated through phosporylation of EGFR (Bhat-Nakshatri et al., 2002). Biswas and his colleagues (2000) studied the involvement of NF-κB in ER negative breast cancer cells with higher expression of EGFR *in vitro* (Biswas et al., 2000) and *in vivo* (Biswas et al, 2001). Biswas et al., (2000) used EGF and anti-EGF to stimulate and inhibit the activities of NF-κB in ER negative breast cancer. Studies found that with the stimulation of EGF, the activity of NF-κB in ER negative breast cancer was increased as well as its transactivator cyclin D1 and pRb (Biswas et al., 2000). A year later, Biswas and his colleagues studied the involvement of NF-κB in ER negative breast cancer *in vivo* using syngeneic A-J mice. They generated ER negative mammary tumor mice by implanting ER negative mouse mammary epithelial tumor cells into syngeneic mice, then stable transfection of dominant negative mutant IκB kinase (IKK) which block the activities of NF-κB. Results showed that after blocking the activity of

NF-κB in ER negative breast tumor mice, the ER negative breast tumor lost tumorigenic ability (Biswas et al., 2001). These studies give evidence to suggest that activation of NF-κB is one of a downstream signaling pathway of EGFR especially in ER negative breast cancer cells (Biswas et al., 2000; Biswas et ala., 2001; Biswas and Iglehart, 2006).

2.9.3 Overexpression of nuclear factor-kappa B (NF- κB)

Transcription factor NF-κB has been under study for many decades. It is a well known transcription factor that is able to control hundreds of human genes directly or indirectly (Courtois & Gilmore, 2006). NF-κB plays an important role in the regulation of inflammation, transformation, cell survival, apoptosis and oncogenesis (Cao & Karin, 2003). It consists of five members: p65 (RelA), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2). All these proteins have conserved an approximate 300 amino acid sequence called the Rel homology domain (RHD) at their N-termini. RHD is responsible for dimerization, DNA binding, nuclear translocation and association with IκB, the inhibitor of NF-κB (Chen et al., 2001). These proteins are categorized as transcriptional active and transcriptional inactive based on their C-terminal region. Transcriptional active proteins, which are p65 (RelA), RelB and c-Rel, contain a transcripting domain

at their C-terminal region, whereas p50 and p52 lack the transcripting domain, but contain multiple copies of ankyrin (ANK) repeats like IκB. These proteins exist in a homo- or hetero- dimer, NF-κBp65/p50-IκBα is the most abundant form of NF-κB found in the cytoplasm. During the resting state, NF-κB binds to one or more members of the inhibitory protein IκB. IκB consists of seven members: IκBα, IκBβ, IκBε, IκBγ, Bcl-3 and the precursor proteins p105 and p100 which are the precursor proteins for p50 and p52 respectively. All these members contain the ANK repeat which mediate the interaction with the RHD sequence of NF-κB family. NF-κB becomes activated once an enzyme called IKK causes the dissociation of IκB in the cytoplasm. After activation, NF-κB translocates into the nucleus and binds to a promoter-specific element that regulates the transcription of NF-κB-dependent genes. Activation of NF-κB almost stimulates the gene transcription, however translocation of NF-κBp50/p50 or p52/p52 homo-dimer results in the repression of gene transcription because they lack the transcripting domain (Chen et al., 2001). Although NF-κBp50/p50 or p52/p52 lacks the transcripting domain, they become transcriptionally active if either p50 or p52 binds to a non-inhibitory IκB member, Bcl-3, which is an oncogene (Chen et al., 2001; Zhou et al., 2005; Liou, 2006) (Figure 2.5).

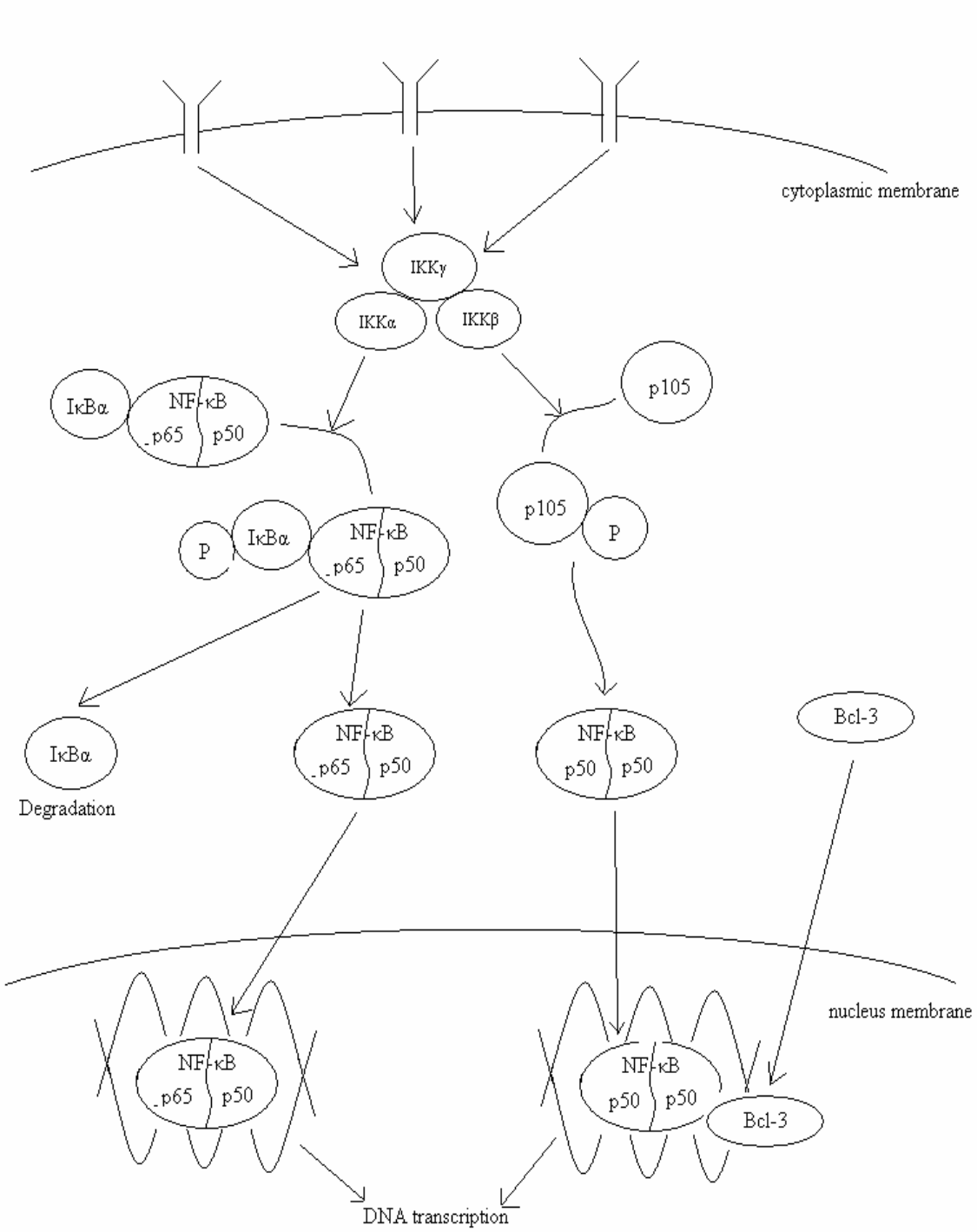


Figure 2.5 Nuclear factor-kappa B (NF- κB) pathway

Activation of NF- κBp65/p50 causes the dissociation of IκBα, then the activated NF-κB p65/p50 translocates into the nucleus and initiates DNA transcription. After activation of NF-κB p50/p50, it translocates into the nucleus, then coactivates with its coactivator Bcl-3 and initiate DNA transcription.
(Modified from IMGENEX, 2008)

2.9.3.1 Role of NF-κB in anti-apoptosis

Many studies have shown that NF-κB has an anti-apoptotic role (Chen et al., 2001; Ling et al., 2003; Lee et al., 2007; Li et al., 2007; Zhao et al., 2007; Kim et al., 2008). The activation of NF-κB is used to act against apoptosis. For example, NF-κB can be activated through the TNF receptor that induces expression of anti-apoptotic protein and mitochondrial membrane stabilizers such as Bcl-xl (Chen et al., 2001; Ling et al., 2003). Knockdown studies of NF-κB subunits p65 and p50 by siRNA showed that apoptosis was enhanced after knockdown of NF-κB (Lee et al., 2007). Another knockout study showed that activation of NF-κB conferred anti-apoptosis ability against radiation-induced apoptosis. A knockout study of peroxisome proliferator-activated receptor (PPAR) alpha in mice showed that this receptor is highly expressed in the kidney and important in the regulation of lipid metabolism and glucose homeostasis. Low-dose radiation applied to knockout and wild-type mice for various durations resulted in the increased activation of NF-κB in a time-dependent manner. The radiation induced apoptosis in knockout mice was suppressed by the activation of NF-κB pathway (Zhao et al., 2007). Recent transfection studies showed that inhibitor of differentiation type 1 (Id-1) conferred anti-apoptotic ability to prostate cancer cells (Ling et al.,

2003), esophageal cancer cells (Li et al., 2007) and breast cancer cells (Kim et al., 2008) through the activation of NF-κB pathway, implicating that Id-1 may be able to regulate the NF-κB pathway.

2.9.3.2 Role of NF-κB in cell cycle regulation

Studies have suggested some involvement of NF- κB pathway in cell cycle regulation (Baldwin et al., 1991; Hinz et al., 1999; Brantley et al., 2001; Joyce et al., 2001; Kountouras et al., 2001). In earlier studies, it was found that NF-κB DNA-binding activity was induced during the transition of the cell cycle from G0 to G1 in mice fibroblasts (Baldwin et al., 1991). The role of NF-κB in the regulation of cell cycle has become more precise since the relationship between NF-κB and cyclin D1 was elucidated (Hinz et al., 1999). It was found that two NF-κB binding sites were localized in the human cyclin D1 promoter, implying that the expression of cyclin D1 was controlled by NF-κB. Moreover the inhibition of NF-κB leads to the reduction of cyclin D1-associated kinase activity and resulted in delayed phosphorylation of the retinoblastoma protein (Hinz et al., 1999). In addition, activation of the NF- κB pathway is one of the important stimulators which induce the proliferation of hepatocytes after a hepatectomy (Kountouras et al., 2001). NF-κB also plays a role in the normal

development of the mammary gland. An animal study investigating IκBα-deficiency showed that without the inhibitor of NF-κB, the increased expression of NF-κB led to an increase in lateral ductal branching and pervasive intraductal hyperplasia compared with wild-type mice (Brantley et al., 2001). Pregnancy is one of the critical stages of mammary gland development (Vander et al., 2001). During pregnancy, two predominant forms of NF-κB, the NF-κBp65/p50 heterodimer and the NF-κBp50/p50 homodimer were detected to be significantly increased (Cao & Karin, 2003). Furthermore, the activation of NF-κB stimulates the proliferation of epithelial cells which leads to the expansion of the lobuloalveolar system (Haffner et al., 2006).

2.9.3.3 Role of NF-κB in carcinogenesis

Since NF-κB plays a role in the process of apoptosis and cell cycle regulation, aberrantly high expression of NF-κB may contribute to uncontrolled cell proliferation and lead to the existence of cells which fail to undergo apoptosis. NF-κB is a well-known transcriptional factor that is linked to tumorigenesis. Overexpression of NF-κB was found in prostate cancer (Chen & Sawyers, 2002), melanoma (Gao et al., 2006), nasopharyngeal cancer (Thornburg et al., 2003), skin cancer (Budunova et al., 1999), leukemia (Nishikori et al., 2003), thyroid

cancer (Namba et al., 2007), colon cancer (Dejardin et al., 1999) and breast cancer (Chen & Sawyers, 2002; Biswas & Iglehart, 2006). Activation of NF-κB in prostate cancer seems to take part in the progression to hormone-independency (Chen & Sawyers, 2002; Paule et al., 2007). Stimulated or ectopic expression of NF-κB p65/p50 contributed to the activation of prostate-specific antigen (PSA) which is a marker of prostate cancer development and progression. Also a higher level of NF-κB was found in an androgen-independent prostate cell line than in an androgen-dependent prostate cancer cell line (Chen & Sawyers, 2002). However the overexpressed subunits of NF-κB were different in nasopharyngeal carcinoma and human melanoma, from that of those found in prostate cancer (Chen & Sawyers, 2002) which indicates that several subunits can be present in different tumor types. Studies reported that the major NF-κB subunit overexpressed in both nasopharyngeal carcinoma (Thornburg et al., 2003) and human melanoma (Gao et al., 2006) was NF-κB p50/p50 homodimer. Gao et al. (2006) showed the important role of NF-κB p105/p50 in the carcinogenesis of melanoma and found that increased NF-κB p50 led to a more aggressive tumor and cell migration. Besides, Thornbury et al. (2003) found that NF-κB p50 as well as its coactivator Bcl-3 were significantly activated in nasopharyngeal carcinoma cells, this suggested

the important involvement of NF-κB p50/p50 homodimer and Bcl-3 in nasopharyngeal carcinogenesis. Bcl-3 is a co-activator of NF-κB p50 and p52 homodimer located at chromosome 19q13 (Courtois & Gilmore, 2006). Chromosomal translocation of the Bcl-3 gene at t(14:19) leads to increased expression of Bcl-3 (Schlette et al., 2005) and results in increased activity (Chapiro et al., 2008). This is frequently associated with chronic lymphocytic leukemia (McKeithan et al., 1997; Michaux et al., 1997; Chen et al., 2001). Besides translocation at t(14:19), it has been suggested that Bcl-3 is an onocogene (Westerheide et al., 2001; Kashatus et al., 2006). Studies revealed that Bcl-3 was able to transactivate the cyclin D1 gene, thus inducing the transition of G1/S phase of the cell cycle, leading to increased cell proliferation (Westerheide et al., 2001; Cao & Karin, 2003). Moreover, Bcl-3 was found to be able to suppress p53, in turn leading to inhibition of the cell cycle arrest usually induced by p53 when DNA damage is encountered, this suppression of cell arrest result in increased somatic hypermutation (Kashatus et al., 2006).

2.9.3.4 NF-κB and Breast cancer

Overexpression of NF-κB has been found to be highly related to beast cancer. The BRCA2 gene is a well-known gene overexpressed or mutated during breast

carcinogenesis which contains a promoter region for NF-κB (Wu et al., 2000). Studies have shown that after co-transfection of NF-κB p65 and p50, the BRCA promoter was found to be increased by up to 16 folds (Wu et al., 2000). Additionally, NF-κB has been reported to induce cyclooxygenase-2 (COX-2) which is related to angiogenesis (Konstantinopoulos et al., 2007). COX-2 was overexpressed in approximately 40% of ER-positive breast cancer patients (Ristimäki et al., 2002; Wu & Kral, 2005), also overexpression of COX-2 was related to angiogenesis and metastasis that correlated with advanced breast cancer (Costa et al., 2002; Half et al., 2002). Many studies revealed that an up-regulated level of NF-κB was observed in breast cancer and was suggested to play a role in the progression of breast cancer to hormone-independency (Chen & Sawyers, 2002). Other studies compared the DNA binding activity of NF-κB in a hormone-dependent and a hormone-independent breast cancer cell line. Results indicated that the DNA binding activity was higher in the hormone-independent breast cancer cell line than in the hormone-dependent breast cancer cell line (Nakshatri et al., 1997).

It is unclear exactly which subunit of NF-κB is involved in breast cancer. Dejardin et al. (1995) suggested that the NF-κB p100/p52 subunit may be

involved in breast cancer progression, this finding was supported by Cogswell *et al*. (2000). Cogswell *et al*. (2000) using both breast cancer cell lines and breast cancer patient samples, these studies found that the nuclear level of NF-κB p65 was expressed minimally, or was actually absent in breast tumors, but c-Rel, p50, p52 and Bcl-3 activation was increased when compared with the adjacent normal breast tissue. A study suggested that NF-κB was involved, especially NF-κB p52 and Bcl-3 in human breast cancer (Cogswell *et al*., 2000). It has been found that ectopic expression of NF-κB p52 in a mouse model contributed to breast tumorigenesis, implying that NF-κB p52 participated in the development of breast cancer (Connelly *et al*., 2007). However, other studies have suggested that the involvement of NF-κBp50 is in breast carcinogenesis (Hu & Kong, 2003). Hu & Kong (2003) suggested that NF-κB p50 was more important than the other family members of NF-κB in chemotherapy resistant breast cancer. Studies semi-quantifying the expression of NF-κB p50 and p65 in breast cancer specimens found that expression of NF-κB p50 was more significant than that of NF-κB p65 in the indication of tamoxifen resistance (Zhou *et al*. 2005). The subunit of NF-κB involved in breast carcinogenesis is still under investigation.

2.10 Inhibitor of differentiation (Id) proteins

Id proteins are a group of proteins which consists of four members named Id-1, Id-2, Id-3 and Id-4. They are located at chromosome 20q11(Id-1), 2p25(Id-2), 1p36.1(Id-3) and 6p21-22(Id-4) in humans and all have a similar molecular size of 13-20kDa but are different in the extensive sequence (Desprez at al., 2003; Wong et al.; 2004). Id protein is one of the members in the helix-loop-helix (HLH) transcription factor family. There are 240 known HLH family members, most of these transcription factors belong to the basic HLH (bHLH) family (Desprez et al., 2003). They act as transcriptional factors that positively or negatively regulate cell growth and differentiation. The presence of the basic amino terminal permits the HLH region to bind to the target DNA sequence which contains E-boxes (CANNTG) or E-box-like protein and activates gene transcription (Lin et al., 2000). The highly conserved HLH region is responsible for binding DNA in a homo- or hetero- manner to form a dimer. However, Id proteins are under the HLH family which lack the basic domain, therefore they can only bind to the target DNA without activating gene transcription. Id proteins were believed to function as an inhibitor of cell differentiation (Benezra et al., 1990; Yokota, 2001; Ling et al., 2004; Di et al., 2006; Ling et al., 2006; Yuen et al., 2006).

2.10.1 Role of Id-1 in normal cell development

Cell differentiation is one of the most important processes in normal cell development. Researchers found that a higher expression of Id proteins were frequently found in precursor cells and were decreased in differentiated cells (Kreider et al., 1992; Wong et al., 2004). It was found that in an interleukin-3 (IL-3) dependent hematopoietic precursor cell line, 32DC13 (G) which will ultimately differentiate into neutrophilic granulocytes after a standard treatment of granulocyte-colony-stimulating factor (G-CSF), expressed Id mRNA. However the Id mRNA level was decreased after treatment with G-CSF. Moreover, when the Id gene was transiently transfected to 32DC13 (G) cells, results showed that the increased level of Id expression led to a decreased ability of the cells to differentiate (Kreider et al., 1992).

Studies of Id protein expression have also suggested a role for Id proteins in the development of the mammary gland (Lin et al., 1999; Desprez et al., 2003; Itahana et al., 2008). The development of the mammary gland occurs throughout life, from the development of the embryo, through puberty, the menstrual cycle, pregnancy, lactation until postmenopausal regression. To maintain this normal mammary gland development, a tight regulation of the

cycle must be employed. Id proteins are important regulators in the normal development of the mammary gland, especially Id-1 and Id-2 (Parrinello et al., 2001; Desprez et al., 2003). Id-1 was suggested as the critical protein involved in the glucocorticoid-induced mammary gland development (Woo et al., 2000). Glucocorticoid was found to induce the reorganization of mammary epithelial cell-cell junction, and is responsible for morphological and structural differentiation during pregnancy and lactation (Buse et al., 1995; Wong et al., 1999). Glucocorticoid was unable to induce a complete reorganization of cell-cell junction in the Scp2 mammary cell line until increased ectopic expression of Id-1. These findings were further confirmed by employing an antisense study against Id-1 (Woo et al., 2000). Moreover, expression of Id-1 was high during the mammary development in virgin mice and during early pregnancy which indicates that periods of high proliferation and invasion is associated with higher levels of Id-1. Interestingly, the expression of Id-1 declined after the mammary epithelial was fully differentiated, for example, during mid-pregnancy (Parrinello et al., 2001). On the other hand, a high expression of Id-2 was found in differentiated mammary epithelial cells *in vivo* and in culture, suggesting that there is an interaction between Id-1 and Id-2 during the development of mammary epithelial cells (Parrinello et al., 2001).

Furthermore, using immunohistochemistry and *in situ* hybridization techniques, investigators were able to demonstrate the expression of Id-1 and Id-2 in mice during pregnancy. The investigators found that during early pregnancy, Id-1 gene expression was increased in the epithelial cells of the growing ductal structure. It was also shown that during late pregnancy the expression of Id-2 was increased while expression of Id-1 decreased, this implicated that Id-1 and Id-2 are participating in different stages of pregnancy. In addition, the expression of Id-1 was negatively correlated with the expression of Id-2 in the development of the mammary gland during pregnancy (Itahana et al., 2008).

2.10.2 Role of Id-1 in cell proliferation

Besides normal development of the mammary gland, Id-1 was found to play a role in the inhibition of cellular senescence. Cellular senescence is a normal cellular state that cells reach at the end of their lifespan. Once cells reach senescence, they stop dividing, and stay in the G1 phase of the cell cycle. This triggers the loss of telomeres and the cell self-destruct by apoptosis (Sedivy, 1998; Ohtani et al., 2001; Ouyang et al., 2002). Knockout mice studies indicated that homogeneous deletion of p53, p16INK4a, p19ARF and p21CIP1 led to decreased cellular senescence in mice (Sedivy, 1998; Wong et al., 2004).

Also, an elevated level of p16INK4a was found in senescent human fibroblast cells (Hara et al., 1996; Wong et al., 2004). p16INK4a is one of the Cdk inhibitors used to suppress the activities of Cdk 4 and Cdk 6. These Cdks are able to initiate the phosphorylation of the retinoblastoma protein (Rb), which means p16INK4a can indirectly inhibit the phosphorylation of tumor suppressor Rb (Ohtani et al., 2001). Studies showed that overexpression of Id-1 was able to inactivate p16INK4a activity through direct interaction with p16INK4a and halt senescence in prostate cancer cells (Ohtani et al., 2001; Ouyang et al., 2002), nasopharyngeal carcinoma cells (Wang et al., 2002) and hepatocellular carcinoma (HCC) cells (Lee et al., 2003). Another study found that Id-1 was able to induce cell proliferation in prostate cancer cells (Ouyang et al. 2001), and an ectopic transfection study of Id-1 in prostate cancer cell lines showed that the increased level of Id-1 led to increased phosphorylation of Rb and down-regulation of p16INK4a (Ouyang et al., 2002). Results implicated that the Id-1 induced cell proliferation may be enhanced through the inactivation of the p16INK4a/pRb pathway (Ouyang et al., 2002). Another ectopic transfection study of Id-1 in a nasopharyngeal carcinoma cell line also showed that the increased expression of Id-1 contributed to increased phosporylation of Rb and induced cell proliferation (Wang et al., 2002). This mechanism of Id-1

in the regulation of the p16INK4a/pRb pathway was supported by Lee et al. (2003) in HCC cell line. Studies have shown that expression of Id-1 was negatively correlated with the expression of p16INK4a in HCC patients and ectopic expression of Id-1 led to an increase cell proliferation, suggesting that Id-1 was able to induce cell proliferation in HCC cells through activation of the p16INK4a/pRb pathway (Lee et al., 2003). However, the story gets more complicated as this mechanism of action may be different in esophageal squamous cell carcinoma (ESCC). Hui et al. (2006) transfected Id-1 into ESCC cell lines and found that Id-1 was able to induce cell growth, stimulate expression of mouse double minute 2 (MDM2) and suppress expression of p21Waf1 protein, but no effect was seen on pRb, CDK4 and p16INK4a expressions. These results suggest that the mechanism of action of Id-1 induced cell proliferation may be different in different types of cancer.

2.10.3 Role of Id-1 in carcinogenesis

Id-1 was believed to play a role in carcinogenesis. Up-regulation of Id-1 was found in many types of cancer such as prostate (Ling et al., 2004), ovarian (Zhang et al., 2004), cervical (Zhang et al., 2007b), endometrial (Wong et al., 2004), nasopharyngeal (Wang et al., 2002), pancreatic (Wong et al., 2004) and

breast (Huh et al., 2006). It was found that ectopic expression of Id-1 was able to stimulate cell proliferation (Ling et al., 2002; Lin et al., 2005), enhance progression of cancer (Lin et al., 2000; Fong et al., 2003), and reduce hormone responsiveness (Ling et al., 2004; Zhang et al., 2004).

It has been reported that Id-1 was able to induce cell proliferation by activating the MAPK signaling pathway (Ling et al., 2002; Lin et al., 2005) and it was able to promote cell survival through activation of the NF-κB signaling pathway in prostate cancer (Ling et al., 2003; Zhang et al., 2007c) and esophageal cancer (Li et al., 2007). Ling et al. (2002) investigated the involvement of the MAPK pathway in Id-1 induced prostate cancer cell proliferation. Raf and Mek1/2 were activated after transfection of Id-1 into a prostate cell line. These results indicate that Id-1 was able to induce cell proliferation, possibly through the activation of the MAPK pathway in the prostate cancer cell line. Moreover, Id-1 was able to activate NF-κB signaling pathway against apoptosis. Researchers used a cytokine or chemotherapeutic drug to induce the cell to undergo apoptosis. Studies found that after transfection of Id-1 into cancer cell lines, the components within NF-κB signaling pathway (p50, p65, Bcl-xl, ICAM) and cell survival were increased. These results suggested that Id-1 may be able

to increase cell survival through activation of NF-κB signaling pathway (Ling et al., 2003; Li et al., 2007; Zhang et al., 2007c). Recently, ectopic expression of Id-1 was studied in MCF-7 breast cancer cell line (Kim et al., 2008). Studies using taxol, which is a mitotic inhibitor used in cancer chemotherapy, to induce apoptosis in MCF-7 and Id-1 transfected MCF-7 cells, showed that ectopic expression of Id-1 conferred cell survival characteristics to MCF-7 cells as indicated in the 3-(4,5-Dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay) and 4'-6-diamidino-2- phenylindole (DAPI) apoptotic cell count. Moreover Western blotting and functional luciferase assay results showed that increased expression of p65 NF-κB and decreased expression of IκB were found in Id-1 transfected clones, suggesting the anti-apoptotic property may be provided through the activation of the NF-κB signaling pathway (Kim et al., 2008). Furthermore, studies showed that Id-1 was able to activate the Akt pathway in esophageal cancer (Li et al., 2007) and breast cancer (Lee et al., 2009). Li 's study (2007) showed that after adding TNF-α to an Id-1 cloned esophageal cancer cell line, HEK293, expression of phosporylated Akt, glycogen synthase kinase 3β, IκB and p65 NF-κB were increased, implying Id-1 was able to protect cancer cells against apoptosis through activation PI3K/Akt/NF-κB). The latter study found that Id-1 activates the Akt pathway through

down-regulation of p53 which led to suppressed activity of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) in breast cancer (Lee et al., 2009). PTEN is an important tumor suppressor gene, down-regulation of PTEN is frequently associated with cancer (Cully et al., 2006).

Apart from proliferation and anti-apoptosis, an increased level of Id-1 was related to the progression of aggressive cancer. Ectopic expression of Id-1 was able to enhance breast cancer cell invasiveness and proliferation in growth factor deficient media. After transfection with Id-1 cDNA into a non-aggressive breast cancer cell line, T47D, the invasiveness and proliferation of T47D was increased when compared with the parental control (Lin et al., 2000). Moreover, studies using immunohistochemical staining found that expression of Id-1 was significantly higher in patients with a higher grade breast cancer than in patients with a lower grade breast cancer (Fong et al., 2003; Jang et al., 2006). The invasive property was confirmed by transfecting Id-1 antisense into breast cancer cell lines. Results showed that MT1-MMP, which correlated with the invasive ability of the cell, was decreased after transfection of Id-1 antisense (Fong et al., 2003). Furthermore, Id-1 was also shown to promote angiogenesis in HCC. Expression of vascular endothelial growth factor (VEGF) is important

in HCC growth and metastasis, studies showed that ectopic expression of Id-1 in HCC cells led to an increased expression of VEGF. The positive correlation between Id-1 and VEGF expression was confirmed by tissue mircoarray in patients with HCC (Lee et al., 2006). In addition, Darby et al. (2008) developed a bone morphogenetic protein (BMP)-6-regulatable gene expression system, the induction of BMP-6 contributed to a significant increase of invasion and migration in both PC3M and DU145 prostate cancer cells. The expression of metalloproteinase (MMP)s and Id-1 were also increased after induction of BMP-6, results implying that the BMP-6 promoted invasion and migration achieved through the stimulation of MMPs and Id-1 (Darby et al., 2008). Besides, Id-1 was able to reduce the hormone-responsiveness in prostate and ovarian cancers, suggesting that Id-1 may be involved in the progression to hormone-independent prostate and ovarian cancers (Ling et al., 2004; Zhang et al., 2004). Studies found that increased level of Id-1 expression was able to activate the expression of EGFR and reduce sex hormone-stimulated growth in bladder (Ding et al., 2006), ovarian (Zhang et al., 2004) and prostate (Ling et al., 2004) cancers. Zhang et al. (2004) suggested that Id-1 may be one of the affecters of EGFR. The reduced growth stimulation from sex hormones may correlate with the activation of EGFR (Ling et al., 2004; Zhang et al., 2004).

Investigators suggested that Id-1 may be an upstream regulator of EGFR in prostate cancer (Ling et al., 2004), and bladder cancer (Ding et al, 2006). It has been found that ectopic expression of Id-1 leads to increased expression of EGFR in the LNCaP prostate cancer cell line (Ling et al., 2004) and RT112 bladder cancer cell line (Ding et al, 2006). In contrast, down-regulating the expression of Id-1 by transfecting antisense oligonucleotides against Id-1 caused a suppression in the expression of EGFR in terms of protein and mRNA levels (Ling et al., 2004). When the relationship of Id-1 and EGFR in bladder cancer was studied using immunohistochemical staining in bladder cancer specimens (Ding et al, 2006) and renal cancer specimens (Li et al., 2007), results showed that up-regulation of Id-1 was associated with increased levels of EGFR in both bladder and renal cancer, however this relationship has not yet been investigated in breast cancer.

Chapter 3: METHODS

3.1 Cell culture

A hormone-dependent human breast cancer cell line, MCF-7 and a hormone-independent human breast cancer cell line MDA-MB231 were obtained from American Type Culture Collection (Rockville, MD). Breast cancer cell line MCF-7 expresses ER and therefore can be stimulated by estrogen, while the MDA-MB231 breast cancer cell line expresses EGFR, but not ER and therefore does not respond to estrogen. All cell lines were maintained in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carksband, CA), 1% penicillin and 1% streptomycin (Invitrogen, Carksband, CA) and incubated in a humidified environment at 37°C supplemented with 5% CO_2. Cells were harvested using 0.5X trysin with a subcultivation ratio of all cell lines of 1:3. Medium was changed once every 3 days.

3.2 MCF-7-Id-1 transfectants

The MCF-7-Id-1 transfectants and vector control have been generated previously. Briefly, retroviral vector containing full length Id-1 cDNA (pBabe-Id-1) or pBabe-puro was transfected into the PG13 packing cell line (obtained from

ATCC) using calcium phosphate method. The culture medium containing infectious viruses was harvested for retroviral infection of MCF-7 cells. Before performing transfection, the sensitivity of the cells to puromycin was tested. The optimal drug concentration at which minimal concentration that was able to kill 100% parental cells was chosen for selecting the transfectants. After transfection, the individual transfectant clones were isolated after 14 days drug selection. The expressions of Id-1 in different isolated clones were examined using Western blotting. Three stable clones expressing different levels of Id-1 were selected and were named as C1, C2 and C3. The vector control was generated from a pool of more than 20 individual transfectant clones of MCF-7-puro.

3.3 Antisense oligonucleotide against Id-1

Transfectant C3 which expressed the highest level of Id-1 was chosen. Cells (2 x 10^5) were seeded onto 6-well plates and cultured for 24 hours. Antisense oligonucleotide against Id-1 or a non-targeting antisense (Invirogen, Carksband, CA) was added to suppress the ectopic expression of Id-1 or as an antisense control respectively. Lipofectamine 2000 (5μl) (Invitrogen, Carksband, CA) was mixed with 250μl RPMI 1640 medium and 5μl of antisense oligonucleotide

was mixed with 250µl RPMI 1640 medium separately, both of the mixtures were incubated at room temperature for 5 minute. The two mixtures were pipetted together and incubated for 20 minutes at room temperature, then added to the cells to give a final concentration of 100nM of siRNA. Cells were incubated for 72 hours. After transfection, whole cell protein and nuclear fragement protein were extracted and the protein levels were evaluated by Western blotting. Cells were harvested and the cell viability was examined by MTT assay.

3.4 Protein Expression assay

3.4.1 Culture conditions

Cells (1 x 10^6) were seeded in a estrogen deprived medium: phenol red free RPMI 1640 containing 5% dextran-coated charcoal-treated FBS (Hyclone, South Logan, UT), 1% penicillin and 1% streptomycin for 72 hours, followed in serum-free medium for another 72 hours. The further incubation in serum-free medium was used to suppress the endogenous expression of Id-1 in all cell lines.

3.4.2 Protein extraction

After incubation, the medium was removed, and the cells washed with phosphate buffered saline (PBS). Cells were harvested, then collected in 15ml centrifuge

tubes and centrifuged to produce a cell pellet. Cell lysates were prepared by resuspending the cell pellet in lysate buffer (Tris-HCl, pH 8.0, NaCl, 0.1% SDS, 1% NP-40, 1mM PMSF, 50μg/ml leupeptin, 50μg/ml aprotinin) and incubating on ice for 20 minutes. Supernatant protein was collected by centrifugation at 2,500rpm for 20 minutes at 4°C. The supernatant was used as the whole cell protein extract for gel eletrophoresis.

3.4.3 Nuclear fragment protein extraction

Nuclear extract was extracted using NE-PER ® Nucleate and cytoplasmic extraction kit (Pierce, Rockford, USA) following the manufacturer's instructions. After incubation, the medium was removed and cells were washed with PBS. Cells were harvested and collected in a 1.7ml eppendorf tube. Cell pellets were produced by centrifuging the tube at 2,500rpm for 3 minutes at 4°C. After centrifugation, the supernatant was removed, then 100μl of reagent CER I was added and the cell pellet resuspended by vortex mixing, then left on ice for 10 minutes. After incubation on ice, 5.5μl ice-cold reagent CER II was added and the tube vortex mixed at high speed and further incubated on ice for 1 minute. After a final vortex at high speed the tube was then centrifuged at ~18,000rpm for 5 minutes at 4°C. The supernatant was removed, the cell pellet was

resuspended in 100 µl ice-cold reagent NER, and vortex mixed at the highest speed, then incubated on ice for 40 minutes with continue vortex mixing every 10 minutes. This supernatant (nuclear extract) was collected by centrifugation at ~18,000rpm for 10 minutes at 4°C. The nuclear extract was stored at -80°C and used for Western blotting or EMSA within two weeks.

3.4.4 Protein concentration measurement

The protein concentration of the whole cell protein and nuclear fragment protein were evaluated by Lowry DC protein assay kit (Bio-Rad). A protein standard curve was plotted using gradient concentration of BSA standard and radioimmunoprecipitation assay (RIPA) buffer as blank. The gradient concentration of BSA standard were listed below:

BSA Standards (mg/ml)	4 mg/ml BSA Stock Solution (µl)	Milli Q (µl)
0.2	1.0	19.0
0.5	2.5	17.5
1.0	5.0	15.0
1.5	7.5	12.5
2.0	10.0	10.0

Whole cell protein and nuclear extract protein were diluted in 1:10 (1.5µl protein + 13.5µl RIPA base buffer solution). All the standard BSA protein and the

protein samples were added into a 96-well plate at a volume of 5µl. Reagent A and reagent S were mixed in a 50:1 ratio (a mixture of phosphotungstric acid and phosphomolybdic acid in phenol). Twenty five microlitres of this mixture was added into the 96-well plate and vortex mixed for 1 minute. Two hundred microlitres of reagent B (sodium 1, 2-naphthoquinone-4-sulfonate) was then added into the 96-well plate and the whole plate placed onto a vortex mixer for at least 30 minutes to allow time for color development. After incubation, the 96-well plate was read by spectrophotometry at a wavelength of 750nm in a Spectrafluor Plus (TECAN) using Magellan programme. Protein concentrations were evaluated by plotting a standard curve using the absorbance of the BSA standard.

3.4.5 Western Blotting

3.4.5.1 Protein gel electrophoresis

A 10% polyacrylamide gel was prepared which contained 10% separation gel and 4% upper stacking gel, the ingredients were listed at Table 3.1. Whole cell protein (30µg)/ nuclear fragment protein (20µg) was loaded onto the 10% polyacrylamide gel, 5µl Bio-Rad protein standard marker (Precision Plus ProteinTM Dual Colr Standards, Bio-Rad) was loaded onto the gel as a standard

protein marker to indicate the molecular weight. The gel was placed into an eletrophoresis tank filled with 1X running buffer (24.9 mM Tris, 191.8 mM glycine and 8.7 mM SDS, pH8.3) and electrophoresis was conducted with a power pack at constant current (45mA) for about 1 hour in 1X running buffer.

3.4.5.2 Blotting

After running the gel, proteins were transferred to a methanol pre-treated PVDF (Amersham, Piscataway, NJ) by making a transfer sandwich using a transfer cassette. The sandwich was made of two filter papers on both sides as illustrated in Figure 3.1. Transfer sandwiches were placed into a transfer tank filled with 1X transferring buffer (25mM Tris, 192mM glycine and 20%(v/v) methanol, pH8.3) and blotting was conducted at a constant voltage (100V) for about 1.5 hours at 4°C.

Table 3.1 The composition of the SDS-PAGE gels

Reagents	10% Separating gel	4% Stacking gel
Milli Q water	3.8ml	3.15ml
1.5M Tris	2ml	N/A
0.5M Tris	N/A	1.25ml
10% SDS	80ul	50ul
40%, 37.5:1 Acrylamide/Bis	2ml	500ul
10% APS	77ul	38ul
TEMED	13ul	12ul
Total Volume	8ml	5ml

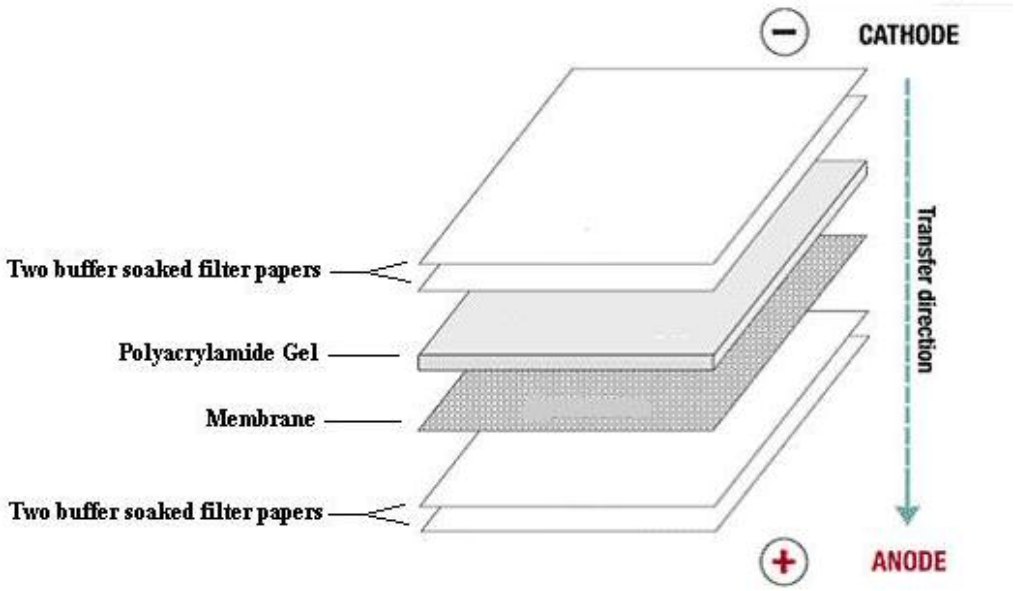


Figure 3.1Transfer sandwich of Western blotting

Two filter papers was added on both sides of the sandwich during transfer (Adapted from Fermentas, 2007, http://www.fermentas.com/techinfo/electrophoresis/pproteintransfer.htm)

3.4.5.3 Immuno-staining

After transfer, the membrane was taken out and non-specific sites on the membrane were blocked with 5% non-fat milk at room temperature for 1 hour. The membrane was then incubated with appropriate primary antibody of Id-1, p65, 1:1000 p50, IκB, Bcl-3, Actin, Histone H1 and EGFR (details were list at Table 3.2) at 4°C overnight. The excess primary antibodies were washed out using 1X TBS (20.1 mM Tris and 136.9 mM NaCl, pH 7.6) for 5 minutes repeated three times. The membrane was then incubated with 1:1000 horseradish peroxidase-conjugated secondary antibodies (Zymed, San Francisco, CA) at room temperature for 2 hours.

Table 3.2 Primary antibodies used in Western blotting

Antibody	Source	Dilution	M / P	Company	Cat. No.
Id-1 (C-20)	Rabbit	1:200	P	Santa Cruz	SC 488
EGFR	Rabbit	1:1000	M	Cell signaling	C74B9
NF-κB p50 (H-119)	Rabbit	1:1000	P	Santa Cruz	SC 7178
NF-κB p65 (C-20)	Rabbit	1:1000	P	Santa Cruz	SC 372
Bcl-3 (H-146)	Rabbit	1:500	P	Santa Cruz	SC 13038
IκB-α (C-21)	Rabbit	1:1000	P	Santa Cruz	SC 371
B-Actin (C4)	Mouse	1:1000	M	Santa Cruz	SC 47778
Histone H1(FL-219)	Rabbit	1:500	P	Santa Cruz	SC 10806

M : Monoclonal
P : Polyclonal

3.4.5.4 Signal development

Chemiluminescent signals were developed using ECL Plus Western blotting detection system (Amersham, Piscataway, NJ) by pouring the reagent mixture (Solution A, ECL Plus Substrate Solution, containing Tris buffer, and Solution B, Stock Acridan solution in Dioxane and Ethanol, in a ratio 4:1) on the membrane and incubated for 5 minutes. The membrane was then covered with an x-ray film in dark. The x-ray film was then placed into the developer, followed by water, fixer and water again for several minutes in dark. All the tests were performed in triplicates.

3.5 NF-kBp50 Transcription Factor ELISA

EMSA which is commonly used to study the interaction between protein-DNA binding, was performed using NF-kBp50 Transcription Factor ELISA kit (EK1110, Panomics, Fremont, CA) following the manufacturer's instructions, the principle of the transcription factor (TF) ELISA kit was in Figure 3.2. Briefly, 20μg of nuclear extract or positive control provided in the kit was incubated with a specific biotinlylated probe and binding buffer. The mixture was incubated for 30 minutes at room temperature, to allow the activated NF-kBp50 molecules from the extract bound to the NF-kBp50 consensus

binding site on the biotinlylated probe. The mixture was then transferred into an ELISA plate coated with streptavidin, the activated NF-kBp50 molecules was then indirectly attached to the assay plate through the binding between the biotinlylated probe and streptavidin. Non-specific molecules were washed away with washing buffer. Activated NF-kBp50 molecules were detected through the primary antibody against NF-kBp50, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. Tetramethylbenzidine (TMB) substrate was added to provide the colorimetric signal and the signal was quantified by spectrophotometry. Six independent experiments were performed.

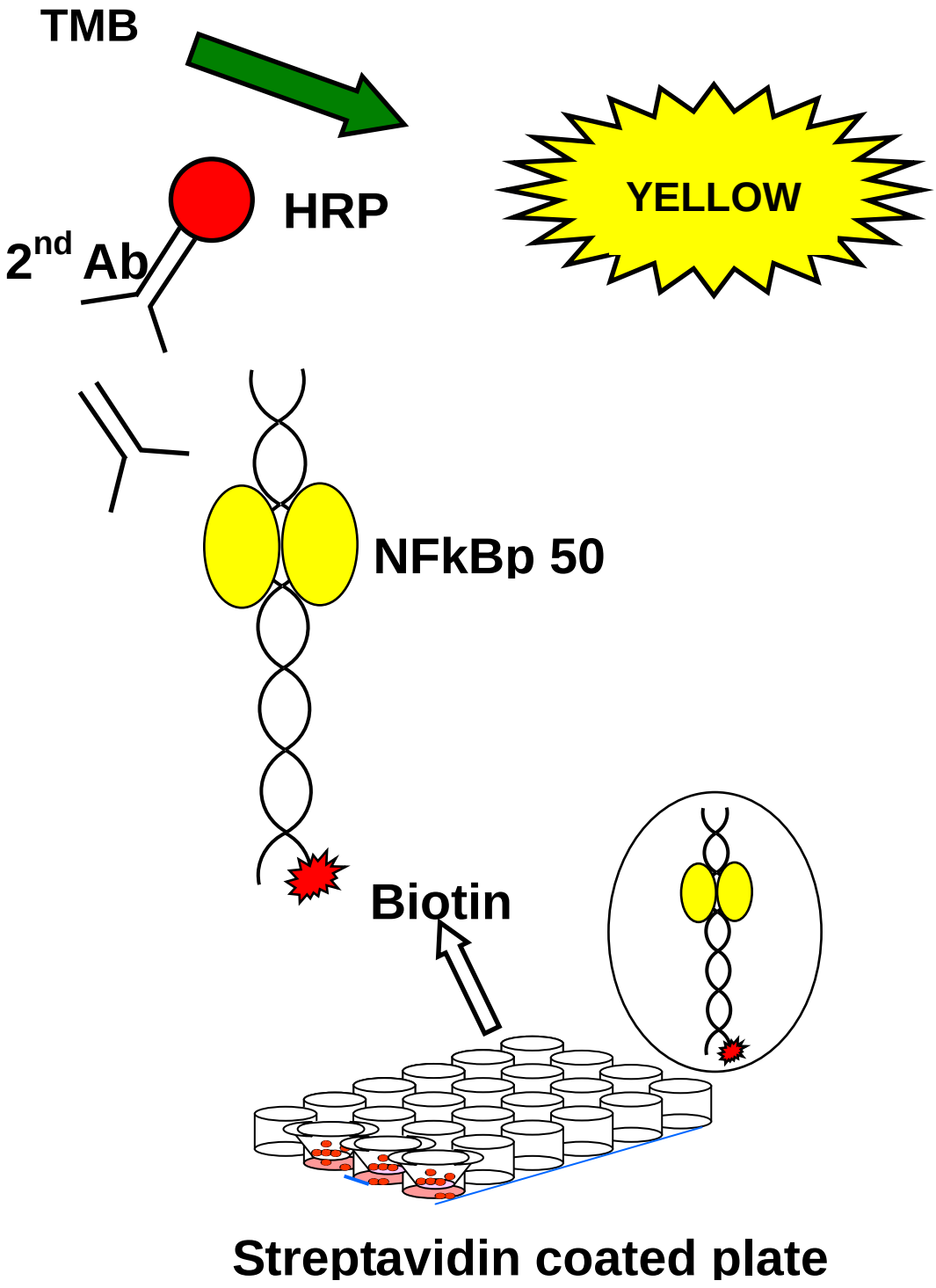


Figure 3.2 The principle of transcription factor (TF) ELISA kit
(Adapted from Panomics, 2009,
http://www.panomics.com/index.php?id=product_23)

3.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

3.6.1 RNA extraction

Total RNA was extracted using TRIZOL (Invirogen, Carksband, CA). After incubation, the medium was removed and washed with PBS. Cells were lysed by adding 1ml TRIZOL reagent into a culture dish. The cell lysate was collected in a 1.7ml eppendorf tube and the mixture was pipetted up and down. Then mixture was incubated at RT for 5 minutes. After incubation, 200µl of chloroform was added for every 1ml TRIZOL reagent, after capping tightly, the tube was shaken for 15 seconds and incubated at RT for 2-3 minutes. The tube was then centrifuged at no more than 12,000rpm for 10 minutes at RT. After centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase, RNA was in the upper aqueous phase. The upper aqueous phase was transferred into a fresh tube, 500µl isopropyl alcohol was added for every 1ml TRIZOL reagent and the incubated for 10 minutes at RT to precipitate the RNA from the aqueous phase. The tube was centrifuged at no more than 12,000rpm for 10 minutes at 4°C, then the supernatant was removed. The RNA pellet was washed by adding 1ml 75% ethanol in every 1ml TRIZOL reagent, it was then centrifuged at 7,500rpm for 5 minutes at 4°C. After centrifugation, the supernatant was removed and the

RNA pellet was allowed to briefly dry on ice. Then, about 5-10µl RNase-free water was added to dissolve the RNA pellet on ice. The RNA can be stored at -80°C and used within one month.

3.6.2 Preparation of cDNA

The quality and concentration of extracted RNA was evaluated by the NanoDrop® spectrophotometer ND-1000. Poly(A)+ RNA was reversely transcribed into cDNA and then amplified using the Fremantas RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's instructions. About 1-5 µl total RNA and 1 µl oligo$(dT)_{18}$ primer (0.5 µg/ µl) were added in a microcentrifuge tube. DEPC-treated water was added to mark up the volume to 12 µl, and the tube was gently mixed. The tube was incubated at 70°C for 5 minutes in the Applied Biosystem GeneAmp® PCR system 9700, at the same time a mastermix was prepared containing 4 µl 5X reaction buffer, 1 µl RiboLock™ Ribonuclease inhibitor (20units/ µl) and 2 µl 10mM dNTP mix. After incubation, the tube was placed on ice and the mastermix was added into the tube. The mixture was mixed gently, then incubated at 37°C for 5 minutes in the Applied Biosystem GeneAmp® PCR system 9700 and then 1 µl RevertAid™ M-MuLV Reverse Transcriptase (200units/ µl) was added, the final

volume was 20 μl. The mixture was further incubated at 42°C for 90 minutes and then 70°C for 10 minutes in the Applied Biosystem GeneAmp® PCR system 9700. cDNA product was stored at -20°C.

3.6.3 Polymerase chain reaction (PCR)

PCR was performed with Applied Biosystem GeneAmp® PCR system 9700. The mixture was prepared as listed in Table 3.3. PCR amplifications were started at 94°C for activation. Denaturation was performed at 94°C and elongation was performed at 72°C. For Id-1 and GADPH, 25 cycles were performed. For EGFR and Bcl-3, 35 cycles were performed. The primers sequences and details of the targeted gene were listed at Table 3.4. GADPH was used as an internal quantitative control. Electrophoresis of the amplified products were performed on 1% agarose gels (Amersham, Piscataway, NJ), stained with ethidium bromide and visualized under CHEMI GENIUS^2 bio imageing system. All the tests were performed in triplicates.

Table 3.3 Preparation of PCR mixture

Reagent	Volume used	Final conc.
10x Gold Buffer	2.5	1X
25mM $MgCl_2$	2.5	2.5mM
2mM dNTPs	2.5	0.2mM
10µM Forward Primer	0.75	0.3µM
10µM Reverse Primer	0.75	0.3µM
5U/µl Ampli Taq	0.1	0.5U
20ng/µl DNA	2.5	-
RNase free water	13.4	-
Total Volume	25µl	-

Table 3.4 The primers used in RT-PCR

Gene	Primer sequences	Tm *	GenBank	Product size
Id-1	Forward: 5’ AGCACCCTGAACGGCGAGAT 3’	65°C	NM_002165	283bp
(Ouyang et al., 2001),	Reverse: 5’ CCCCCTCCCAAAGTCTCTG 3’			
EGFR	Forward: 5’ CTCACGCAGTTGGGCACTTT 3’	60°C	NM_005228	261bp
(Kitazaki et al., 2005)	Reverse: 5’ TCATGGGCAGCTCC TTCAGT 3’			
Bcl-3	Forward: 5’ GCAGATCTTGGAC TCATGAGG 3’	60°C	NM_005178	199bp
(Rocha et al., 2003)	Reverse: 5’ CTGGGGTCAGAGT CCTGGGAG 3’			
GADPH	Forward: 5’ GCCACATCGCTC AGACAC 3’	60°C	NM_002046	452bp
	Reverse: 5’ AGG AGGCATTGCTGATGATCTT 3’			

Tm* : Annealing temperature of the primers

3.7 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay)

Cell viability was measured using the MTT proliferation assay (Sigma, St Louis, MO) according to the manufacturer's instructions. Cells were seeded onto 96-well plates and cultured for an appropriate period of time. Cells were then washed with PBS and incubated with 100μl MTT reagent in a dilution ratio 1:20 in phenol red free RPMI 1640 medium for 4 hours in dark to allow the metabolic reaction between mitochondrial succinate dehydrogenase from living cells and the MTT reagent to take place. The yellow MTT complexes were reduced into an insoluble, dark purple colored formazan product by the presence of mitochondrial succinate dehydrogenase in living cells. The purple colored formazan product was centrifuged at 2500 rpm for 5 minutes. The formazan was then dissolved with DMSO, and absorbance was then measured at 570nm in a Spectrafluor Plus (TECAN) using Magellan programme within 15 minutes.

3.8 Cell proliferation assay on MCF-7-Id-1 transfectants

The effect of ectopic expression of Id-1 on cell proliferation in the absence of estrogen was examined. Cells (3000) of three MCF-7-Id-1 transfectants and the mock-control pBabe were seeded onto 96-well plates and cultured in an

estrogen deprived medium for 1, 3 and 5 day(s). Cell viabilities were evaluated using the MTT assay at Day 1, 3 and 5. The relative percentage cell viability was calculated using Day 1 as a baseline. The change in absorbance was calculated as below:

[(Absorbance of the transfectant at that day – Absorbance of the transfectant at Day 1) / Absorbance of the transfectant at Day 1] x 100%

The effect of ectopic expression of Id-1 on cell proliferation was confirmed by antisense oligonucleotide against Id-1, using the transfectant C3 which expressed the highest level of Id-1. Antisense oligonucleotide against Id-1 was added to suppress the expression of Id-1, a non-targeting oligonucleotide was also added as a antisense control. Cells ($2x10^5$) were seeded onto 6-well plates, the medium was changed to estrogen deprived medium after 24 hours. Then the mixture 100nm antisense oligonucleotide against Id-1 or non-targeting antisense control and 5μl lipofamine in transfectant C3 was added. After 72 hours transfection, cells were collected and $1x10^4$ cells were seeded onto 96-well plates. The medium was changed after 24 hours to the estrogen deprived medium for 1, 3, 5 day(s). As in the growth assay above for the MCF-7-Id-1 transfectants, cells viability was evaluated using the MTT assay.

3.9 Study the involvement of NF-κB on MCF-7-Id-1 transfectants

3.9.1 Cell inhibition assay

Cells ($1x10^4$) of MCF-7-Id-1 and pBabe control were seeded onto 96-well plate. Cells were incubated in an estrogen deprived medium with the addition of different volumes (10μM, 25μM) of parthenolide or the vehicle (DMSO) for 1, 3, 5 days. Cells were then harvested and examined using MTT assay at Day 1, 3 and 5. The percentage inhibition was calculated as below:

[(Absorbance of the relative conc. of DMSO - Absorbance of the transfetcant under relation conc. of parthenolide) / Absorbance of the relative conc. of DMSO] x 100%

3.9.2 Cell cycle analysis

Cells (4×10^5) were seeded onto a 60mm dish, the medium was changed after 24 hours into estrogen deprived medium with the addition of either 10μM or 25μM parthenolide, or relative conc. of vehicle (DMSO). After 24 hours incubation, cells were harvested and collected in a 1.7ml eppendorf tube. Cell pellets were obtained by centrifugating the eppendorf tube at 2500rpm for 5 minutes at 4°C. Cell pullets were resuspended in 200μl PBS and fixed in 1ml ice-cold 70% ethanol overnight at 4°C. The fixed cells were collected by centrifuging the

tube at 2500rpm for 5 minutes at 4°C. After removing the supernatant, cell pellets were resuspended in 500µl cold PBS. The resuspended cells were treated with 5µl RNase/ Propidium iodide (PI) buffering reagent (BD PharmingenTM, San Diego, CA) at room temperature for 30 minutes to allow PI to intercalate into double-stranded nucleic acids of the living cells and to provide a fluorescent signal for detection. Cell cycle analysis was performed on a Cytomics FC 500 Flow with CXP software version 2.0 (Beckman Coulter). Results were analyzed using Modfit LT 3.0 software. Three independent experiments were performed and the mean of the results obtained was used in data analysis.

3.10 Statistical analysis

All statistical analyses were performed using one-way ANOVA test (Kruskal-Wallis test) to compare the variable of the tranfectants and controls by using GraphPad PRISM 4.00 version. The significance of the P value was expressed based on a "Michekin Guide" scale as significant (* $P<0.05$), highly significant (** $P<0.01$) and extremely significant (*** $P<0.001$) (Motulsky, 1995).

Chapter 4: RESULTS

4.1 Id-1 protein expression in the MCF-7-Id-1transfectants

Protein expressions of Id-1 in MCF-7-Id-1 transfectants (C1, C2 & C3) and the controls were examined using Western blotting. Results showed that all transfectants expressed a higher concentration of Id-1 protein than the controls. Moreover, C3 had the highest expression of Id-1 while C1 had the lowest expression of Id-1 protein level (Figure 4.1).

Transfectant C3 expressing the highest level of Id-1 was chosen to examine the effect of the antisense oligonucleotide in suppressing the expression of Id-1. Protein expression results showed that Id-1 was decreased after transfection of antisense against Id-1 (C3 siRNA) compared with transfectant C3 transfected with non-targeting antisense control (C3 si-CON). Results also showed that the Id-1 expression level was similar between untreated transfectant C3 (C3) and transfectant C3 transfected with non-targeting antisense control (Figure 4.2).

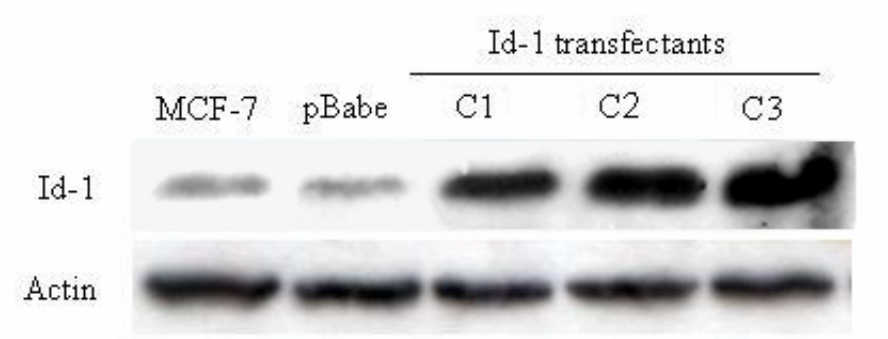


Figure 4.1 Expression of Id-1 protein in the MCF-7- Id-1 transfectants. Western blotting results showed that three MCF-7-Id-1 transfectants expressed different Id-1protein level. All these MCF-7-Id-1 transfectants expressed higher level of Id-1 than the mock-control and parental control. Actin was used as an internal control.

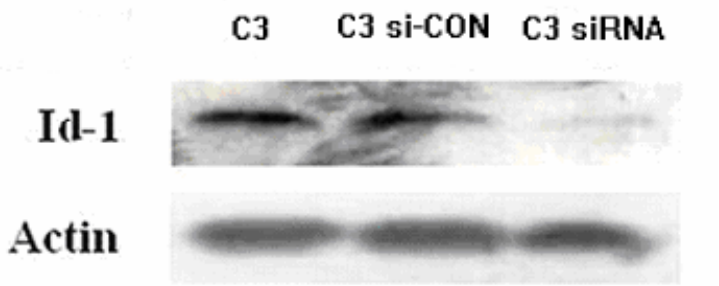


Figure 4.2 Expression of Id-1 in transfectant C3 after transfection of antisense oligonucleotide against Id-1.

After transfection of antisense oligonucleotide against Id-1, Id-1 protein expression was decreased compared with the controls. Actin was used as an internal control.

4.2 Effect of ectopic expression of Id-1 on cell proliferation of MCF-7-Id-1 transfectants in the absence of estrogen

After culturing under estrogen deprived conditions for different periods, results showed that relative percentage cell viability of all MCF-7-Id-1 transfectants were significantly higher than that of the mock-control pBabe. Also the relative percentage cell viability was positively associated with the protein expression of Id-1, highest in transfectant C3 and lowest in transfectant C1 (Figure 4.3).

With regard to the relative percentage cell viability of transfectant C3 after transfection of antisense oligonucleotide against Id-1 (C3 siRNA), expression of Id-1 in transfectant C3 was significantly decreased compared with transfectant C3 transfected with non-targeting antisense control (C3 si-CON). No significant difference was seen between untreated transfectant C3 (C3) and C3 si-CON at Day 3. Moreover, relative percentage cell viability of the C3 siRNA was similar to that of pBabe (Figure 4.4). The effect of Id-1 on cell proliferation was confirmed by transfection of antisense oligonucleotide against Id-1.

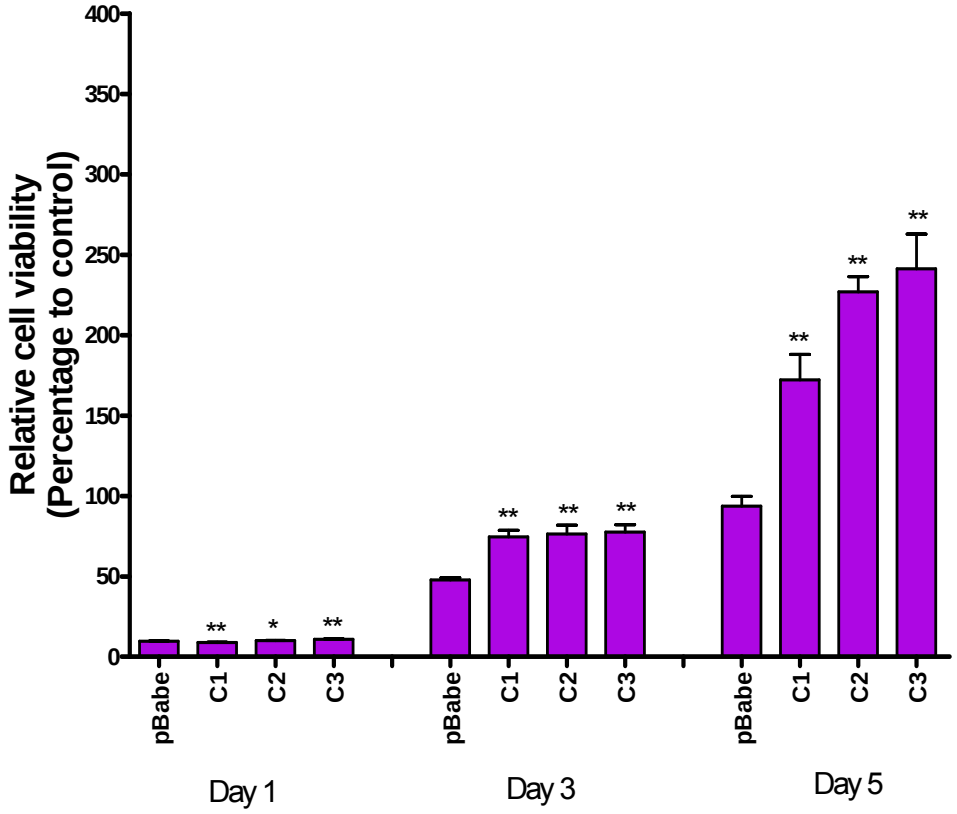


Figure 4.3 Relative cell viability of MCF-7-Id-1 transfectants and pBabe control in the absence of estrogen.

The cell viability of all three transfectants (C1, C2 and C3) were significantly higher than the mock-control pBabe. Data showed means + SD, (n=6).

* — P value <0.05 (statistical significant)

** — P value <0.01 (highly statistical significant)

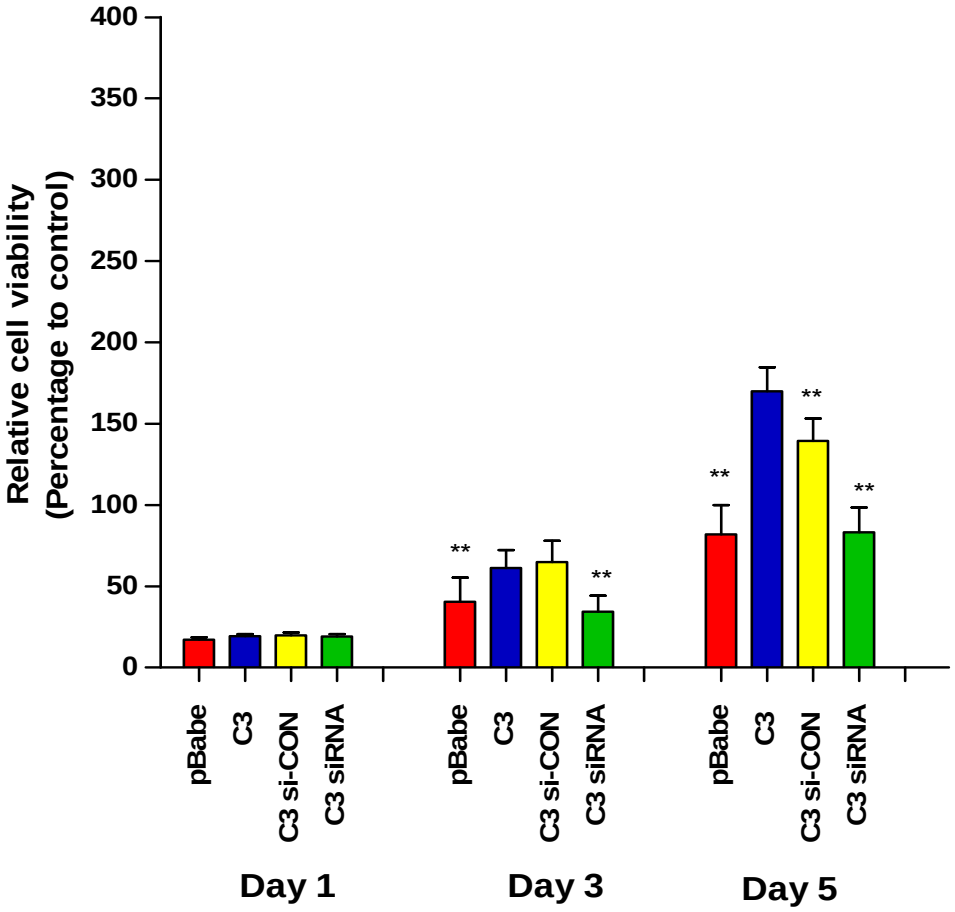


Figure 4.4 Relative cell viability of transfectant C3 after transfection of antisense oligonucleotide.

The relative cell viability was significantly decreased after transfection of antisense oligonucleotide against Id-1 compared with the controls. Data showed means +SD, (n=6).

* — P value <0.05 (statistical significant)

** — P value <0.01 (highly statistical significant)

4.3 Effect of ectopic expression of Id-1 on EGFR

Western blotting results showed that MDA-MB231 displayed the highest levels of both Id-1 and EGFR. The expression levels of EGFR in MCF-7-Id-1 transfectants were higher than that of the parental control and mock-control. Also the expressions of EGFR in MCF-7-Id-1 transfectants were positively associated with their Id-1 expression levels in terms of protein (Figure 4.5) and RNA (Figure 4.6). When transfectant C3 was transfected with antisense oligonucleotide against Id-1 (C3 siRNA), expression of Id-1 and EGFR were both reduced compared with transfectant C3 without any antisense transfection (C3 si-CON) (Figure 4.7).

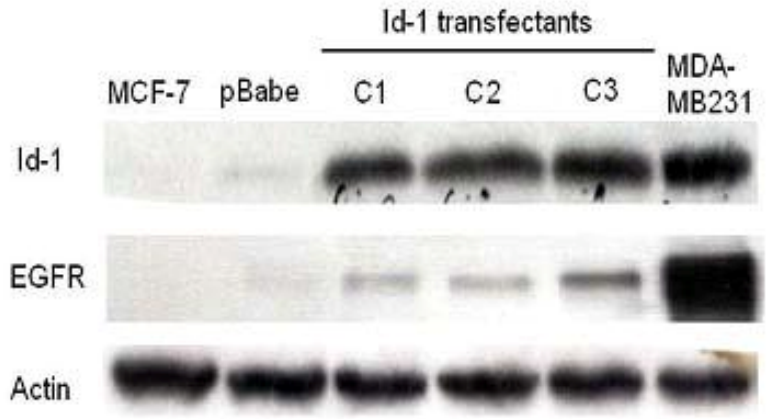


Figure 4.5 Protein expression of EGFR in MCF-7-Id-1 transfectants and controls.

Protein expressions lof EGFR in different MCF-7-Id-1 transfectants were positively associated with their Id-1 expression. Actin was used as an internal control.

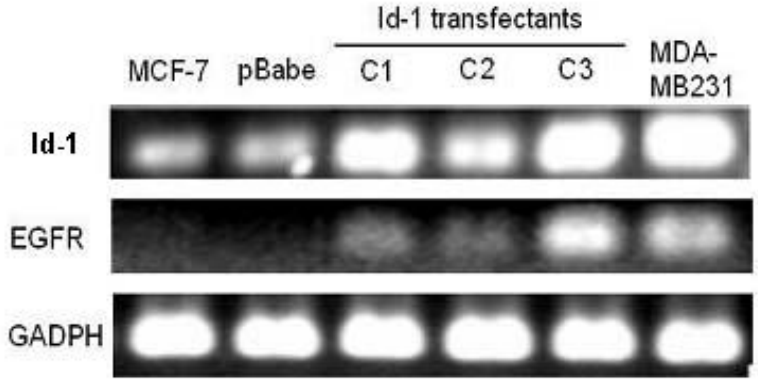


Figure 4.6 RNA expression of EGFR in MCF-7-Id-1 transfectants and controls. RNA expressions of EGFR in different MCF-7-Id-1 transfectants were positively associated with their Id-1 expression. GADPH was added as an internal control.

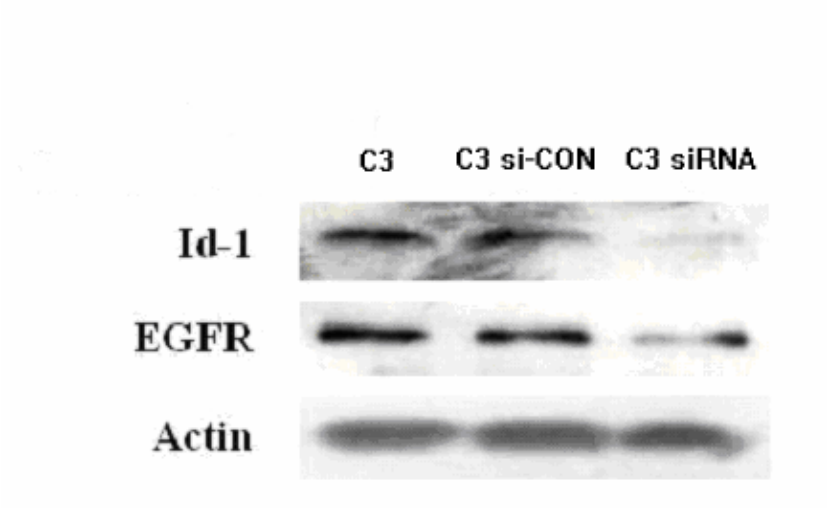


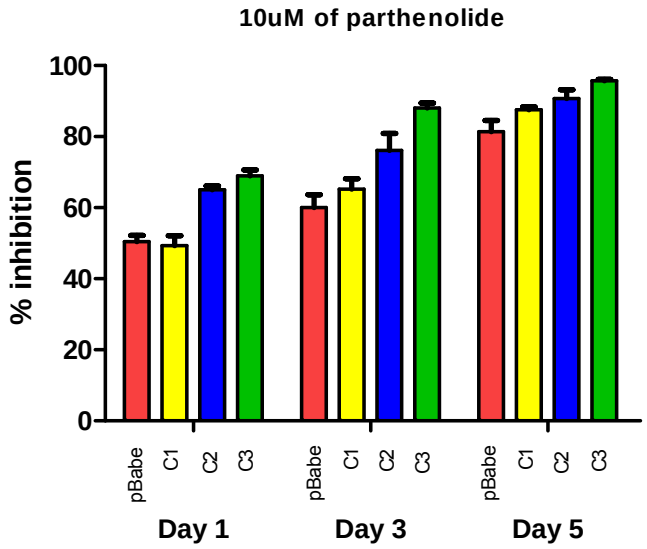
Figure 4.7 Protein expression of EGFR in transfectant C3 with/ without transfection of antisense oligonucleotide.
Protein expression of Id-1 and EGFR were both reduced after transfection of antisense oligonucleotide against Id-1 compared with the controls. Actin was used as an internal control.

4.4 Impact of NF-κB pathway inhibition on MCF-7-Id-1 transfectants

4.4.1 MTT cell inhibition assay

After applying different concentrations of parthenolide (10μM or 25μM) on the cells, more than 50% inhibition was found compared with DMSO control in all cell lines at Day 1, 3 and 5 when using the MTT assay. Moreover, percentage inhibition was positively correlated with the protein expression level of Id-1 in which transfectant C3 expressed the highest and transfectant C1 expressed the lowest level of Id-1 (Figure 4.8).

A.



B.

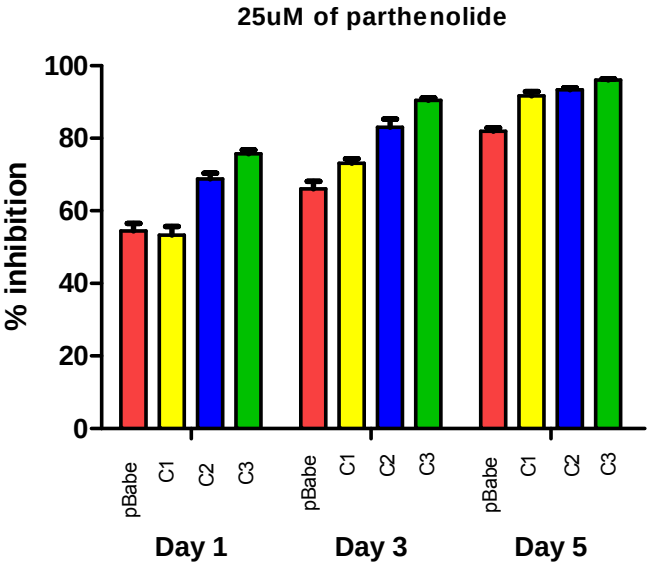


Figure 4.8 Percentage inhibition in MCF-7-Id-1 transfectants after applying A. 10μM or B. 25μM of NF-κB pathway inhibitor, parthenolide.
Percentage inhibition was positively correlated with their protein expression level of Id-1. Error bars represented means + SD (n=6).

4.4.2 Cell cycle analysis

Since even at Day 1 showed more than 50% inhibition in all cell lines compared with DMSO controls, this timepoint was chosen and used for cell cycle analysis. After applying 10μM parthenolide, cell cycle analysis revealed a decreased percentage of S phase in all cell lines compared with the vehicle. Also the percentage S phase in the MCF-7-Id-1 transfectants were lower than that of the control after applying 10μM parthenolide. However, after applying 25μM parthenolide, cell cycle distribution was undetectable in all cell lines (Figure 4.9), while another peak appeared before the cell cycle in the X-axis. The peak represents a population of smaller size fragments, debris or dead/apoptotic cells. Figure 4.10 was the same as figure 4.9, but the X-axis was extended to the right to give better illustration of the population of cell in the peak which became smaller in size after the application of 25μM parthenolide, implying that the cells were dead (Figure 4.10).

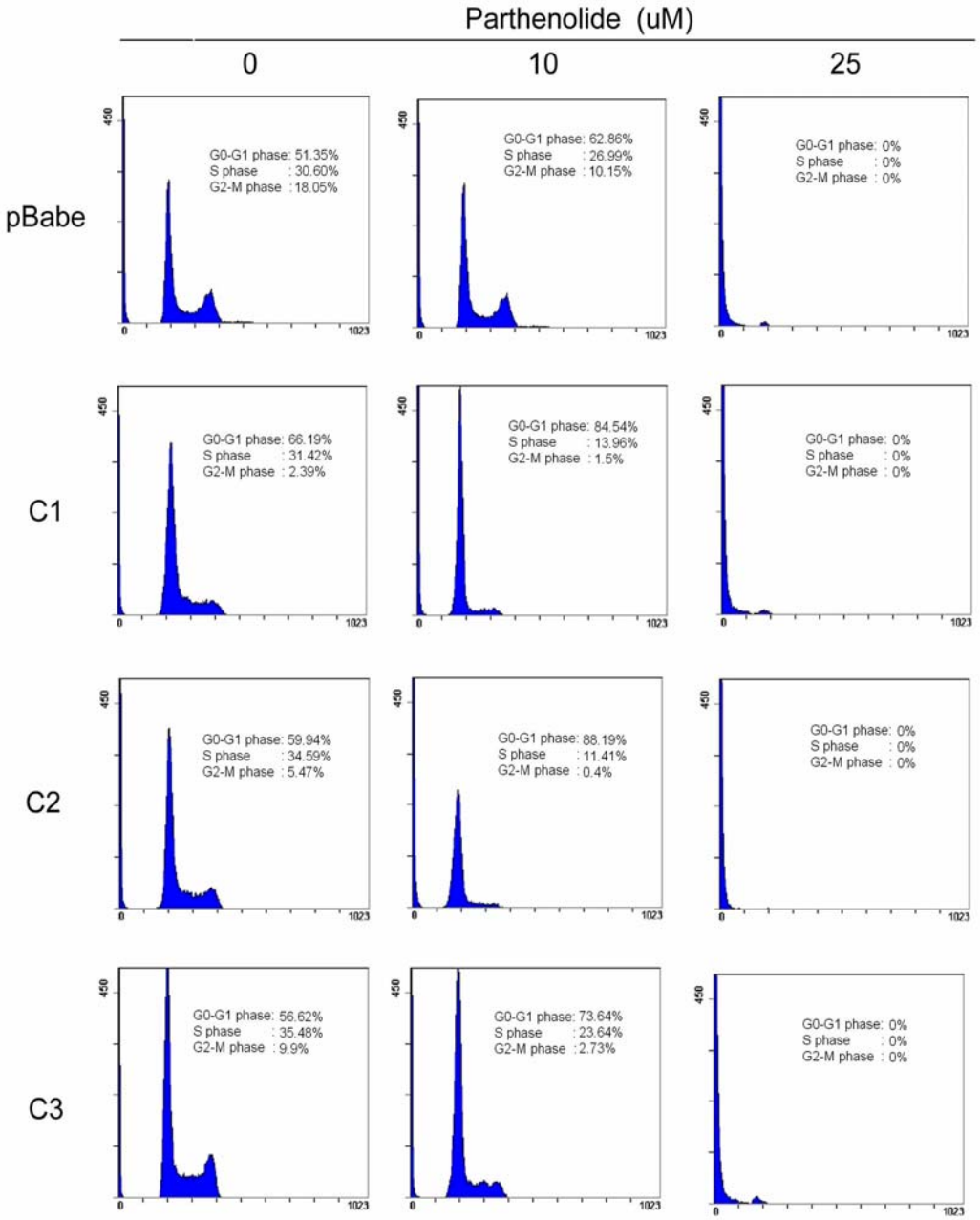


Figure 4.9 Representative cell cycle distributions of MCF-7-Id-1 transfectants after applying NF-κB pathway inhibitor.

After adding 10μM parthenolide, % S phase of all cell lines were reduced, however cell cycle was undetectable after adding 25μM parthenolide. The percentage cell distribution was the average of three individual experiments.

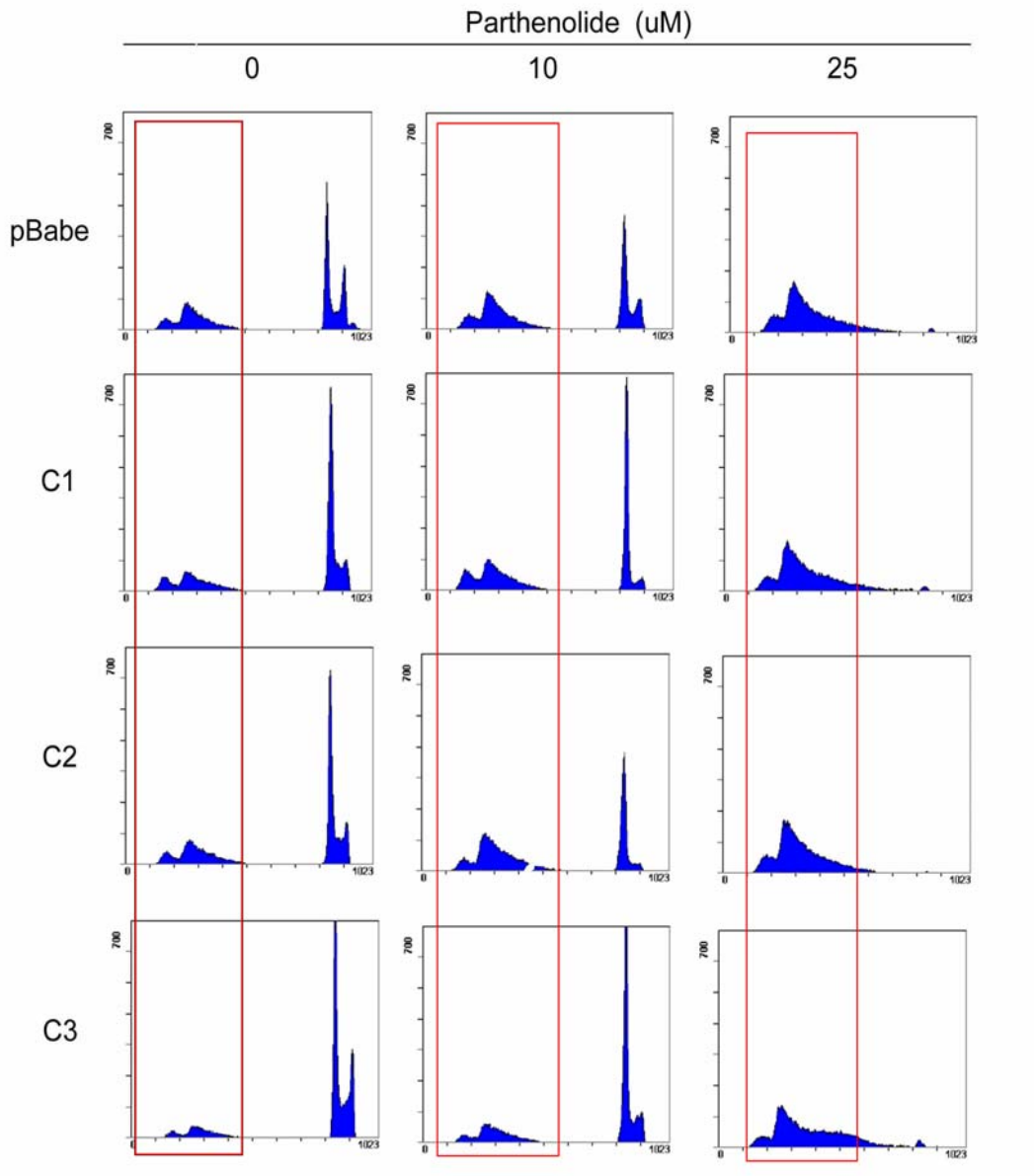


Figure 4.10 Dead cells of MCF-7-Id-1 transfectants after applying different concentrations of NF-κB pathway inhibitor.

Figure was the same as figure 4.9, but the X-axis was extended to the right to indicate the smaller or dead cell. Figure showed that after addition of 25μM parthenolide, a peak which represents dead cell was found in all cell lines.

4.5 Effect of ectopic expression of Id-1 on NF-κB pathway

Whole cell lysates and nuclear fragment extracts were collected and evaluated using Western blotting. As mentioned in the literature review, subunits of NF-κB p65 and p50, the commonest members activated in human epithelial cells, were the main focus of this part of the study.

4.5.1 Expression of NF-κB p65 and p50 in whole cell lysate

Results showed that the protein expression levels of NF-κB p65 and p50 were similar in the whole cell protein of all cell lines (Figure 4.11).

4.5.2 Expression of NF-κB p65 and p50 in nuclear fragment extract

Results of the Western blotting using nuclear fragment protein showed that the expression levels of NF-κB p50 in MCF-7-Id-1 transfectants were higher than that of the MCF-7 controls, but no observable difference was found in NF-κB p65 expression among different cell lines (Figure 4.12).

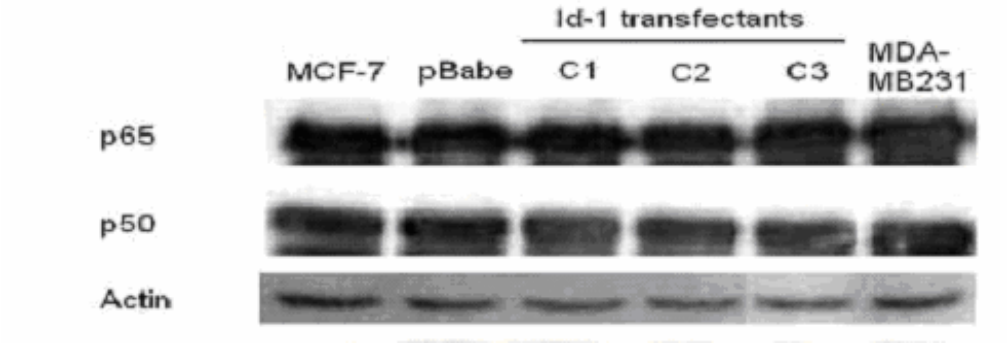


Figure 4.11 Expression of NF-κB p65 and p50 subunits in whole cell protein extract.

No observable expression difference of NF-κB p65 and p50 subunits in whole cell protein extract was found between MCF-7-Id-1 transfectants and the controls. Actin was used as an internal control.

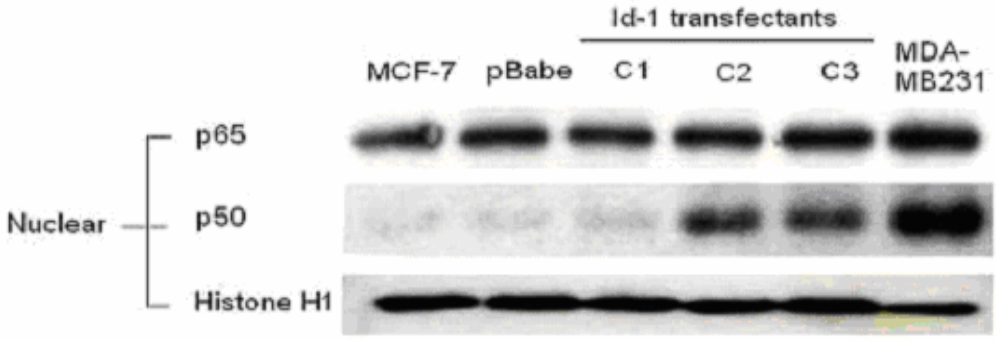


Figure 4.12 Expression of NF-κB p65 and p50 subunits in nuclear protein extract. Expression of NF-κB p50 subunits in nuclear protein extract of MCF-7-Id-1 transfectants were higher than that of the MCF-7 controls, while no observable difference of NF-κB p65 expression was found between MCF-7-Id-1 transfectants and controls. Histone H1 was used as an internal control for nuclear protein extract.

4.5.3 Expression of I-κB in whole cell lysate

In order to examine whether the activation of NF-κB p65/p50 heterodimer was involved in the MCF-7-Id-1 transfectant, the expression of IκBα, the inhibitor of NF-κB p65/p50 heterodimer, was examined. Results showed that the expression of IκBα was lower in MDA-MB231 compared with other cell lines. No observable difference was found between MCF-7-Id-1 transfectants and the MCF-7 controls (Figure 4.13).

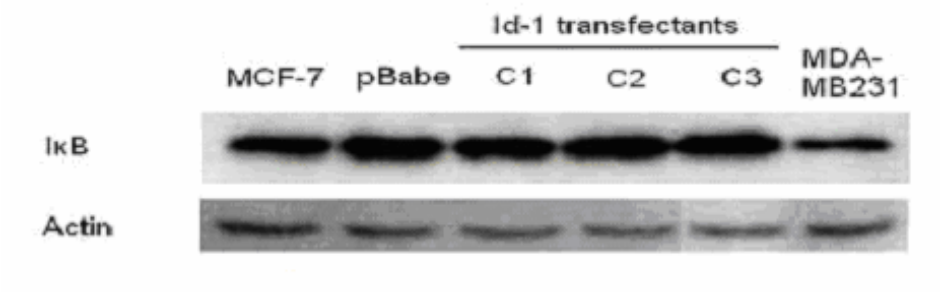


Figure 4.13 Expression of IκB in whole cell protein extract.
Protein expression of IκB was lower in MDA-MB231, but no observable difference was found between MCF-7-Id-1 transfectants and MCF-7 controls. Actin was used as an internal control.

4.5.4 Expression of Bcl-3 in nuclear fragment extract and RNA extract

Bcl-3 is a coactivator of NF-κB p50/p50, with the purpose to examine whether NF-κB p50/p50 homodimer was activated in the MCF-7-Id-1 transfectants, the expression of Bcl-3 was examined. Results showed that the expression level of Bcl-3 was positively associated with the expression of NF-κB p50 in the MCF-7 cell lines in terms of nuclear fragment protein (Figure 4.14) and RNA level (Figure 4.15).

When transfectant C3 was transfected with antisense oligonucleotide against Id-1, the expression level of Bcl-3 was reduced in both whole cell protein (Figure 4.16) and nuclear fragment protein (Figure 4.17) when compared with transfectant C3 after transfection with non-targeting antisense control. The reduced expression of NF-κB p50 was only observed in nuclear protein extract after transfection of antisense oligonucleotide against Id-1 (Figure 4.17).

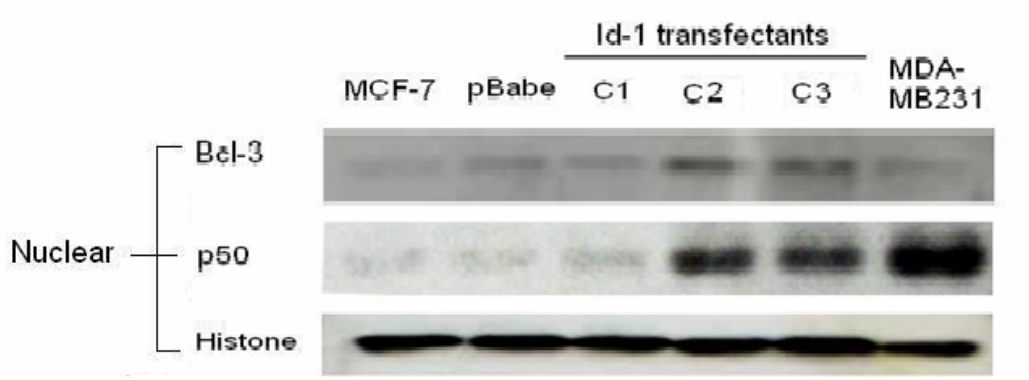


Figure 4.14 Expression of Bcl-3 in nuclear protein extract.
Protein expression of Bcl-3 was positively associated with their expression of NF-κB p50 in nuclear protein extract except MDA-MB231. Histone H1 was used as an internal control.

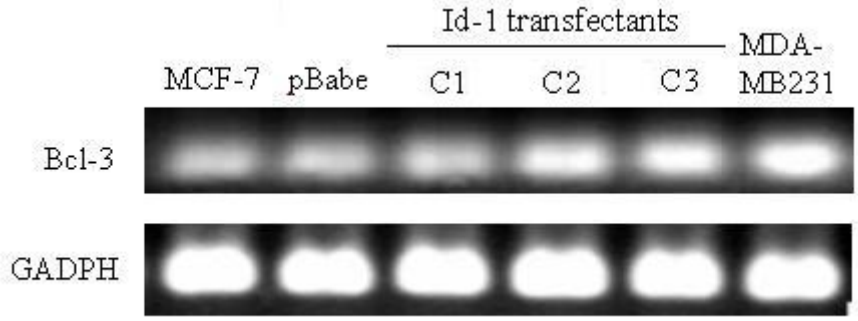


Figure 4.15 RNA expression of Bcl-3.
RNA expressions of Bcl-3 in MCF-7-Id-1 transfectants were higher than that of the MCF-7 controls. GADPH was used as an internal control.

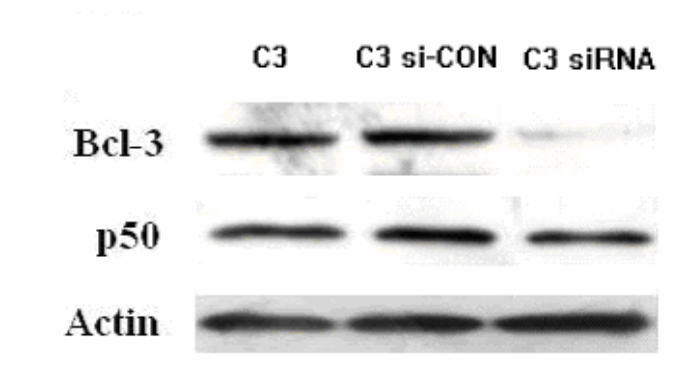


Figure 4.16 Expression of Bcl-3 and p50 after applying antisense oligonucleotide against Id-1 in whole cell protein extract.
Expression of Bcl-3 in whole cell protein extract was reduced after transfection of antisense oligonucleotide against Id-1 compared with the controls. Actin was used as an internal control.

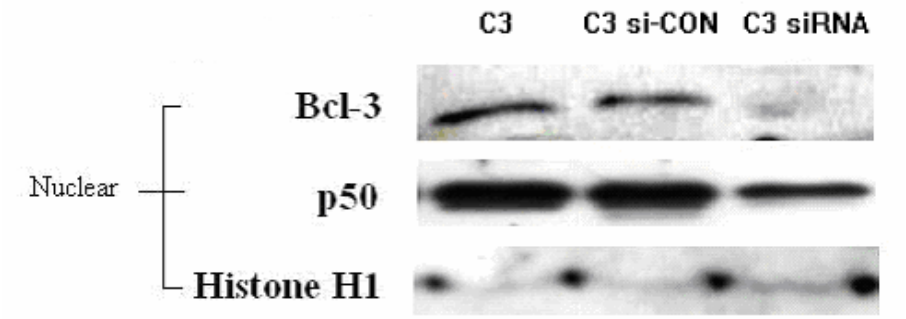


Figure 4.17 Expression of Bcl-3 and NF-κB p50 after applying antisense oligonucleotide against Id-1 in nuclear protein extract.
Expression of Bcl-3 and NF-κB p50 in nuclear protein extract were both reduced after transfection of antisense oligonucleotide against Id-1 compared with the controls. Histone H1 was used as an internal control.

4.5.5 Expression of NF-κB p52 in nuclear fragment extract

Since there is a possibility that Bcl-3 will bind with NF-κB p52/p52 homodimer, the expression of p52 in nuclear fragment extract of all cell lines were examined. Results showed that the expression of NF-κB p52 in all cell lines were low and no association with the level of Id-1 in MCF-7-Id-1 transfectants was found (Figure 4.18).

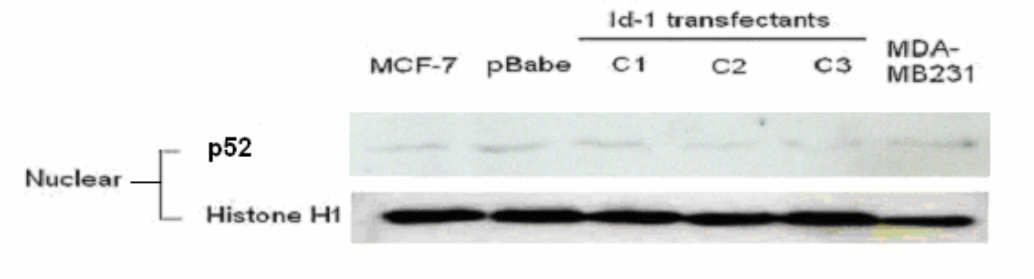


Figure 4.18 Expression of NF-κB p52 in nuclear protein extract.
Expression of NF-κB p52 in nuclear protein extract was low and no associateion was found between MCF-7-Id-1 transfectants and controls. Histone H1 was used as an internal control.

4.5.6 Activity of NF-κB p50/p50 on MCF-7-Id-1 transfectants

To evaluate the activated NF-κB p50 in the nuclear fragment extract was bound to the NF-κB p50 nucleotide, EMSA of NF-κB p50/p50 was performed using an ELISA method. Results showed that activity of all MCF-7-Id-1 transfectants were significantly higher than the MCF-7 control (Figure 4.19), with transfectant C3 showing the highest activity, and transfectant C1 and C2 showing similar activity, with C2 being slightly higher than C1.

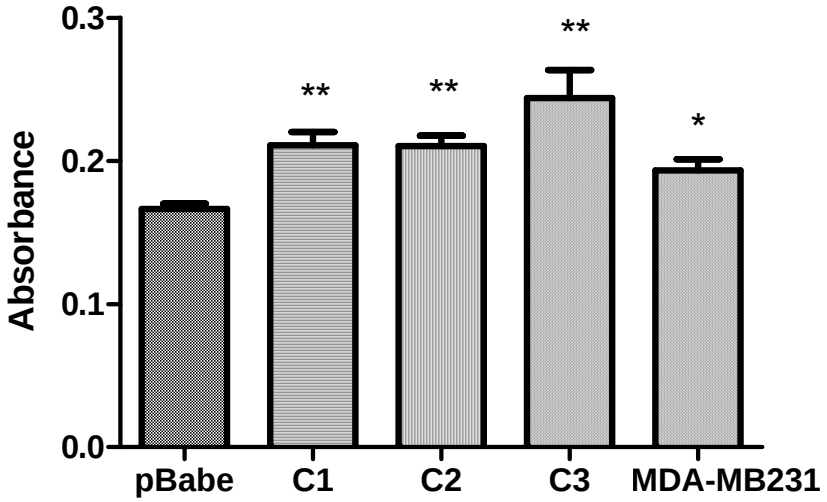


Figure 4.19 Activities of NF-κB p50/p50 in different MCF-7-Id-1 transfectants using an ELISA method.

Activities of NF-κB p50/p50 in MCF-7-Id-1 transfectants were significantly higher than that of the controls. Error bars represent means +SD (n=6).

* — P value <0.05 (statistical significant)

** — P value <0.01 (highly statistical significant)

Chapter 5: DISCUSSIONS

5.1 Id-1 able to induce cell proliferation in the absence of estrogen

In the growth study, cells were cultured in estrogen deprived conditions by using charcoal absorbed FBS. MCF-7 is a hormone-dependent breast cancer cell line which is ER positive and able to be stimulated by estrogen (ATCC, 2008). Results in this study showed that without the stimulation of estrogen, the percentage cell growth of the Id-1 transfectants was significantly higher than that of the mock-control MCF-7 breast cancer cell (Figure 4.3) and that the growth of transfectants was positively associated with their protein expression of Id-1 (Figure 4.1, 4.2, 4.3 & 4.4). This indicates that Id-1 was able to increase cell proliferation in the absence of estrogen. Lack of growth factors causes cell arrest at the restriction point, but once the restriction point has been passed, cells will be able to undergo DNA synthesis and cell division (Heath, 2001). Higher cell proliferation rate in transfectants than in the controls suggested that Id-1 may give an advantage to the cells to pass through the restriction point and bypass the exogenous stimulatory influence of estrogen. These results were supported by other investigators that ectopic expression of Id-1 was able to increase cell growth and enhance the G1/S phase transition in the cell cycle in esophageal cancer (Hui et al., 2006) and in prostate cancer (Ouyang et al., 2002).

Moreover, ectopic expression of Id-1 also enabled cyclin D1 to be upregulated in breast cancer (Lee et al., 2009). Cyclin D1 is important in the G1/S phase transition, up-regulation of cyclin D1 leads to an increase in the transition through increased cell proliferation (Perry et al., 1998; Biswas et al., 2000; Kaldis, 2006). Furthermore, an increased level of Id-1 was able to delay cellular senescence in prostate cancer cells through the inhibition of p16INK4a (Ouyang et al., 2002). These studies may provide evidence that Id-1 is able to endow a growth advantage to breast cancer cells in the absence of estrogen. Results also implied that Id-1 may be able to contribute to the development of hormonal resistance. However, the pathways through which Id-1 acted to induce cell proliferation without the stimulation of estrogen is still unknown.

5.2 Id-1 able to modulate the expression of EGFR

In order to investigate the possible role of Id-1 in the induction of cell proliferation in the absence of estrogen, the expression of EGFR was also studied. Results showed that ectopic expression of Id-1 in MCF-7 cells led to an increased expression of EGFR in terms of protein (Figure 4.5) and mRNA levels (Figure 4.6). In addition, after transfection of antisense oligonecleotide against Id-1, the expression of EGFR was also reduced (Figure 4.7), implying

that EGFR may be one of the downstream effectors of Id-1 in breast cancer. In fact, the relationship between expression of Id-1 and EGFR has been confirmed by an immunohistochemical staining study conducted on 96 cases of invasive ductal carcinoma. Results using semi-quantitative McCarty's H scoring system showed that the correlation coefficient (r) between Id-1 and EGFR is 0.336 with P value = 0.001, implying an extremely statistical significant positive correlation between Id-1 and EGFR expression among these breast cancer cases (unpublished results from our own research).

The finding mentioned above is corroborated by studies of other investigators that expression of Id-1 was positively associated with the expression of EGFR in the bladder (Ding et al, 2006) and in renal cancer (Li et al., 2007). In addition, EGFR has been suggested to be one of the downstream effectors of Id-1 in prostate cancer (Ling et al., 2004) and bladder cancer (Ding et al, 2006). Moreover, a recent study reported that increased expression of Id-1 led to suppression of expression of PTEN and p53 (Lee et al., 2009). These tumor suppressor genes have been reported to inhibit expression of EGFR (Bouali et al., 2009). If Id-1 was able to suppress both expressions of PTEN and p53, this implies that Id-1 may be able to overexpress EGFR.

In breast cancer, overexpression of EGFR is associated with ER negative breast cancer (Davidson et al., 1987; Klijn et al., 1992; Rajkumar & Gullick, 1994; Biswas et al., 2000; Mclnerney et al., 2001; Hicks & Tubb, 2005; Nieto et al., 2007). Biswas et al. (2000) suggested that EGF is a major growth-stimulating factor for ER negative breast cancer cells based on the inverse correlation found between ER and EGFR. Previous findings of our research group using the same cell lines as in the present study has shown that ectopic levels of Id-1 could down-regulate the expression of ER and reduce the growth responsiveness to estrogen stimulation (unpublished results). So, coupled with the results of this study where we found that Id-1 provided a growth advantage to breast cancer cells in the absence of estrogen, and was able to modulate the expression of EGFR, we may suggest that Id-1 can provide stimulation to other proliferation factors and enable the cells to bypass the stimulation of estrogen through increased expression of EGFR. These findings provide additional evidence to support the hypothesis that Id-1 may be an upstream regulator of EGFR and overexpression of EGFR is able to provide growth ability to the cells in the absence of estrogen.

5.3 NF-κB pathway may be involved in Id-1 induced cell proliferation

NF-κB pathway has been linked to drug resistance and hormone-independent cancer (Nakshatri et al., 1997; Chen & Sawyers, 2002), and is one of the signaling pathways of EGFR. Activation of both EGFR and NF-κB were found in ER negative breast cancer (Biswas et al., 2000; Biswas et al., 2001; Biswas and Iglehart, 2006). The activity of NF-κB was found to be inhibited by ER (Nakshatri et al., 1997; Biswas & Iglehart, 2006). In the present study, estrogen deprived medium was used to minimize the exogenous activation of ER in the investigation of the involvement of the NF-κB pathway in the Id-1 induced cell proliferation. An inhibitor of NF-κB, parthenolide which can suppress the activation of NF-κB by specific blocking of IKK (Zhou et al., 2005) was applied and resulted in growth inhibition of more than 50% and decreased S phases in all 4 cell lines (Figures 4.8 & 4.9). These findings suggest that without the stimulation of estrogen, cell proliferation may rely on the activation of the NF-κB pathway. Results also showed that the percentage inhibition of MCF-7-Id-1 transfectants were positively associated with their expression level of Id-1, implying that Id-1 may be able to activate the NF-κB pathway. Since a negative correlation has been found between ER and the activities of NF-κB (Nakshatri et al., 1997; Biswas & Iglehart, 2006), this revelation that the NF-κB

pathway is involved in the Id-1 induced cell proliferation agrees with the previous finding of our research group that Id-1 was able to suppress the protein expression of ER.

The transcription factor NF-κB consists of five members: p65, RelB, c-Rel, p50 and p52 (NF-κB2), where NF-κB p65 and p50 are the most common subunit activated in human epithelial cells (Chen et al., 2001), and it was these subunits which were examined in this study. Western blotting results showed that the expressions of NF-κB p65 and p50 were similar between all cell lines using whole cell protein (Figure 4.11). It was not surprising that no observable difference was found between different MCF-7-Id-1 transfectants and the controls, because NF-κB is abundant in the cytoplasm. The inactivated form of NF-κB binds with a corresponding inhibitor in the cytoplasm during the resting state, upon activation by a corresponding kinase the inhibitor is unmasked, and the activated NF-κB will then be translocated into the nucleus. Thus, NF-κB subunits involved in the Id-1 induced cell proliferation was assessed using nuclear fragment protein extract. The nuclear protein results showed that expressions of NF-κB p50 were higher in MCF-7-Id-1 transfectants than that of the MCF-7 controls, although no observable difference was found between the

expressions of NF-κB p65 in all of the cell lines (Figure 4.12). Since NF-κB p50 is an essential subunit responsible for dimerization (Liou, 2006), results in this study suggest that the increased level of NF-κB p50 could exist as NF-κB p65/p50 heterodimer or p50/p50 homodimer. Since NF-κB p65/p50 is the most common dimer in the NF-κB pathway, it was the first to be investigated.

In the resting state, NF-κB p65/p50 heterodimer was found to bind with IκBα in the cytoplasm. Once IκBα is phosphorylated by the IκB kinase complex, IKK, it becomes separated from NF-κB p65/p50 heterodimer and will be degraded, and so the activation of NF-κB p65/p50 heterodimer is negatively associated with the expression of IκBα. In this study, the expression of IκBα was found to be lower in MDA-MB231 than in other cell lines, but no difference was found between MCF-7-Id-1 transfectants and the MCF-7 controls (Figure 4.13). Findings implied that the NF-κB p65/p50 heterodimer was activated more in MDA-MB231.

Since IκBα was found to be similar between MCF-7-Id-1 transfectant and the MCF-7 control. It suggests that the NF-κB dimer involved in the Id-1 induced cell proliferation was not NF-κB p65/p50, but rather, the dimer NF-κB p50/p50,

and so, the corresponding coactivator of NF-κB p50/p50, Bcl-3 was examined. Bcl-3 is a noninhibitory family protein of IκB, and a coactivator of homodimer NF-κB p50/p50 or NF-κB p52/p52 subunits (Courtois & Gilmore, 2006). Results showed that expression of Bcl-3 was higher in the MCF-7-Id-1 transfectants than that of the MCF-7 controls (Figures 4.14 & 4.15). The expression of NF-κB p52 in the nuclear extract has also been examined, however, the expression of NF-κB p52 was very low and no association with the level of Id-1 in the MCF-7-Id-1 transfectants was found (Figure 4.18). Since Bcl-3 can be coexpressed with either NF-κB p50/p50 or NF-κB p52/p52 (Chen et al., 2001; Zhou et al., 2005; Liou, 2006), results of NF-κB p52 excluded the possibility of Bcl-3 being coactivated with NF-κB p52/p52. These findings suggested that the NF-κB dimer involved in the Id-1 induced cell proliferation is likely to be NF-κB p50/p50 homodimer in association with Bcl-3. In the TF ELISA results, activity of the MCF-7-Id-1 transfectants were significantly higher than that of the pBabe control (Figure 4.19), which implies that NF-κB p50 is more readily available to DNA in the MCF-7-Id-1 transfectants than in the controls and that NF-κB p50/p50 homodimer activation was positively correlated with their Id-1 expression level.

Taken together, overall results of Western blotting on NF-κB subunits (Figures 4.11, 4.12, 4.13, 4.14, 4.15, 4.16, 4.17 & 4.18) and EMSA (Figure 4.19) suggested that the involvement of NF-κB p65/p50 in MDA-MB231, a well developed hormone-independent breast cancer cell line which over-expresses Id-1, but less likely to be involved in our MCF-7-Id-1 transfectants. This may be due to the possibility that our MCF-7-Id-1 transfectants are only at the stage of becoming less hormone-dependent under the effect of ectopic Id-1. Conversely, the involvement of the NF-κB p50/p50 subunit was better demonstrated in the MCF-7-Id-1 transfectants, but not so clearly in the MDA-MB231 cell line. Regardless of whether it was NF-κB p50/p50 or NF-κB p65/p50 subunit involvement, it was the NF-κB p50 protein that has increased expression with ectopic Id-1 expression.

Studies have found that Id-1 was able to activate the NF-κB p65/p50 subunit in prostate cancer (Ling et al., 2003), esophagus cancer (Li et al., 2007) and breast cancer (Kim et al., 2008). Studies using cytokines such as TNF-α (Ling et al., 2003) or taxol (Kim et al., 2008) to induce apoptosis showed that Id-1 has an anti-apoptotic effect through the activation of NF-κB p65/p50 subunit (Ling et al., 2003; Li et al., 2007; Kim et al., 2008). Other studies using MCF-7 cells

suggested that the anti-apoptotic property was independent of the activity of NF-κB p50/p50 homodimer when TNF-α was used to stimulate apoptosis (Zhang et al., 2007c). Since we studied the proliferation effect of Id-1 on NF-κB in the absence of estrogen, whereas other studies were demonstrating the anti-apoptotic role of Id-1 on the NF-κB pathway, this may explain why our results were different from others.

Elevated levels of NF-κB p50/p50 homodimer have been found to be associated with the carcinogenesis of skin (Bndunova et al., 1999), nasopharyngeal (Thornburg et al., 2003) and breast (Hu & Kong, 2003). Constitutive expression of NF-κB p50 was found to be related to tamoxifen resistance (Zhou et al., 2005), one of the progression stages from hormone-dependent to hormone-independent. Our study also suggested that Id-1 induced NF-κB p50 activity may contribute to progression of breast cancer from hormone-dependent to hormone-independent. Although NF-κB p50/p50 themselves lack the active transcriptional domain (Chen et al., 2001), it becomes functionally active when NF-κB p50 binds to Bcl-3 (Zhou et al., 2005; Liou, 2006).

In this study, Bcl-3 was found to be positively correlated with their NF-κB p50 expression level in the nuclear fragment protein extract (Figure 4.14). Moreover, antisense oligonucleotide transfection was able to suppress the expression of Bcl-3 (Figure 4.17), implicating that the expression of Bcl-3 was affected by ectopic Id-1. Bcl-3 is a non-inhibiting member of the IκB family and functions as an oncogene to increase cellular proliferation and survival (Westerheide et al., 2001; Kashatus et al., 2006). Studies have found that Bcl-3 was able to increase cell proliferation through the induction of cyclin D1, and also shortened the G1 phase of the cell cycle (Westerhedie et al., 2001; Cao & Karin, 2003). Taken together, Id-1 induced cell proliferation may act through NF-κB p50/p50 homodimer and Bcl-3, but not the NF-κB p65/50 subunit.

5.4 Further study

Findings of this study support the involvement of Id-1 in the progression of breast cancer from hormone-dependent to hormone-independent, however further study is needed. In order to clarify the role of Id-1 in the progression of breast cancer from hormone-dependent to hormone-independent, tamoxifen resistance is suggested to be investigated in the MCF-7-Id-1 transfectants. If MCF-7-Id-1 transfectants become less hormone-dependent, the resistance to

tamoxifen will be increased. In addition, the suppression of Id-1 in MDA-MB231 hormone-independent breast cancer cell line should be investigated. Since we found that the expression of Id-1 was higher in MDA-MB231 than in MCF-7-Id-1 transfectants, if a higher level of Id-1 can enable the cells to progress towards hormone-independency, suppressing the expression of Id-1 in MDA-MB231 may make these cells less hormone-independent.

5.5 Conclusion

To conclude, in the current study, it was found that Id-1 can promote cell proliferation in the absence of estrogen, possibly through the activation of EGFR, NF-κB p50/p50 and Bcl-3. Together with the previous findings of our research team (unpublished results), we propose that Id-1 induces cell proliferation in MCF-7 cells via suppression of ER expression as well as overexpression of EGFR, and then with the activation of EGFR on the NF-κB p50/p50 with the coactivator of Bcl-3. Figure 5.1 represents the suggested pathway of how Id-1 effects cell proliferation.

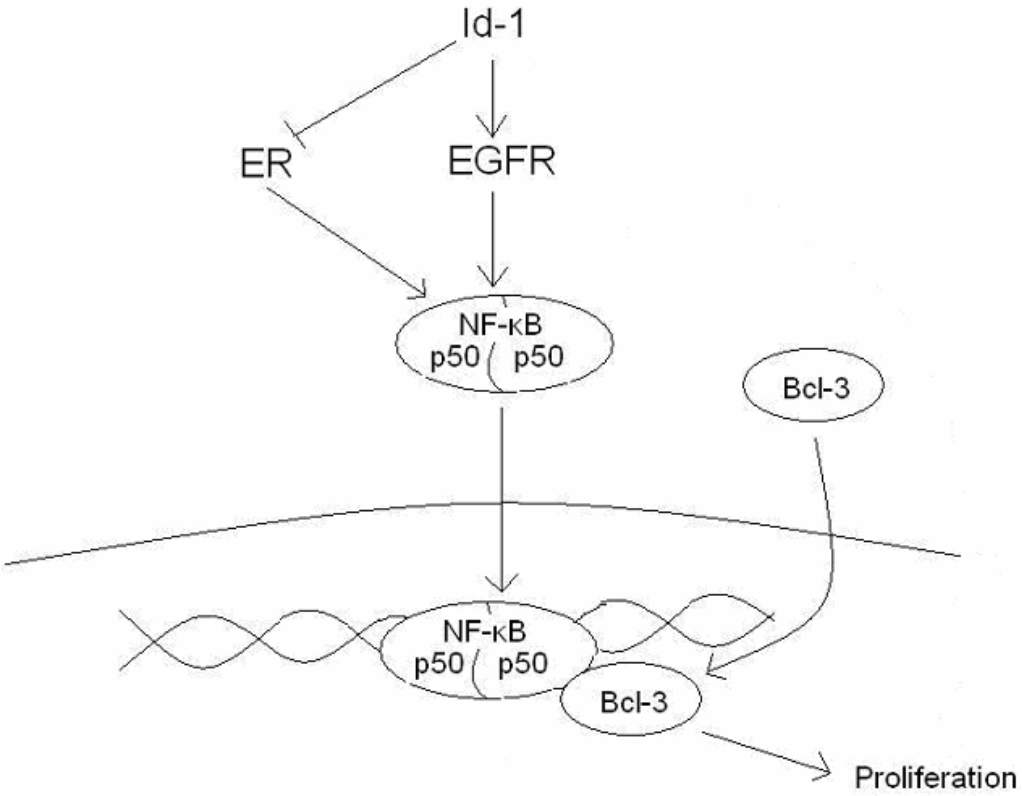


Figure 5.1 The possible pathways involved in Id-1 induced cell proliferation

References:

American Cancer Society. (2006) Breast Cancer Facts & Figures 2007-2008.

Baldwin AS Jr, Azizkhan JC, Jensen DE, Beg AA, Coodly LR. (1991) Induction of NF-kappa B DNA-binding activity during the G0-to-G1 transition in mouse fibroblasts. Molecular and cellular biology. 11(10):4943-51.

Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. (1990) The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell. 61(1):49-59.

Bertwistle D and Ashworth A. (1999) The pathology of familial breast cancer: How do the functions of BRCA1 and BRCA2 relate to breast tumour pathology? Breast cancer research. 1(1):41-7.

Bhat-Nakshatri P, Sweeney CJ, Nakshatri H. (2002) Identification of signal transduction pathways involved in constitutive NF-kappaB activation in breast cancer cells. Oncogene. 21(13):2066-78.

Biswas DK and Iglehart JD. (2006) Linkage between EGFR family receptors and nuclear factor kappaB (NF-kappaB) signaling in breast cancer. Journal of cellular physiology. 209(3):645-52.

Biswas DK, Cruz AP, Gansberger E, Pardee AB. (2000) Epidermal growth factor-induced nuclear factor kappa B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. Proceedings of the national academy of zciences of the United States of America. 97(15):8542-7.

Biswas DK, Dai SC, Cruz A, Weiser B, Graner E, Pardee AB. (2001) The nuclear factor kappa B (NF-kappa B): a potential therapeutic target for estrogen receptor negative breast cancers. Proceedings of the national academy of sciences of the United States of America. 98(18):10386-91.

Biswas DK, Iglehart JD. (2006) Linkage between EGFR family receptors and nuclear factor kappaB (NF-kappaB) signaling in breast cancer. Journal of cellular physiology. 209(3):645-52.

Biswas DK, Reddy PV, Pickard M, Makkad B, Pettit N, Pardee AB. (1998) Calmodulin is essential for estrogen receptor interaction with its motif and activation of responsive promoter. Journal of biological chemistry. 273(50):33817-24.

Blagosklonny MV, Pardee AB. (2002) The restriction point of the cell cycle. Cell cycle. 1(2):103-10.

Bouali S, Chrétien AS, Ramacci C, Rouyer M, Marchal S, Galenne T, Juin P, Becuwe P, Merlin JL. (2009) P53 and PTEN expression contribute to the inhibition of EGFR downstream signaling pathway by cetuximab. Cancer gene therapy. 16(6):498-507.

Bowcock AM (1999) Breast cancer: molecular genetics, pathogenesis, and therapeutics. Human press, Totowa, N.J.

Brantley DM, Chen CL, Muraoka RS, Bushdid PB, Bradberry JL, Kittrell F, Medina D, Matrisian LM, Kerr LD, Yull FE. (2001) Nuclear factor-kappaB (NF-kappaB) regulates proliferation and branching in mouse mammary epithelium. Molecular biology of the Cell. 12(5):1445-55.

Breastcancer.org (2009) Stages of Breast Cancer
http://www.breastcancer.org/symptoms/diagnosis/staging.jsp

Budunova IV, Perez P, Vaden VR, Spiegelman VS, Slaga TJ, Jorcano JL. (1999) Increased expression of p50-NF-kappaB and constitutive activation of NF-kappaB transcription factors during mouse skin carcinogenesis. Oncogene. 18(52):7423-31.

Buse P, Woo PL, Alexander DB, Cha HH, Reza A, Sirota ND, Firestone GL. (1995) Transforming growth factor-alpha abrogates glucocorticoid-stimulated tight junction formation and growth suppression in rat mammary epithelial tumor cells. Journal of biological chemistry. 270(12):6505-14.

Cao Y and Karin M. (2003) NF-kappaB in mammary gland development and breast cancer. Journal of mammary gland biologicl neoplasia. 8(2):215-23.

Carmichael AR, Bates T. (2004) Obesity and breast cancer: a review of the literature. Breast. 13(2):85-92.

Cavalieri E, Frenkel K, Liehr JG, Rogan E, Roy D. (2000) Estrogens as endogenous genotoxic agents- DNA adducts and mutations. Journal of the national cancer institute. monographs. 27: 75-93.

Chakravarti D, Mailander PC, Li KM, Higginbotham S, Zhang HL, Gross ML, Meza JL, Cavalieri EL, Rogan EG. (2001) Evidence that a burst of DNA depurination in SENCAR mouse skin induces error-prone repair and forms mutations in the H-ras gene. Oncogene. 2001 Nov 29;20(55):7945-53.

Chapiro E, Radford-Weiss I, Bastard C, Luquet I, Lefebvre C, Callet-Bauchu E, Leroux D, Talmant P, Mozziconacci MJ, Mugneret F, Struski S, Raynaud S, Andrieux J, Barin C, Jotterand M, Mossafa H, Ramond S, Terré C, Lippert E, Berger F, Felman P, Merle-Béral H, Bernard OA, Davi F, Berger R, Nguyen-Khac F. (2008) The most frequent t(14;19)(q32;q13)-positive B-cell malignancy corresponds to an aggressive subgroup of atypical chronic lymphocytic leukemia. Leukemia. 22(11):2123-7.

Chen CD and Sawyers CL. (2002) NF-kappa B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. Molecular and cellular biology. 22(8):2862-70.

Chen F, Castranova V, Shi X. (2001) New insights into the role of nuclear factor-kappaB in cell growth regulation. The American journal of pathology. 159(2):387-97.

Cheung HW, Ling MT, Tsao SW, Wong YC, Wang X. (2004) Id-1-induced Raf/MEK pathway activation is essential for its protective role against taxol-induced apoptosis in nasopharyngeal carcinoma cells. Carcinogenesis. 25(6):881-7.

Ciardiello F and Tortora G. (2001) A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clinical cancer research. 7(10):2958-70.

Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin AS Jr. (2000) Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. Oncogene. 19(9):1123-31.

Connelly L, Robinson-Benion C, Chont M, Saint-Jean L, Li H, Polosukhin VV, Blackwell TS, Yull FE. (2007) A transgenic model reveals important roles for the NF-kappa B alternative pathway (p100/p52) in mammary development and links to tumorigenesis. Journal of biology chemistry. 282(13):10028-35.

Corzo C, Corominas JM, Tusquets I, Salido M, Bellet M, Fabregat X, Serrano S, Sole F. (2006) The MYC oncogene in breast cancer progression: from benign epithelium to invasive carcinoma. Cancer genetics and cytogenetics. 165(2): 151-156.

Costa C, Soares R, Reis-Filho JS, Leitão D, Amendoeira I, Schmitt FC. (2002) Cyclo-oxygenase 2 expression is associated with angiogenesis and lymph node metastasis in human breast cancer. Journal of clinical pathology. 55(6):429-34.

Courtois G and Gilmore TD. (2006) Mutations in the NF-kappaB signaling pathway: implications for human disease. Oncogene. 25(51):6831-43.

Crook T, Brooks LA, Crossland S, Osin P, Barker KT, Waller J, Philp E, Smith PD, Yulug I, Peto J, Parker G, Allday MJ, Crompton MR, Gusterson BA. (1998) p53 mutation with frequent novel condons but not a mutator phenotype in BRCA1- and BRCA2-associated breast tumours. Oncogene. 17(13):1681-9.

Crook T, Crossland S, Crompton MR, Osin P, Gusterson BA. (1997) p53 mutations in BRCA1-associated familial breast cancer. Lancet. 350(9078):638-9.

Cully M, You H, Levine AJ, Mak TW. (2006) Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nature reviews. cancer. 6(3):184-92.

Darby S, Cross SS, Brown NJ, Hamdy FC, Robson CN. (2008) BMP-6 over-expression in prostate cancer is associated with increased Id-1 protein and a more invasive phenotype. The journal of pathology. 214(3):394-404.

Davidson NE, Gelmann EP, Lippman ME, Dickson RB.(1987) Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Molecular endocrinology. 1:216-223.

Davis PL, Miron A, Andersen LM, Iglehart JD, Marks JR. (1999) Isolation and initial characterization of the BRCA2 promoter. Oncogene. 18(44):6000-12.

De Laurenzi V and Melino G. (2000) Apoptosis: The little devil of death. Nature. 406: 135-136.

Dejardin E, Bonizzi G, Bellahcène A, Castronovo V, Merville MP, Bours V. (1995) Highly-expressed p100/p52 (NFKB2) sequesters other NF-kappa B-related proteins in the cytoplasm of human breast cancer cells. Oncogene. 11(9):1835-41.

Dejardin E, Deregowski V, Chapelier M, Jacobs N, Gielen J, Merville MP, Bours V. (1999) Regulation of NF-kappaB activity by I kappaB-related proteins in adenocarcinoma cells. Oncogene. 18(16):2567-77.

Desprez PY, Sumida T, Coppé JP. (2003) Helix-loop-helix proteins in mammary gland development and breast cancer. Journal of mammary gland biology and neoplasia. 8(2):225-39.

Di K, Ling MT, Tsao SW, Wong YC, Wang X. (2006) Id-1 modulates senescence and TGF-beta1 sensitivity in prostate epithelial cells. Biology of the cell. 98(9):523-33.

Ding Y, Wang G, Ling MT, Wong YC, Li X, Na Y, Zhang X, Chua CW, Wang X, Xin D. (2006) Significance of Id-1 up-regulation and its association with EGFR in bladder cancer cell invasion. International journal of oncology. 28(4):847-54.

Dorai H and Sampath TK. (2001) Bone morphogenetic protein-7 modulates genes that maintain the vascular smooth muscle cell phenotype in culture. The Journal of bone and joint surgery. American volume. 83-A Suppl 1(Pt 1):S70-8.

Fan S, Wang J, Yuan R, Ma Y, Meng Q, Erdos MR, Pestell RG, Yuan F, Auborn KJ, Goldberg ID, Rosen EM. (1999) BRCA1 inhibition of estrogen receptor

signaling in transfected cells. Science. 284(5418):1354-6.

Fong S, Itahana Y, Sumida T, Singh J, Coppe JP, Liu Y, Richards PC, Bennington JL, Lee NM, Debs RJ, Desprez PY. (2003) Id-1 as a molecular target in therapy for breast cancer cell invasion and metastasis. Proceedings of the national academy of sciences of the United States of America. 100(23):13543-8.

Gao K, Dai DL, Martinka M, Li G. (2006) Prognostic significance of nuclear factor-kappaB p105/p50 in human melanoma and its role in cell migration. Cancer research. 66(17):8382-8.

Gill JK, Press MF, Patel AV, Bernstein L. (2006) Oral contraceptive use and risk of breast carcinoma in situ (United States). Cancer Causes Control. 17(9):1155-62.

Greenblatt MS, Chappuis PO, Bond JP, Hamel N, Foulkes WD. (2001) TP53 mutations in breast cancer associated with BRCA1 or BRCA2 germ-line mutations: distinctive spectrum and structural distribution. Cancer Research. 61(10):4092-7.

Haffner MC, Berlato C, Doppler W. (2006) Exploiting our knowledge of NF-kappaB signaling for the treatment of mammary cancer. Journal of mammary gland biology and neoplasia. 11(1):63-73.

Half E, Tang XM, Gwyn K, Sahin A, Wathen K, Sinicrope FA. (2002) Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma in situ. Cancer Research. 62(6):1676-81.

Han DF, Zhou X, Hu MB, Xie W, Mao ZF, Chen DE, Liu F, Zheng F. (2005) Polymorphisms of estrogen-metabolizing genes and breast cancer risk: a multigenic study. Chinese medical journal. 118: 1507-1516.

Hara E, Smith R, Parry D, Tahara H, Stone S, Peters G. (1996) Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. Molecular and cellular biology. 16: 859-867.

Head JE and Johnston SR. (2003) Protein farnesyltransferase inhibitors. Expert opinion on emerging drugs. 8(1):163-78.

Henderson BE, Feigelson HS (2000) Hormonal carcinogenesis. Carcinogenesis. 21: 427-433.

Hicks DG and Tubbs RR. (2005) Assessment of the HER2 status in breast cancer by fluorescence in situ hybridization: a technical review with interpretive guidelines. Human pathology. 36(3):250-61.

Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Strauss M. (1999) NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. Molecular and cellular biology. 19(4):2690-8.

Hong Kong Cancer Registry Hospital Authority. (2007) Fast stats for female breast cancer.

Hong Kong Hospital Authority, (2007) HA clinical Practice Guideline: Management of breast cancer.

Horseman ND. (1999) Prolactin and mammary gland development. Journal of mammary gland biology and neoplasia. 4(1):79-88.

Hu Q and Kong X (2003) The expression of NF-kappa B family protein and its relationship with drug resistance in breast cancer cell lines. The Chiese-German journal of clinical oncology. 2(4): 216-218.

Huh JI, Calvo A, Charles R, Green JE. (2006) Distinct tumor stage-specific inhibitory effects of 2-methoxyestradiol in a breast cancer mouse model associated with Id-1 expression. Cancer research. 66(7):3495-503.

Hui CM, Cheung PY, Ling MT, Tsao SW, Wang X, Wong YC, Cheung AL. (2006) Id-1 promotes proliferation of p53-deficient esophageal cancer cells. International journal of cancer. 19(3):508-14.

IMGENEX (2008) NF-κB Family Pathway.

Itahana Y, Piens M, Fong S, Singh J, Sumida T, Desprez PY. (2008) Expression of Id and ITF-2 genes in the mammary gland during pregnancy. Biochemical and biophysical research communications. 372(4):826-30.

Jang KS, Han HX, Paik SS, Brown PH, Kong G. (2006) Id-1 overexpression in invasive ductal carcinoma cells is significantly associated with intratumoral microvessel density in ER-negative/node-positive breast cancer. Cancer letter. 244(2):203-10.

Johnston SR, Saccani-Jotti G, Smith IE, Salter J, Newby J, Coppen M, Ebbs SR, Dowsett M. (1995) Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. Cancer research. 55(15): 3331-8.

Joyce D, Albanese C, Steer J, Fu M, Bouzahzah B, Pestell RG.(2001) NF-kappaB and cell-cycle regulation: the cyclin connection. Cytokine and growth factor review. 12(1):73-90.

Kaldis P. (2006) Cell cycle regulation. Springer. Berlin. New York.

Karnik PS, Kulkarni S, Liu XP, Budd GT, Bukowski RM. (1994) Estrogen receptor mutations in tamoxifen-resistant breast cancer. Cancer research. 54:349–53.

Kashatus D, Cogswell P, Baldwin AS. (2006) Expression of the Bcl-3 proto-oncogene suppresses p53 activation. Genes and development. 20(2):225-35.

Kim H, Chung H, Kim HJ, Lee JY, Oh MY, Kim Y, Kong G. (2008) Id-1 regulates Bcl-2 and Bax expression through p53 and NF-kappaB in MCF-7 breast cancer cells. Breast cancer research treatment. 112(2):287-96.

Kitazaki T, Oka M, Nakamura Y, Tsurutani J, Doi S, Yasunaga M, Takemura M, Yabuuchi H, Soda H, Kohno S. (2005) Gefitinib, an EGFR tyrosine kinase inhibitor, directly inhibits the function of P-glycoprotein in multidrug resistant cancer cells. Lung Cancer. 49(3):337-43.

Kiyota A, Shintani S, Mihara M, Nakahara Y, Ueyama Y, Matsumura T, Todd R,

Wong DT. (2000) Expression of a truncated epidermal growth factor receptor in oral squamous cell carcinomas. Cancer letter. 161(1):9-15.

Klijn JG, Berns PM, Schmitz PI, Foekens JA. (1992) The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endocrine review. 13(1):3-17.

Konstantinopoulos PA, Vandoros GP, Karamouzis MV, Gkermpesi M, Sotiropoulou-Bonikou G, Papavassiliou AG. (2007) EGF-R is expressed and AP-1 and NF-kappaB are activated in stromal myofibroblasts surrounding colon adenocarcinomas paralleling expression of COX-2 and VEGF. Cellular oncology. 29(6):477-82.

Korc M. (1998) Role of growth factors in pancreatic cancer. Surgical oncology clinics of North America. 7(1):25-41.

Kountouras J, Boura P, Lygidakis NJ. (2001) Liver regeneration after hepatectomy. Hepatogastroenterology. 48(38):556-62.

Kreider BL, Benezra R, Rovera G, Kadesch T. (1992) Inhibition of myeloid differentiation by the helix-loop-helix protein Id. Science. 255(5052):1700-2.

Kumar V and Chambon P. (1988) The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell. 55(1):145-56.

Kuske B, Naughton C, Moore K, Macleod KG, Miller WR, Clarke R, Langdon SP, Cameron DA. (2006) Endocrine therapy resistance can be associated with high estrogen receptor alpha (ERalpha) expression and reduced ERalpha phosphorylation in breast cancer models. Endocrine-related cancer. 13(4):1121-33.

Lee JY, Kang MB, Jang SH, Qian T, Kim HJ, Kim CH, Kim Y, Kong G. (2009) Id-1 activates Akt-mediated Wnt signaling and p27(Kip1) phosphorylation through PTEN inhibition. Oncogene. 28(6):824-31.

Lee TK, Man K, Ling MT, Wang XH, Wong YC, Lo CM, Poon RT, Ng IO, Fan

ST. (2003) Over-expression of Id-1 induces cell proliferation in hepatocellular carcinoma through inactivation of p16INK4a/RB pathway. Carcinogenesis 24:1729–36.

Lee TK, Poon RT, Yuen AP, Ling MT, Wang XH, Wong YC, Guan XY, Man K, Tang ZY, Fan ST. (2006) Regulation of angiogenesis by Id-1 through hypoxia-inducible factor-1alpha-mediated vascular endothelial growth factor up-regulation in hepatocellular carcinoma. Clinical cancer research. 12(23):6910-9.

Lee UJ, Choung SR, Prakash KV, Lee EJ, Lee MY, Kim YJ, Han CW, Choi YC. (2007) Dual knockdown of p65 and p50 subunits of NF-kappaB by siRNA inhibits the induction of inflammatory cytokines and significantly enhance apoptosis in human primary synoviocytes treated with tumor necrosis factor-alpha. Molecular biology reports. 35(3):291-8.

Leicht DT, Balan V, Kaplun A, Singh-Gupta V, Kaplun L, Dobson M, Tzivion G. (2007) Raf kinases: function, regulation and role in human cancer. Biochimica et biophysica acta. 1773(8):1196-212.

Leung G, Tsao SW, Wong YC. (2002) The effect of flutamide and tamoxifen on sex hormone-induced mammary carcinogenesis and pituitary adenoma. Breast cancer research and treatment. 72(2):153-62.

Leung G, Tsao SW, Wong YC. (2003) Sex hormone-induced mammary carcinogenesis in female Noble rats: detection of differentially expressed genes. Breast cancer research and treatment. 77(1):49-63.

Lev-Schelouch D, Sperber F, Gat A, Klausner J, Gutman M. (2003) Paget's disease of the breast. Harefuah. 142(6):433-7, 485.

Li B, Cheung PY, Wang X, Tsao SW, Ling MT, Wong YC, Cheung AL. (2007) Id-1 activation of PI3K/Akt/NF{kappa}B signaling pathway and its significance in promoting survival of esophageal cancer cells. Carcinogenesis. 28(11):2313-20.

Li X, Zhang Z, Xin D, Chua CW, Wong YC, Leung SC, Na Y, Wang X. (2007) Prognostic significance of Id-1 and its association with EGFR in renal cell cancer. Histopathology. 50(4):484-90.

Li Y and Brown PH. (2009) Prevention of ER-negative breast cancer. Recent Results Cancer research.181:121-34.

Liehr JG. (2000) Is estradiol a genotoxic mutagenic carcinogen? Endocrine review. 21: 40-54.

Lin CQ, Parrinello S, Campisi J, Desprez PY. (1999) Lin CQ, Parrinello S, Campisi J, Desprez PY. Endocrine-related cancer. 6(1):49-50.

Lin CQ, Singh J, Murata K, Itahana Y, Parrinello S, Liang SH, Gillett CE, Campisi J, Desprez PY. (2000) A role for Id-1 in the aggressive phenotype a nd steroid hormone response of human breast cancer cells. Cancer research. 60(5):1332-40.

Lin JC, Chang SY, Hsieh DS, Lee CF, Yu DS. (2005) Modulation of mitogen-activated protein kinase cascades by differentiation-1 protein: acquired drug resistance of hormone independent prostate cancer cells. The journal of urology. 174(5):2022-6.

Ling MT, Wang X, Lee DT, Tam PC, Tsao SW, Wong YC. (2004) Id-1 expression induces androgen-independent prostate cancer cell growth through activation of epidermal growth factor receptor (EGF-R). Carcinogenesis. 25(4):517-25.

Ling MT, Wang X, Ouyang XS, Lee TK, Fan TY, Xu K, Tsao SW, Wong YC. (2002) Activation of MAPK signaling pathway is essential for Id-1 induced serum independent prostate cancer cell growth. Oncogene. 21(55):8498-505.

Ling MT, Wang X, Ouyang XS, Xu K, Tsao SW, Wong YC. (2003) Id-1 expression promotes cell survival through activation of NF-kappaB signalling pathway in prostate cancer cells. Oncogene. 22(29):4498-508.

Ling MT, Wang X, Zhang X, Wong YC. (2006) The multiple roles of Id-1 in cancer progression. Differentiation. 74(9-10):481-7.

Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, Perou CM. (2006) Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Modern pathology. 19(2):264-71.

Lo HW and Hung MC. (2006) Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. British journal of cancer. 94(2):184-8.

Lobanova YS, Scherbakov AM, Shatskaya VA, Krasil'nikov MA (2009) NF-jB suppression provokes the sensitization of hormone-resistant breast cancer cells to estrogen apoptosis. Molecular and cellular biochemistry. 324:65–71.

Macedo LF, Sabnis G, Brodie A. (2009) Aromatase inhibitors and breast cancer. Annals of the New York Academy of Sciences. 1155:162-73.

Matsumoto A, Deyama Y, Deyama A, Okitsu M, Yoshimura Y, Suzuki K. (1998) Epidermal growth factor receptor-mediated expression of NF-kappaB transcription factor in osteoblastic MC3T3-E1 cells cultured under a low-calcium environment. Life science. 62(17-18):1623-7.

McCarty KS Jr, Miller LS, Cox EB, Konrath J, McCarty KS Sr. (1985) Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. Archives of pathology & laboratory medicine. 109(8):716-21.

McInerney JM, Wilson MA, Strand KJ, Chrysogelos SA. (2001) A strong intronic enhancer element of the EGFR gene is preferentially active in high EGFR expressing breast cancer cells. Journal of cellular biochemistry. 80(4):538-49.

McKeithan TW, Takimoto GS, Ohno H, Bjorling VS, Morgan R, Hecht BK, Dubé I, Sandberg AA, Rowley JD. (1997) BCL3 rearrangements and t(14;19) in chronic lymphocytic leukemia and other B-cell malignancies: a molecular and cytogenetic study. Genes Chromosomes Cancer. 20(1):64-72.

Michaux L, Dierlamm J, Wlodarska I, Bours V, Van den Berghe H, Hagemeijer

A. (1997) t(14;19)/BCL3 rearrangements in lymphoproliferative disorders: a review of 23 cases. Cancer genetics cytogenetics. 94(1):36-43.

Morcel K, Rouquette S, Dugast C, Bendavid C, Audrain O, Levêque J. (2008) Breast cancer prevention: from chemoprevention to prophylactic surgery. Journal de gynécologie, obstétrique et biologie de la reproduction. 37(7):661-71.

Motulsky H. (1995) Intuitive Biostatistics. Oxford University Press, Oxford.

Moudgil VK (1994) Steroid hormone receptors: Basic and clinical aspects. Birkhäuser. Boston.

Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ Jr, Sledge GW Jr. (1997) Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Molecular and cellular biology. 17(7):3629-39.

Namba H, Saenko V, Yamashita S. (2007) Nuclear factor-kB in thyroid carcinogenesis and progression: a novel therapeutic target for advanced thyroid cancer. Arquivos brasileiros de endocrinologia e metabologia. 51(5):843-51.

Nieto Y, Nawaz F, Jones RB, Shpall EJ, Nawaz S. (2007) Prognostic significance of overexpression and phosphorylation of epidermal growth factor receptor (EGFR) and the presence of truncated EGFRvIII in locoregionally advanced breast cancer. Journal of clinical oncology. 25(28):4405-13.

Nishikori M, Maesako Y, Ueda C, Kurata M, Uchiyama T, Ohno H. (2003) High-level expression of BCL3 differentiates t(2;5)(p23;q35)-positive anaplastic large cell lymphoma from Hodgkin disease. Blood. 101(7):2789-96.

Obata H, Biro S, Arima N, Kaieda H, Kihara T, Eto H, Miyata M, Tanaka H. (1996) NF-kappa B is induced in the nuclei of cultured rat aortic smooth muscle cells by stimulation of various growth factors. Biochemical and biophysical research communications. 224(1):27-32.

Ohtani N, Zebedee Z, Huot TJ, Stinson JA, Sugimoto M, Ohashi Y, Sharrocks AD, Peters G and Hara E (2001) Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. Nature, 409: 1067-1070.

Ouyang XS, Wang X, Lee DT, Tsao SW, Wong YC. (2001) Up-regulation of TRPM-2, MMP-7 and ID-1 during sex hormone-induced prostate carcinogenesis in the Noble rat. Carcinogenesis. 22(6):965-73.

Ouyang XS, Wang X, Ling MT, Wong HL, Tsao SW, Wong YC (2002) Id-1 stimulates serum independent prostate cancer cell proliferation through inactivation of p16(INK4a)/pRB pathway. Carcinogenesis 23:721–5.

Parrinello S, Lin CQ, Murata K, Itahana Y, Singh J, Krtolica A, Campisi J, Desprez PY. (2001) Id-1, ITF-2, and Id-2 comprise a network of helix-loop-helix proteins that regulate mammary epithelial cell proliferation, differentiation, and apoptosis. Journal of biological chemistry. 276(42):39213-9.

Paule B, Terry S, Kheuang L, Soyeux P, Vacherot F, de la Taille A. (2007) The NF-kappaB/IL-6 pathway in metastatic androgen-independent prostate cancer: new therapeutic approaches? World journal of urology. 25(5):477-89.

Perry JE, Grossmann ME, Tindall DJ. (1998) Epidermal growth factor induces cyclin D1 in a human prostate cancer cell line. Prostate. 35(2):117-24.

Phillips LS, Millikan RC, Schroeder JC, Barnholtz-Sloan JS, Levine BJ. (2009) Reproductive and Hormonal Risk Factors for Ductal Carcinoma In situ of the Breast. Cancer epidemiology, biomarkers & prevention. 18(5):1507-14.

Planas-Silva MD and Weinberg RA. (1997) The restriction point and control of cell proliferation. Current opinion in cell biology. 9(6):768-72.

Poch B, Gansauge F, Schwarz A, Seufferlein T, Schnelldorfer T, Ramadani M, Beger HG, Gansauge S. (2001) Epidermal growth factor induces cyclin D1 in human pancreatic carcinoma: evidence for a cyclin D1-dependent cell cycle progression. Pancreas. 23(3):280-7.

Poirier-Solomon L. (2002) HRT. Findings from the Women's Health Initiative. Diabetes Forecast. 55(12):34-6.

Potten C and Wilson J (2004) Apoptosis: the life and death of cells. Cambridge University Press, Cambridge, U.K.

Preston- Martin S, Pike MC, Ross RK, Jones PA, Henderson BE (1990) Increased cell division as a cause of humen cancer. Cancer research. 50: 7415-7421.

Rajkumar T and Gullick WJ. (1994) The type I growth factor receptors in human breast cancer. Breast cancer research and treatment. 29(1):3-9.

Ristimäki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J. (2002) Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer research. 62(3):632-5.

Rocha S, Martin AM, Meek DW, Perkins ND. (2003) p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. Molecular and cellular biology. 23(13):4713-27.

Russo J, Russo IH. (2006) The role of estrogen in the initiation of breast cancer. The Journal of steroid biochemistry and molecular biology. 102(1-5):89-96.

Sarvilinna N, Eronen H, Miettinen S, Vienonen A, Ylikomi T. (2006) Steroid hormone receptors and coregulators in endocrine-resistant and estrogen-independent breast cancer cells. International journal of cancer. 118(4):832-40.

Sasano H, Suzuki T, Nakata T, Moriya T. (2006) New development in intracrinology of breast carcinoma. Breast cancer. 13(2):129-36.

Schlette E, Rassidakis GZ, Canoz O, Medeiros LJ. (2005) Expression of bcl-3 in chronic lymphocytic leukemia correlates with trisomy 12 and abnormalities of chromosome 19. American journal of clinical pathology. 123(3):465-71.

Sedivy JM (1998) Can ends justify the means? : telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells. Proceedings of the national academy of sciences of the United States of America. 95: 9078-9081.

Sherr CJ (1994) G1 phase progression; cycling on cue. Cell 79: 551-555.

Stein GS and Pardee AB (2004) Cell cycle and growth control: biomolecular regulation and cancer 2nd edition, cell cycle regulatory cascades. Hoboken. John Wiley & Sons, Inc. Canada. 3:95-128.

Steiner E, Klubert D, Knutson D. (2008) Assessing breast cancer risk in women. American family physician. 78(12):1361-6.

Stewart ZA and Pietenpol JA (2001) Syk: a new player in the field of breast cancer. Breast cancer research. 3: 5-7.

Stoll BA. (2000) Adiposity as a risk determinant for postmenopausal breast cancer. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 24(5):527-33.

Sun L and Carpenter G. (1998) Epidermal growth factor activation of NF-kappaB is mediated through IkappaBalpha degradation and intracellular free calcium. Oncogene. 16(16):2095-102.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature. 397: 441-446.

Thornburg NJ, Pathmanathan R, Raab-Traub N. (2003) Activation of nuclear factor-kappaB p50 homodimer/Bcl-3 complexes in nasopharyngeal carcinoma. Cancer research. 63(23):8293-301.

Tsai SY, Tsai MJ, O'Malley BW. (1989) Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements. Cell. 57(3):443-8.

van den Brandt PA, Spiegelman D, Yaun SS, Adami HO, Beeson L, Folsom AR, Fraser G, Goldbohm RA, Graham S, Kushi L, Marshall JR, Miller AB, Rohan T, Smith-Warner SA, Speizer FE, Willett WC, Wolk A, Hunter DJ. (2000) Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk. American journal of epidemiology. 152(6):514-27.

Vander SL (2001) Human Physiology 8theidition, McGraw Hill, New York.

Venkitaraman AR. (2001) Functions of BRCA1 and BRCA2 in the biological response to DNA damage. Journal of cell science. 114(Pt 20):3591-8.

Veronesi U. (1990) Breast cancer, Springer-Verlag, European.

Wang JY and Casero RA. (2006) Polyamine Cell Signaling: Physiology, Pharmacology, and Cancer Research. Human Press, Totowa, New Jersey.

Wang X, Xu K, Ling MT, Wong YC, Feng HC, Nicholls J, Tsao SW. (2002) Evidence of increased Id-1 expression and its role in cell proliferation in nasopharyngeal carcinoma cells. Molecular carcinogenesis. 35(1):42-9.

Weber W, Gill GN, Spiess J. (1984) Production of an epidermal growth factor receptor-related protein. Science. 224(4646):294-7.

Wells A. (1999) EGF receptor. The international journal of biochemistry & cell biology. 31(6): 637-643.

Wesierska-Gadek J, Schreiner T, Maurer M, Waringer A, Ranftler C. (2007) Phenol red in the culture medium strongly affects the susceptibility of human MCF-7 cells to roscovitine. Cellular & molecular biology letters. 12(2):280-93.

Westerheide SD, Mayo MW, Anest V, Hanson JL, Baldwin AS Jr.(2001) The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G(1) transition. Molecular and cellular biology. 21(24):8428-36.

Winchester DJ, Winchester DP, Hudis CA, Norton L. (2006) Breast cancer 2nd Edition. B.C. Decker, Inc. Hamilton.

Wong V, Ching D, McCrea PD, Firestone GL. (1999) Glucocorticoid down-regulation of fascin protein expression is required for the steroid-induced formation of tight junctions and cell-cell interactions in rat mammary epithelial tumor cells. Journal of biological chemistry. 274(9):5443-53.

Wong YC, Wang X, Ling MT. (2004) Id-1 expression and cell survival. Apoptosis. 9(3):279-89.

Woo PL, Cercek A, Desprez PY, Firestone GL. (2000) Involvement of the helix-loop-helix protein Id-1 in the glucocorticoid regulation of tight junctions in mammary epithelial cells. Journal of biology chemistry. 275(37):28649-58.

Wu JT and Kral JG. (2005) The NF-kappaB/IkappaB signaling system: a molecular target in breast cancer therapy. The journal of surgical research. 123(1):158-69.

Wu K, Jiang SW, Thangaraju M, Wu G, Couch FJ. (2000) Induction of the BRCA2 promoter by nuclear factor-kappa B. Journal of biological chemistry. 275(45):35548-56.

Yokota Y. (2001) Id and development. Oncogene. 20(58):8290-8.

Yu Y and Feig LA. (2002) Involvement of R-Ras and Ral GTPases in estrogen-independent proliferation of breast cancer cells. Oncogene. 21(49):7557-68.

Yue W, Santen RJ, Wang JP, Li Y, Verderame MF, Bocchinfuso WP, Korach KS, Devanesan P, Todorovic R, Rogan EG, Cavalieri EL. (2003) Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. The journal of steroid biochemistry and molecular biology. 86(3-5):477-86.

Yuen HF, Chua CW, Chan YP, Wong YC, Wang X, Chan KW. (2006) Id proteins expression in prostate cancer: high-level expression of Id-4 in primary prostate cancer is associated with development of metastases. Modern pathology. 19(7):931-41.

Zhang SM, Lee IM, Manson JE, Cook NR, Willett WC, Buring JE. (2007a) Alcohol consumption and breast cancer risk in the Women's Health Study. American journal of epidemiology. 165(6):667-76.

Zhang X, Ling MT, Feng H, Wong YC, Tsao SW, Wang X. (2004) Id-I stimulates cell proliferation through activation of EGFR in ovarian cancer cells. British journal of cancer. 91(12):2042-7.

Zhang X, Ling MT, Wang Q, Lau CK, Leung SC, Lee TK, Cheung AL, Wong YC, Wang X. (2007b) Identification of a novel inhibitor of differentiation-1 (ID-1) binding partner, caveolin-1, and its role in epithelial-mesenchymal transition and resistance to apoptosis in prostate cancer cells. Journal of biological chemistry. 282(46):33284-94.

Zhang X, Ling MT, Wong YC, Wang X. (2007c) Evidence of a novel antiapoptotic factor: Role of inhibitor of differentiation or DNA binding (Id-1) in anticancer drug-induced apoptosis. Cancer Science. 98 (3), 308¡V314.

Zhang X, Moréra S, Bates PA, Whitehead PC, Coffer AI, Hainbucher K, Nash RA, Sternberg MJ, Lindahl T, Freemont PS. (1998) Structure of an XRCC1 BRCT domain: a new protein-protein interaction module. The EMBO journal. 17(21):6404-11.

Zhao W, Iskandar S, Kooshki M, Sharpe JG, Payne V, Robbins ME. (2007) Knocking out peroxisome proliferator-activated receptor (PPAR) alpha inhibits radiation-induced apoptosis in the mouse kidney through activation of NF-kappaB and increased expression of IAPs. Radiation research. 167(5):581-91.

Zhou Y, Eppenberger-Castori S, Eppenberger U, Benz CC. (2005) The NFkappaB pathway and endocrine-resistant breast cancer. Endocrine-related cancer. 12(1): S37-46.

Zhou Y, Yau C, Gray JW, Chew K, Dairkee SH, Moore DH, Eppenberger U, Eppenberger-Castori S, Benz CC. (2007) Enhanced NF kappa B and AP-1 transcriptional activity associated with antiestrogen resistant breast cancer. BMC Cancer. 7:59.

Zidan A, Christie Brown JS, Peston D, Shousha S. (1997) Oestrogen and progesterone receptor assessment in core biopsy specimens of breast carcinoma. Journal of clinical pathology. 50(1):27-9.