Exploring the tumor suppressive roles of Estrogen Receptor β in lung and breast cancer

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A Dissertation Presented to

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Foti Nikollo

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Dedicated to my parents, Vasilios and Eleftheria.

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Abstract

Estrogens represent a subclass of steroid hormones that by regulating cell growth and differentiation, influence normal physiology as well as pathology. The effects of estrogen are mediated by two members of the nuclear receptor superfamily, Estrogen Receptor α (ERα) and ERβ. A plethora of studies have shown that ERα and ERβ exert opposite effects on cancer development and progression by eliciting distinct transcriptional responses and differentially influencing cellular processes such as cell proliferation, apoptosis, and migration. The present study focused on the potential role of ERβ in affecting development and progression of the two most commonly diagnosed cancers in men and women, lung and breast cancer, respectively. Our studies revealed that upregulation of wild-type ERβ (ERβ1), but not the splice variant ERβ2, reduces proliferation and enhances apoptosis in non-small cell lung cancer (NSCLC) cells. ERβ1 was found to induce apoptosis by stimulating the intrinsic apoptotic pathway that involved upregulation of the pro-apoptotic factor BIM and downregulation of components of the growth factor signaling pathway. Manipulation of EGFR and RAS expression and activity in ERβ1-expressing cells revealed the central role of oncogenic RAS signaling in ERβ1-mediated pro-apoptotic phenotype and EGFR regulation. In addition, our studies demonstrated that ERβ1 sensitizes NSCLC cells to chemotherapeutic agents. Upregulation of ERβ1 decreased the viability of doxorubicin- and etoposide-treated NSCLC cells by inducing

G_2/M phase cell cycle arrest. In response to treatment, ERβ1-expressing cells had increased p-Chk1 levels, an indicator of activated DNA damage response, compared with the control cells. Finally, we showed that ERβ1 represses epithelial to mesenchymal transition (EMT) and invasion of basal-like breast cancer cells both *in vitro* and *in vivo*. ERβ1 impeded EMT by downregulating EGFR. EGFR downregulation in ERβ1-expressing cells was associated with the stabilization of the ubiquitin ligase c-Cbl-EGFR complexes that led to increased ubiquitylation and degradation of the activated receptor. In conclusion, our studies have unveiled the important role of ERβ in regulating crucial processes of lung and breast cancer development and progression and propose ERβ as a potential biomarker for predicting metastasis in breast cancer and response to treatment in NSCLC.

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List of Abbreviations

AF-1	Activation Function 1
AF-2	Activation Function 2
ALK	Anaplastic Lymphoma Kinase
BCL-2	B-Cell Lymphoma 2
BIM	BCL-2 Interacting Mediator of cell death
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
c-Cbl	Cellular-Casitas B-lineage Lymphoma
CDK4	Cyclin Dependent Kinase 4
c-MYC	Cellular-Myelocytomatosis
CYP19	Cytochrome P450 family 19
DsRed	Discosoma sp. Red fluorescent protein
DTT	Ditheiothreitol
DUTT1	Deleted in U-Twenty Twenty 1
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol Tetraacetic Acid
EML4	Echinoderm Microtubule associated protein like 4
ERBB	Erythroblasric leukemia viral oncogene
ERK	Extracellular signal-regulated Kinase
FBS	Fetal Bovine Serum
FHIT	Fragile Histidine Triad
FLK1	Fetal Liver Kinase 1
FUS-1	Fused in Sarcoma 1
GRB2	Growth factor Receptor-Bound protein 2
HER-2	Human Epidermal growth factor Receptor 2
IGF1R	Insulin-like Growth Factor 1 Receptor
LKB1	Liver Kinase B1
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen/Extracellular signal-regulated Kinase
NFκB	Nuclear Factor kappa B
PARP	Poly (ADP-ribose) polymerase
PIK3CA	Phosphatidylinositol-4,5-biphosphate 3-kinase
PMSF	Phenylmethanesulfonylfluoride
PTEN	Phosphatase and Tensin homolog
RAF-1	v-Raf-1 murine leukemia viral oncogene homolog 1
RAS	Rat Sarcoma

RPMI-1640	Roswell Park Memorial Institute-1640
RASFF1A	Ras association domain-containing protein 1
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
siRNA	Small interfering RNA
STK11	Serine/Threonine Kinase 11
TBST	Tris Buffer Saline - Tween-20
TGFα	Tumor Growth Factor α
TGF-β	Tumor Growth Factor β
VEGF	Vascular Epithelial Growth Factor
WNT-1	Wingless-related integration site 1

Chapter 1

Introduction

1.1 Cancer

Cancer is a complex disease characterized by uncontrolled cell divisions capable of invading other tissues. Common causes of cancer include environmental factors namely: tobacco smoking, infectious agents, chemicals and radiations, and genetic factors including inherited or acquired mutations, hormones, and immune system pathologies. These factors act simultaneously or in sequence to promote development and progression of cancer (American Cancer Society, 2014).

Cancer is the second most common cause of death surpassed only by heart disease. American cancer society estimates that roughly 1.5 million people will be diagnosed with cancer in the U.S. in 2014 and half a million of cancer patients will die. A significant portion of these cancers is preventable. A major portion of the cancer-related deaths each year is attributed to tobacco smoking- or heavy drinking-related cancers that can easily be prevented, as well as to physical inactivity, poor nutrition, infectious agents, and sun exposure that can be prevented by behavioral change and use of vaccines and antibiotics (Siegel et al., 2014, American Cancer Society, 2014).

Screenings have proven to be of great value as they can detect cancers at early stages when the disease is manageable, preventing further progression. Advances in early detection and better treatment options have increased the 5-year survival rate for all cancers diagnosed from 49% (1975-1977) to 68% (2003-2009). Unfortunately, cancer treatments are often unaffordable for a major portion of the patients diagnosed with the disease. The National Institutes of Health estimated that the costs for cancer treatment in U.S. for 2009 reached $216.6 billion. In addition to the high cost of treatments, lack of health insurance make it difficult for a significant number of Americans to receive optimal cancer treatment (American Cancer Society, 2014, Siegel et al., 2014).

The most commonly diagnosed cancers in men and women are lung and breast cancer, respectively. Lung cancer is the major cause of cancer-related deaths for both males and females. More than 200,000 new cases of lung cancer are expected in the U.S. in 2014, accounting for 13% of diagnosed cancers (Siegel et al., 2014). On the other hand, more than 200,000 women will be diagnosed with invasive breast cancer in the U.S. in 2014 (American Cancer Society, 2014, Siegel et al., 2014); however breast cancer incidence in women has dropped 7% since 2002, mainly attributed to the reduction in use of hormonal replacement therapy (HTR) (Hermsmeyer et al., 2011). The high incidence of lung and breast cancer urges for a better understanding of the environmental factors that are responsible for the onset as well as the genetic alterations that drive the development and progression of these deadly diseases.

1.2 Lung Cancer

Lung cancer is a deadly disease that affects millions of people worldwide. The five-year overall survival rate for patients diagnosed with lung cancer is 15%. Among them, only 1%-2% diagnosed with advanced stage lung cancer survive that long (Yuan et al., 2014). The primary reason for the high mortality rate is attributed to the fact that most of the lung cancer cases are diagnosed at an advanced stage when the tumors are highly undifferentiated with limited treatment options; thus, the development of detection methods that can detect lung cancer at early stages could benefit patients to survive longer (Wistuba and Gazdar, 2006). Nevertheless, early detection of lung cancer from the histological and biological point of view is a very challenging task, mainly due to its complex nature and multiple preneoplastic pathways involved (Minna et al., 2002).

1.2.1 Histology of lung cancer

Histologically, the two major forms of lung cancer are non-small cell lung cancer (NSCLC) that comprises approximately 80% of the lung cancer cases, and small cell lung cancer (SCLC) that accounts for approximately 20% of all lung cancer cases. NSCLC is further divided into three major subtypes: adenocarcinoma, squamous-cell carcinoma, and large-cell carcinoma, with the first two constituting the majority of diagnosed lung cancer cases (Herbst et al., 2008). Lung cancers may arise centrally from the major bronchi or peripherally from the small bronchi, bronchioles, or alveoli of the distant airway of the lung. Commonly, squamous-cell carcinomas and SCLCs arise centrally, whereas

adenocarcinomas and large-cell carcinomas are found in the periphery of the lung (Wistuba and Gazdar, 2006).

As with other epithelial cancers, lung cancer is thought to develop through progressive pathological changes, the preneoplastic or premalignant lesions (Colby et al., 1998, Wistuba and Gazdar, 2006). Squamous-cell carcinoma is believed to arise from mucosal changes in the large airways that lead to squamous dysplasia and carcinoma in situ (CIS) (Colby et al., 1998). However, there is little evidence on the development and progression of adenocarcinomas, large-cell carcinomas, and SCLC (Colby et al., 1998, Kerr, 2001). The limited available data suggest that adenocarcinomas arise from preneoplastic lesions, including atypical adenomatous hyperplasia (AAH) in the peripheral airway cells (Kerr, 2001). In the case of SCLC, the evidence is even less. There are no preneoplastic lesions associated with SCLC; recent data suggest that it may arise from multifocal lesions indicative of extensive genetic mutations due to tobacco-related carcinogens (Wistuba et al., 2002). However, these preneoplastic lesions are thought to be responsible for only a subset of lung cancers.

1.2.2 Origin of Lung Cancer

Conclusive evidence that indicates the specific respiratory epithelial cell type from which each lung cancer subtype develops has not been established. Historically, many different cell types that constitute the lung have been implicated with the origin and evolution of lung cancer (Hanna and Onaitis, 2013). Of them, tissue stem cells seem to be the most

attractive candidates for the cell of origin of lung tumors. Tissue stem cells have a long life span that allows them to accumulate genetic mutations able to drive tumorigenesis (Smalley and Ashworth, 2003). Indeed, lineage tracing experiments indicated that type II alveolar cells were able to develop into adenocarcinomas in response to constitutively active K-Ras^{G12D} mutant (Xu et al., 2012). Type II alveolar cells have self-renewal capacity and give rise to type I cells (Adamson and Bowden, 1974, Evans et al., 1975). However, these studies need validation in human subjects.

Differentiated and committed progenitor cells have also been proposed to be the cell(s) of origin of lung cancer, yet their involvement is not clear, and commonly depends on the acquisition of a specific genetic mutation or a combination that might confer self-renewal capacities and quick turnover rates (Smalley and Ashworth, 2003).

1.2.3 Genetic susceptibility and molecular pathology of lung cancer

Development of lung cancer is attributed to both environmental and genetic factors. Tobacco-smoking is the major risk factor for the development of lung cancer with risk increasing both with the duration and quantity of smoking. Other major risk factors include radon gas and asbestos inhalation, mainly through occupational exposure.

Genetic susceptibility significantly contributes to the development of lung cancer ,particularly for those who develop lung cancer at an early stage (American Cancer Society, 2014). Germ-line mutations in p53, Rb, EGFR, and other genes that cause inherited cancer syndromes are associated with increased lung

cancer risk (Herbst et al., 2008). Increased lung cancer risk is also associated with compromised DNA repair capacity due to germ-line mutations in the genes that contain the DNA damage response machinery (Herbst et al., 2008).

Additionally, clinical studies have shown that lung tumors exhibit multiple genetic and epigenetic autosomal changes. The majority of them are found in known tumor suppressor genes and oncogenes (Zochbauer-Muller and Minna, 2000). Notably, these genetic aberrations occur both in the malignant and the histologically normal lung epithelium, consistent with the notion of diffused tissue injury (Spira et al., 2007). Some of the major oncogenes involved in lung tumorigenesis include *CMYC*, mutated *RAS*, *CyclinD1*, *BCL-2*, and mutations in the members of the *ErbB* family including *EGFR* and *ErbB2* (Lynch et al., 2004, Paez et al., 2004, Stephens et al., 2004). *ErbB* family mutations are predominantly found in adenocarcinomas, East Asia patients, and non- or light-smokers.

Early events in the development of NSCLC include loss of heterozygosity on the short arm of chromosome 3 where several putative tumor suppressor genes reside (e.g. *DUTT1*, *FHIT*, *RASFF1A*, and *FUS-1*). Loss of heterozygosity at chromosomal regions 9p21 (*p16*) and 17p13 (*p53*) also occurs at the early stages of lung cancer (Minna et al., 2002).

Studies have shown that different gene alteration patterns occur between SCLC and NSCLC as well as between adenocarcinomas and squamous-cell carcinomas, adding to the wide heterogeneity observed in lung carcinomas (Shivapurkar et al., 1999, Virmani

et al., 1998). Allelic loss analysis at different chromosomal regions and methylation status of tumor suppressor genes from patients with SCLC and NSCLC has shown a different pattern of inactivation during the development of these malignancies (Bhattacharjee et al., 2001, Toyooka et al., 2003, Wistuba et al., 1999). For instance, methylation of *p16* occurs early in the development of squamous-cell carcinoma and is associated with recurrence after resection (Brock et al., 2008), whereas it is very rare in adenocarcinomas and then only in high-grade AAH (Licchesi et al., 2008). On the other hand, *RAS* and *ErbB* family mutations are mutually exclusive and predominantly found in adenocarcinoma subtypes, indicating two different molecular pathways in the development of lung adenocarcinomas (Gazdar et al., 2004). Notably, tobacco smoking favors *RAS* mutations whereas unknown mutagens favor ERBB gene family mutations in non-smokers (Shigematsu et al., 2005a, Shigematsu et al., 2005b). These mutations confer a growth advantage to cancer cells and render them addicted to Ras and EGFR signaling for their survival and growth (Gazdar et al., 2004).

1.2.4 Treatment of lung cancer

Treatment options for lung cancer depend on the stage at which the disease is diagnosed. The most common treatment for early stage lung cancer is surgery frequently followed by adjuvant therapy and radiotherapy. Locally advanced or metastatic lung cancers are primarily treated by neoadjuvant chemotherapy consisting of platinum-based doublets (Group, 2008).

The identification of *EGFR* gene amplification and mutations in patients with advanced NSCLC and the fact that these alterations confer sensitivity to tyrosine kinase inhibitors (TKIs) has revolutionized the field of lung cancer treatment. Independent studies have identified somatic mutations of *EGFR* in patients with lung adenocarcinoma (10% of the patients in the United States and 30%-50% of patients in Asia) that predict sensitivity to TKIs such as gefitinib and erlotinib (Sequist et al., 2007, Sequist and Lynch, 2008). Epidemiological studies have established three factors that are independently and collectively associated with response of *EGFR* alterations to TKIs: whether the patient is non-smoker, female, or Asian (Shepherd et al., 2005). The majority (80%) of *EGFR* mutations occur as in-frame deletions within exon 19 or the L858R mutant within exon 21. Amplification of *EGFR* is also associated with sensitivity to TKIs (Hirsch et al., 2008). Treatment of patients with advanced or metastatic NSCLC harboring sensitive EGFR mutations with TKIs alone or in combination with cytotoxic agents has been shown to significantly improve survival (Mok et al., 2009, Zhou et al., 2011).

After the success of EGFR TKIs, there has been a tremendous effort to identify new targets amenable to therapeutic interventions. These efforts resulted in several drugs that target key survival pathways of lung cancer cells. A great example of bench-to-bedside success is the identification of anaplastic lymphoma kinase - echinoderm microtubule-associated protein like 4 (ALK-EML4) translocation as a cancer driver in a significant subset of advanced-stage lung cancers. This discovery led to the development of ALK inhibitors currently under clinical development (Steuer and Ramalingam, 2014).

Other key targets of various agents currently under clinical assessment for the treatment of locally advanced or metastatic lung cancer include: MAPK, protein kinase B (AKT), RAF, c-MET, fibroblast growth factor receptor (FGFR), PIK3CA, PTEN, and rearranged during transfection (RET) (Zhang et al., 2014).

Advances in the identification of novel regulatory enzymes of RAS activity and function have revived the efforts for new K-RAS-targeted therapies, although it is considered traditionally as a difficult molecule to target (Riely et al., 2009, Vasan et al., 2014). Additionally, the use of RAS as a prognostic factor in NSCLC remains elusive. Several studies have identified mutant RAS as a negative prognostic factor associated with poor overall outcome, while other reports noted no prognostic value (Riely et al., 2009). On the other hand, RAS mutations have been shown to have predictive value as markers for therapy. Early stage NSCLC patients with RAS mutations are unlikely to benefit from treatment with chemotherapy agents. Importantly, EGFR inhibitors are rendered ineffective by RAS mutants that might result in decreased efficacy of chemotherapy drugs when combined (Riely et al., 2009).

1.2.5 Resistance to therapy

Unfortunately, despite the initial response of lung tumors to the available therapies (cytotoxic agents, radiotherapy, targeted therapies) they eventually become resistant and relapse. There are several pathways implicated in the development of resistance. Intracellularly, resistance may result from inactivation of the drug by detoxifying

enzymes, decrease in drug activation, or binding to the target and decreased drug accumulation due to increased efflux or decreased uptake. The most studied mechanism of drug resistance acquisition is activation of ATP-binding cassette transporters such us P-glycoprotein and multidrug resistance proteins. These transporters pump the chemotherapy drugs outside the tumor cells, lowering the intracellular concentration of the drug. Additionally, increased DNA damage repair, resistance to apoptosis either by upregulating antiapoptotic or downregulating pro-apoptotic factors, increased tolerance to DNA damage and altered cell cycling or expression of transcription factors are also pathways employed by the cancer cells to increase resistance to drugs (Almeida et al., 2008, Stewart et al., 2010, Zhang et al., 2014).

1.2.5.1 Molecular pathways of therapy resistance

Activation of various overlapping survival pathways is another way cells acquire drug resistance. Several of these pathways, including MAPK, protein kinase B (AKT), mammalian target of rapamycin (mTOR), NFκB, and notch signaling pathways, are implicated in resistance to chemotherapy agents (Donev et al., 2011). Furthermore, several miRNAs have recently been proposed to play a significant role in lung cancer drug resistance (Catuogno et al., 2013, Garofalo et al., 2012, Romano et al., 2012).

Recently, cancer stem-like cells (CSLC) or cancer stem cells (CSC), a small population of tumor cells that is inherently more resistant to therapeutic agents than the rest of the tumor cells, have recently been linked to drug resistance. The drug resistance

pathways employed by these cells involve: altered cell cycle kinetics, increased DNA repair potential, resistance to apoptosis, and increased expression of multi-drug resistance protein transporters (Morrison et al., 2011, Vermeulen et al., 2012). Failure to eliminate these cells is believed to be one of the major reasons for tumor recurrence after years from a successful first round of therapy. However, the extent of the CSLCs or CSCs involvement is not fully understood, since a high percentage of lung tumors presented in the clinic are intrinsically resistant to the conventional therapies due to one or more of the mechanisms discussed above.

1.2.5.2 Resistance to TKIs

Resistance to TKIs, either intrinsic or acquired, has also been documented. The most common form of acquired resistance is the EGFR T790M gatekeeper point mutation in exon 20 that reduces drug binding (Yu et al., 2013). Other mechanisms involve: *EGFR*, *c-MET*, and *ERBB2* amplification, *PIK3CA* mutations, and NFκB activation that result in suppressed TKI-induced apoptosis (Zhang et al., 2014). Finally, RAS mutations that activate downstream effectors of the growth factor signaling and epithelial to mesenchymal transition (EMT) have also been linked to acquired resistance to TKIs (Shien et al., 2013, Zhang et al., 2014).

1.3 Breast Cancer

1.3.1 Risk factors

Known risk factors for the development of breast cancer include: age, ethnicity, early puberty and late menopause, lack of or late childbearing, and absence of breastfeeding (American Cancer Society, 2014). The periods of puberty, pregnancy, and post-pregnancy are responsible for dramatic changes in the mammary gland structure accompanied with increased cell expansion and differentiation. In addition, exposure to estrogen and progesterone as well as lifestyle factors such as obesity, alcohol consumption and lack of exercise have been linked to breast tumorigenesis (American Cancer Society, 2014).

Hereditary forms of breast cancer are also present in the population and account for 5% of the breast cancer cases. Most often, they are caused by mutations in key tumor suppressor genes with high-penetrance including BRCA1, BRCA2, p53, PTEN, and STK11/LKB1. Nevertheless, the occurrence of the majority of breast cancers is attributed to low-penetrance gene mutations during mammary gland development and the carcinogenic input from the environment (Baselga and Norton, 2002). Notably, distinct breast cancer subtypes are associated with different risk factors (Baselga and Norton, 2002).

1.3.2 Breast cancer biology

Breast cancer is a heterogeneous disease with distinct clinical, histopathological, and molecular characteristics. Breast tumors with similar histological features often display different clinical outcomes (Sotiriou and Pusztai, 2009, Weigelt et al., 2010). To date, treatment decisions are based on clinicopathological criteria, such as age, tumor size, histological grade, lymphovascular invasion, and expression status of estrogen receptor (ER), progesterone receptor (PR) and HER-2 (ErbB2) (Wesolowski and Ramaswamy, 2011). Although this approach has improved survival of the average population, certain populations do not benefit from the chosen therapy. The cause is most likely the dysregulation of specific genes and signaling pathways. Studies of breast cancer biology at the molecular level have increased our knowledge of the intrinsic variations occurring in the different subtypes.

1.3.2.1 Breast cancer intrinsic molecular subtypes

Undoubtedly, gene expression profiling studies have contributed immensely in understanding the complexity of breast cancer. A series of seminal studies using gene expression profiling showed that breast cancer is divided into distinct molecular subtypes, often with the same histopathological features (Sorlie et al., 2001, Perou et al., 2000, Zhao et al., 2004). Analysis of a large cohort of breast tumors and hierarchical clustering identified at least five distinct molecular subtypes. These intrinsic subgroups are different in terms of biology, survival, response to therapy, and recurrence rate. The

intrinsic subgroups are primarily separated based on the ERα status to ERα-positive tumors (also called luminal tumors) and ERα-negative tumors. ERα-positive tumors have a gene profile resembling that of the luminal epithelial cells, which line the mammary ducts (Sorlie et al., 2003). Luminal tumors are further subdivided into Luminal A and B based on the expression of genes associated with proliferation and HER-2 expression. Among the two, Luminal A subtype is less proliferative and has better prognosis, while Luminal B subtype is more aggressive (Hu et al., 2006).

The ERα-negative tumors are divided into three molecular subtypes (HER-2-enriched, basal-like, and normal-like), and correlate with poorer prognosis compared to the luminal types. HER-2-positive tumors express high levels of the HER-2 amplicon but lack the expression of ER and PR. They are associated with increased proliferation and poor outcome (Hu et al., 2006, Perou et al., 2000, Sorlie et al., 2006). Basal-like tumors have a gene expression profile similar to the basal epithelial cells and express high levels of keratins 5 and 17 as well as laminin (Rakha et al., 2008b). They were initially speculated to arise from the myoepithelial progenitors. However, recent studies showed that their gene signature is closer to that of the luminal progenitor cells (Lim et al., 2009, Prat and Perou, 2009). Normal-like breast tumors express genes that are specifically expressed in the adipose tissue and their gene profile is closer to the basal-like rather than the luminal cells (Sorlie et al., 2001). A sixth subgroup, termed claudin-low, was added to include tumors with low expression of tight junction genes and endothelial markers. Claudin-low tumors are mostly triple-negative (Prat et al., 2010). The normal-like and claudin-low

subtypes are believed to arise from the mammary stem cells due to their gene signature similarities (Lim et al., 2009).

Since the identification of the intrinsic molecular subtypes, a plethora of gene signatures have emerged that associate with prognosis and response to treatment (Sotiriou and Pusztai, 2009). Additionally, single sample predictors were developed to assess the molecular subtype of single tumors (Parker et al., 2009). Despite the enormous information obtained by these studies their use in clinical setups has not been established. The main reason being the lack of robust and independently validated methods for the identification of the intrinsic subtypes (Colombo et al., 2011).

1.3.3 Treatment of breast cancer

1.3.3.1 Endocrine therapy

Breast cancer treatment decisions are based on the disease stage and pathologic features such as expression of ER, PR, HER-2 and lymph node involvement. Disease stage is determined by tumor size, number, location of the involved lymph nodes and the presence or absence of distant metastasis (Moulder and Hortobagyi, 2008). The majority of breast tumors (approximately 70%) are ER/PR-positive, which makes them excellent candidates for endocrine therapy with tamoxifen. Tamoxifen is a selective estrogen receptor modulator (SERM) developed in the 1970s that competitively inhibits estrogen-ER binding and is to-date the most successful targeted cancer therapy. Adjuvant therapy with tamoxifen has significantly contributed to the reduced breast cancer-related deaths

and disease recurrence rates observed annually (Musgrove and Sutherland, 2009). Due to the success of tamoxifen, more effective endocrine therapies have been developed that target estrogen synthesis or ER signaling including aromatase inhibitors or other SERMs and pure anti-estrogens, respectively (Musgrove and Sutherland, 2009).

1.3.3.2 HER2-targeted therapy

HER2-overexpression occurs in 15-30% of breast cancer patients. The first line of targeted therapy for HER-2-positive tumors is trastuzumab (Herceptin). Herceptin is a recombinant, humanized monoclonal antibody against the extracellular domain of HER-2. Herceptin functions by sequestering HER-2 homo- or heterodimerization (Moulder and Hortobagyi, 2008). Herceptin has proven effective as a single agent or in combination with chemotherapy for the treatment of metastatic breast cancer with HER-2 amplification. Other members of the *ErbB* family are also potential targets for the treatment of breast cancer. Inhibition of interaction of HER-2 with EGFR by using small-molecule tyrosine kinase inhibitors, has been shown to inhibit proliferation and induce apoptosis in breast cancer cell lines. In this setting, the use of lapatinib, a dual small-molecule tyrosine kinase inhibitor of EGFR and HER-2, as a single agent or in combination with Herceptin and/or chemotherapy, improved outcome and augmented chemotherapy response in metastatic breast cancer (Moulder and Hortobagyi, 2008).

1.3.3.3 Treatment of triple-negative breast cancer

Triple-negative breast cancers comprise 10-20% of the cases and are characterized by the lack of ER, PR and HER-2 expression. They have very aggressive behavior with visceral metastasis including the central nervous system and poor outcome (Di Cosimo and Baselga, 2010). The majority of the triple-negative breast cancers display basal-like molecular features and often having BRCA1 mutations. Unfortunately, there is no specific molecular target identified for triple-negative breast cancers. However, they respond very well to chemotherapeutic agents such as anthracyclins and taxane-based regimens.

Additionally, EGFR, PARP, and VEGF inhibitors have been used for the treatment of triple-negative breast cancer. EGFR is often overexpressed in triple-negative breast cancer, and combinatorial treatment with anti-EGFR compounds and cytotoxic agents have shown promising results in clinical trials. Furthermore, the use of PARP inhibitors in BRCA-mutated breast tumors have produced striking results in pre-clinical models and several PARP inhibitors are currently in clinical trials (Di Cosimo and Baselga, 2010).

1.3.4 Resistance to therapy

Despite the initial breast cancer response, resistance eventually develops. Several pathways have been implicated in breast cancer systemic or targeted therapy resistance. Some of them include activation of PI3K pathway, cross-talk between ER and receptor tyrosine kinases (RTKs), CyclinD1 and CDK4 overexpression, and aberrations in the FGFR pathway, as well as disruption of the p53 pathways (Ignatiadis and Sotiriou, 2013).

Currently, inhibitors for the pathways involved in endocrine resistance are under clinical investigation (Zardavas et al., 2013).

1.4 The *ErbB* Family of Receptor Tyrosine Kinases

ErbB family is part of the RTK superfamily of transmembrane proteins with cytoplasmic activity localized in the cell membrane. Their primary role is to mediate important growth factor signals from the extracellular environment into the cell. They play an important role in organ development and determination of several cell lineages.

ErbB gene family consists of four members: *ErbB1* (HER1/EGFR), *ErbB2* (HER-2/neu), *ErbB3* (HER-3), and *ErbB4* (HER-4). All members have similar molecular structures: They consist of a cysteine-rich ligand-binding extracellular domain, a single α-helix transmembrane domain, a cytoplasmic domain with tyrosine kinase activity, and a carboxy-terminal signaling domain (Yarden and Pines, 2012, Yarden and Sliwkowski, 2001). All *ErbB* proteins are kinase active, except from *ErbB3* that has a kinase-dead intracellular domain. ErbB proteins are ligand-activated, with the exception of ErbB2 that does not have any known ligand. The first ligand to be identified was epidermal growth factor (EGF) that binds to EGFR. Other known ligands include TGFα, amphiregulin, betacellulin, heparin-binding growth factor, epiregulin, and neuregulin (Yarden and Sliwkowski, 2001).

Upon ligand binding, ErbB receptors can homodimerize and/or heterodimerize with other family members that leads to activation of the tyrosine kinase domain. This process results

in autophosphorylation of the cytoplasmic domain and functions as a docking platform for adaptor proteins that couple the receptors to downstream signaling pathways (Yarden and Pines, 2012, Yarden and Sliwkowski, 2001). Intracellular signaling is primarily mediated by the RAS-MEK-ERK, PI3K-PTEN-AKT, and signal transducer and activator of transcription (STAT) pathways. ErbB signaling leads to increased proliferation, inhibition of apoptosis, angiogenesis, and metastasis (da Cunha Santos et al., 2011).

1.4.1 ErbB family in cancer

ErbB family members are often deregulated during cancer development and progression. EGFR, the most studied member, is a potent oncogene often overexpressed or mutated in many types of epithelial cancer, including breast and NSCLC (Yarden and Pines, 2012, Yarden and Sliwkowski, 2001). Retrospective studies have shown that EGFR is overexpressed in 62% of NSCLC and correlates with poor prognosis (Sharma et al., 2007). EGFR overexpression in breast cancer is an indicator for recurrence after surgical resection and correlates with decreased disease-free and overall survival in advanced breast cancer (Sharma et al., 2007, Yarden and Sliwkowski, 2001). Cognate ligands of EGFR including EGF and TGFα are also found overexpressed in cancer cells or histologically normal surrounding cells indicating autocrine or paracrine loops that can lead to receptor hyperactivation (Sharma et al., 2007). Improper activation of EGFR leads to increased proliferation, inhibition of apoptosis, and cellular transformation that contribute to tumor progression. In addition, gain-of-function mutations in the kinase domain of EGFR render

the receptor constitutively active. Cumulatively, these findings make EGFR a promising target for developing novel anticancer therapeutic agents.

1.5 Steroid Hormones and Receptors

Hormones are chemical messengers produced and released by cells or glands that alter the function of nearby (autocrine or paracrine) or distant cells (endocrine). Hormones are classified based on their structure to steroids, polypeptides, amino acids, and fatty acid-derived compounds. They are formed and stored in endocrine cells and released to the bloodstream via exocytosis upon external stimulation. Hormone actions are mediated by corresponding hormone receptors (Nussey and Whitehead, 2001).

Steroid hormones comprise a class of hormones, which are lipophilic derivatives of cholesterol. They are primarily synthesized in the ovaries (estrogen, progesterone), testes (testosterone), and adrenal cortex (aldosterone, mineralocorticoids, and glucocorticoids), while their effects are carried out by the steroid hormone receptors (Nussey and Whitehead, 2001). Steroid hormones are important for regulating physiological processes while they play a role in disease development including cancer.

1.5.1 Steroid hormone receptors

Steroid hormone receptors are a class of the nuclear receptor superfamily. Nuclear receptors (NRs) are transcription factors able to transduce the biological message of ligands directly to the transcriptional machinery. Their ligands include but are not limited

to steroids, retinoids, thyroid hormones, and lipophilic molecules. Upon ligand binding, NRs undergo specific conformational changes that leads to dimerization and translocation to the nucleus where they recognize and bind to cognate DNA sequences, namely the hormone receptor response elements, and orchestrate the transcription of specific target genes. NRs are pivotal for normal cellular functions including differentiation, cellular growth, and lineage specificity (Evans, 1988, Laudet, 1997).

NRs are a diverse class of transcription factors evolved from a common ancestor (Detera-Wadleigh and Fanning, 1994, Laudet, 1997). They are modular in structure and share common structural elements despite their diversity. NRs are divided into 5 structural domains: N-terminal domain (NTD) or A/B, DNA binding domain (DBD) or C, hinge region or D, ligand-binding domain (LBD) or E, and C-terminal domain (CTD) or F. Functionally, NR superfamily consists of three major domains: (a) amino-terminal transactivation domain, which contains AF-1 transactivation domain that exerts ligand-independent functions and is poorly conserved among the NR superfamily members, (b) central DNA-binding domain, which is the most conserved among NR family members and consists of two zinc-fingers responsible for DNA binding, dimerization, and co-activator recruitment and (c) carboxy-terminal ligand-binding domain that contains ligand-binding domain and AF-2 transactivation domain that is functional upon ligand binding. This functional domain determines the ligand and is the least conserved among the NR members. It also contains the nuclear localization signal as well as the dimerization

and co-regulator binding sites (Kumar et al., 1987, Schwabe et al., 1993, Green and Chambon, 1987, Evans, 1988, Laudet, 1997).

1.6 Role of Estrogens in Cancer

Estrogen biosynthesis occurs via aromatization of testosterone by the rate-limiting enzyme cytochrome P-450 encoded by *CYP19* (aromatase) gene in humans (Simpson et al., 2000). Estrogens play a major role in the development of female sexual characteristics during embryogenesis and maturation of sex organs during puberty. In premenopausal women, estrogen is mainly formed in the ovaries and in lesser amounts by the peripheral tissues. After menopause, when ovaries cease to act, estrogen is synthesized only in the peripheral sites, which include adipose tissue as well as the bone, the vascular endothelium, the aortic smooth muscle cells, and the brain (Simpson et al., 2000). Although it is considered a feminine hormone, a small amount of estrogen is found in circulation of males throughout their life and is thought to play an essential role in the development and maintenance of male fertility (Taylor et al., 2012). Indeed, male aromatase knock-out (ArKO) mice develop a progressive infertility that is evidenced by the disrupted morphology of the testes, arrest of spermatogenesis, and reduced sperm production (Robertson et al., 1999, Jones and Simpson, 2000).

The role of estrogen and estrogenic signaling in promoting cancer growth of hormone-responsive tissues such as the breast, the ovary, and the endometrium has been very well documented (Colditz, 1998, Ho, 2003, Russo and Russo, 2006, Shang, 2006). It is

well-established that sustained exposure to exogenous and endogenous estrogens causes breast cancer (Hankinson et al., 2004). In addition, estrogens seem to be involved in the pathogenesis of hormone-unresponsive tissues such as the lung. Recent clinical studies have found a correlation between circulating estrogens and lung cancer pathogenesis. For instance, high serum estrogen levels correlate with poor survival in male and female lung cancer patients (Olivo-Marston et al., 2010). Furthermore, postmenopausal women have enhanced survival compared to men and premenopausal women (Moore et al., 2003). In line with this observation, premenopausal women are diagnosed with more poorly differentiated and advanced stage lung cancer compared to postmenopausal women, while HRT might increase the risk of lung cancer development (Chlebowski et al., 2009). However, a direct link between estrogens and lung cancer incidence remains unconfirmed.

1.6.1 Estrogen receptors

Estrogen activities in target tissues are mediated through the estrogen receptors. Humans have two estrogen receptor genes (*ESR1* and *ESR2*), located on different chromosomes that encode ERα and ERβ, that belong to the nuclear receptor superfamily of transcription factors (Enmark et al., 1997, Gosden et al., 1986). ERα is a 66 kDa protein that was cloned in 1985 (Walter et al., 1985). ERβ was cloned later on in 1996 from rat prostate and encodes a 59.5 kDa protein (Kuiper et al., 1996). Since their discovery several ERα and ERβ variants have been identified (Herynk and Fuqua, 2004).

A significant number of ERα and ERβ isoforms has been detected in normal and cancer tissues that differ from the wild-type receptors in structure and function and arise from alternative splicing or promoter usage (Mangelsdorf et al., 1995). ERα variants have been detected in cancer cell lines and tissue samples from breast, endometrial, and ovarian cancers, while their expression correlated with the clinical outcome (Herynk and Fuqua, 2004, Thomas and Gustafsson, 2011). On the other hand, only five ERβ (ERβ1 – ERβ5) isoforms have been identified, most of which have been detected in cancer tissues. The ERβ isoforms are mainly the result of alternative splicing occurring in the last exon although alternative promoter usage has been reported to generate some of the variants. ERβ variants have been reported to modulate transcriptional activity of both ERα and wild-type ERβ and are associated with clinical outcome (Green et al., 2008, Thomas and Gustafsson, 2011).

The expression pattern of estrogen receptors in humans differs significantly. ERα is mainly expressed in the ovaries, breast, and uterus, whereas ERβ is more ubiquitous and is found in the brain, lung, prostate, breast, ovaries, and testes. Both ERs have similar structures and can be divided into 6 regions (A-F) and three functional domains (Figure 1.1). The highest similarity among the receptors is observed in the DBD domain (97%) while LBD and NTD domains are less conserved (59% and 20%, respectively) (Pettersson and Gustafsson, 2001). ERs and their variants show unique binding affinities to a plethora of agonists, antagonists, and SERMs. For instance, unlike 17β-estradiol (E2), which binds ERα and ERβ with similar affinity, phytoestrogens such as genistein and the androgen

metabolite 5α-adrostane-3β,17β-diol (3β-Adiol) have much higher affinity for ERβ (Cerillo et al., 1998, Montano et al., 1998, Paech et al., 1997, Webb et al., 2003, Weihua et al., 2002). Furthermore, ERs respond differently to various SERMs. In the presence of tamoxifen, ERα recruits co-repressors whereas ERβ does not (Smith et al., 1997, Webb et al., 2003). These studies demonstrate that ERs have unique functions depending on the ligand they interact with and the co-factors recruited (Bjornstrom and Sjoberg, 2005).

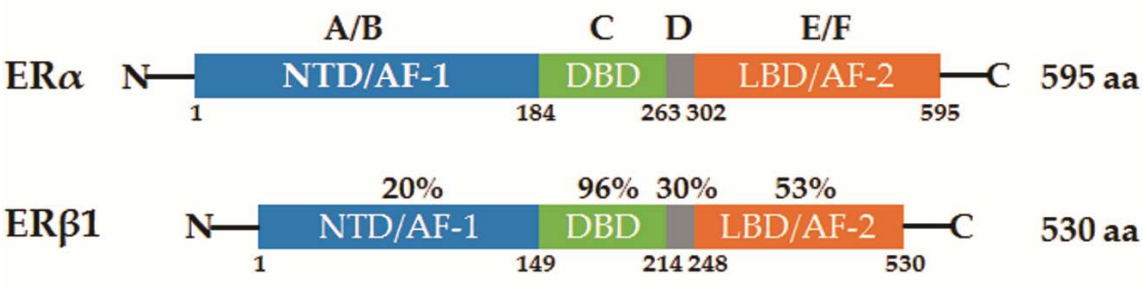


Figure 1.1 Structural and functional domains of the ERs. The strucural domains of ERs (A-F) and their relative amino acid positions are shown. The percentage amino acid homologies between ERα and ERβ1 are also shown. ERs are composed of 3 functional domains: The N-terminal domain (NTD) that contains the activation function 1 (AF-1), the DNA-binding domain (DBD), and the ligand-binding domain (LBD) that contains the activation function 2 (AF-2).

1.6.2 Mechanisms of estrogen receptor actions

The classical model for ER action involves the binding of ligand to the LBD of the receptor, which induces conformational changes of the protein unique to the ligands. The ligand-bound receptors dimerize and bind directly to DNA through their DBD at sequence-specific motifs known as estrogen response elements (EREs), or indirectly by tethering to DNA bound proteins such as activating protein 1 (AP1) and specificity protein 1 (SP1).

Upon binding to the DNA, ERs recruit co-activators or co-repressors of the p160 family of co-regulators such as the steroid receptor co-activator (SRC) 1 and 3, as well as the silencing mediator of retinoid and thyroid receptor (SMRT/NCOR2) that form multi-protein complexes based on the ligand-induced conformational changes. These complexes modulate the activity of the receptors that activates or represses transcription of target genes (Bjornstrom and Sjoberg, 2005, Heldring et al., 2007).

ERs can elicit transcriptional responses in the absence of ligand through crosstalk with other signaling pathways. For instance, active growth factor signaling through EGFR and IGFR can stimulate protein kinase cascades that phosphorylate and activate ERs in the absence of ligand (Britton et al., 2006). Furthermore, ample evidence suggests that membrane-bound and cytoplasmic ER mediate the rapid, non-genomic effects of estrogens. Non-genomic actions of ERs involve activation of MAPK, PI3K, endothelial nitrogen oxide synthase (eNOS), ErbB2, EGFR, IGFIR, caveolin 1, SRC, and G proteins shortly after treatment with estrogens that regulates transcription via activation of additional transcription factors (Bjornstrom and Sjoberg, 2005, Thomas and Gustafsson, 2011).

1.7 Estrogen Receptors in Cancer

Perturbation of estrogen signaling has been associated with cancer initiation, progression, and response to therapy (Hankinson et al., 2004). The unique transcriptional responses elicited by ERα and ERβ upon binding to estrogens and SERMs in breast, prostate, and

endometrial cancer, in combination with variations in ERα/ERβ expression ratio in these tissues, suggest different roles for the two receptors in cancer biology and therapy. Further support of this notion came from several studies in animal and cell models where ER signaling is regulated by specific ligands or expression of receptor subtype is specifically disrupted and have shown that ERα and ERβ have opposing roles on cell proliferation and apoptosis (Fox et al., 2008, Thomas and Gustafsson, 2011). In addition, clinical studies have indicated expression changes of wild-type ERα and ERβ as well as their splice variants in cancer tissues depending on tumor type and disease stage (Ellem and Risbridger, 2007, Musgrove and Sutherland, 2009, Speirs et al., 2008, Wong et al., 2005). This load of evidence provides support for an important role for ERs in cancer and suggests that targeting or restoring ER protein levels and activity in cancer tissues can improve outcome of patients with hormone-dependent cancers.

1.7.1 Role of ERs in breast cancer

The role of ERα in breast cancer biology has been widely studied. ERα is often found overexpressed in breast cancer and it is considered a cancer driver. Increased expression of ERα has been reported to stimulate cell proliferation by upregulating MYC and cyclin D1 (Dubik and Shiu, 1992, Planas-Silva et al., 2001). Furthermore, the expression of ERα correlates with better response to tamoxifen treatment making it the primary target of anti-estrogen therapy. On the other hand, loss of ERα delays the onset of WNT1- and ErbB2-induced mammary tumors.

In addition to wild-type ERα, ERα splice variants are also implicated in breast cancer development and response to therapy. ERα-36 associates with resistance to tamoxifen treatment and has been shown to mediate tamoxifen-induced cell proliferation by activating ERK signaling (Shi et al., 2009). ERβ has been shown to elicit opposite effects from ERα in breast cancer. For instance, ERβ inhibits the expression of ERα target genes that promote cell proliferation and suppresses tumor growth and angiogenesis in xenograft models when expressed in ERα-positive cells (Lindberg et al., 2003, Strom et al., 2004, Williams et al., 2008). Furthermore, ERβ expression in triple-negative breast cancer correlates with better survival and better response to tamofixen monotherapy (Honma et al., 2008). The ERβ splice variant ERβ2, which differs from ERβ1 in 26 C-terminal amino acids, has been associated with reduced metastasis and vascular invasion when localized in the nucleus, but with worse outcome and resistance to chemotherapy when found in the cytoplasm (Shaaban et al., 2008).

1.7.2 Role of ERs in lung cancer

The role of ERs in lung cancer pathogenesis has not been very well studied. Early findings have found expression of both ERs in the normal human lung (Brandenberger et al., 1997). Moreover, ERβ knockout mice display lung abnormalities with alveolar collapse, reduced expression of key regulators of surfactant homeostasis, and alterations in the extracellular matrix (Patrone et al., 2003, Morani et al., 2006). Interestingly, ERα knockout mice do not have this phenotype. These studies revealed ERβ to be an important component of lung

development and homeostasis. In cancerous lung, clinical studies have indicated an association of ERα expression with poor prognosis among patients with NSCLC and EGFR mutations. Reduced expression of both ERs is associated with increased cell proliferation, whereas ERα overexpression and ERβ negativity correlates with poor prognosis and higher risk even at early clinical stage (Raso et al., 2009, Chen et al., 2008). On the other hand, ERβ expression correlates with better prognosis and reduced mortality in male NSCLC patients. ERβ positivity inversely correlates with lymph node metastases and tumor size. Furthermore, nuclear expression of ERβ in NSCLC patients with EGFR mutations correlates with good differentiation and increased disease-free survival. Intriguingly, expression of ERβ predicts better response to TKIs among NSCLC patients with EGFR mutations (Nose et al., 2009, Nose et al., 2011, Schwartz et al., 2005, Skov et al., 2008, Abe et al., 2010). On the contrary, a few *in vitro* studies have proposed a proliferative and anti-apoptotic role of ERβ in NSCLC cell lines. In these studies, cytoplasmic ERβ seems to promote proliferation of NSCLC cells when treated with ERβ-specific ligands (Hershberger et al., 2005, Pietras et al., 2005). All these studies support an important role for ERs in lung carcinogenesis, disease progression, and response to treatment. However, the molecular mechanisms through which ERs exert their functions in lung cancer are still under investigation.

Chapter 2

Materials and Methods

2.1 Cells and Reagents

Non-small cell lung cancer cell lines A549, H1299, H661, and H358, and breast cancer cell lines MDA-MB-231, Hs578T, and MCF-7 were purchased from American Type Culture Collection (ATCC). H1299, H661, H358, MDA-MB-231, and Hs578T cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) and A549 and MCF-7 in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich, St.Louis, MO, USA), 13.5 mM D-Glucose and 50 μg/mL Kanamycin at 37°C in a humidified incubator with 5% CO_2. In ligand experiments, cells were cultured in phenol red-free media containing 0%, 0.5%, 2%, or 5% dextran-coated charcoal (DCC)-treated FBS. Cells were either treated with 10 nM of 17β-estradiol (E2) or 10 nM 5α-androstane-3β,17β-diol (3β-Adiol). In the TGF-β and EGF experiments cells were treated with recombinant human TGF-β1 (5 ng/ml; R & D Systems, Minneapolis, MN USA) for one to three days or EGF (10 ng/ml; Sigma-Aldrich) for 24 hours (h). Doxorubicin and etoposide were purchased from Cell Signaling Technologies (Boston, MA, USA). Primary antibodies against ERβ1 (Clone 14C8) and p84 were purchased from Genetex (Irvine, CA, USA). For validation purposes two additional ERβ antibodies were used, a monoclonal anti-ERβ antibody (clone 68-4; Millipore) and a C-terminus rabbit polyclonal anti-ERβ antibody

that recognizes only the ERβ1 isoform (Invitrogen). The primary antibodies for EGFR, phospho-ERK1/2, Caspase 3, p27, p21, ERα, E-cadherin, α-Tubulin, Ubiquitin, and c-Myc were purchased from Santa Cruz Biotechnologies (Dallas, TX, USA) and the primary antibodies against Bim, ERK1/2, phosphor-Akt(S473), pan-Akt, RAS, cleaved Caspase 3, phospho-Chk1 (S345), and Cyclin D2 were purchased from Cell Signaling Technologies. Anti-c-Cbl antibody was purchased from BD Biosciences. β-actin and Flag primary antibodies were obtained from Sigma-Aldrich (Appendix I). Recombinant ERβ1 was purchased from Invitrogen and used as positive control in **sodium dodecyl sulfate polyacrylamide gel electrophoresis** (SDS-PAGE) experiments.

2.2 Plasmid Constructions and Transfections

ERβ1 and ERβ2 expression constructs were generated by cloning the full-length ERβ1 or ERβ2 in the pIRESneo3 expression vector (Clontech, Mountain View, CA, USA). H1299 and A549 cells were transfected with empty pIRESneo vector or the recombinant pIRESneo-ERβ1 or pIRESneo-ERβ2 plasmids. H661 were infected with lentiviruses containing the empty plenti6/V5 vector or the recombinant pLenti6/V5-D-FLAG-ERβ1 as described previously (Hartman et al., 2009). H1299 cells were transiently transfected twice with ERβ-specific siRNAs (Invitrogen), target sequences 1# 5′-TTAGCGACGTCTGTCGCGTCTTCAC-3′, 2# 5′-TATTGACCGCTACCTGGTGATTTCC-3′. An siRNA targeting luciferase was used as a control (Cat. No. 12935-146, Invitrogen). For the expression of wild-type EGFR, cells were stably transfected with the pBABE-EGFR

construct (Addgene, plasmid #11011, Cambridge, MA USA), using the empty pBABE vector (Addgene, plasmid # 1764) as a control. Mutant NRAS (61K) was purchased from Addgene (Plasmid # 12543). The coding sequence of mutant NRAS was subcloned in to the pIRESpuro3 vector (Clontech). The myc epitope was tagged on the C-terminus of mutant N-Ras using the following primers: FW 5′-GTACGACCGGTGCCACCATGACTGAGTACAAACTGGT-3′ and RV 5′-AGCAGGATCCTTACAGATCTTCTTCAGAAATAAGTTTTTGTTCCATC-ACCACACATGGCA-3′. ERβ1-expressing H1299 cells were stably transfected with an empty pIRESpuro vector or the pIRESpuro-N-RAS(61K) plasmid. For ERE-luciferase reporter assays, cells were incubated in DCC-FBS media for 48 h and transfected with 800 ng DNA per well (3-ERE-TATA-LUC reporter plasmid, β-gal plasmid) using Lipofectamine 2000 (Invitrogen). Cells were mock treated (EtOH) or treated with E2 for 24 h in 2% DCC-FBS media. Luciferase reporter activity was normalized to β-galactosidase enzyme activity.

2.3 Cell Survival Assay

Cell survival assays were performed in order to assess the survival of NSCLC cells after treatment with cytotoxic agents. Control and ERβ1-expressing H1299, H358, and H661 cells were seeded onto 96-well plates in 10% FBS-containing medium at a density of 5000 cells/well and treated with increasing concentrations of doxorubicin (0-10 μM) in quintuplicates. Seventy-two hours later the surviving portion of the cells was measured

by the Cell Titer-Blue cell viability assay, following manufacturer's protocol (Promega). The IC_{50} values for control and ERβ1-expressing cells was calculated using GraphPad Prism 5 after taking the mean of three independent experiments.

2.4 Clonogenic Survival Assay

Clonogenic survival assays were performed in order to assess the clonogenicity of control and ERβ1-expressing cells. Following growth in 5% DCC-FBS media for 48 h, cells were harvested by trypsinization and replated at the density of $1x10^3$ cells per 60-mm dish in triplicates and were either mock (EtOH)-treated or E2-treated. After 14 days, cells were washed, fixed in an acetic acid:methanol (3:1) solution, and stained with 0.5% Crystal Violet in 25% methanol solution. Surviving colonies in each dish were counted and the plating efficiency was calculated using the equation: plating efficiency = (number of colonies counted/number of cells plated) *X* 100. Then, the fraction of the cells surviving the expression of ERβ1 and/or the treatment with E2 was determined by normalizing the plating efficiency of the ERβ1 expressing and/or E2-treated cells to that of the plates with control untreated cells, which was set to 100%.

2.5 Proliferation Assay

Proliferation assays were performed in order to determine the proliferation capacity of control and ERβ1-expressing cells upon treatment with E2. H1299 and H661 cells were seeded onto 12-well plates at a density of $1X10^4$ cells per well in 5% DCC–FBS media. Cells

were incubated for 5 days with EtOH or E2. Cells were then fixed in ice-cold methanol and stained with 0.1% crystal violet in phosphate-buffered saline (PBS). Plates were air-dried and the stained cells were solubilized in 10% SDS solution overnight. The optical density of the extracted dye was measured with a spectrophotometer at 590 nm. Optical density measurements were used to generate proliferation curves.

2.6 RNA Extraction and Real-Time PCR

Total RNA was isolated using Aurum Total RNA mini kit (Biorad, Hercules, CA, USA) and reverse-transcribed to cDNA using the iScript cDNA synthesis kit (Biorad). Real-Time PCR was performed using the iTaq SYBR Green kit (Biorad). All quantitative data were normalized to *GAPDH* and *36B4*. The sequences for the primers used are listed in Appendix II.

2.7 Immunoblotting and Immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, and 1% NP-40) containing protease (1 mM EDTA, Roche protease inhibitor mixture, and 2 mM PMSF) and phosphatase inhibitors (1 mM NaF, 1 mM Na3VO4, and Sigma phosphatase inhibitor mixture). For separation of cytoplasmic and nuclear fractions, cells were suspended in a cold buffer containing 10 mM Hepes pH 7.0, 10 mM KCI, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. After 15 minutes incubation on ice, the homogenate was mixed with 10% NP-40

and centrifuged for 30 sec. The nuclear pellet was resuspended in a cold buffer containing 10 mM Hepes-KOH pH 7.9, 400 mM NaCI, 0.1 mM EDTA, 5% glycerol, 1 mM DTT, and 0.5 mM PMSF, and the nuclear extract was isolated by centrifugation. The lysates were subjected to SDS–PAGE and the proteins were transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in TBST (0.05% Tween-20) for 3 hours at room temperature and probed with the primary antibodies overnight at 4°C. The membranes were incubated with secondary antibodies for 2 hours at room temperature (RT). Proteins were visualized using enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences). Band intensities were quantified by densitometry using the ImageJ software. For ubiquitylation analysis, cells were lysed in RIPA buffer, briefly sonicated, and cleared by centrifugation at 4°C. Supernatants were incubated with anti-EGFR antibody overnight at 4°C and A/G agarose beads for 2 h at 4°C. The immunocomplexes were washed three times, boiled in 2× sample buffer and immunoblotted with anti-ubiquitin antibody. For the EGFR-c-Cbl co-immunoprecipitations, cells were lysed in a buffer containing 50 mM Hepes pH 7.4, 150 mM NaCI, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% glycerol including protease and phosphatase inhibitors. Lysates were incubated on ice for 30 minutes without sonication, cleared by centrifugation and the cleared lysates were subjected to immunprecipitation and immunoblotting as described.

2.8 Immunofluorescence

Cells were plated onto 18 mm^2 coverslips, fixed in 3% paraformaldehyde (PFA) and 2% sucrose for 15 minutes at RT, after which they were permeabilized in 20 mM Tris HCI pH 7.5, 75 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$ and 0.5% Triton-X-100 for 15 minutes at RT and blocked with 5% goat serum in PBS for 1 hour at RT. Slides were incubated with an E-cadherin antibody (BD Biosciences) at 4°C overnight, washed, incubated with secondary antibody, and photographed using an OLYMPUS BX51 microscope equipped with an OLYMPUS XM10 camera (OLYMPUS, Center Valley, PA USA).

2.9 Flow Cytometry

Flow cytometry was performed in order to determine the cell cycle kinetics of control and ERβ1-expressing cells. After treatment, cells were trypsinized and fixed in 70% ice-cold ethanol overnight at 4°C. Cells were resuspended in a propidium iodide (100 μg/ml) / RNase A solution (50 μg/ml) and analyzed on a FACSAria II cell sorter (BD Biosciences). Cell cycle data analysis was performed using FlowJo software (Tristar Inc.).

2.10 Migration and Invasion Assays

In the wound-healing assay, after cells were allowed to form monolayers at 24-well plates, they were scratched with a pipette tip to form the wound. Twelve hours later, images of the wound were taken using a 10X objective in an OLYMPUS IX51 microscope equipped

with an OLYMPUS camera (OLYMPUS, Center Valley, PA USA). Cells in the wound area from five independent fields were counted.

In the invasion assay, cells were seeded in matrigel-coated 6.5 mm Transwell chambers (8 μm pore size; BD Biosciences, San Jose, CA USA). Six hours later, the cells that translocated to the lower compartment of the wells and attached to the lower surface of the filter were fixed in methanol and stained with crystal-violet. The stained cells from five independent fields in each Transwell were counted.

2.11 Caspase-3/7 Assay

A549 cells were plated onto 96-well plate at a density of 1 $X10^4$ per well in triplicates. After treatment with EtOH, E2 or 3β-Adiol for 24 hours, cells were washed with PBS, lysed and the luminescent signal generated by the cleavage of the proluminescent caspase-3/7 substrate, that is, proportional to the caspase-3/7 activity, was measured according to the manufacturer's protocol (Promega).

2.12 RAS Activity Assay

RAS activity was assessed using a Pan-RAS Activation Kit from Cell Biolabs. The active RAS–GTP form interacts with the effector RAF-1. The RAS–GTP was pulled down from cell lysates using the RAS-binding domain of RAF-1 immobilized to agarose beads. The levels of precipitated RAS–GTP were measured by immunoblotting using an anti–Pan RAS antibody (Cell Biolabs).

2.13 Zebrafish Xenotransplantation Study

Animal work was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Houston. Two different zebrafish lines were used in these studies including the transgenic strain expressing enhanced green fluorescent protein (EGFP) under the Flk1 promoter, *Tg(Flk-1;EGFP)*, which was a gift from Dr. Daniel Wagner (Rice University) and allows for visualization of the vascular system as well as the pigmentation mutant *casper* line that demonstrates a complete lack of all melanocytes and iridophores in both embryogenesis and adulthood that was purchased from Zebrafish International Resource Center (ZIRC). The casper *Tg(Flk-1;EGFP)* zebrafish was created by crossing *Tg(Flk-1;EGFP)* with the casper line and is almost completely transparent. Control (Lenti) and ERβ1-expressing (ERβ1) MDA-MB-231 cells were stably transfected with either the pAmCyan vector or the pCMCV-DsRed vector (Clontech, Mountain View, CA USA). A tumor cell suspension (5 nL) of approximately 300 to 500 cells containing a mixture of equal numbers of either DsRed-Lenti:AmCyan-ERβ1 cells or AmCyan-Lenti:DsRed-ERβ1 cells were injected into the perivitelline cavity of each 48 hour post-fertilization casper *Tg(Flk-1;EGFP)* anesthetized embryo using a pressure injector (Harvard Apparatus, Holliston, MA USA) and Manipulator (MM33-Right, Märzhäuser Wetzlar, Germany). Glass needles (1.00 mm in diameter, Sutter Instrument Company, Novato, CA USA), were used for the microinjection. Injected embryos were kept at 32°C and were examined every day for tumor invasion using a fluorescent microscope (OLYMPUS IX51) equipped with an OLYMPUS XM10 camera.

Chapter 3

ERβ Regulates NSCLC Phenotypes by Controlling Oncogenic RAS Signaling

3.1 Introduction

Lung cancer is the most frequent cause of cancer death for both men and women worldwide. The major risk factor is tobacco smoking; however, there is a significant proportion of non-smokers that develop lung cancer (Parkin et al., 2001). Observations from population-based clinical studies propose a role for female steroid hormones in lung tumor development and progression. The non-smoking-related lung cancer is more common in women; premenopausal women develop less differentiated lung cancer compared with postmenopausal women who have lower levels of circulating estrogen (Uramoto et al., 2006). Interestingly, local production of estradiol has been observed in NSCLC. Its concentration is higher in cancer tissues compared with non-neoplastic lung tissues and its intratumoral concentration has been associated positively with aromatase expression and markers of tumor growth in a group of male and postmenopausal female patients with NSCLC (Niikawa et al., 2008). Consistent with the clinical studies, treatment with estrogen was reported to promote progression of p53-defective mouse lung tumors that express mutant *k-ras* (Hammoud et al., 2008).

Estrogens regulate various physiological processes including cell growth, differentiation, and development (Matthews and Gustafsson, 2003, Ascenzi et al., 2006). Estrogens mediate their actions through two members of the nuclear receptor superfamily, estrogen receptor (ER) α and β. In response to ligand binding or in a ligand-independent manner ERs can regulate gene expression either by acting as transcription factors at sequence-specific response elements known as estrogen response elements (EREs) or by interacting with and activating other transcription factors (Thomas and Gustafsson, 2011). ERs demonstrate different tissue distribution, and perturbation of ER subtype-specific expression has been detected in various pathological conditions including cancer. Whereas ERα is overexpressed in a significant proportion of breast cancers, both ERs have been detected in non-small cell lung cancer (NSCLC) cells (Thomas and Gustafsson, 2011). However, the role of ERs in NSCLC remains poorly understood because previous studies produced contradictory data ((Schabath et al., 2004, Schwartz et al., 2007, La Vecchia, 2006). Although two cell-based studies have reported an increased NSCLC cell proliferation in response to treatment with ERβ ligands, clinical studies demonstrated a correlation between ERβ positivity and better outcome of lung cancer patients (Hershberger et al., 2009, Karachaliou et al., 2013, Kawai et al., 2005a, Nose et al., 2009, Nose et al., 2011, Schwartz et al., 2005, Zhang et al., 2009). In particular, increased expression of wild-type ERβ (ERβ1) has been associated with better prognosis and reduced mortality and inversely associated with lymph node metastases and tumor size in patients with NSCLC (Abe et al., 2010, Schwartz et al., 2005, Skov et al., 2008). In

addition, the correlation of ERβ with increased disease-free survival in patients with NSCLC carrying EGFR mutations and better response to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) proposed an antitumorigenic function of ERβ in NSCLC that involves potential regulation of growth factor signaling (Nose et al., 2009, Nose et al., 2011).

EGFR that is expressed in high levels in 62% of NSCLCs correlates with poor prognosis (Hirsch et al., 2003, Ohsaki et al., 2000). Furthermore, NSCLCs often produce EGFR ligands such as epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α), suggesting the function of an autocrine growth-stimulatory mechanism that supports the EGFR oncogenic actions (Putnam et al., 1992, Rusch et al., 1993). Antibody-based therapies that target the EGFR ligand-binding domain and disrupt the autocrine receptor-activating mechanisms have been associated with improved survival in patients with lung cancer (Mendelsohn, 1992, Sharma et al., 2007). In addition to receptor overexpression, somatic mutations in the tyrosine kinase domain that result in constitutive activation of EGFR were identified in NSCLCs (Uramoto et al., 2006). The EGFR-TKIs gefitinib and erlotinib that bind preferentially to the tyrosine-kinase domain of some of the EGFR mutants and inhibit the activity of the receptor have been associated with improved clinical outcome in patients with EGFR-mutant lung tumors ((NGM), 2013). Unfortunately, intrinsic and acquired resistance limits the effectiveness of these drugs (Hynes and Lane, 2005). Among the mechanisms that account for the resistance of lung tumors to EGFR inhibitors is the presence of somatic mutations in genes encoding

components of the growth factor signaling such as RAS, a GTPase that acts as a signaling molecule downstream of EGFR (Pao et al., 2005). RAS and EGFR mutations are mutually exclusive in NSCLC (Pao et al., 2005). Ninety percent of RAS mutations in lung adenocarcinoma represent alterations in K-RAS and most of them involve single substitutions at residues 12 and 13 (Prior et al., 2012). Certain K-RAS mutations have been associated with worse prognosis in lung cancer (oncogenic mutations in G12 residues) and worse response of NSCLC and colorectal cancer to treatment with EGFR-TKIs and the EGFR-directed antibodies, respectively (Bokemeyer et al., 2009, Lievre et al., 2008, Mascaux et al., 2005, Pao et al., 2005, Van Cutsem et al., 2008).

Multiple clinical and laboratory-based studies have demonstrated anti-proliferative and pro-apoptotic functions for ERβ in various types of cancers including breast, colon, prostate, and ovarian cancer (Thomas and Gustafsson, 2011). In contrast, the role of ERβ in lung cancer development and progression is still poorly understood. In this study, we investigated whether ERβ elicits antitumorigenic actions in NSCLC cells that may account for the correlation between ERβ and better survival observed in patients with NSCLC.

3.2 Results

3.2.1 ERβ1 inhibits cell growth and induces apoptosis in NSCLC cells

Oncogenic mutations that frequently occur in lung cancer are attractive targets for anticancer therapy. Although a few targets like EGFR have been successfully targeted, direct inhibition of some mutant genes, such as K-RAS remains elusive. Patients with K-RAS mutations tend to have poor prognosis and do not respond to EGFR-TKIs (Karachaliou et al., 2013, Pao and Chmielecki, 2010). Interestingly, a positive correlation between the expression of ERβ and better survival has been observed in patients with NSCL tumors that carry EGFR mutations (Nose et al., 2009, Nose et al., 2011). However, the molecular basis for this correlation is unknown. We hypothesized that ERβ regulates cell survival in NSCLC and that high expression of ERβ in NSCLC cells is associated with decreased cell proliferation and induction of apoptosis. To test this hypothesis, we stably expressed ERβ1 in three NSCLC cell lines that express very low levels of endogenous ERβ1 (Figure 3.1C). These include the H1299 and A549 cells that carry N-RAS and K-RAS mutations respectively, and the H661 cells that express wild-type RAS. To achieve comparable expression of ERβ1 in the cell lines, A549 and H1299 cells were stably transfected with the pIRES-ERβ1 plasmid, whereas the H661 cells that proved difficult to transfect were stably infected with lentivirus containing the pLenti-FLAG-ERβ1 plasmid (Figure 3.1C). As shown in Figure 3.2D, ERβ1 was localized in the nucleus of the NSCLC cells. Induction of ERβ1 expression inhibited cell growth in all three NSCLC cell lines (Figure 3.1A and Figure 3.2). Cell survival assays revealed that ERβ1 decreased cell

growth even in the absence of ligand and that addition of E2 in cell culture further enhanced the cytotoxic effect of ERβ1 in H1299 but not in A549 and H661 cells (Figure 3.1A and Figure 3.3). The ligand-independent anti-tumorigenic function of ERβ1 that was observed in NSCLC cells has also been previously described in breast, colon, and prostate cancer cells (Dey et al., 2012, Hartman et al., 2009, Thomas et al., 2012, Thomas and Gustafsson, 2011). Interestingly, the effect of ERβ1 was more potent in H1299 and A549 cells that express mutant RAS compared to H661 cells that carry wild-type RAS, suggesting that ERβ1 may suppress the growth of NSCLC cells by targeting oncogenic RAS signaling (Figure 3.1A). To elucidate the mechanism through which ERβ1 inhibited NSCLC cell growth, control and ERβ1-expressing cells were analyzed for cell cycle progression and apoptosis by flow cytometry. As shown in flow cytometry histograms in Figure 3.1B, upregulation of ERβ1 inhibited cell cycle progression by arresting the H1299 cells in G1 phase. In addition to G1/S phase cell cycle arrest, ERβ1 enhanced apoptosis as shown by the higher percentage of ERβ1-expressing H1299 and A549 cells in sub-G1 fraction that is indicative of apoptosis (Figure 3.1B). These results suggest that upregulation of ERβ1 in NSCLC cells inhibits cell growth by inducing cell cycle arrest and apoptosis.

Figure 3.1 ERβ1 inhibits the growth of NSCLC cells. (A) Control and ERβ1-expressing, RAS mutated H1299 and A549 NSCLC cells and wild-type RAS H661 large-cell lung carcinoma cells were treated with 10 nM E2 or ethanol (vehicle) as a control in 5% DCC-containing media. 14 days later, surviving cell colonies were stained and counted and the fraction of the cells (surviving fraction) surviving ERβ1 expression and/or E2 treatment was estimated as described in Materials and Methods. The values in the graphs represent the mean (surviving fraction) of three separate experiments with the standard deviation (SD) and *p* value (*) ≤ 0.05% indicated. **(B)** Upper panel: Following incubation in 2% DCC-containing media for 48 hours (h), 5 x 10^5 control and ERβ1-expressing A549 cells were treated with 10 nM E2 in serum-free medium for 48 h. Lower panel: Control and ERβ1-expressing H1299 cells were initially incubated in 2% DCC-containing medium for 48 h. 10 nM E2 was added to the cell culture in serum-free medium (Time: 0 h) and the cells were collected at this time point and after incubation with E2 for 12 and 24 h. Cells were stained with propidium iodide and the DNA content was analyzed by flow cytometry. The percentage of control and ERβ1-expressing cells in sub-G1 fraction which is indicative of apoptosis is shown as mean of three independent experiments. Immunoblots of ERβ1 in lysates from H1299 stably transfected with pIRES empty vector (control) or pIRES-ERβ1 construct and H661 cells infected with lentivirus carrying pLenti empty vector (control) or pLenti-flag-ERβ1 construct. Recombinant full-length ERβ1 protein (rERβ1, Invitrogen) was loaded as positive control.

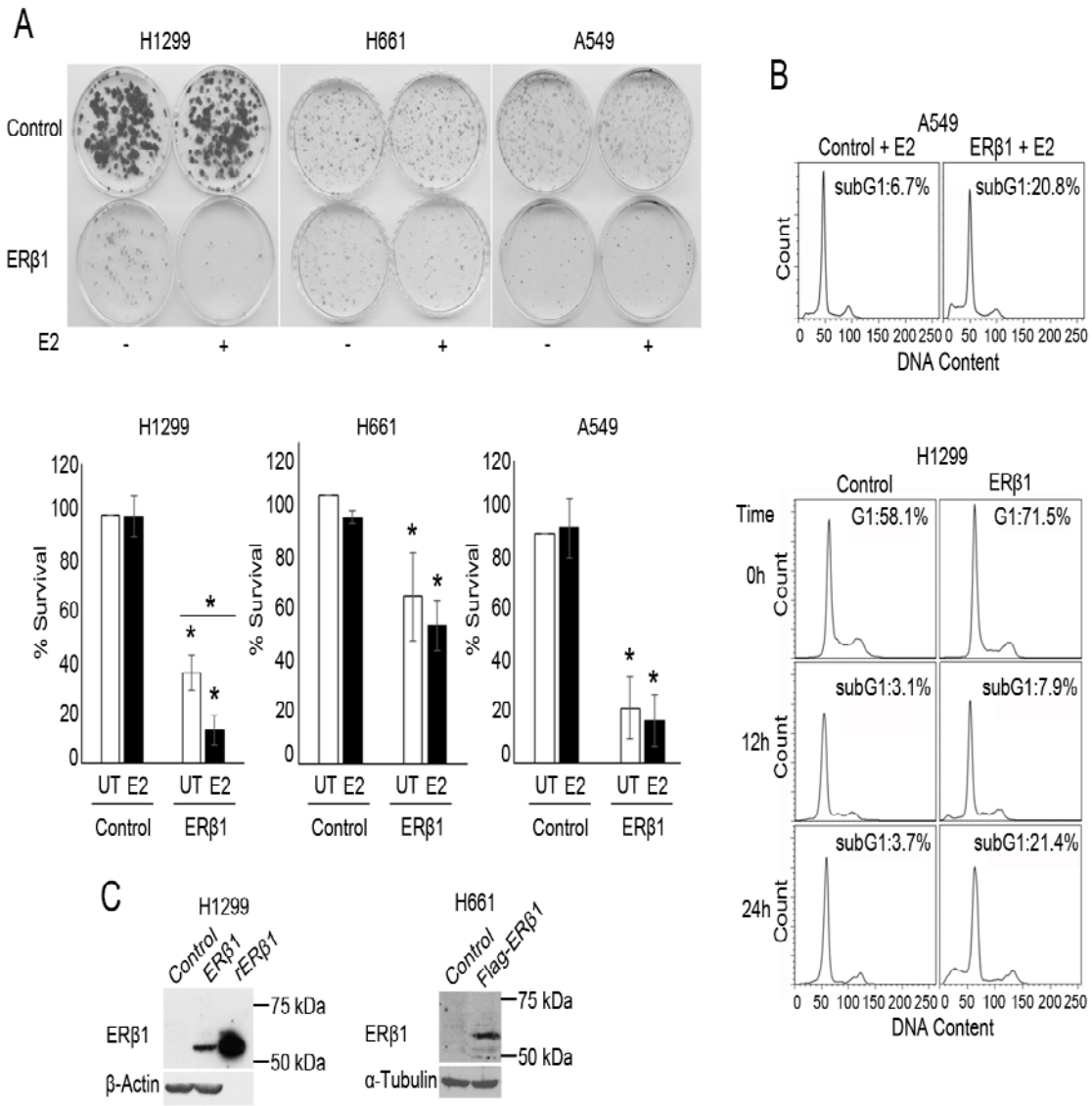
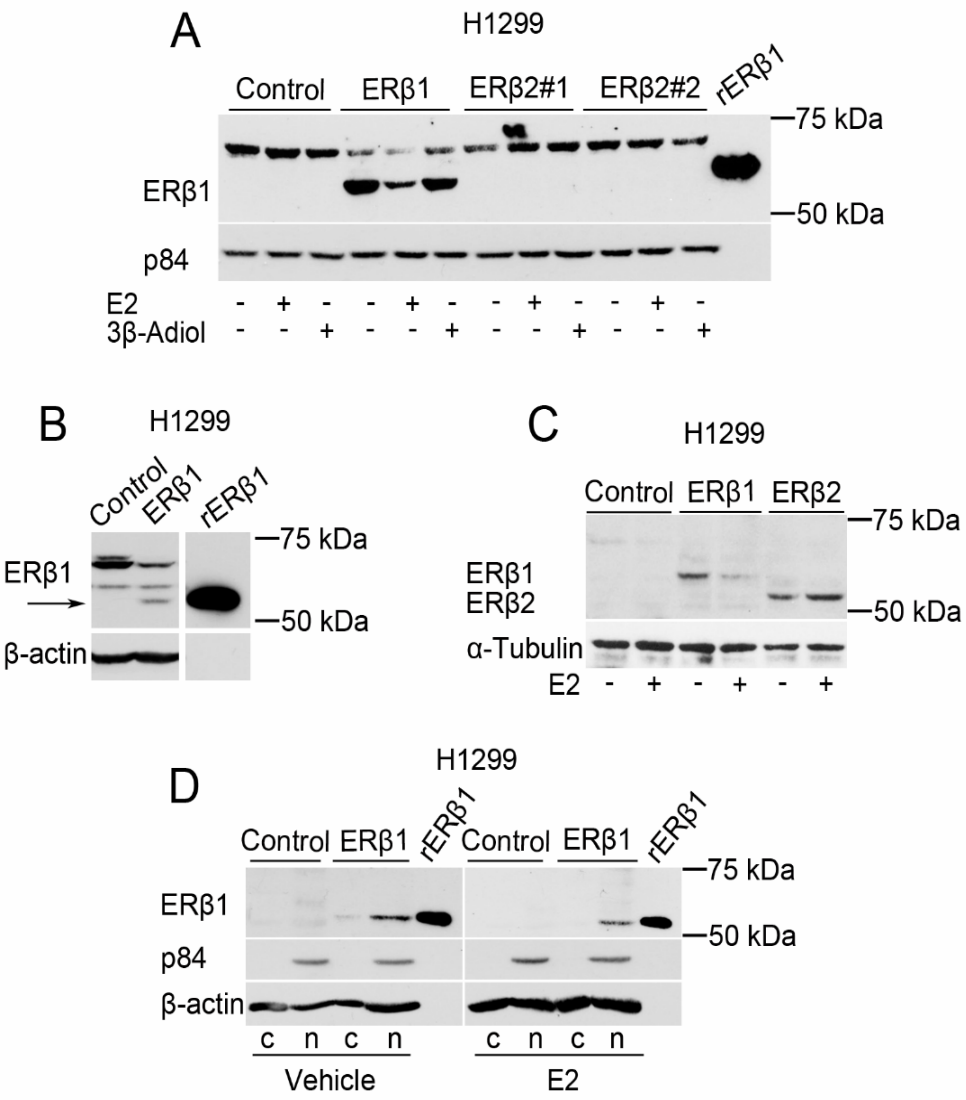

A
H1299
H661
A549
Control
ERβ1
E2
-
+
-
+
-
+
B
A549
Control + E2
ERβ1 + E2
subG1:6.7%
subG1:20.8%
Count
0 50 100 150 200 250 0 50 100 150 200 250
DNA Content
H1299
H661
A549
% Survival
120
100
80
60
40
20
0
UT E2
UT E2
Control
ERβ1
H1299
Control
ERβ1
Time
0h
12h
24h
G1:58.1%
G1:71.5%
subG1:3.1%
subG1:7.9%
subG1:3.7%
subG1:21.4%
Count
0 50 100 150 200 250 0 50 100 150 200 250
DNA Content
C
H1299
Control
ERβ1
rERβ1
ERβ1
β-Actin
75 kDa
50 kDa
H661
Control
Flag-ERβ1
ERβ1
α-Tubulin
75 kDa
50 kDa

Figure 3.2 Evaluation of ERβ1 expression in NSCLC cell lines.
(A) Immunoblot of ERβ1 in lysates from control, ERβ1 or ERβ2-expressing H1299 cells (clones #1 and #2) following treatment with 10 nM E2 or 10 nM 3β-Adiol. Membrane was probed with a rabbit polyclonal antibody against the C-terminus of ERβ (Invitrogen) that recognizes only the ERβ1 isoform and not the splice variant ERβ2 that has a unique C-terminal amino acid sequence. Recombinant full-length ERβ1 protein (rERβ1) was loaded as positive control. (B) Immunoblot of ERβ1 in lysates from control and ERβ1-expressing H1299 cells using a rabbit monoclonal antibody against the N-terminus of ERβ (clone 68-4, Millipore). The bands in two vertically sliced images correspond to protein samples run in the same gel. (C) ERβ1 and ERβ2 levels in control, ERβ1 or ERβ2-expressing H1299 cells following treatment with 10 nM E2. Membrane was probed with an antibody against the N-terminus of ERβ (14C8, GeneTex) that recognizes both ERβ1 and ERβ2. Notice in A and C that only ERβ1 that contains a fully functional ligand binding domain is downregulated in the presence of E2 in contrast to ERβ2 that is known to bind E2 with much lower affinity than ERβ1 (1). (D) Immunoblot of ERβ1 in nuclear (n) and cytoplasmic (c) extracts from control and ERβ1-expressing H1299 cells following treatment with 10 nM E2. p84 was used as a loading control for the nuclear fraction. Recombinant full-length ERβ1 protein (rERβ1) was loaded as positive control.


A
H1299
Control
ERβ1
ERβ2#1
ERβ2#2
rERβ1
75 kDa
50 kDa
ERβ1
p84
E2
3β-Adiol
B
H1299
Control
ERβ1
rERβ1
75 kDa
50 kDa
ERβ1
β-actin
C
H1299
Control
ERβ1
ERβ2
75 kDa
50 kDa
ERβ1
ERβ2
α-Tubulin
E2
D
H1299
Control
ERβ1
rERβ1
Control
ERβ1
rERβ1
75 kDa
50 kDa
ERβ1
p84
β-actin
c n c n
c n c n
Vehicle
E2

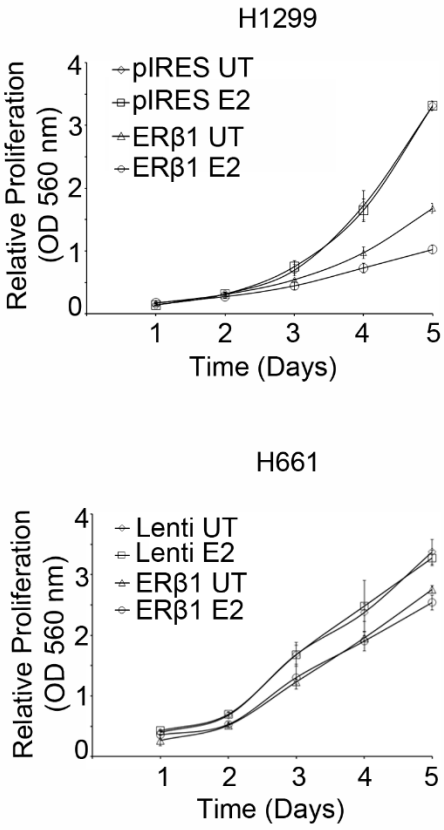


Figure 3.3 ERβ1 reduces cell proliferation in NSCLC cells.
Cell proliferation was monitored in control and ERβ1-expressing H1299 (upper panel) and H661 (bottom panel) cells following treatment with or without 10 nM E2 for the indicated times.

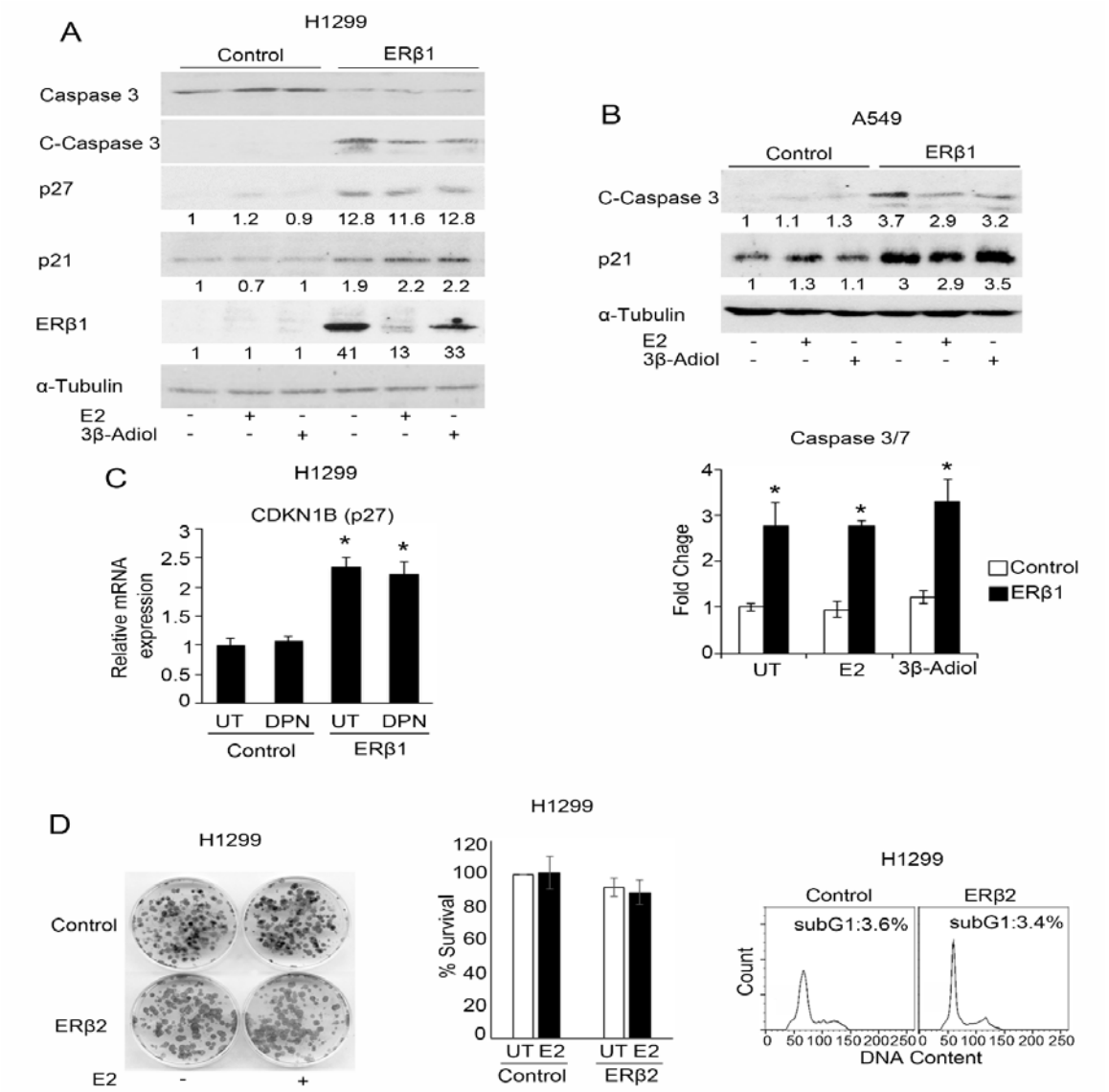
3.2.2 ERβ1 increases the expression of cell cycle inhibitors and activates pro-apoptotic factors in NSCLC cells

To investigate the molecular mechanism involved in the ERβ1-induced cell cycle arrest and apoptosis, control and ERβ1-expressing H1299 and A549 cells following treatment with E2 or 3β-Adiol for 24h were initially assessed for the expression of the cell cycle regulatory proteins p21(Waf1/cip1) and p27(kip1). p21 and p27 retard cell cycle progression by inhibiting the activity of cyclin-dependent kinases (CDKs) (Besson et al., 2008). We treated the cells with the same concentration of the androgen metabolite 3β-Adiol. 3β-Adiol that displays high affinity for ERβ has been shown to elicit anti-proliferative effects in prostate cancer and to affect cell proliferation in other reproductive tissues (Omoto et al., 2005). Although expression of the enzymes 5α-reductase and 3β-hydroxysteroid dehydrogenase that participate in the generation of 3β-Adiol has been described in NSCLC cells and tissues, its presence and role in lung cancer is still poorly understood (Kapp et al., 2012). As shown in Figure 3.4A and B, ERβ1-expressing H1299 and A549 cells had higher levels of p21 and/or p27 compared to control cells. In line with the immunoblotting results, gene expression analysis by qRT-PCR indicated that ERβ1 upregulates p27 mRNA levels (Figure 3.4C). This is consistent with previous studies showing upregulation of p21 and/or p27 by ERβ1 in breast, prostate, and colon cancer cells (Dey et al., 2014, Hartman et al., 2009, Treeck et al., 2010). In addition to cell cycle inhibitors, pro-apoptotic factors were analyzed in control and ERβ1-expressing cells. Caspase 3 that is activated by proteolytic cleavage is one of the executioner caspases of

the intrinsic apoptotic pathway (Slee et al., 2001). To examine whether the intrinsic apoptotic pathway is involved in the ERβ1-induced apoptosis, the cleavage of caspase 3 was evaluated in control and ERβ1-expressing H1299 and A549 cells. Immunoblotting and a luciferase-based assay for the assessment of caspase 3/7 activity revealed increased cleavage and activity of caspase 3 in two ERβ1-expressing NSCLC cell lines, both in the absence and presence of the ligands E2 or 3β-Adiol (Fig 4A and B, upper and middle panel). As shown in Figure 3.4A and B, although no significant differences in the levels of cleaved caspase 3, p21 and p27 were observed between E2- and 3β-Adiol-treated cells, E2 treatment caused downregulation of ERβ1 to a higher extent than 3β-Adiol. This ligand-mediated downregulation of ERs has been associated with the activation of the receptors (Hauser et al., 2000, Pan et al., 2011). Importantly, in contrast to ERβ1, stable expression of the ERβ splice variant ERβ2, that differs from ERβ1 in 26 C-terminal amino acids, failed to significantly decrease cell growth and it did not enhance apoptosis in H1299 cells (Figure 3.4D and Figure 3.2C). This suggests that the pro-apoptotic phenotype observed in ERβ1-expressing cells was due to specific upregulation of the fully functional ERβ1. These results indicate that ERβ1 inhibits the growth of NSCLC cells by upregulating cell cycle inhibitors and stimulating the intrinsic apoptotic pathway.

Figure 3.4 ERβ1 regulates pro-apoptotic factors and cell cycle components in NSCLC cells.

(A) H1299 cells stably transfected with an empty vector (Control) or ERβ1 plasmid were either mock treated or treated with 10 nM E2 or 10 nM 3β-Adiol for 24 h and analyzed for the expression of cleaved-caspase 3, caspase 3, p21 and p27 by immunoblotting. The p21, p27 and ERβ1 band intensities were analyzed by densitometry and normalized to α-tubulin. The numbers under each immunoblot show the fold change compared to the intensities of the untreated control cells that were given a value of 1. Results are representative of three independent experiments. **(B)** Upper panel: cleaved-caspase 3 and p21 levels in A549 cells stably transfected with an empty vector (Control) or ERβ1 plasmid after treatment with 10 nM E2 or 10 nM 3β-Adiol for 24 h. The band intensities were analyzed as described above. Lower panel: caspase 3/7 activity in control and ERβ1-expressing A549 cells in the absence or presence of 10 nM E2 or 10 nM 3β-Adiol. Graph shows the mean ± SD of three independent experiments. *P-value* (*) ≤ 0.05. (**C)** mRNA expression analysis of CDKN1B (p27) in control and ERβ1-expressing H1299 cells treated with 10 nM of the ERβ specific agonist diarylpropionitrile (DPN) for 24 h. The graph shows the mean of three experiments normalized to vehicle-treated control cells with SD and *P-value* (*) ≤ 0.05 indicated. (**D)** Left and middle panel: following incubation for 14 days in 5% DCC media containing vehicle or 10 nM E2, control and ERβ2-expressing H1299 cells were fixed and stained. Surviving cell colonies were counted and plotted as mean ± SD of three experiments normalized to vehicle-treated control cells. *P-value* (*) ≤ 0.05. Right panel: DNA analysis of control and ERβ2-expressing H1299 cells that were initially incubated in 2% DCC media for 48 h and then in serum-free media containing 10 nM E2 or vehicle for 24 h. The percentage of the cells in sub-G1 fraction is shown as mean of three independent experiments.

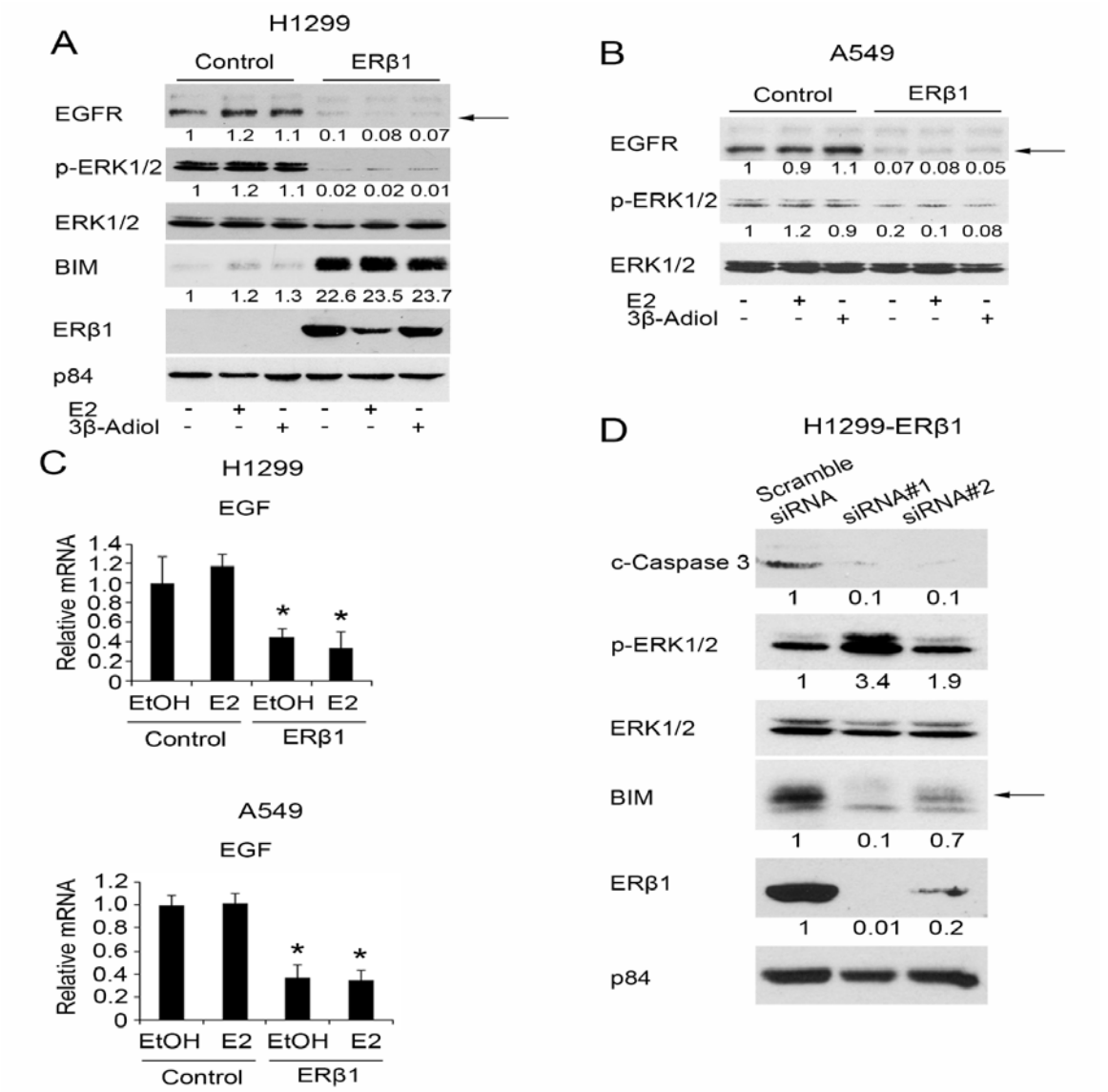

A
H1299
Control
ERβ1
Caspase 3
C-Caspase 3
p27
1 1.2 0.9 12.8 11.6 12.8
p21
1 0.7 1 1.9 2.2 2.2
ERβ1
1 1 1 41 13 33
α-Tubulin
E2 - + - - + -
3β-Adiol - - + - - +
B
A549
Control
ERβ1
C-Caspase 3
1 1.1 1.3 3.7 2.9 3.2
p21
1 1.3 1.1 3 2.9 3.5
α-Tubulin
E2 - + - - + -
3β-Adiol - - + - - +
C
H1299
CDKN1B (p27)
Relative mRNA expression
UT DPN UT DPN
Control
ERβ1
Caspase 3/7
Fold Chage
UT
E2
3β-Adiol
Control
ERβ1
D
H1299
Control
ERβ2
E2 - +
H1299
% Survival
UT E2 UT E2
Control
ERβ2
H1299
Control
ERβ2
subG1:3.6%
subG1:3.4%
Count
DNA Content

3.2.3 ERβ1 activates the intrinsic apoptotic pathway in NSCLC cells by inhibiting growth factor signaling

EGFR signaling is associated with the progression and resistance of NSCLC to targeted therapy (Hynes and Lane, 2005). EGFR and downstream components activate pro-survival signaling pathways mainly by regulating protein abundance of members of the BCL-2 family of pro-apoptotic factors (Cragg et al., 2007). Although H1299 and A549 NSCLC cells do not carry EGFR mutations, they overexpress an active form of the receptor as a result of constitutive secretion of EGFR ligands (Putnam et al., 1992). We hypothesized that ERβ1 induces apoptosis in NSCLC cells by regulating EGFR signaling. Immunoblotting analysis revealed reduced expression of EGFR and decreased activity of ERK1/2 that act downstream of EGFR in ERβ1-expressing cells compared to the control cells (Figure 3.5A and B). This effect was observed in the absence of ligands, and the levels of EGFR and phosphorylated ERK1/2 were similar between E2- and 3β-Adiol-treated cells. ERK1/2 are components of the pro-survival signaling pathway that inhibits apoptosis by promoting proteasomal degradation of the pro-apoptotic BCL-2 family member BIM (Hubner et al., 2008). As expected, increased expression of BIM was found in ERβ1-expressing cells compared to the control cells (Figure 3.5A). Furthermore, inhibition of ERK1/2 has been reported to impair the production of EGFR ligands (Toulany et al., 2007). As shown in Figure 3.5C, assessment of the expression of the EGFR intrinsic ligand, EGF, by qRT-PCR revealed reduced EGF mRNA levels in ERβ1-expressing cells. To strengthen our results connecting the pro-apoptotic phenotype in

NSCLC with the specific upregulation of ERβ1, ERβ1 was depleted in ERβ1-expressing H1299 cells by siRNA knockdown. Immunoblotting analysis showed that downregulation of ERβ1 using two specific ERβ siRNAs rescued ERK1/2 phosphorylation and decreased BIM expression and caspase 3 cleavage (Figure 3.5D). Taken together, these results suggest that ERβ1 stimulates pro-apoptotic pathways in NSCLC cells by inactivating EGFR signaling.

Figure 3.4 EFigure 3.5 ERβ1 regulates growth factor signaling in NSCLC cells. EGFR, phospho(p)-ERK1/2 and BIM protein levels in control and ERβ1-expressing H1299 cells treated with vehicle, 10 nM E2 or 10 nM 3β-Adiol for 24 h. The band intensities were quantified and normalized to ERK1/2 (p-ERK1/2) and p84 (EGFR, BIM) intensities. Results are representative of three independent experiments. (**B)** EGFR and p-ERK1/2 levels in control and ERβ1-expressing A549 cells treated with 10 nM E2 or 3β-Adiol for 24 h. Representative immunoblots from three independent experiments are shown. Quantification of the band intensities of EGFR and p-ERK1/2 (normalized with those of ERK1/2) is shown. **(C)** mRNA expression analysis of EGF in lysates from control and ERβ1-expressing H1299 (top panel) and A549 (bottom panel) cells treated with vehicle or 10 nM E2 for 24 h. Data were normalized to vehicle-treated control cells and shown as mean ± SD of three independent experiments. *P-value* (*) ≤ 0.05. **(D)** ERβ1-expressing H1299 cells were transiently transfected with control or two ERβ specific siRNAs (#1 and #2) and analyzed for cleaved-caspase 3, p-ERK1/2 and BIM expression by immunoblotting. Band intensities were quantified and analyzed as described in A and B.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H1299
Control
ERβ1
EGFR
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p-ERK1/2
1 1.2 1.1 0.02 0.02 0.01
ERK1/2
BIM
1 1.2 1.3 22.6 23.5 23.7
ERβ1
p84
E2 - + - - + -
3β-Adiol - - + - - +
B
A549
Control
ERβ1
EGFR
1 0.9 1.1 0.07 0.08 0.05
p-ERK1/2
1 1.2 0.9 0.2 0.1 0.08
ERK1/2
E2 - + - - + -
3β-Adiol - - + - - +
C
H1299
EGF
Relative mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
EtOH E2 EtOH E2
Control ERβ1
A549
EGF
Relative mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
EtOH E2 EtOH E2
Control ERβ1
D
H1299-ERβ1
Scramble siRNA
siRNA#1
siRNA#2
c-Caspase 3
1 0.1 0.1
p-ERK1/2
1 3.4 1.9
ERK1/2
BIM
1 0.1 0.7
ERβ1
1 0.01 0.2
p84

3.2.4 ERβ1 decreases the activity of ERK1/2 by inactivating mutant RAS

To investigate whether repression of EGFR signaling is essential for the inactivation of ERK1/2 and the enhanced apoptosis observed in ERβ1-expressing NSCLC cells, control and ERβ1-expressing H1299 cells were treated with EGF and analyzed for the activity of factors downstream of EGFR signaling. Upon ligand binding, EGFR is activated, and through its interaction with the adaptor proteins GRB2-associated-binding protein 1 (GAB1) and growth factor receptor-bound protein 2 (GRB2), activates the PI3K-AKT and the ERK pathways, respectively. Recruitment of GRB2 to EGFR results in activation of the RAS-RAF signaling cascade, which in turn activates ERK1/2 (Er et al., 2013). As expected, treatment of H1299 cells with EGF rapidly induced EGFR phosphorylation at Tyr1068, which is indicative of EGFR activation and significantly increased the activity of AKT, as shown by its increased phosphorylation at S473 in both control and ERβ1-expressing cells (Figure 3.6A). In contrast, EGFR activation did not reverse the ERβ1-mediated decrease in ERK1/2 phosphorylation, suggesting that ERβ1 decreases the activity of ERK1/2 by acting on a component downstream of EGFR. To confirm this, we overexpressed EGFR in ERβ1-expressing H1299 cells. As shown in Figure 3.6B, EGFR overexpression significantly increased the phosphorylation of AKT, but did not reverse the ERβ1-mediated inactivation of ERK1/2 and the increased expression of BIM. Similarly, treatment of ERβ1-expressing cells that overexpress EGFR with EGF, although profoundly increasing the levels of phosphorylated AKT at S473, failed to significantly increase the activity of ERK1/2 (Figure 3.6C). Consistent with the effect on ERK signaling, overexpression of

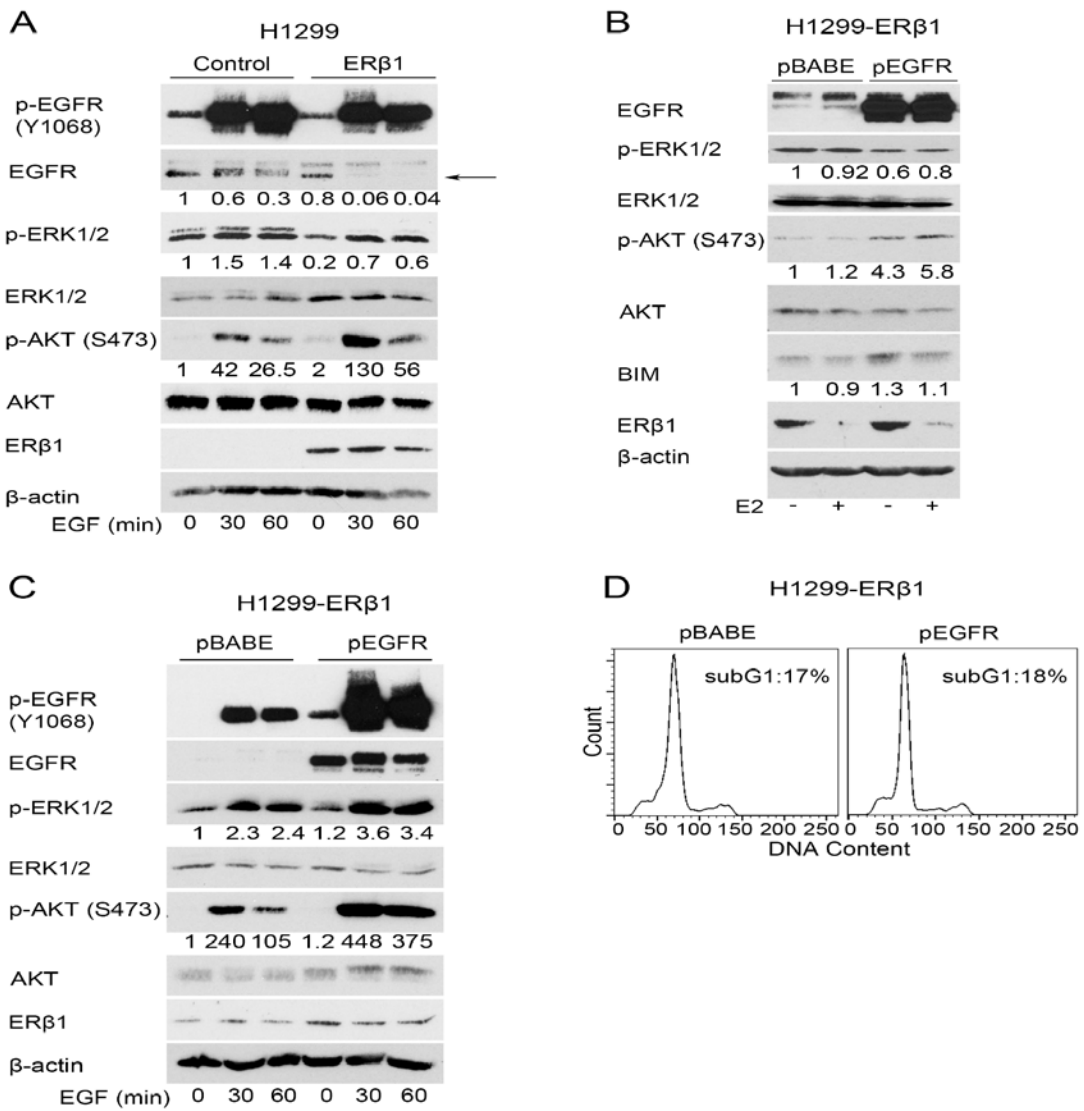
EGFR failed to rescue the H1299 cells from the ERβ1-induced apoptosis, strengthening our hypothesis that ERβ1 induces apoptosis by inhibiting growth factor signaling downstream of receptor tyrosine kinases (Figure 3.6D).

Downstream of EGFR, RAS activates ERK1/2 by interacting with and regulating the activity of RAF. RAS is one of the most frequently mutated genes in NSCLC. Ninety percent of RAS mutations in lung adenocarcinoma represent alterations in K-RAS, and K-RAS mutations correlate with a worse prognosis in lung cancer and are implicated in the resistance to EGFR-TKIs (Pao et al., 2005). We investigated whether RAS is involved in the ERβ1-mediated regulation of EGFR and ERK1/2. We overexpressed mutant N-RAS in ERβ1-expressing H1299 cells and, following incubation in complete media or media lacking growth factors, we analyzed these cells as well as control and ERβ1-expressing cells for EGFR expression and ERK1/2 phosphorylation. Upregulation of mutant N-RAS was found to rescue EGFR for the ERβ1-mediated downregulation, suggesting that the decreased expression of EGFR in ERβ1-expressing cells was RAS-dependent. This effect was more potent in the presence of growth factors suggesting a ligand-mediated regulation of EGFR by RAS and ERβ1 (Figure 3.6A and Figure 3.7A, upper panel). Previous studies have reported increased endocytic trafficking and degradation of EGFR by the downstream AKT in the presence of EGF (Er et al., 2013). In addition, we have previously shown increased degradation of EGFR in ERβ1-expressing triple-negative breast cancer cells in the presence of EGF (Thomas et al., 2012). Taken together, these results suggest that ERβ1 may induce degradation of EGFR in NSCLC cells by regulating

the activity of the downstream RAS. In contrast to restoring EGFR levels, overexpression of mutant N-RAS did not reverse the ERβ1-mediated inactivation of ERK1/2 suggesting that ERβ1 may block the activity of RAS (Figure 3.7A, upper panel). Indeed, as shown in the bottom panel of Figure 3.7A, ERβ1 decreased the activity of RAS in H1299 cells suggesting that ERβ1 may elicit tumor suppressive actions in NSCLC cells by inactivating mutant RAS.

Figure 3.6 EGFR inhibition is not essential for the ERβ1-mediated regulation of ERK activity.

(A) Control and ERβ1-expressing H1299 cells were treated with 100 ng/ml EGF for the indicated times and analyzed for the expression of p-EGFR-Y1068, p-ERK1/2 and p-AKT-S473 by immunoblotting. The EGFR, p-ERK1/2 and p-AKT band intensities were normalized to those of ERK1/2 and AKT, respectively. **(B)** ERβ1-expressing H1299 cells were stably transfected with an empty pBABE vector or pBABE-EGFR plasmid, treated with 10 nM E2 and analyzed for EGFR, p-ERK1/2, ERK1/2, p-AKT, AKT and BIM expression by immunoblotting. Note that E2 treatment causes downregulation of ERβ1 which has been associated with the activation of the receptor. Quantification of the band intensities of p-ERK1/2 and p-AKT (normalized to those of ERK1/2 and AKT) is shown. (**C)** ERβ1-expressing H1299 cells that overexpress EGFR were treated with 100 ng/ml EGF for the indicated times and analyzed for the expression of p-EGFR, p-ERK1/2 and p-AKT by immunoblotting. The intensities of p-ERK1/2 and p-AKT bands were normalized to ERK1/2 and AKT band intensities. **(D)** ERβ1-expressing H1299 cells that carry an empty pBABE vector or overexpress EGFR were analyzed for DNA content by flow cytometry. The mean of sub-G1 cell percentage from three experiments is shown.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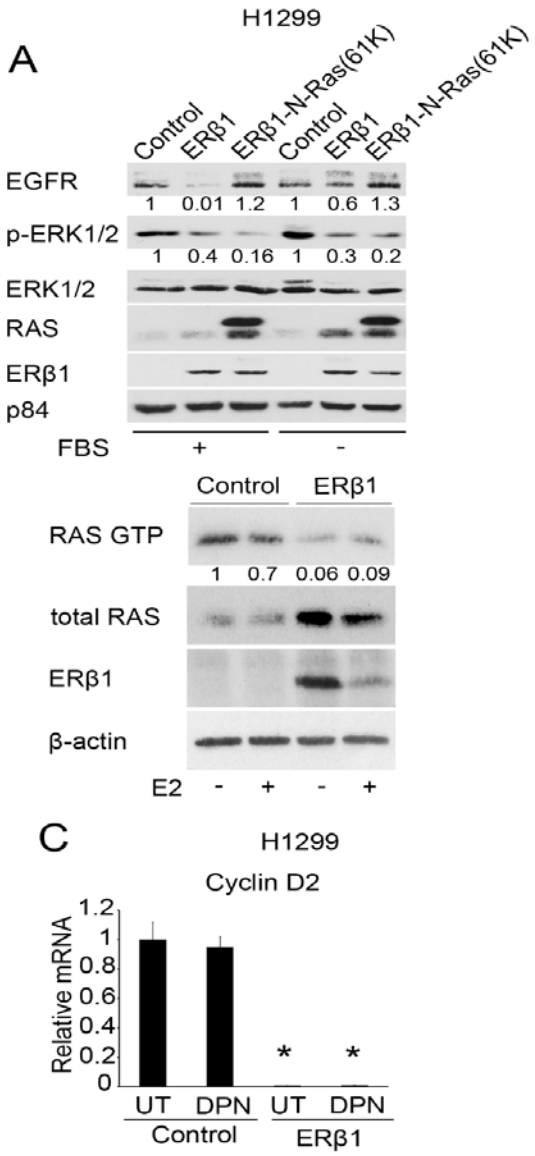
H1299
Control
ERβ1
p-EGFR (Y1068)
EGFR
1 0.6 0.3 0.8 0.06 0.04
p-ERK1/2
1 1.5 1.4 0.2 0.7 0.6
ERK1/2
p-AKT (S473)
1 42 26.5 2 130 56
AKT
ERβ1
β-actin
EGF (min) 0 30 60 0 30 60
B
H1299-ERβ1
pBABE
pEGFR
EGFR
p-ERK1/2
1 0.92 0.6 0.8
ERK1/2
p-AKT (S473)
1 1.2 4.3 5.8
AKT
BIM
1 0.9 1.3 1.1
ERβ1
β-actin
E2 - + - +
C
H1299-ERβ1
pBABE
pEGFR
p-EGFR (Y1068)
EGFR
p-ERK1/2
1 2.3 2.4 1.2 3.6 3.4
ERK1/2
p-AKT (S473)
1 240 105 1.2 448 375
AKT
ERβ1
β-actin
EGF (min) 0 30 60 0 30 60
D
H1299-ERβ1
pBABE
pEGFR
subG1:17%
subG1:18%
Count
0 50 100 150 200 250 0 50 100 150 200 250
DNA Content

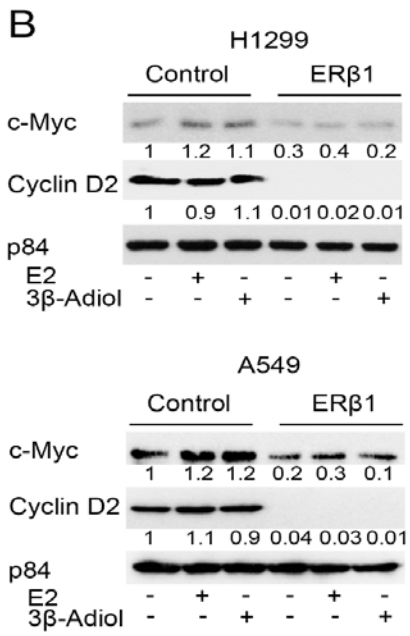
3.2.5 ERβ1 regulates the expression of RAS/ERK signaling mediators

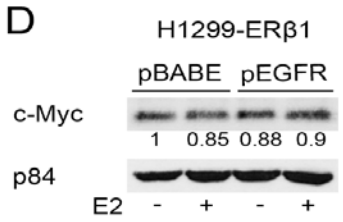
Several factors have been shown to facilitate the maintenance of RAS-dependent tumors (Karachaliou et al., 2013). The inhibition of the transcription factor MYC triggers rapid regression of mutant RAS-induced tumors *in vivo* (Soucek et al., 2008). We investigated whether ERβ1 affects c-MYC, which in response to diverse extracellular and intracellular signals acts downstream of RAS/ERK to promote cell growth. As shown in Figure 3.7B and C, upregulation of ERβ1 decreased the expression of c-MYC and that of the c-MYC target gene Cyclin D2 in H1299, and A549 cells, both in the absence or presence of ERβ ligands. Similarly to the expression of cleaved caspase 3, no difference was observed in the levels of Cyclin D2 between E2- and 3β-Adiol-treated cells. As in the case of RAS and ERK1/2 activity, EGFR upregulation in ERβ1-expressing H1299 cells did not affect the expression of c-MYC strengthening our findings that EGFR downregulation is not the critical event in the ERβ1-mediated inhibition of the signaling that stimulates cell growth (Figure 3.7D). These results demonstrate that ERβ1 downregulates the effectors of the RAS/ERK pathway in NSCLC cells by reducing the activity of RAS independently of EGFR.

Figure 3.6 EGFR in Figure 3.7 ERβ1 decreases the activity of RAS and regulates RAS signaling-associated factors.

(A) Top panel: Control, ERβ1-expressing and ERβ1-expressing H1299 cells that overexpress mutant N-RAS were incubated in 10% FBS-containing media (+) or 1% DCC-containing media (-) for 24 h and analyzed for the expression of EGFR, p-ERK1/2 and RAS by immunoblotting. Bottom panel: RAS activity was assessed in control and ERβ1-expressing H1299 cells following treatment with 10 nM E2. The band intensities were quantified and normalized to p84 and total RAS intensities. (**B)** Control and ERβ1-expressing cells (H1299 top panel, A549 bottom panel) were treated with 10 nM E2 or 3β-Adiol and analyzed for the expression of c-MYC and Cyclin D2 by immunoblotting. **(C)** Cyclin D2 mRNA levels in control and ERβ1-expressing H1299 cells following treatment with 10 nM DPN. Graph shows the mean ± SD of three independent experiments normalized to vehicle-treated control cells. *P-value* (*) ≤ 0.05. **(D)** c-MYC expression in ERβ1-expressing H1299 cells that carry an empty control vector (pBABE) or overexpress EGFR. In B and D, the intensities of c-MYC and Cyclin D2 bands were normalized to p84 intensities and adjusted to those of control untreated cells.


A
H1299
Control
ERβ1
ERβ1-N-Ras(61K)
Control
ERβ1
ERβ1-N-Ras(61K)
EGFR
1 0.01 1.2 1 0.6 1.3
p-ERK1/2
1 0.4 0.16 1 0.3 0.2
ERK1/2
RAS
ERβ1
p84
FBS + -
Control ERβ1
RAS GTP
1 0.7 0.06 0.09
total RAS
ERβ1
β-actin
E2 - + - +
C
H1299
Cyclin D2
Relative mRNA
1.2
1
0.8
0.6
0.4
0.2
0
* *
UT DPN UT DPN
Control
ERβ1


B
H1299
Control ERβ1
c-Myc
1 1.2 1.1 0.3 0.4 0.2
Cyclin D2
1 0.9 1.1 0.01 0.02 0.01
p84
E2 - + - - + -
3β-Adiol - - + - - +
A549
Control ERβ1
c-Myc
1 1.2 1.2 0.2 0.3 0.1
Cyclin D2
1 1.1 0.9 0.04 0.03 0.01
p84
E2 - + - - + -
3β-Adiol - - + - - +


D
H1299-ERβ1
pBABE pEGFR
c-Myc
1 0.85 0.88 0.9
p84
E2 - + - +

3.3 Discussion

The discovery of oncogenic mutations in NSCLC has improved the knowledge of the aberrant molecular signaling found in this lung cancer subtype and led to the development of biomarkers with associated targeted therapeutics ((NGM), 2013). Although EGFR mutations and anaplastic lymphoma kinase (ALK) translocations were successfully targeted with EGFR-TKIs and crizotinib, respectively, direct blockade of mutant K-RAS, which accounts for about 30% of all mutations in lung adenocarcinoma, remains inefficient (Alamgeer et al., 2013, Pao and Chmielecki, 2010). In addition to the overactive growth factor signaling, dysregulation of other pathways that regulate cell growth such as those mediated by estrogen receptors has been linked to lung cancer development and progression. Interestingly, lower levels of circulating estrogen in women with lung cancer over the age of 60 correlated with better survival and hormone replacement therapy has been associated with shorter median survival (K. S. Albain, 2007, Chlebowski et al., 2009). The adverse effects of estrogen could be mediated by either ERα or ERβ since both ERs are expressed in lung tumors according to the NCBI EST profile's databases and studies that analyzed ER protein expression in human lung cancers ((NCBI), Kawai et al., 2005a). However, the correlation of ERβ1 with better outcome and that of ERα with worse survival and poorer prognosis in patients with NSCLC suggest that ERα, that promotes cell proliferation in breast cancer, may also mediate the tumorigenic actions of estrogen in lung tissue (Kawai et al., 2005a, Raso et al., 2009, Nose et al., 2009, Nose et al., 2011, Kawai et al., 2005b, Thomas and Gustafsson, 2011). In

addition to the protective function proposed in lung cancer, ERβ1 is known to inhibit the growth of breast, ovarian, colon, and prostate cancer cells (Thomas and Gustafsson, 2011). Although treatment of NSCLC cells with ERβ agonists has been reported to stimulate cell proliferation, the role of ERβ in regulating cell survival and apoptosis in lung cancer still remains unclear (Hershberger et al., 2009, Zhang et al., 2009).

In this study we carried out experiments to determine functions of ERβ1 in NSCLC cells that may account for its association with the better clinical outcome of patients with NSCLC. Given that mutant RAS correlates with worse prognosis and is implicated in the acquired resistance to EGFR-TKIs, we investigated the role of ERβ1 in regulating cell survival in NSCLC that express wild-type and mutant RAS (Karachaliou et al., 2013). Immunoblotting analysis, based on the use of appropriate controls and different ERβ antibodies that had previously been validated for their specificity, revealed that the NSCLC cells we studied express very low (no detectable) levels of ERβ1 (Figure 3.1C and Figure 3.2) (Thomas et al., 2012, Thomas and Gustafsson, 2011). Induction of ERβ1 expression in these cells profoundly decreased cell growth. The growth inhibitory effects of ERβ1 were mostly observed in the absence of ligand, which is consistent with previous studies demonstrating ligand-independent anti-tumorigenic actions of ERβ1 in different types of cancer cells (Dey et al., 2012, Hartman et al., 2009, Thomas et al., 2012, Thomas and Gustafsson, 2011, Tremblay et al., 1999). However, treatment with E2 further suppressed cell growth in one of the NSCLC cell lines. The same treatment caused downregulation of ERβ1 that has been associated with receptor activation (Hauser et al.,

2000, Pan et al., 2011). This ligand-dependent ERβ1-mediated regulation of cell survival that was observed only in survival assays after long-term treatment of the cells with E2 may suggest the use of specific ERβ1 agonists as potential treatment modality for the clinical management of NSCLC. Further analysis of the NSCLC cells revealed that ERβ1 induces G1/S cell cycle arrest and apoptosis by increasing the levels of the pro-apoptotic marker cleaved caspase 3 and the cell cycle inhibitors p21 and p27.

Interestingly, the cell growth inhibitory effects of ERβ1 were more potent in NSCLC cells that express mutant RAS suggesting the involvement of RAS and growth factor signaling in the ERβ1-mediated regulation of NSCLC cell survival. Upon growth factor binding, EGFR is activated and, through binding the GRB2, promotes the recruitment of guanine nucleotide exchange factors (GEFs) to the plasma membrane where RAS is localized as a result of farnesylation. The increased interaction of GEFs with RAS facilitates the formation of the active GTP-bound state RAS and the subsequent activation of the downstream ERK pathway (Hynes and Lane, 2005). We investigated whether inactivation of the EGFR/RAS/ERK signaling axis was associated with the induction of apoptosis in ERβ1-expressing cells. Indeed, decreased protein levels of EGFR and activity of ERK1/2 were detected in ERβ1-expressing NSCLC cells. These results together with the upregulation of the pro-apoptotic marker BIM that predicts response to EGFR-TKI treatment and is degraded in response to ERK1/2 activation strengthened the inhibition of EGFR-RAS pathway by ERβ1 in NSCLC cells (Toulany et al., 2007, Hubner et al., 2008, Lee et al., 2013a).

Importantly, knockdown of the transfected ERβ1 in NSCLC cells reversed the pro-apoptotic phenotype as shown by the decreased levels of the pro-apoptotic cleaved caspase 3 and BIM and restored ERK1/2 activity. In addition, upregulation of the ERβ splice variant ERβ2 did not significantly affect cell growth and apoptosis in NSCLC cells, strengthening the association of the pro-apoptotic phenotype and the inhibition of the growth factor signaling in NSCLC cells with the specific upregulation of ERβ1. The expression of ERβ2 has been associated with various clinical outcomes in cancer. In particular, it has been correlated to increased survival and invasiveness of prostate and ovarian cancer cells. In breast cancer, nuclear ERβ2 has been associated negatively with metastasis and cytoplasmic ERβ2 with worse outcome (Leung et al., Shaaban et al., 2008). ERβ2 has been suggested to elicit its biological functions by modulating the transcriptional activity of wild-type ERα and ERβ through heterodimerization or by interacting with the membrane and cytoplasmic signaling cascade (Thomas and Gustafsson, 2011). Upregulation of ERβ2 had no significant impact on the survival of NSCLC cells that do not express wild-type ERα and ERβ. However, it might differentially affect the phenotype of NSCLC cells that co-express ERα and ERβ1 by modulating their activity. In such cellular context, in contrast to ERβ1, ERβ2 may increase the survival and metastatic potential of NSCLC cells and tumors.

To provide more insights into the mechanism through which ERβ1 regulates EGFR-RAS pathway in NSCLC cells, we modified the expression of EGFR and mutant RAS in ERβ1-expressing cells. Restoring EGFR activity by EGF treatment or EGFR

upregulation in ERβ1-expressing NSCLC cells, although increasing the activity of the PI3K/AKT pathway, failed to reverse the ERβ1-mediated downregulation of phospho-ERK1/2 and the subsequent upregulation of BIM and enhanced apoptosis. This suggests that direct blockade of oncogenic RAS downstream of EGFR and not downregulation of EGFR may be essential for the apoptosis observed in ERβ1-expressing NSCLC cells. Indeed, induction of ERβ1 expression decreased the activity of RAS and upregulation of mutant RAS in ERβ1-expressing cells reversed the EGFR downregulation indicating the central role of oncogenic RAS inhibition in ERβ1-mediated phenotype in NSCLC cells including the regulation of EGFR (Figure 3.8). The downregulation of EGFR by ERβ1 in the presence of EGF suggests that ERβ1 may induce degradation of EGFR by regulating the activity of downstream RAS. This is consistent with previous studies showing increased degradation of EGFR in cells with altered activity of the downstream AKT (Er et al., 2013). The inhibition of oncogenic RAS by ERβ1 in lung cancer cells was further supported by the downregulation of the effector of the RAS/ERK signaling c-MYC and its direct target Cyclin D2 independent of EGFR in ERβ1-expressing NSCLC cells (Soucek et al., 2008). In addition to lung cancer, certain K-RAS mutations have been associated with worse prognosis in colorectal cancer (Lievre et al., 2008, Mascaux et al., 2005, Pao et al., 2005). Interestingly, ERβ1 has been shown to inhibit the proliferation of the mutant RAS SW480 colon adenocarcinoma cells by downregulating c-MYC and increasing the expression of p27 (Hartman et al., 2009). Given that the expression of both factors was altered in ERβ1-expressing NSCLC cells in which the activity of RAS decreased, it is

possible that an oncogenic RAS inactivation could account for the ERβ1-mediated tumor repressive functions in colon cancer cells.

Although ERβ1 has been previously reported to inhibit the growth of breast, colon, ovarian, and prostate cancer cells, this is the first demonstration that ERβ1 decreases lung cancer cell survival by regulating oncogenic RAS (Dey et al., 2012, Hartman et al., 2009, Thomas et al., 2012, Thomas and Gustafsson, 2011). These results may shed more light into the mechanisms that regulate resistance to targeted therapy in lung cancer cells and explain the association between ERβ1 and outcome of NSCLC patients observed in clinical studies. Further understanding of the mechanisms that suppress oncogenic RAS and decrease cell survival in ERβ1-expressing cells is necessary to establish ERβ1 as a tumor suppressor in NSCLC and as a factor with potential utility in the prognosis and treatment of the disease.

3.4 Summary

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths for both males and females worldwide. In addition to the aberrant growth factor signaling, deregulation of other pathways, such as those mediated by estrogens and their receptors, have been linked to lung cancer initiation and progression. Interestingly, both estrogen receptors have been detected in patients with lung cancer however, their role is poorly understood. In this study, the potential role of ERβ in influencing NSCLC cell growth was examined. It was found that upregulation of wild-type ERβ (ERβ1) suppressed

proliferation and enhanced apoptosis of NSCLC cells. Downregulation of EGFR and inactivation of RAS as well as the downstream mediators ERK1/2 were involved in the ERβ1-induced apoptosis. Manipulation of EGFR and RAS expression and activity in ERβ1-expressing cells revealed the central role of oncogenic RAS in ERβ1-mediated pro-apoptotic phenotype and EGFR regulation.

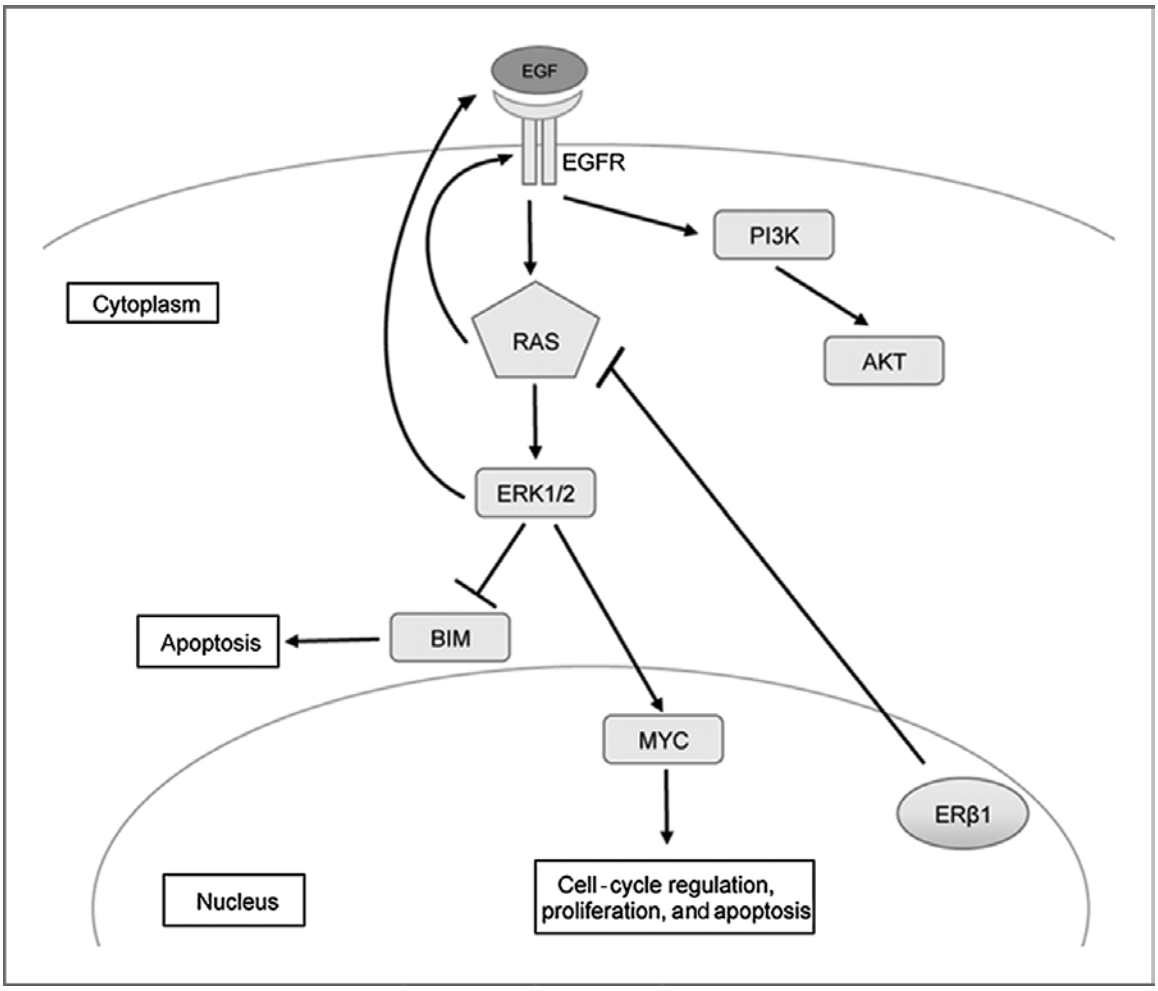


Figure 3.8 Proposed mechanism employed by ERβ1 to regulate NSCLC cell growth and apoptosis.

Aberrant EGFR/RAS signaling leads to constitutive activation of ERK1/2 that promote cell growth and inhibit apoptosis by activating downstream effectors such as c-MYC. By decreasing the activity of RAS, ERβ1 downregulates the active forms of ERK1/2, reduces the expression of c-MYC and upregulates BIM that results in cell growth inhibition and induction of apoptosis. ERβ1 may also downregulate EGFR by blocking the oncogenic RAS in NSCLC cells.

Chapter 4

ERβ1 Increases the Sensitivity of NSCLC Cells to Chemotherapy-induced Cell Death

4.1 Introduction

Lung cancer results in the death of hundreds of thousands of people worldwide every year (Siegel et al., 2014). Non-small cell lung cancer (NSCLC) comprises more than 85% of the diagnosed lung cancers and has a 5-year survival rate of 18% (DeSantis et al., 2014). Despite the advances in early-detection techniques and treatment options, NSCLC is often diagnosed at an advanced stage and has poor prognosis. The standard of care for advanced NSLC includes adjuvant chemotherapy and antineoplastic agents. However, the efficacy of these treatment methods is limited due to intrinsic or acquired resistance (Stewart et al., 2010, Zhang et al., 2014). Several mechanisms have been implicated in the development of drug resistance in cancer. These include increased drug inactivation by detoxifying enzymes, decreased drug activation or binding to target, increased DNA damage tolerance and repair, increased resistance to apoptosis, and activation of survival signaling pathways that counteract the effects of the drugs (Almeida et al., 2008, Stewart et al., 2010). Interestingly, several of the factors implicated in drug resistance are currently under investigation for their use as biomarkers for better selecting patients that will

respond to first-line chemotherapy (Lwin et al., 2013). Thus, developing novel therapeutic approaches aimed to overcome resistance or predict response to the available treatment is crucial for the clinical management of the disease.

Cytotoxic agents elicit their effects through various mechanisms that result in the activation of DNA-damage response and induction of apoptosis. DNA-damaging agents arrest cells in different phases of the cell cycle depending on the status of tumor suppressor protein p53; non-tumor cells that normally express functional p53 are primarily arrested in the G_1 phase due to p53-mediated upregulation of p21, whereas p53-defective tumor cells are arrested in S- and G_2/M-phase checkpoints. Interestingly, expression of wild-type p53 in tumor cells has been reported to impair the apoptotic response to chemotherapy by inducing cell senescence (Jackson et al., 2012). Cancer cells have often deregulated DNA damage response mechanisms (Dixon and Norbury, 2002, Vogelstein et al., 2000, Vousden and Lu, 2002). The combination of p53 deficiency with further disruption of the DNA damage response renders cancer cells sensitive to continuous exposure to chemotherapeutic agents and leads to increased accumulation of DNA damage that activates the apoptotic machinery. This observation has led to several therapeutic strategies that target the residual G_2/M checkpoint in order to specifically sensitize cancer cells to DNA-damaging agents (DiPaola, 2002, Dixon and Norbury, 2002).

The potential role of estrogens in lung cancer development and response to therapy has recently been under investigation (Thomas and Gustafsson, 2011). Several studies have suggested that estrogens and estrogenic compounds may be responsible for

resistance to chemotherapeutic agents (Teixeira et al., 1995, Zampieri et al., 2002). Estrogens elicit their effects in target tissues by binding to ERα and ERβ (Thomas and Gustafsson, 2011). The expression of ERs has been shown to correlate with disease outcome in NSCLC. In particular, expression of ERα in patients with NSCLC has been associated with poor prognosis and correlates with EGFR mutations (Kawai et al., 2005b). Interestingly, the expression of wild-type ERβ (ERβ1) has been associated with better survival among men with NSCLC, while ERβ1 positivity is a favorable predictor of response of patients with lung adenocarcinoma to tyrosine kinase inhibitors (Kawai et al., 2005a, Nose et al., 2009, Nose et al., 2011, Skov et al., 2008). In various tissues (e.g., prostate, breast, colon), ERβ1 has been shown to inhibit cell proliferation by activating pro-apoptotic pathways and down-regulating anti-apoptotic factors both in the presence or absence of a ligand (Thomas and Gustafsson, 2011). More importantly, ERβ has been shown to increase the efficacy of chemotherapeutic agents. Expression of ERβ1 or the ERβ splice variant ERβ5 has been shown to sensitize breast cancer cells to doxorubicin and cisplatin, suggesting that ERβ could be used as a potential biomarker for predicting response to chemotherapy drugs (Lee et al., 2013b, Thomas and Gustafsson, 2011). In contrast to breast cancer, the role of ERβ in modulating the effectiveness of chemotherapeutic agents in NSCLC has not been studied. Here, we show that ERβ1 increases the sensitivity of NSCLC cells to chemotherapeutic agents by inducing G_2/M cell cycle arrest and/or enhancing apoptosis.

4.2 Results

4.2.1 ERβ1 sensitizes p53-defective NSCLC cells to chemotherapeutic agents

The role of ERβ in regulating responses of NSCLC cells to cytotoxic agents has not been examined. Provided that induction of ERβ1 expression in NSCLC cells inhibits cell growth by disrupting oncogenic RAS signaling (Nikolos et al., 2014), we asked whether ERβ1 sensitizes NSCLC cells to chemotherapy-induced cell death. To answer this question, we stably expressed ERβ1 in p53-defective H1299, H358, and H661 NSCLC cells and assessed their survival in the presence of increasing concentrations of doxorubicin. Interestingly, expression of ERβ1 in H1299, H358, and H661 significantly enhanced cell death in response to doxorubicin treatment as indicated by the smaller IC_{50} value in ERβ1-expressing cells compared with the control cells (Figure 4.1 A-C). The effect of ERβ1 on drug-induced cell death was concentration-dependent in all three NSCLC cell lines tested (Figure 4.1 A-C left panels).

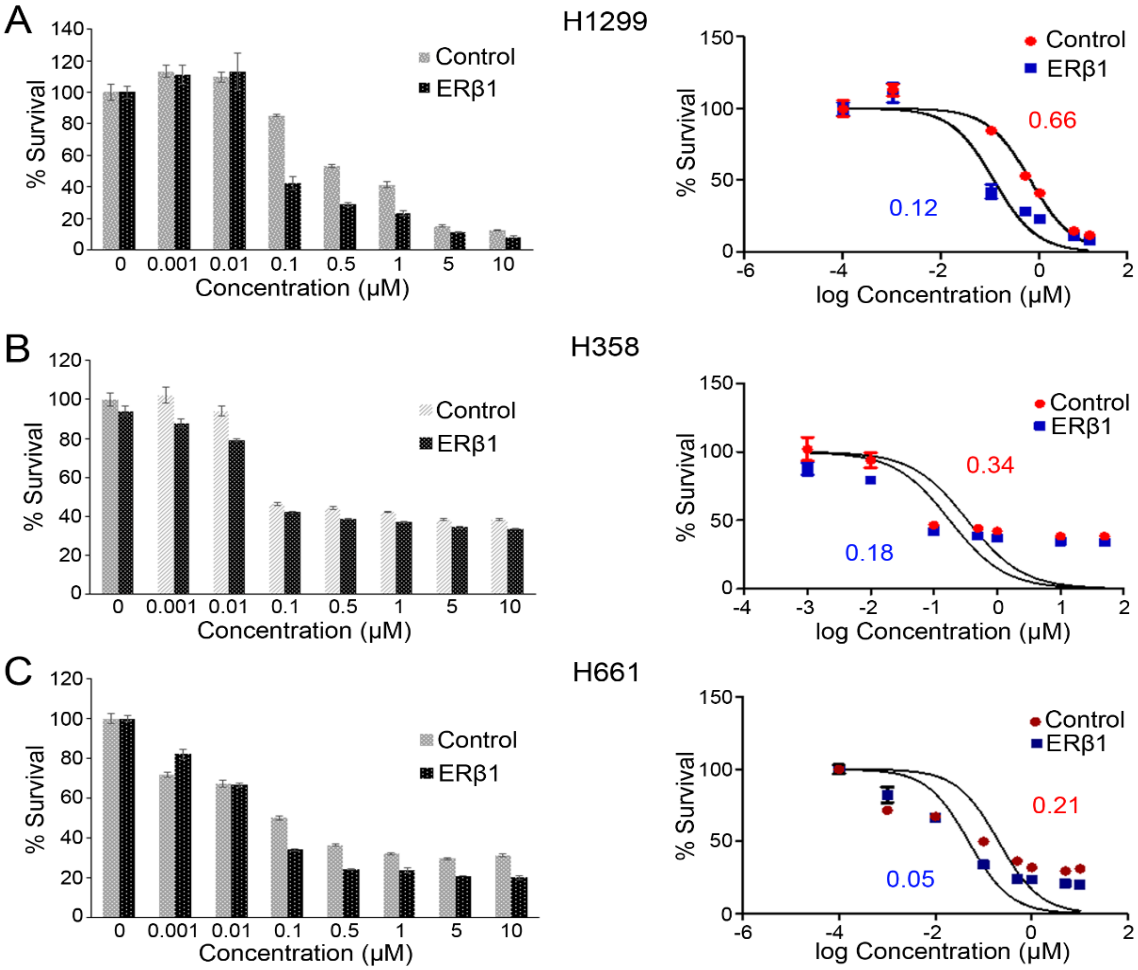


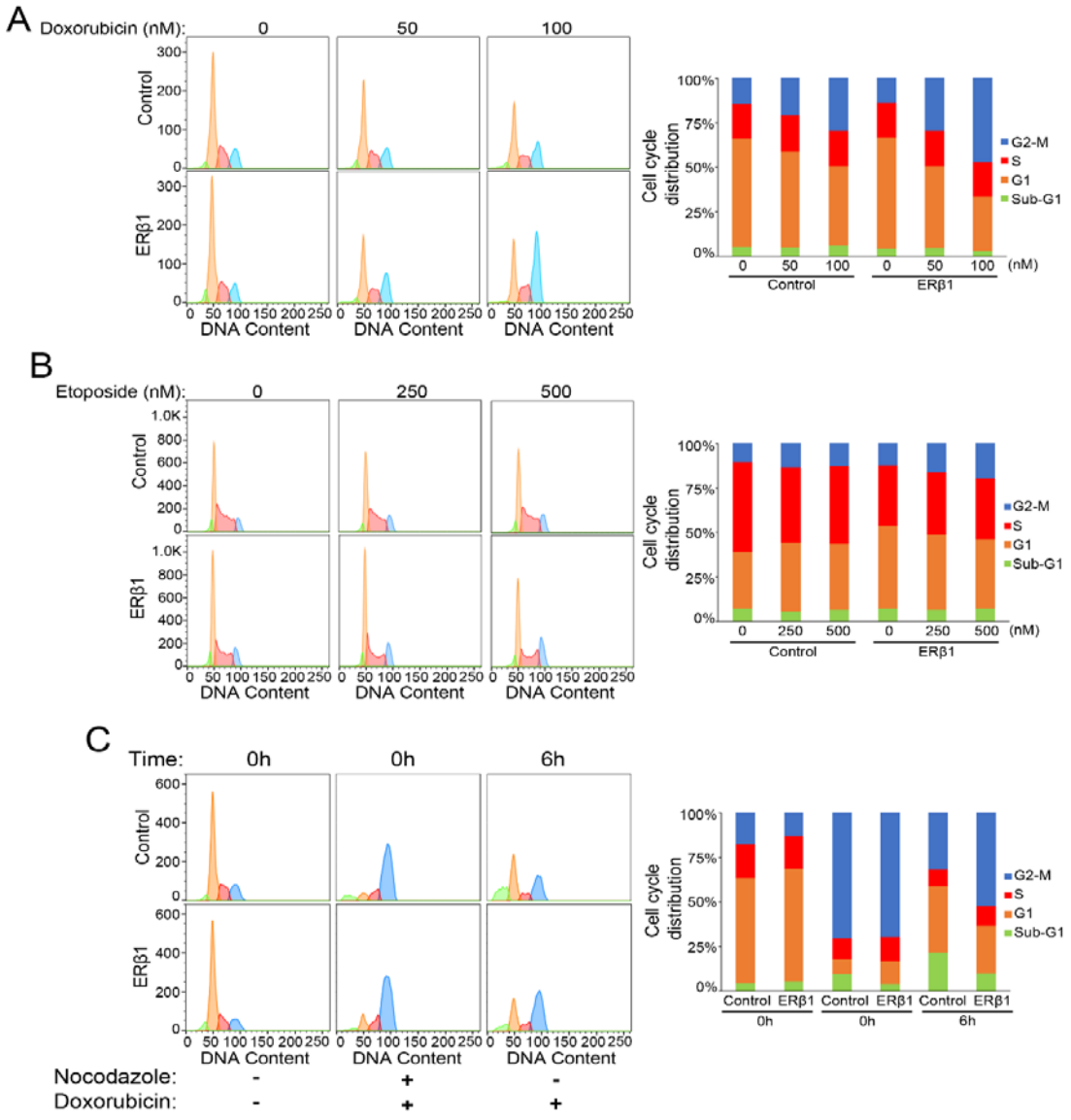
Figure 4.1 ERβ1 sensitizes NSCLC cells to chemotherapeutic drugs. (A-C, left panels) Cell viability assay in control and ERβ1-expressing H1299, H358, and H661 p53-defective NSCLC cells following treatment with increasing concentrations of the topoisomerase II inhibitor doxorubicin. Bar graphs represent mean of three independent experiments with standard error of the mean (SEM) and $p<0.05$. (A-C, right panels) Calculation of inhibitory concentration 50 (IC50) for doxorubicin in control and ERβ1-expressing H1299, H358, and H661 cells. IC50 values represent mean of three independent experiments and $p<0.05$.

4.2.2 ERβ1 induces G2/M checkpoint arrest in H1299 cells

In order to examine the mechanism involved in ERβ1-mediated increased cell sensitivity to DNA-damaging agents, p53-defective control and ERβ1-expressing H1299 cells were exposed to two different concentrations of the topoisomerase II inhibitors doxorubicin and etoposide for 48 hours. Cells were analyzed by flow cytometry for their DNA content. p53-deficient cells respond to DNA damage by arresting in S and G_2/M phases of the cell cycle (Dixon and Norbury, 2002). As expected, cell cycle analysis showed that treatment with doxorubicin and etoposide arrested control and ERβ1-expressing cells in the G_2/M phase of the cell cycle in a concentration-dependent manner (Figure 4.2A and B Left panels). However, the significantly higher percentage of ERβ1-expressing cells in the G_2/M phase compared with the control cells suggests further activation of G_2/M checkpoint by ERβ1 (Figure 4.2A and B right panels).

To further confirm these results, control and ERβ1-expressing H1299 cells were treated with nocodazole in the presence of doxorubicin in order to arrest cells at the G_2/M checkpoint. After 18 hours, cells were released from the nocodazole block and treated additionally for 6 hours with doxorubicin. As seen in Figure 4.2C (right panel), higher percentage of ERβ1-expressing cells remained in the G_2/M phase of the cell cycle compared with the control cells 6 hours post-nocodazole block. Taken together, these results strongly suggest that ERβ1 sensitizes H1299 cells to chemotherapy-induced cytotoxicity by prolonging G_2/M-phase cell cycle arrest.

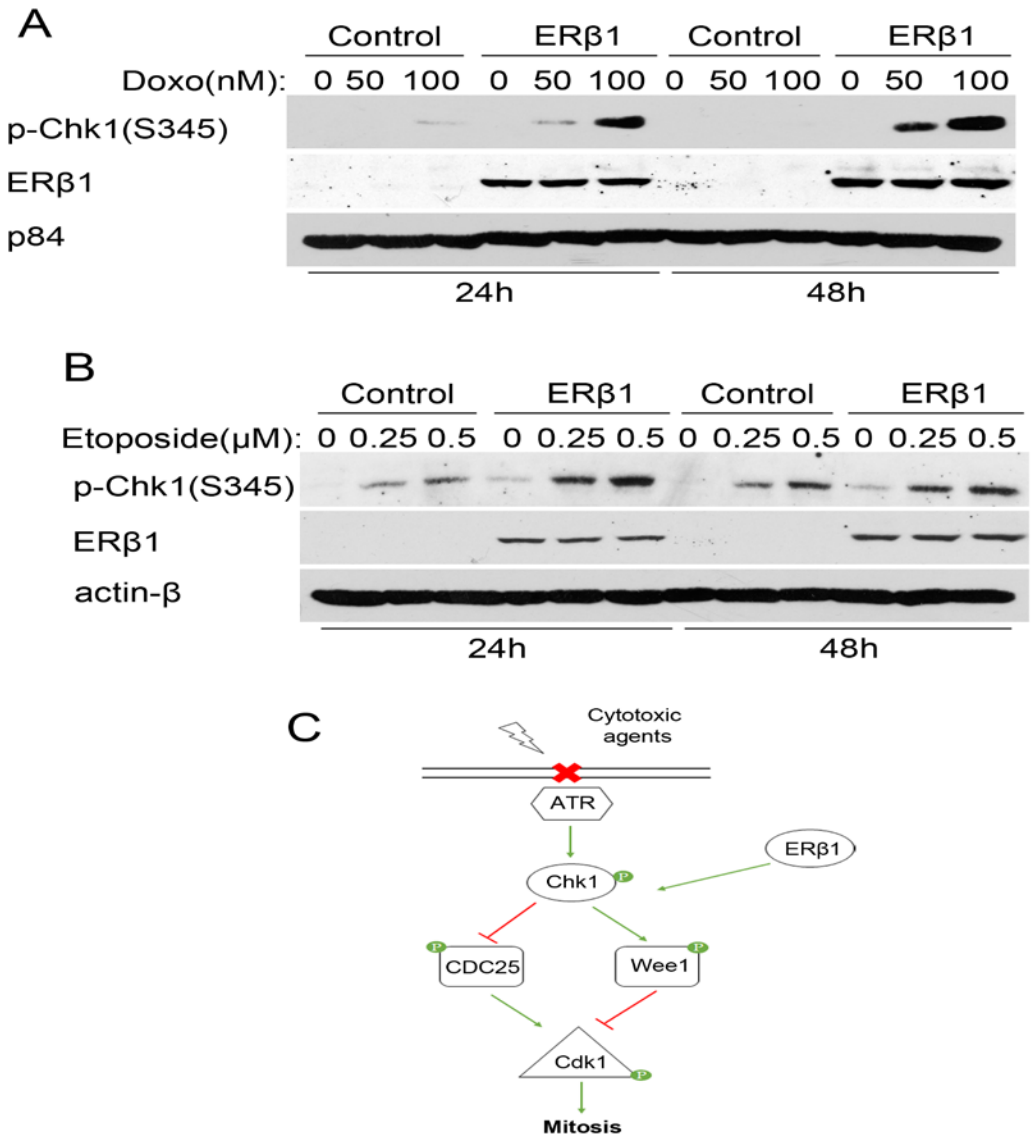
Figure 3.4 EFigure 3.5 ERβ Figure 4.2 ERβ1 induces G_2/M-phase cell cycle arrest in H1299 cells in response to treatment with chemotherapeutic agents. (A-B) Control and ERβ1-expressing H1299 cells were treated with two different concentrations of doxorubicin and etoposide (a topoisomerase II inhibitor) for 48 hours. Cell cycle was analyzed by flow cytometry after staining of the cells with propidium iodide (PI). Bar graphs on the left represent quantitation data of the percentage of the cells in different phases of the cell cycle from three independent experiments. $p<0.05$. (C) Control and ERβ1-expressing cells were either left untreated (left panel) or treated with 30 nM nocodazole and 100 nM doxorubicin. 18 hours later nocodazole block was removed (middle panel) and cells were additionally treated with 100 nM doxorubicin for 6 hours. Untreated and treated cells were subjected to cell cycle analysis by flow cytometry after they were stained with PI. The percentage of the cells in different phases of the cycle from three independent experiments was quantified and the mean is represented in the bar graph on the left.


A
Doxorubicin (nM):
0
50
100
Control
ERβ1
DNA Content
Cell cycle distribution
G2-M
S
G1
Sub-G1
(nM)
B
Etoposide (nM):
0
250
500
C
Time:
0h
0h
6h
Nocodazole:
Doxorubicin:

4.2.3 ERβ1 regulates the G_2/M checkpoint components

Upon DNA damage, sensor proteins such as Rad3 detect the sites of damaged DNA and activate the DNA damage response pathways. A key component of the DNA damage response machinery is Chk1 that is activated via phosphorylation at S345 (Kuntz and O'Connell, 2009, Zhou and Elledge, 2000). At the G_2/M checkpoint, S345-p-Chk1 controls cell cycle progression by phosphorylating CDC25 and Wee1 that leads to cell cycle arrest (Figure 4.3C). In order to further elucidate the mechanism of ERβ1-mediated cell sensitization to chemotherapeutic agents, control and ERβ1-expressing H1299 cells were treated with two different concentrations of doxorubicin and etoposide for 24 and 48 hours and the phosphorylation status of Chk1 was examined. ERβ1-expressing cells displayed increased phosphorylated levels of Chk1 at S345 upon treatment with both doxorubicin and etoposide when compared with the control cells (Figure 4.3A and B) in a dose- and time-dependent manner. Importantly, these results further confirm the involvement of ERβ1 in the prolonged activation of the G_2/M checkpoint corroborated by the cell cycle analysis data.

Figure 4.3 ERβ1 modulates components of the DNA damage response machinery. (A-B) Immunoblots of p-Chk1 (S354) and ERβ1 from control and ERβ1-expressing H1299 cells after treatment with two different concentrations of doxorubicin and etoposide for 24 and 48 hours. (C) Cartoon model of G2/M checkpoint activation upon DNA damage.


A
Control
ERβ1
Control
ERβ1
Doxo(nM):
0 50 100 0 50 100 0 50 100 0 50 100
p-Chk1(S345)
ERβ1
p84
24h
48h
B
Control
ERβ1
Control
ERβ1
Etoposide(μM):
0 0.25 0.5 0 0.25 0.5 0 0.25 0.5 0 0.25 0.5
p-Chk1(S345)
ERβ1
actin-β
24h
48h
C
Cytotoxic agents
ATR
ERβ1
Chk1
CDC25
Wee1
Cdk1
Mitosis

4.2.4 ERβ1 induces apoptosis in H358 cells upon treatment with cytotoxic agents

In order to investigate whether ERβ1 sensitizes H358 cells to chemotherapy agents by prolonging G_2/M checkpoint activation, control and ERβ1-expressing H358 cells were treated with two different concentrations of doxorubicin and etoposide for 48 hours. Surprisingly, cell cycle analysis revealed that expression of ERβ1 significantly increased the percentage of apoptotic cells in the presence of chemotherapeutic agents (indicated by the sub-G_1 cell population) compared with the control cells but not the percentage of the G_2/M population (Figure 4.4A and B). These results suggest that ERβ1 induces apoptosis in H358 upon treatment with cytotoxic drugs. However the mechanisms involved are yet to be determined. Taken together, these results suggest that ERβ1 employs various mechanisms of action depending on the cell type in order to confer sensitivity to chemotherapeutic agents.

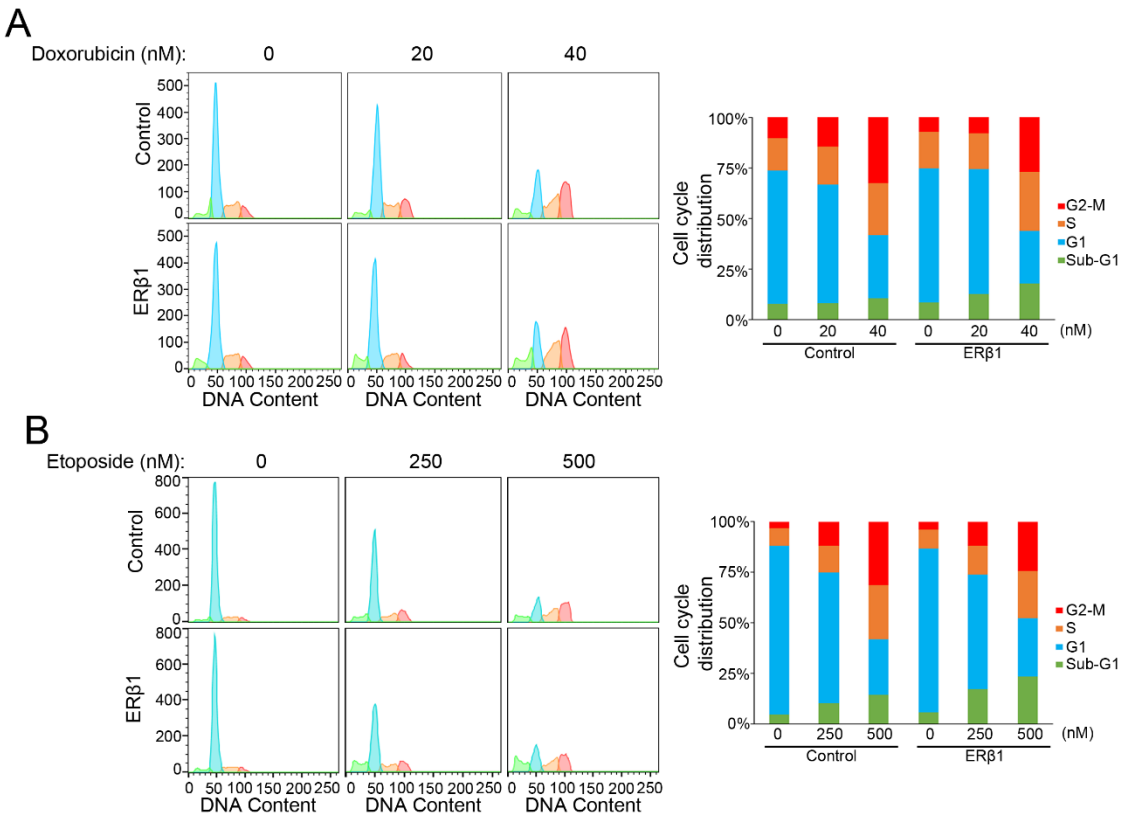


Figure 4.4 ERβ1 induces apoptosis in H358 cells in response to treatment with chemotherapeutic agents. (A-B) Control and ERβ1-expressing p53-null H358 cells after treatment with two different concentrations of doxorubicin and etoposide for 48 hours. Cell cycle was analyzed by flow cytometry after staining of the cells with PI. Bar graphs on the right represent quantitative data of the percentage of cells in different phases of the cell cycle from three independent experiments. $p<0.05$

4.3 Discussion

Defects in the pathways that facilitate repair of DNA damage are common feature in human cancers rendering cancer cells vulnerable to DNA-damaging agents. Strategies are currently under development that aim to exploit this vulnerability by targeting the remaining DNA damage response pathways, which is thought to increase the tumor-specific toxicity of chemotherapeutic agents particularly in the case of p53-deficient tumors (Dixon and Norbury, 2002).

Increased expression of ERβ has been positively associated with disease outcome in patients with NSCLC (Nose et al., 2011, Thomas and Gustafsson, 2011). Furthermore, ERβ1 regulates NSCLC cell growth *in vitro* by suppressing mutant Ras signaling (Nikolos et al., 2014). Taken together, these data suggest that ERβ1 is an important factor in NSCLC biology; thus, we sought to investigate whether ERβ1 sensitizes NSCLC cells to chemotherapeutic agents by modulating DNA damage response mechanisms. Here, we report that expression of ERβ1 in p53-defective NSCLC cells significantly decreased cell survival following DNA damage with chemotherapeutic agents. p53-defective cells respond to DNA damage by arresting into the S- or G_2/M phase of the cell cycle in order to repair the damage (Dixon and Norbury, 2002). So, we investigated whether ERβ1 decreases the survival of p53-deficient NSCLC cells in response to chemotherapy treatment by modulating the activation of the G_2/M checkpoint. Cell cycle analysis revelead that in response to doxorubicin and etoposide treatment ERβ1 arrests H1299 cells in the G_2/M phase in a concentration-dependent manner. Induction of G_2/M arrest by

nocodazole block followed by release of the cells confirmed that ERβ1 prolonged the G_2/M phase arrest of the cells (Figure 4.2C).

Chk1 is a key mediator of DNA damage response machinery conserved among species (Zhou and Elledge, 2000). In p53-defective cells, activation of Chk1 by DNA damage results in G_2/M arrest of the cell cycle. Immunoblot analysis revealed that following treatment with doxorubicin and etoposide ERβ1-expressing cells have increased levels of phosphorylated (active) Chk1 at S345 compared with the control cells. The increase in S345-p-Chk1 was time- and concentration-dependent. Taken together, cell cycle analysis and immunobloting results support that ERβ1 decreases the viability of H1299 cells after DNA damage by prologing activation of the G_2/M chekpoint that results in cells being held in the G_2/M phase of the cell cycle.

Interestingly, the decreased survival of ERβ1-expressing H358 cells in response to doxorubicin and etoposide treatment was not associated with the delay of the cells in the G_2/M phase. On the contrary, ERβ1 enhanced chemotherapy-induced cytotoxicity in H358 cells by activating apoptosis (Figure 4.4). However, the mechanistic details of the ERβ1-induced apoptosis are still under investigation. These data suggest that ERβ1 utilizes various mechanisms in order to sensitize cells to chemotherapy-induced cell death.

Taken together, our results support a role for ERβ1 in modulating DNA damage response pathways in NSCLC. Assesing the expression of ERβ1 in patients with NSCLC might hold a predictive value for the successful response to chemotherapy of p53-defective cancers.

4.4 Summary

Chemotherapy drugs are the standard of care for patients with advanced metastatic NSCLC. However, a significant portion of patients do not respond to chemotherapeutic agents due to intrinsic resistance of the tumor cells, and those who initially respond eventually become resistant. Thus, developing novel therapeutic approaches aimed to overcome resistance or predict response to the available treatment is crucial for the clinical management of NSCLC. In this study the potential role of ERβ in sensitizing NSCLC cells to cytotoxic agents was investigated. It was found that upregulation of ERβ1 decreased the viability of doxorubicin- and etoposide-treated p53-defective NSCLC cells by inducing G_2/M phase cell cycle arrest and/or enhancing apoptosis. In response to treatment, ERβ1-expressing cells had increased p-Chk1 levels, an indicator of activated DNA damage response, compared with the control cells. Taken together, these results support a role for ERβ1 in modulating DNA damage response pathways in NSCLC. Assessing the expression of ERβ1 in patients with NSCLC might hold a predictive value for the successful response of p53-defective cancers to chemotherapeutic agents.

Chapter 5

ERβ1 Represses Basal-like Breast Cancer EMT by Regulating EGFR Degradation

5.1 Introduction

In the recent years, several gene expression studies have identified various intrinsic subtypes of breast cancer with distinct outcome and response to therapy (Perou et al., 2000, Prat and Perou, 2011). Among them, basal-like breast cancers, which show a partial overlap with the triple negative breast cancers, are characterized by the expression of myoepithelial markers of the normal breast such as EGFR, p63, and cytokeratins CK14, CK5/6 and CK17 (Perou et al., 2000, Sorlie et al., 2003). Based on gene expression profiling, approximately 75% of triple-negative breast cancers are classified as basal-like tumors. Basal-like tumors are often resistant to chemotherapy and develop metastases to the lung and brain (Kreike et al., 2007, Perou et al., 2000, Rakha et al., 2008a). Lately, basal-like phenotypes have been shown to correlate with epithelial to mesenchymal transition (EMT) (Sarrio et al., 2008).

EMT is a cellular process characterized by the loss of cell adhesion and is a crucial step in tumor metastasis (Thiery, 2002, Yori et al., 2011). Cell adhesion is lost due to downregulation of cell junction proteins such as CD44 and E-cadherin (Hazan et al., 2004,

Maeda et al., 2005). The decrease in E-cadherin is mediated by increased expression or activation of a number of transcriptional repressors such as SLUG, SNAIL, ZEB-1, ZEB-2, and TWIST (Bolos et al., 2003, Comijn et al., 2001, Eger et al., 2005, Yang et al., 2004). Expression of microRNA 200 family and microRNA 205A as well as upregulation of EGFR signaling have been shown to influence expression of E-cadherin by regulating the transcriptional repressors ZEB-1 and ZEB-2 (Shin and Blenis, 2010, Shin et al., 2010). Thus, by promoting migration and invasion during the progression of breast carcinomas, EMT is an essential process for breast cancer metastasis.

The role of estrogen receptors in regulating EMT has not been fully elucidated (Thomas and Gustafsson, 2011). Recent reports have suggested a role for ERα in regulating breast cancer migration and invasion (Wang et al., 2007, Ye et al.). In addition, the expression of ERβ1 and its splice variants ERβ2 and ERβ5 has been associated with the regulation of migration and invasion in prostate cancer (Leung et al., Mak et al.). Downregulation of ERβ1 has been shown to promote EMT in prostate cancer cells that correlated with the loss of ERβ1 in high Gleason grade invasive prostate carcinoma (Mak et al.). Approximately 60% of basal-like breast cancers have been shown to express wild-type ERβ (ERβ1). Clinical studies have shown an inverse correlation between ERβ1 positivity and expression of EGFR, an essential component of basal-like cancers that promotes proliferation and EMT (Marotti et al., 2010). Intriguingly, patients with triple-negative breast cancer that were treated with adjuvant tamoxifen had significantly better survival when the tumors were ERβ1 positive (Honma et al., 2008). Given the

downregulation of ERβ1 during breast cancer progression, we hypothesized that ERβ1 functions to maintain an epithelial phenotype in breast cancer and investigated whether ERβ1 suppresses invasiveness of breast cancer cells by regulating EMT (Leygue et al., 1996).

5.2 Results

5.2.1 ERβ1 is required for the epithelial breast cancer phenotype

Basal-like cancers are high-grade (grade III), ERα negative invasive breast tumors that express EMT markers and show cadherin switching as a consequence of tumor de-differentiation (Sarrio et al., 2008). Recent reports showed that ERβ1 expression declines from breast ductal carcinoma in situ (DCIS) to invasive cancer and represses mesenchymal characteristics in invasive prostate cancer (Mak et al., Shaaban et al., 2003, Skliris et al., 2003). We hypothesized that ERβ1 regulates EMT in breast cancer and that low ERβ1 expression in a proportion of basal-like cancers is associated with mesenchymal characteristics and poor clinical outcome. To test this hypothesis, we stably expressed ERβ1 in the invasive triple-negative breast cancer MDA-MB-231 and Hs578T cells and compared the expression levels achieved in these cells with the endogenous expression of ERs in MCF-7 cells (Figure 5.1).

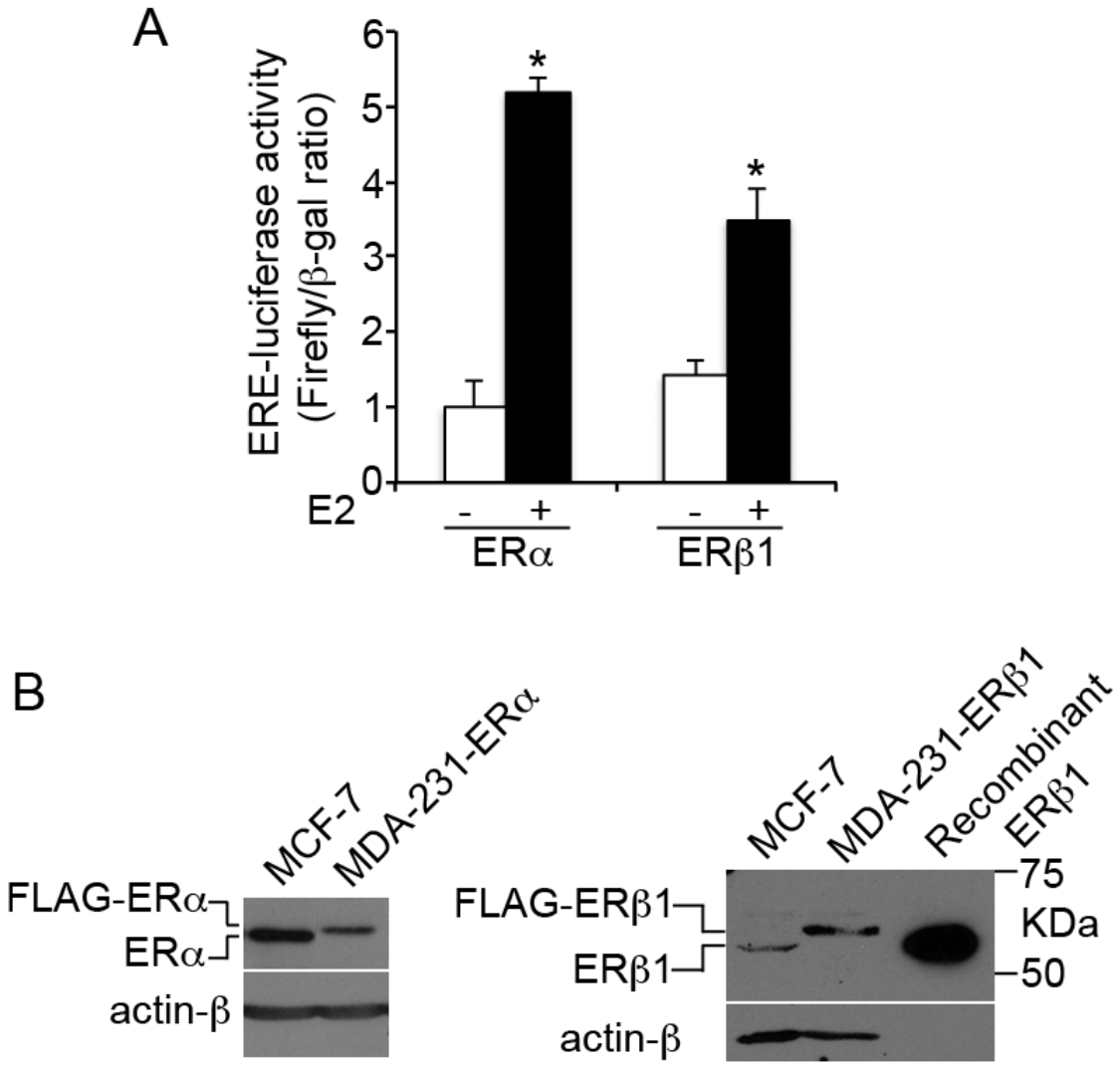


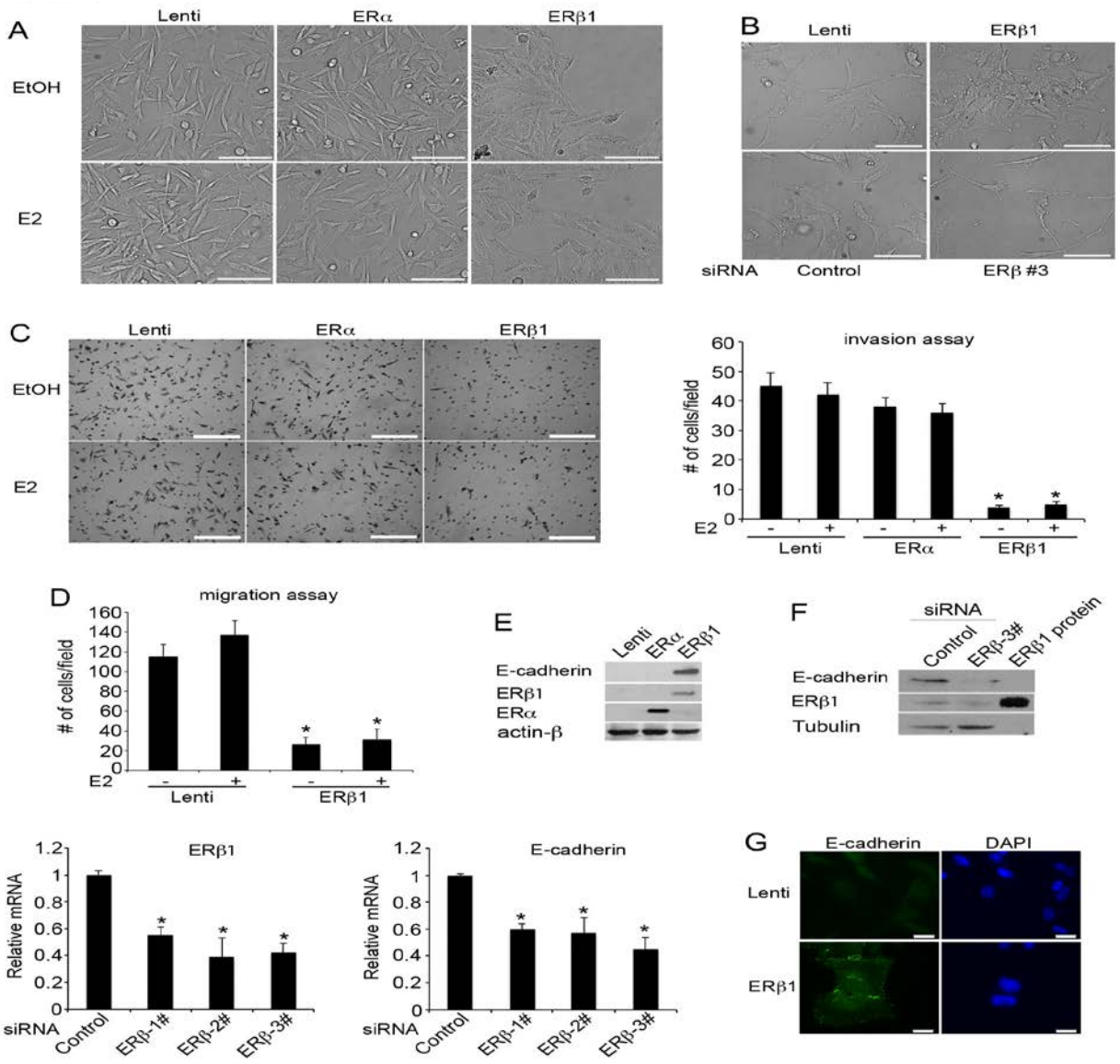
Figure 5.1 Functional analysis of ERα and ERβ1 in MDA-MB-231 cells.
(**A**) Luciferase reporter assay in ERα and ERβ1-expressing MDA-MB-231 cells demonstrating similar activation of an ERE-luciferase reporter following incubation with 10 nM E2. The graph represent the mean of three independent experiments with standard error of the mean (SEM) and *P<0.05 indicated. (**B**) Immunoblots of ERα and ERβ1 in parental MCF-7 and ERα- and ERβ1-expressing MDA-MB-231 cells. MCF-7 cells express relatively high levels of ERα and relatively low levels of ERβ1. The immunoblots indicate lower expression of ERα in ERα-expressing MDA-MB-231 cells compared to the endogenous ERα in MCF-7 cells and higher expression of ERβ1 in ERβ1-expressing MDA-MB-231 cells compared to endogenous ERβ1 in MCF-7 cells.

The gene expression profile of MDA-MB-231 and Hs578T cells resembles the claudin-low breast cancer subtype; however, as basal-like tumors, they display low expression of the luminal and HER2 gene clusters and express low amounts of ERβ1 (Prat et al., 2010). Expression of ERβ1, induced morphological changes in MDA-MB-231 and Hs578T cells characterized by the loss of the "fibroblastoid-like" phenotype and the acquisition of an epithelial-like compact morphology (Figure 5.2A and B, upper panel). The morphology of MDA-MB-231 and Hs578T cells altered in ERβ1-expressing cells in the absence of ligand, and treatment with 17β-estradiol failed to induce additional changes (Figure 5.2A). Furthermore, a more spindle-shaped morphology was observed when endogenous ERβ1 was knocked down with ERβ siRNA in Hs578T cells (Figure 5.2B, lower panel). Consistent with the changes in the morphology, induction of ERβ1 expression in MDA-MB-231 cells repressed invasion and migration (Figure 5.1C and D), cellular processes characteristic of EMT (Yang and Weinberg, 2008). Although induction of ERβ1 and ERα expression resulted in a similar activation of an ERE-luciferase reporter, ERα failed to promote epithelial morphology and reduce the invasiveness of MDA-MB-231 cells (Figure 5.2A and C; Figure 5.1). Similar to the impact on the cellular morphology and invasiveness, only ERβ1 inhibited cadherin switching, as shown by the up-regulation of epithelial E-cadherin in ERβ1-expressing MDA-MB-231 and Hs578T cells (Figure 5.2E). The positive correlation between ERβ1 and E-cadherin expression was confirmed by the decrease of E-cadherin mRNA and protein levels when ERβ1 was knocked down in MDA-MB-231 cells (Figure 5.2F). In line with the results from the immunoblotting analysis,

immunofluorescence showed higher expression of E-cadherin on the cell surface of the ERβ1-expressing cells compared with the control cells (Figure 5.2G). This suggests that ERβ1 up-regulates the functional form of E-cadherin that promotes cell-cell adhesion. No alteration in the levels of the mesenchymal marker vimentin was detected in ERβ1-expressing MDA-MB-231 cells suggesting that ERβ1 induces cell-cell adhesion in these cells by primarily regulating the expression of E-cadherin (Figure 5.2B).

Figure 5.2 ERβ1 inhibits invasion and migration in breast cancer cells by regulating EMT.

(**A**) Bright field microscopy of Control (Lenti), ERα- and ERβ1-expressing MDA-MB-231 cells upon treatment for 24h with EtOH or 17β-estradiol (E2) (scale bars, 50 μm). (**B**) Bright field microscopy of Control (Lenti) and ERβ1-expressing Hs578T cells (upper panel) and Hs578T cells that were transiently transfected with a siRNA targeting luciferase (Control) or a specific ERβ siRNA (siRNA 3#) (lower panel) (scale bars, 100 μM). (**C**) Control (Lenti), ERα- and ERβ1-expressing MDA-MB-231 cells following treatment with EtOH or E2 were assessed for invasion by using matrigel-coated Transwell chambers. The cells that translocated to the lower surface of the filter are shown (left panel) (scale bars, 500 μm). The graph represents the mean (cell number per field) of three independent experiments with the SEM and P-value (*) ≤0.05% indicated. (**D**) Control (Lenti) and ERβ1-expressing MDA-MB-231 cells were incubated with E2 for 24 h and assessed for migration using wound-healing assay. The bar graph shows the mean (cells migrated into the wound) of three separate experiments with SEM and P-value (*) ≤0.05% indicated. (**E**) Immunoblot of E-cadherin protein levels in control (Lenti), ERα or ERβ1-expressing MDA-MB-231 cells. (**F**) E-cadherin expression was analyzed by immunoblotting in MDA-MB-231 cells transfected with control or ERβ siRNA (3#) (upper panel) and qPCR in MDA-MB-231 cells transfected with control or three specific ERβ siRNAs (lower panel). The graph indicates the mean of three separate experiments with SEM and P-value (*) ≤0.05%. (**G**) E-cadherin was visualized by immunofluorescence in control (Lenti) and ERβ1-expressing cells (scale bars, 20 μm).


A
Lenti
ERα
ERβ1
EtOH
E2
B
Lenti
ERβ1
siRNA
Control
ERβ #3
C
Lenti
ERα
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EtOH
E2
invasion assay
# of cells/field
E2
Lenti
ERα
ERβ1
D
migration assay
# of cells/field
E2
Lenti
ERβ1
E
Lenti
ERα
ERβ1
E-cadherin
ERβ1
ERα
actin-β
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siRNA
Control
ERβ-3#
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E-cadherin
Relative mRNA
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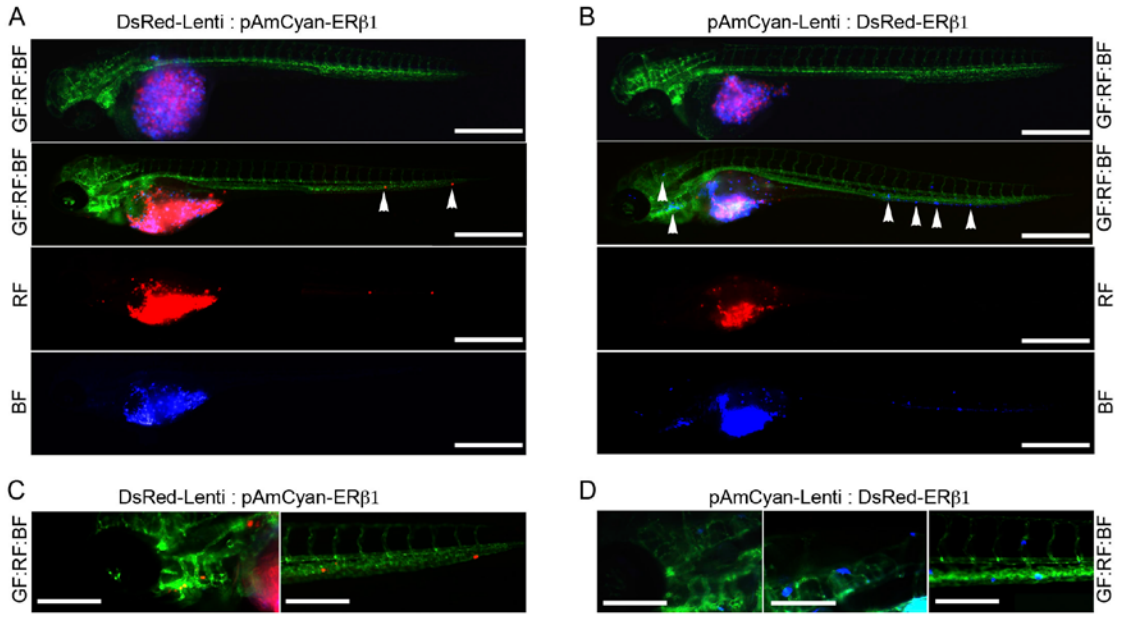
ERβ-1#
ERβ-2#
ERβ-3#
G
E-cadherin
DAPI
Lenti
ERβ1

5.2.2 ERβ1 inhibits invasion of breast cancer cells in vivo

To study the role of ERβ1 in regulating early events of the metastatic cascade, we used a zebrafish tumor model. The Tg(flk1:EGFP)/casper zebrafish embryos that lack pigmentation and express green fluorescent protein (GFP) in the vascular system for direct visualization of vascular development (Lee et al., 2009) were implanted with the highly metastatic human MDA-MB-231 cells. Both control (Lenti) and ERβ1-expressing MDA-MB-231 cells were stably transfected with either DsRed or AmCyan fluorescent proteins. A pool of either control-DsRed and ERβ1-AmCyan cells or control-AmCyan and ERβ1-DsRed cells were injected into the perivitelline cavity at 48 hours post-fertilization (hpf), at which time the immune system of the fish is not yet developed. The zebrafish were first imaged 3 h after implantation (Figure 5.3A and B, upper panels) and invasion and dissemination of DsRed and AmCyan cells were monitored daily. At five days post-injection (dpi), both DsRed and AmCyan MDA-MB-231 control cells had significantly disseminated away from the primary injection site, including the head and the tail regions, whereas ERβ1-expressing MD-MB-231 cells labeled with either DsRed or AmCyan remained at the primary site (Figure 5.3 A-D). Out of 45 embryos that were injected with both control and ERβ1-expressing cells, 27 embryos had disseminated control cells, and only 2 embryos had disseminated control and ERβ1-expressing cells. However, in these two zebrafish, the ratio of control:ERβ1 disseminated cells was more than 8:1 (Figure 5.3E). Our results show that the difference in metastatic potential between the control and the ERb1-expressing cells is due to their different capacity to invade and disseminate.



E

Type of fluorescent cells injected	# of fish studied	# of fish with disseminated cells at 5 dpi*	Location of disseminated cells		
			Head	Trunk	Back
Lenti (DsRed); ERβ1 (AmCyan)	25	10 (DsRed)	5	3	5
Lenti (AmCyan); ERβ1 (DsRed)	20	17 (AmCyan) 2 (DsRed)	4 -	3 2	16 -

*dpi: days post injection

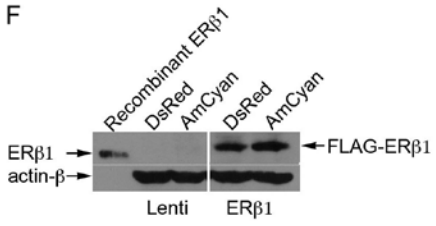


Figure 5.3 ERβ1 inhibits tumor cell invasion, dissemination and micrometastasis *in vivo*.

Control (Lenti) and ERβ1-expressing MDAMB- 231 cells were stably transfected with pAmCyan or pCMCV-DsRed vector. A tumor cell suspension containing equal numbers of either DsRed-Lenti:AmCyan-ERβ1 cells (**A**) or AmCyan-Lenti:DsRed-ERβ1 cells (**B**) were injected into perivitelline space of 48 hpf embryos and tumor cell invasion and dissemination were detected using fluorescent microscopy at 5 dpi. The upper panels show the zebrafish 3 hpi. Arrowheads indicate disseminated tumor cells (Scale bar, 500 μm). (**C** and **D**) High magnification micrographs of A and B, respectively (scale bar, 100 μm). (**E**) Table showing the number of zebrafish injected with either DsRed-Lenti:AmCyan-ERβ1 or AmCyan-Lenti:DsRed-ERβ1 MDA-MB-231 cells, the number of zebrafish with disseminated human tumor cells and the number of the zebrafish with disseminated cells in different regions of the body. (**F**) DsRed-Lenti, AmCyan-Lenti, DsRed- ERβ1 and AmCyan-ERβ1 MDA-MB-231 cells were analyzed for ERβ1 expression by immunoblotting. (BF, blue filter; RF, red filter; GF, green filter).

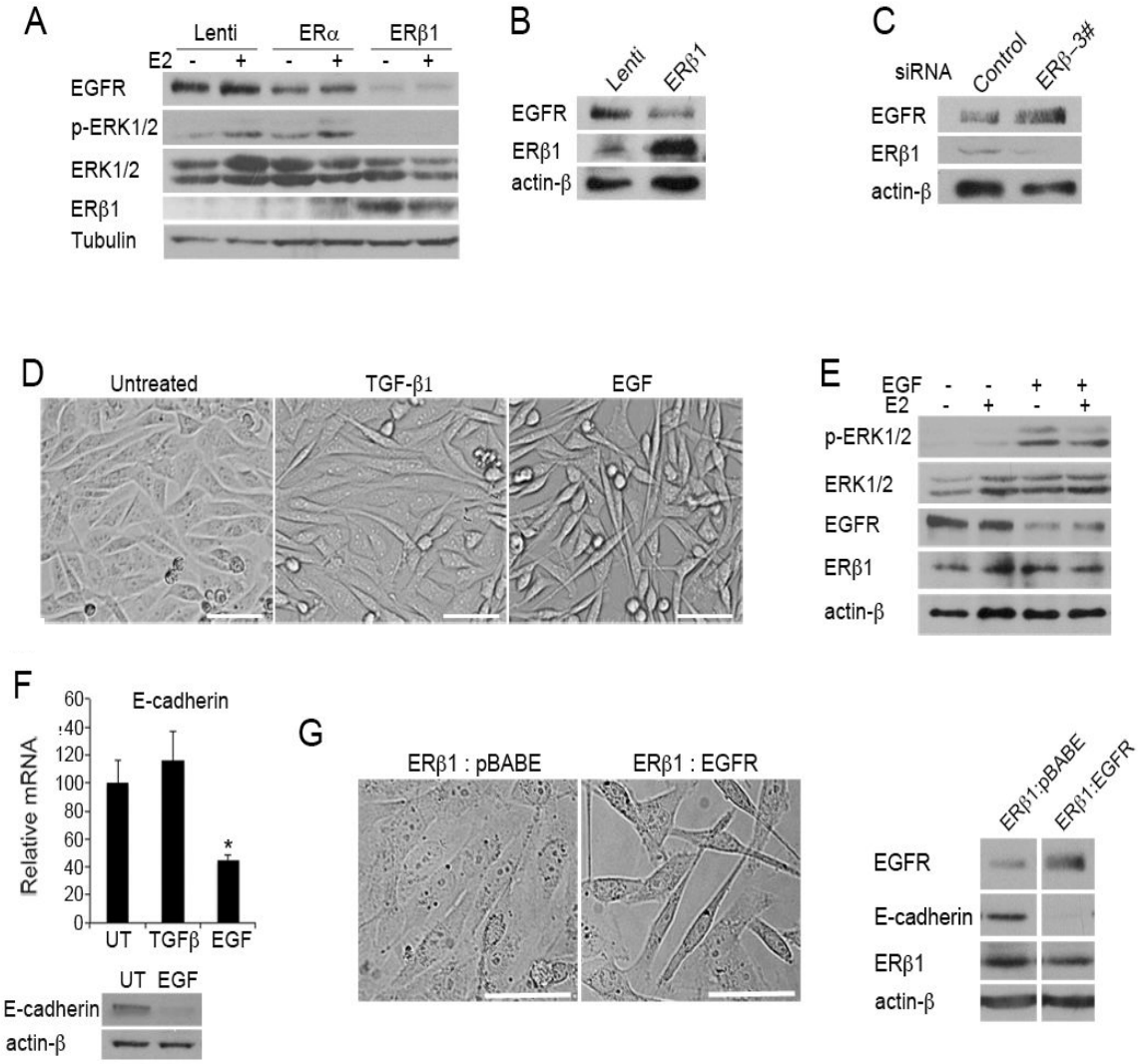
5.2.3 ERβ1 inhibits EMT by repressing EGFR signaling

EGFR that is overexpressed in MDA-MB-231 and Hs578T cells has been associated with poor survival in basal-like breast cancers. Overexpression of EGFR is known to promote migration in breast cancer cells (Hirsch et al., 2006, Nielsen et al., 2004). Activation of EGFR following ligand binding results in phosphorylation and activation of extracellular signal-regulated kinases (ERKs) (Ramos, 2008). Activation of ERK2 has recently been shown to promote EMT by inducing the expression of the transcriptional repressors of E-cadherin ZEB-1 and ZEB-2 (Shin and Blenis, 2010, Shin et al., 2010). Provided the induction of E-cadherin expression observed in ERβ1-expressing MDA-MB-231 and Hs578T cells, we examined whether ERβ1 inhibits EMT by down-regulating EGFR signaling. Induction of ERβ1 expression caused a strong reduction in the EGFR protein levels in MDA-MB-231 and Hs578T cells and decreased the phosphorylation of ERK1/2 as assessed by immunoblotting (Figure 5.4 A and B). Furthermore, reduction of endogenous ERβ1 expression in MDA-MB-231 cells led to up-regulation of EGFR (Figure 5.4C). To test whether the ERβ1-EGFR interaction is a critical regulator of EMT in basal-like breast cancer cells, we treated the ERβ1-expressing cells with EGF or the EMT inducer TGF-β1 for 24 h. For the same purpose, we stably transfected the ERβ1-expressing MDA-MB-231 cells with an empty vector or a plasmid that encodes wild-type EGFR. As expected, treatment of the cells with EGF restored the phosphorylation of ERK1/2, decreased the cell-cell contact and suppressed E-cadherin levels observed in the ERβ1-expressing cells (Figure 5.4 D-F). In contrast, treatment of the cells with TGF-β1, for the same time period

as for EGF, failed to reverse the ERβ1-induced phenotype in MDA-MB-231 cells (Figure 5.4D and F). As in the case of EGF treatment, EGFR overexpression induced a more fibroblastoid morphology in ERβ1 expressing cells, which was accompanied by down-regulation of E-cadherin (Figure 5.4G).

Figure 5.4 EGFR is involved in ERβ1-induced epithelial cell morphology and E-cadherin expression.

(**A**) EGFR, total ERK1/2 and phospho-ERK1/2 levels in control (Lenti), ERα- and ERβ1 expressing MDA-MB-231 cells following incubation with or without E2 for 24 h. (**B**) EGFR protein levels in control (Lenti) and ERβ1-expressing Hs578T cells. (**C**) EGFR protein levels in MDA-MB-231 cells transiently transfected with control or ERβ siRNA (3#). (**D**) ERβ1-expressing MDA-MB-231 cells were incubated in absence or presence of 5 ng/ml TGF-β1 or 10 ng/ml EGF for 24 h and photographed (scale bars, 50 μm). (**E**) ERβ1-expressing MDA-MB-231 cells were incubated in absence or presence of 10 ng/ml EGF for 24 h and analyzed for the expression of EGFR, total ERK1/2 and phospho-ERK1/2 by immunoblotting. Note that the decreased EGFR levels following EGF treatment is due to increased degradation. (**F**) E-cadherin mRNA and protein levels in ERβ1-expressing MDA-MB-231 cells following incubation with 5 ng/ml TGF-β1 or 10 ng/ml EGF for 24 h. The graph shows the mean of three separate experiments with SEM and P-value (*) ≤0.05% indicated. (**G**) ERβ1-expressing MDA-MB-231 cells were stably co-transfected with an empty pBABE vector (ERβ1:pBABE cells) or the pBABE-EGFR plasmid (ERβ1:EGFR cells), photographed and analyzed for EGFR, E-cadherin and ERβ1 expression by immunoblotting (scale bars, 50 μm).


A
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E2 - + - + - +
EGFR
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ERβ1
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B
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EGFR
ERβ1
actin-β
C
siRNA
Control
ERβ-3#
EGFR
ERβ1
actin-β
D
Untreated
TGF-β1
EGF
E
EGF - - + +
E2 - + - +
p-ERK1/2
ERK1/2
EGFR
ERβ1
actin-β
F
E-cadherin
Relative mRNA
60
40
120
100
80
60
40
20
0
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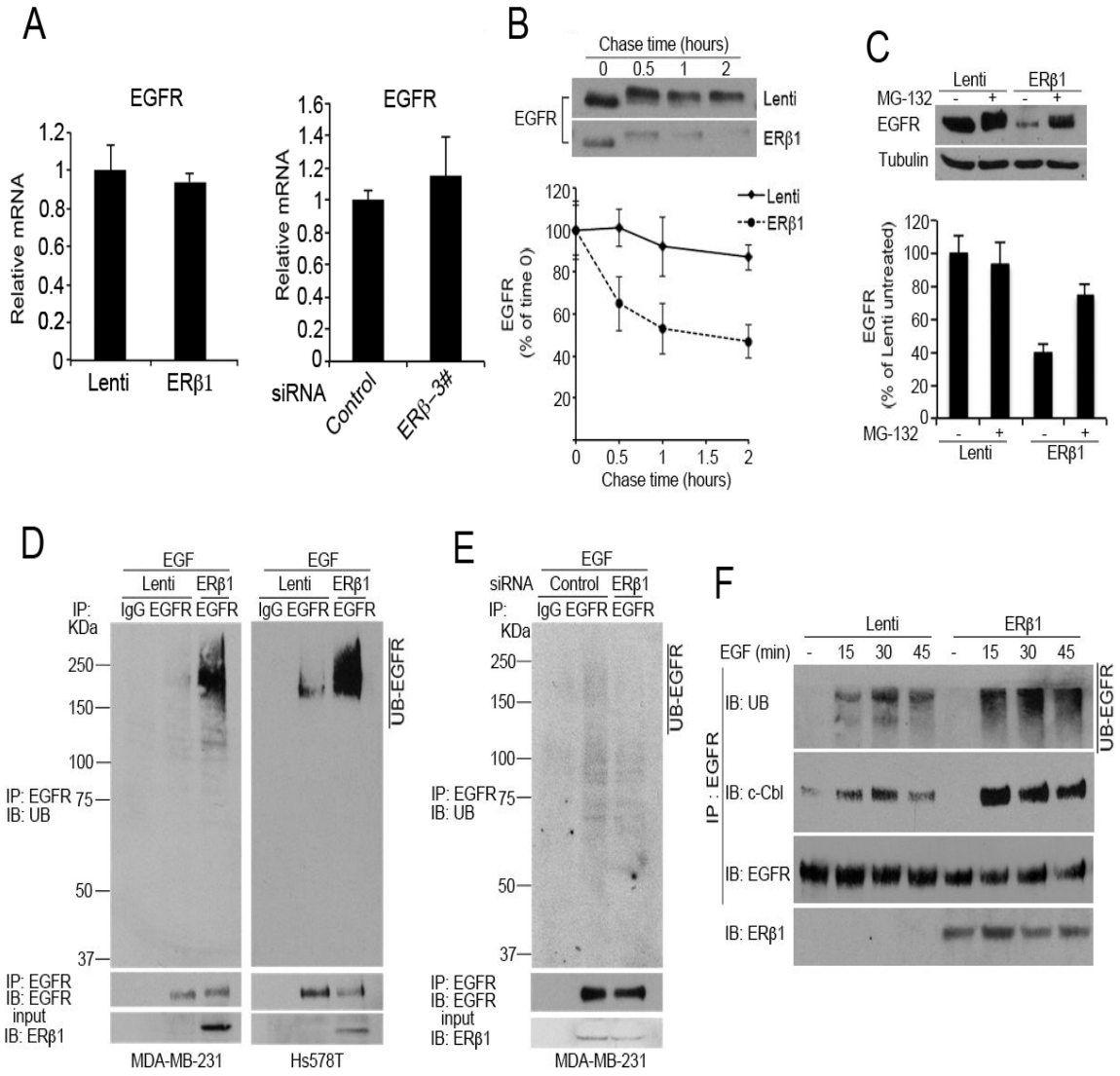
UT TGFβ EGF
UT EGF
E-cadherin
actin-β
G
ERβ1 : pBABE
ERβ1 : EGFR
ERβ1:pBABE
ERβ1:EGFR
EGFR
E-cadherin
ERβ1
actin-β

5.2.4 ERβ1 induces EGFR degradation by enhancing the interaction of EGFR with c-Cbl

Analysis of EGFR mRNA by qPCR revealed the same levels in control and ERβ1-expressing cells, as well as in cells where ERβ1 had been knocked down (Figure 5.5A), suggesting that ERβ1 does not regulate the transcription of *EGFR* gene. Given that ERβ1 altered only the protein but not the mRNA levels of EGFR, we set out to investigate whether ERβ1 regulates EGFR at a post-transcriptional level. Specifically, we hypothesized that ERβ1 induces degradation of the EGFR protein. EGFR degradation occurs through a process that includes ubiquitylation of the receptor, accelerated endocytosis, and degradation by proteasomal and lysosomal hydrolases (Levkowitz et al., 1999). In chase experiments, expression of ERβ1 reduced the half-life and accelerated EGFR protein turnover (Figure 5.5B). Treatment of the cells with the proteasome inhibitor MG-132 restored the ERβ1-dependent reduction in EGFR protein abundance (Figure 5.5C), confirming that EGFR down-regulation in ERβ1-expressing cells was due to increased degradation. Provided that ubiquitylation is an important step in the degradation of EGFR, we carried out ubiquitylation assays to test whether ERβ1 induces ubiquitylation of EGFR. Interestingly, the levels of the ubiquitylated EGFR were dramatically increased in ERβ1-expressing MDA-MB-231 and Hs578T cells (Figure 5.5D). Furthermore, the ubiquitylated EGFR was decreased when ERβ1 was knocked down in MDA-MB-231 cells (Figure 5.5E). Ubiquitylation of the activated EGFR is mediated primarily by members of the Cbl family of RING domain E3 ubiquitin ligases, including

the c-Cbl (Pennock and Wang, 2008). Hence, we examined whether ERβ1 promotes ubiquitylation of EGFR by inducing its association with c-Cbl. In control MDA-MB-231 cells, immunoprecipitation of EGFR under non-denaturing conditions showed a rapid but transient recruitment of c-Cbl to EGFR, with a barely detectable c-Cbl-EGFR association at 45 minutes following EGF induction. ERβ1-expressing MDA-MB-231 cells showed increased and more sustained c-Cbl-EGFR association with high amounts of c-Cbl recruited to EGFR even at 45 minutes following EGF induction (Figure 5.5F). These results strengthen our hypothesis that ERβ1 down-regulates EGFR by inducing its degradation.

Figure 5.5 ERβ1 induces ubiquitylation and degradation of EGFR.
(**A**) Left: control and ERβ1-expressing MDA-MB-231 cells were analyzed for EGFR expression by TaqMan mRNA assay. Right: MDA-MB-231 cells were transiently transfected with control or ERβ siRNA (3#) and analyzed for EGFR expression by TaqMan mRNA assay. Graphs represent the mean of three separate experiments with SEM and p value (*) ≤ 0.05% indicated. (**B**) Control (Lenti) and ERβ1 expressing MDA-MB-231 cells were incubated in the presence of 100 μM cycloheximide and 10 ng/ml EGF for the indicated times and analyzed for EGFR expression by immunoblotting. Treatment with EGF induces phosphorylation of EGFR and this accounts for the retarded electrophoretic mobility of EGFR at times 0.5 to 2. Lower panel: the graph represents the quantification of EGFR protein abundance from three independent experiments with SEM and P value (*) ≤0.05% indicated. (**C**) Control (Lenti) and ERβ1-expressing MDA-MB-231 cells were incubated in absence or presence of 1 μM MG-132 for 6 h and analyzed for EGFR expression by immunoblotting. Lower panel: the bar graph represents the quantification of EGFR protein levels with SEM and P-value (*) ≤0.05% indicated. (**D**) Control (Lenti) and ERβ1-expressing MDA-MB-231 and Hs578T cells were incubated in the presence of 10 ng/ml EGF for 20 minutes. Lysates were immunoprecipitated with anti-EGFR antibody, followed by immunoblotting with the indicated antibodies. The bottom panel is the input control of cell lysates. (**E**) MDA-MB-231 cells were transiently transfected with control or ERβ siRNA (3#). 72 h after the transfection, cells were incubated with 10 ng/ml EGF for 20 minutes and analyzed as described in C. (**F**) Control (Lenti) and ERβ1-expressing MDA-MB-231 cells were serum starved, challenged with 10 ng/ml EGF for the indicated times and lysed under nondenaturing conditions. EGFR immunoprecipitates were probed with antibodies against EGFR and c-Cbl. The bottom panel is the input control of cell lysates.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EGFR
Relative mRNA
Lenti
ERβ1
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Relative mRNA
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Control
ERβ-3#
B
Chase time (hours)
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EGFR
Lenti
ERβ1
EGFR (% of time 0)
Chase time (hours)
C
Lenti
ERβ1
MG-132
EGFR
Tubulin
EGFR (% of Lenti untreated)
D
EGF
Lenti
ERβ1
IP:
IgG EGFR EGFR
KDa
UB-EGFR
IP: EGFR
IB: UB
IP: EGFR
IB: EGFR
input
IB: ERβ1
MDA-MB-231
Hs578T
E
EGF
siRNA
Control
ERβ1
IP:
IgG EGFR EGFR
KDa
UB-EGFR
IP: EGFR
IB: UB
IP: EGFR
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IB: UB
IB: c-Cbl
IB: EGFR
IB: ERβ1
UB-EGFR

5.3 Discussion

Although basal-like breast cancers in general are associated with relatively poor prognosis, nevertheless they include subgroups that have diverse response to chemotherapy and risk of developing distant metastases (Kreike et al., 2007, Prat and Perou, 2011, Sarrio et al., 2008). Interestingly, triple-negative cancers that were treated with tamoxifen and stained positive for ERβ1 had an inverse correlation with the expression of EGFR, a central marker for the classification of basal-like cancers (Honma et al., 2008, Marotti et al., 2010, Nielsen et al., 2004).

One process that has been attributed to primary tumor metastasis is EMT. Here we examined whether by regulating EMT ERβ1 can influence invasion and metastasis in basal-like cancers. The results indicate that ERβ1 represses the mesenchymal spindle-shaped morphology of the MDA-MB-231 and Hs578T cells and increases cell-cell contact. ERβ1 altered the morphology of these cells in the absence of ligand. This is in agreement with our previous data showing increased transcriptional activity following expression of ERβ1 in MDA-MB-231 cells in the absence of ERβ agonists. The increased transcriptional activity in the absence of ligand was correlated with the phosphorylation of ERβ1 at Ser-87 (Thomas et al., 2011). As a result of the changes in the morphology, ERβ1 inhibited migration and reduced the invasiveness of MDA-MB-231 cells *in vitro* as well as dissemination to distant sites after implantation of ERβ1-expressing cells into zebrafish embryos, suggesting that ERβ1 functions as a crucial anti-migratory factor. Provided that expression of EMT markers and cadherin switching have been reported to correlate with

the basal-like phenotypes in *in vitro* model systems and in specimens from patients (Sarrio et al., 2008), we examined whether ERβ1 inhibits invasion and migration by regulating EMT in cells with basal characteristics.

Overexpression of EGFR promotes migration and invasion of basal cells and its expression correlates with poor survival in basal-like cancers (Hirsch et al., 2006, Nielsen et al., 2004). ERβ1 was found to induce the expression of E-cadherin that correlates with the epithelial breast cancer phenotype. ERK2 - a downstream effector of EGFR - has recently been shown to regulate ZEB1/2 transcriptional repressors of E-cadherin expression in human mammary cells (Shin and Blenis, 2010, Shin et al., 2010). Thus, we tested whether repression of EGFR and ERK1/2 signaling are involved in ERβ1-mediated up-regulation of E-cadherin and the subsequent inhibition of cell migration and invasion. Indeed, ERβ1 induced a decrease in EGFR protein levels without altering the transcription of the EGFR gene followed by down-regulation of the phosphorylated ERK1/2 forms. Induction of EGFR signaling in ERβ1-expressing cells through up-regulation of EGFR or treatment of the cells with EGF reversed the ERβ1-dependent epithelial phenotype, suggesting that EGFR is a critical factor in the ERβ1-mediated regulation of EMT.

Provided that ERβ1-mediated down-regulation of EGFR was transcription independent, we examined whether ERβ1 promotes degradation of the tyrosine kinase receptor. EGFR degradation is a complex process that involves ubiquitylation of the activated receptor by the E3 enzyme Cbl and subsequent proteolysis by proteosomal and lysosomal hydrolases (Levkowitz et al., 1999). ERβ1 was found to induce ubiquitylation

and degradation of EGFR by enhancing the EGFR-c-Cbl association. Ubiquitylation is an important process of a negative regulatory circuit that terminates EGFR signaling by targeting the receptor for degradation (Frosi et al., 2010). Our data show for the first time that ERβ1, by inducing these negative feedback pathways, is likely to exert a role of EGFR inhibitor and tumor suppressor function.

Interestingly, it has recently been shown that ERβ decreases the expression of insulin-like growth factor II mRNA binding protein 3 (IMP-3) by repressing EGFR transcription in MDA-MB-231 cells (Samanta et al., 2012). In our study, the transcription of EGFR was not altered when ERβ1 was expressed or knocked down in MDA-MB-231 and Hs578T basal-like cells. Instead, as mentioned above, ERβ1 promotes degradation of EGFR by inducing its ubiquitylation in both MDA-MB-231 and Hs578T cells.

Our data suggest that the low ERβ1 levels may be the primary cause of low E-cadherin expression and induction of EMT in some breast cancers. Provided that EMT correlates with a group of basal-like breast cancers that often develop metastases in distant sites (Sarrio et al., 2008), ERβ1 may play a crucial role in repressing invasive behavior and inhibiting metastasis in this subset of breast cancers. Our data show that ERβ1 impedes EMT and suppresses invasion by downregulating EGFR, which is expressed in basal-like cancers. These results strengthen the possibility that ERβ1 can help to identify patients with basal-like cancer with lower risk to develop metastasis.

5.4 Summary

Epithelial to mesenchymal transition (EMT) is a feature of the basal-like breast cancers. Sixty percent of basal-like cancers have been shown to express wild-type estrogen receptor beta (ERβ1). However, it is still unclear whether the ERβ expression is related to EMT, invasion, and metastasis in breast cancer. In the present study, we examined whether ERβ1 through regulating EMT can influence invasion, and metastasis in basal-like cancers. Our results indicate that ERβ1 inhibits EMT and invasion in basal-like breast cancer cells when they grow either *in vitro* or *in vivo* in zebrafish. The inhibition of EMT correlates with an ERβ1-mediated increased expression of E-cadherin. Downregulation of the basal marker EGFR through stabilization of the ubiquitin ligase c-Cbl complexes and subsequent ubiquitylation and degradation of the activated receptor is involved in the ERβ1-mediated repression of EMT and induction of EGFR signaling abolished the ability of ERβ1 to sustain the epithelial phenotype. Taken together, the results of our study strengthen the association of ERβ1 with the regulation of EMT and propose the receptor as a potential crucial marker in predicting metastasis in breast cancer.

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Appendix I: List of antibodies

Antibody	Company	Catalog No.
ERβ (14C8)	Genetex	GTX70174
ERβ (68-4)	Millipore	05-824
ERβ1 (D7N)	Invitrogen	51-7700
p84	Genetex	GTX70220
EGFR	Santa Cruz Biotechnologies	sc-03
phospho-ERK1/2	Santa Cruz Biotechnologies	sc-7383
Caspase 3	Santa Cruz Biotechnologies	sc-7272
$p27^{Kip1}$	Santa Cruz Biotechnologies	sc-1641
$p21^{WAF1/CIP1}$	Santa Cruz Biotechnologies	sc-817
ERα	Santa Cruz Biotechnologies	sc-542
E-cadherin	Santa Cruz Biotechnologies	sc-7870
α-Tubulin	Santa Cruz Biotechnologies	sc-5546
Ubiquitin	Santa Cruz Biotechnologies	sc-8017
c-Myc	Santa Cruz Biotechnologies	sc-40
Bim	Cell Signaling Technologies	2933
ERK1/2	Cell Signaling Technologies	9102
phospho-Akt(S473)	Cell Signaling Technologies	4056
pan-Akt	Cell Signaling Technologies	4685
RAS	Cell Signaling Technologies	3965
cleaved Caspase 3	Cell Signaling Technologies	9664
phospho-Chk1 (S345)	Cell Signaling Technologies	2341
Cyclin D2	Cell Signaling Technologies	3741
c-Cbl	BD Biosciences	610442
β-actin	Sigma-Aldrich	A5316
Anti-FLAG (M2)	Sigma-Aldrich	F3165

Appendix II: List of primers used for qPCR

Gene	Sequence
ERβ1 FW	GCTCAATTCCAGTATGTACC
ERβ1 RV	GGACCACATTTTTGCACT
E-cadherin FW	CCCACCACGTACAAGGGTC
E-cadherin RV	CTGGGGTATTGGGGGCATC
EGFR FW	CGAGACCCCCAGCGCTACCT
EGFR RV	CGGCATCCACCACGTCGTCC
36B4 FW	GCAATGTTGCCAGTGTCTGT
36B4 RV	GCCTTGACCTTTTCAGCAAG
CDKN1B FW	CCCTTTCAGAGACAGCTGATAC
CDKN1B RV	ACCAGATCTCCCAAATGAGAA
GAPDH FW	TGATGACATCAAGAAGGTGGTGAAG
GAPDH RV	CCTTGGAGGCCATGTGGGCCAT
EGF FW	CTGCCTCCATGATGGTGTGT
EGF RV	CTCGGTACTGACATCGCTCC
CCND2 FW	TGCAGAAGGACATCCAACCC
CCND2 RV	GCCAAGAAACGGTCCAGGTA