Y-BOX BINDING PROTEIN-1 (YB-1) IS ESSENTIAL FOR THE GROWTH AND SURVIVAL OF HER-2 OVER-EXPRESSING BREAST CANCER CELLS

by

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ABSTRACT

The human epidermal growth factor receptor (HER-2) is over-expressed in 20-30% of breast carcinomas and is a prognostic marker for poor patient outcome. We previously identified the transcription/translation factor Y-box binding protein-1 (YB-1) to be a novel substrate of AKT which binds to epidermal growth factor receptor (EGFR) and HER-2 promoters once phosphorylated (Wu J et al. 2006). YB-1 is over-expressed in approximately 40% of breast cancers; its expression is strongly correlated with HER-2 and is associated with poor patient survival.

In order to gain a deeper understanding of the functional role of YB-1 in HER-2 over-expressing breast cancer, we silenced the expression of this factor in BT474-m1 and MDA-MB-453 cells. The loss of YB-1 inhibited the growth of BT474-m1 and MDA-MB-453 cells in monolayer and/or in soft agar. Consistent with this, we found a decrease in the expression of YB-1 responsive gene *egfr* and/or *her-2* in BT474-m1 and MDA-MB-453 cells, which could begin to explain how growth is promoted by this factor. Furthermore, loss of YB-1 expression induced apoptosis in BT474-m1 cells.

Beyond its role in tumor growth, YB-1 is also strongly linked to drug resistance. We therefore addressed whether it could play a part in Herceptin sensitivity. Herceptin is currently being used to treat patients with HER-2 positive breast cancer; however, only 30% of the patients respond to the therapy and many of them develop resistance within the first year of treatment. Therefore, it is of utmost importance to understand the biology of HER-2 over-expressing breast cancer to develop novel therapies that can benefit more patients. First we established that Herceptin inhibited BT474-m1 cell growth in anchorage-independent conditions whereas MDA-MB-453 cells were resistant to this treatment. We subsequently demonstrated that knock-down of YB-1 increased sensitivity of BT474-m1 cells to Herceptin while MDA-MB-453 cells failed to respond to the combination treatment. The mechanism for Herceptin resistance in MDA-MB-453 cells still remains elusive and requires further investigation. Thus far, we conclude that YB-1 is needed for the growth and survival of HER-2 positive BT474-m1 and MDA-MB-453 breast cancer cells by inducing members of the HER family.

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cellular cytoxicity
AH	Atypical hyperplasia
BDP1	Brain derived phosphatase1
BIG	Breast internation group
CDK	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
CGH	Comparative genomic hybridization
CRS	Cytoplasmic retention site
CSD	Cold shock domain
CTD	C-terminal domain
DbpA	DNA binding protein A
DbpB	DNA binding protein B
DbpC	DNA binding protein C
DCIS	Ductal carcinoma in situ
DMSO	Dimethyl Sulfoxide
4E-BP1	4E-binding protein 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELB	Egg lysis buffer
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERK1/2	Extracellular-signal-regulated kinase 1/2

FBS	Fetal bovine serum
FBX33	F-box protein 33
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
GSK3	Glycogen synthase kinase 3
HB-EGF	Heparin-binding epidermal growth factor
HDAC	Histone deacetylase complex
HER-2	Human epidermal growth factor receptor-2
HERA	Herceptin® adjuvant trial
HIV-I	Human T-cell lymphotropic virus type I
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IL-6	Interleukin-6
IRE	Iron responsive element
LOH	Loss of heterozygosity
MAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MDR-1	Multidrug resistance 1
MHC	Major histocompatibility complex
MMP-2	Matrix metalloproteinase-2
MMP-12	Matrix metalloproteinase-12
mRNP	Messenger ribonucleoprotein particles
MRP 1	Multidrug resistance-related protein 1

MVP	Major vault protein
NCoR	Nuclear receptor corepressor
NK cell	Natural killer cell
NLS	Nuclear localization signal
NRG	Neregulin
NSCLC	Non-small-cell lung cancer
NSEP-1	Nuclease-sensitive element protein-1
P-AKT	Phosphorylated AKT
PCNA	Proliferating cell nuclear antigen
PDK1	3-phosphoinositide-dependent protein kinase-1
PH	Pleckstrin homology
PKC	Protein kinase C
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PMSF	Phenylmethylsulfonyl fluoride
PTB	Protein tyrosine binding
PTEN	Phosphatase and tensin homologue
RIPA	Radioimmunoprecipitation assay
RTK	Receptor tyrosine kinase
SERD	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator
SH2	SRC-homology-2

TGF-α	Transforming growth factor-α
TKI	Tyrosine kinase inhibitor
TMA	Tumor tissue microarray
UDH	Usual ductal hyperplasia
UV	Ultraviolet
YB-1	Y-box binding protein-1
YRE	YB-1 responsive element

CHAPTER 1

INTRODUCTION

Cancer is defined as a group of diseases resulting from uncontrolled growth and spread of genetically abnormal cells that can localize to other areas of the body commonly leading to fatality. To date, millions of people around the world have been affected by this disease. Cancer accounts for 1 in every 4 deaths in the United States (American Cancer Society) and is the second most common cause of death after heart disease (www. cancer. org).

The development of cancer can be attributed to internal factors such as inherited genetic mutations, hormonal status, immune condition or external factors such as exposure to radiation, carcinogenic chemicals or oncogenic viruses. All these events could act in concert or in sequence to promote carcinogenesis (American Cancer Society). Cancer is a complicated disease in that its cause is multi-factorial and many steps lead to its development and dissemination. During the course of malignant transformation, a series of genetic events involving loss of heterozygosity (LOH) of tumor suppressor genes and mutations in oncogenes enable the cells to rapidly proliferate, ignore apoptotic signals, increase nutrient supply by angiogenesis, and in some cases, metastasize from the primary site and spread to other vital organs of the body, increasing the chance of mortality (Hanahan et al. 2000).

1.1 Breast Cancer

According to the Canadian Breast Cancer Society, breast cancer is the most commonly diagnosed and the second leading cause of cancer-related deaths in women in North America. It was estimated that 22,300 women will be diagnosed with and 5,300 of the patients will die of this disease in the year 2007. The cancer cells can originate from both the ducts or the lobules giving rise to ductal or lobular carcinoma, respectively, with the former being more frequent (www. cancer.ca).

Histopathological studies indicate that premalignant breast lesions from usual ductal hyperplasia (UDH), atypical hyperplasia (AH) to ductal carcinoma in situ (DCIS) are

associated with an increased risk of developing invasive breast cancer, and that malignant lesions are clonally derived from DCIS (Gong et al. 2001).

Germline mutations leading to loss in heterozygosity (LOH) in tumor suppressor genes such as *brca1 and brca 2* (Miki et al. 1994), *p53* (Sidransky et al. 1992) and *pten* (Liaw et al. 1997) are known for the association with an inherited predisposition to breast cancer. Proteins encoded by these genes negatively regulate cell growth by controlling apoptosis, cell cycle progression or other important growth signaling pathways; therefore, inactivation in both alleles results in complete loss of function of these genes leading to uncontrolled cell proliferation. In addition, activating mutations on one allele of oncogenes such as *c-myc* (Escpt et al. 1986), *cyclin D* (Buckley et al. 1993) and *erbb2* (Slamon et al.1987) could also lead to the development of breast cancer. Activation or over-expression of these proteins enhances signaling through mitogenic and survival pathways or accelerates cell cycle progression promoting growth of cancer cells.

1.2 Classification of Breast Cancer

Breast cancer can be categorized into different subtypes according to the specific molecular markers that are expressed by the tumor cells. These subclasses of breast cancer could be considered as biologically distinct disease entities that may require different therapeutic strategies (Sorlie et al. 2007).

Breast cancer is typically divided into 2 main groups: estrogen receptor-positive (ER+) or estrogen receptor-negative (ER-). The ER+ breast cancer, which represents greater than 70% of all breast cancer cases, is generally characterized as being more differentiated, less aggressive and with lower levels of growth factor receptors compared to the ER- breast tumors (reviewed in The Basic Science of Oncology, 2005). Within the ER+ group are the luminal subtypes A and B, which are distinguished by their levels of cytokeratin-8 and cytokeratin-18 expression. The ER- breast cancer includes 3 distinct subgroups: the ErbB2+, basal-like and the “normal breast-like”. Compared to the “normal breast-like” ER- breast tumors, the ErbB2+ and basal-like breast cancer are more well-known for their aggressiveness in the development of the disease. Patients with the ErbB2+ or basal-like subtype of breast cancers have the shortest survival time, which is related to the highly invasive and metastatic nature of the tumors (Sorlie et al. 2003). While ErbB2+ subtype

over-expresses ERBB2 protein, or the human epidermal growth factor receptor-2 (HER-2), the basal-like tumors are characterized by their high levels of cytokeratin-5, cytokeratin-7 and their "triple negative" phenotype: estrogen receptor (ER)-negative, progesterone receptor (PR)-negative and human epidermal growth factor receptor-2 (HER-2)-negative (Perou et al. 2000).

1.3 Standard Treatments for Breast Cancer

1.3.1 Chemotherapy

For breast cancers that have not metastasized to distant sites such as lungs, liver or bone, the patient can expect a 98% chance of survival 5 years after diagnosis. However, survival becomes far less likely (26%) when the tumor cells have spread to distant sites. Therefore, early detection becomes crucial to successful treatment of the disease.

The front line treatment for breast tumors that have not metastasized is radiation therapy and surgical removal of the tumor with clear margins by lumpectomy or removal of the breast by mastectomy (Baselga and Norton 2002). Chemotherapeutic agents can also be prescribed as adjuvant therapy to eliminate residual cancer cells that may have spread to other sites thereby improving disease-free and overall survival. Commonly used chemotherapeutic drugs are the alkylating agents (eg. cyclophosphamide), antimetabolites (eg. 5-fluorouracil), topoisomerase inhibitors (eg. etoposide) and anti-microtubular agents (eg. paclitaxel) that, despite having different mechanism of actions, work under the same principle of interfering DNA synthesis and/or cell replication, a process that is important not only for cancer cells, but also certain normal but rapidly proliferating cells. Therefore, adverse side-effects such as hair loss, vomiting and nausea, are frequently associated with these traditional anti-cancer drugs.

1.3.2 Hormonal Therapy

The primary risk factors for breast carcinoma are female gender and increasing age. Prolonged exposure to estrogen, a hormone integral to normal development of the mammary gland, due to early menarche, late menopause and obesity in post-menopausal women have also been proposed to increase the risk of breast cancer (reviewed in The Basic Science of Oncology, 2005). Addition of estrogen to estrogen receptor (ER)-positive breast cancer cells

has been shown to induce cell proliferation (Anderson et al. 1998), implicating that estrogen responsiveness or its mechanism of action could play a role in the pathogenesis of the breast cancer. Hormonal therapy, which aims to inhibit the proliferative effects of estrogen, has thus become a suitable treatment for breast cancer patients with tumors that are ER-positive.

There are two ways that hormonal therapy inhibits the biological effects of estrogen: 1) decrease the level of circulating and/or local estrogen or, 2) block estrogen action at the receptor level. Aromatase inhibitors such as formestane, exemestane (steroidal) or fadrozole, anastrozole, or letrozole (non-steroidal) inhibit the conversion of weak androgens to estrogens and therefore decrease the production of estrogen. Upon binding of estrogen, ER is phosphorylated and undergoes receptor dimerization. Subsequently, coactivators such as AIB1 (SRC3) and CBP/p300 are recruited to form a multi-protein complex that promotes transcription of ER target genes (McKenna et al. 1999). In contrast, Selective Estrogen Receptor Modulators (SERMs) such as tamoxifen, binds to ER and induces conformational changes in the receptor such that the corepressor (NCoR) and deacetlyases (HDACs) are recruited and transcription is consequently inhibited (Smith et al. 1997). Raloxifene is another example of a SERM that has undergone much investigation for its efficacy in breast cancer treatment. In contrast to tamoxifen, which has both estrogenic and anti-estrogen properties, depending on the specific tissues or cell types examined (McKenna and O'Malley, 2000), fulvestrant is a pure anti-estrogen in that it inactivates the ER complex, down-regulates ER expression by increasing its degradation (O'Regan and Jordan, 2001), and is classified as a Selective Estrogen receptor Downregulator (SERD).

1.3.3 Molecular Targeted Therapy

The recent advances in understanding of oncogenic signaling pathways at the molecular level have prompted the design of improved therapeutics that aim to inhibit individual proteins essential for cancer cell growth. This treatment, termed molecular targeted therapy, offers a new promising avenue for the development of novel anti-cancer compounds.

In molecular targeted therapy, the drug effect is achieved due to the differential expression or activation of receptors or intracellular proteins in normal compared to malignant cells. Alteration or aberrant expression of oncogenic proteins tends to be present in

the majority of tumor cells but not normal cells. Targeting these proteins with specific inhibitors could therefore selectively suppress or eliminate cancer cells. Recently, much endeavor has been made to design drugs that act against known proteins or to identify novel molecular targets useful for future diagnostic and treatment purposes. Imatinib (Gleevac), erlotinib (Tarceva) and gefitinib (Iressa) are a few of the examples of drugs that have been shown to demonstrate therapeutic efficiencies in certain human malignancies.

1.4 The HER Receptor Family Members

1.4.1 Biological Functions of the HER(ErbB)

The subclass I receptor tyrosine kinase (RTK) ErbB superfamily consists of EGFR/ErbB1, HER-2/ErbB2, HER-3/ErbB3 and HER-4/ErbB4. The ErbB family members and their associated ligands are involved in a wide range of cellular processes and mediate cell-cell interactions in organogenesis and adulthood (Yarden et al. 2001). Studies from knockout and transgenic mice have helped elucidate the biological functions of individual ErbB. EGFR knockout mice had impaired development in organs involved in eye opening and tooth growth. The neregulin-responsive receptors such as HER-2, HER-3 and HER-4 are implicated in cardiac and neural development. Inactivation of HER-2 and HER-4 in mice resulted in death at embryonic day 10.5 with cardiac defects (Burden et al. 1997). The HER-3 deficient mice, survived to embryonic day 13.5 but suffered from defective cardiac formation (Britsch et al. 1998). Together, these studies emphasize the importance of ErbB family members in development of different organs.

1.4.2 Protein Structure of the HER

All the ErbB family members are composed of an extracellular ligand-binding region, a single-membrane spanning region and a cytoplasmic tyrosine-kinase-containing domain (Yarden et al. 2001). The extracellular component of the receptors is comprised of domains I~IV where domains II and IV are cysteine-rich and each contains about 10 disulfide linkages (Roskoski Jr. 2004). Ligand binding to domains I and III of the receptor induces a substantial domain rearrangement releasing the intramolecular linkage between domain II and IV; the exposed domain II can then participate in receptor-receptor dimerization.

X-ray crystallographic structures of ErbB2 have revealed unusual properties of this receptor (Burgess et al. 2003; Chopin et al. 2003). In the absence of ligand, the ectodomain of ErbB2 lacks the domain II/domain IV tether and resembles the extended conformation of EGFR making it poised for dimerization with other family members. This receptor must therefore rely on ligand dependent activation of other EGFR family members that lead to HER-2 heterdimerization and activation (Yarden et al. 2001; Citri et al. 2003). The tyrosine kinase domain is highly homologous among EGFR, HER-2 and HER-4 with approximately 80% amino acid identity, suggesting the essential role of this region in mediating signaling in the ErbBs (Earp et al. 1995). HER-3 is the only member of the family that does not have intrinsic kinase activity and therefore signals by heterodimerization (Citri et al. 2003). The basic structures of the ErbB family members and their respective ligands are illustrated in Fig 2.

1.4.3 HER-Mediated Signaling

The EGF family of ligands can be divided into three groups. The first group includes EGF, transforming growth factor-α (TGF-α) and amphiregulin, all of which bind specifically to EGFR. The second group includes the dual specificity ligands such as betacellulin, heparin-binding EGF (HB-EGF) and epiregulin, which bind both EGFR and ErbB4. The last group of ligands, neregulins (NRGs), are categorized into 2 subgroups. While NRG1 and NRG2 bind both ErbB3 and ErbB4, NRG3 and NRG4 bind only ErbB4 (Hynes et al. 2005).

Upon binding by growth factors, the receptors dimerize and autophosphorylate to become fully activated. Adaptor proteins and specific kinases are recruited to the C-terminal phosphorylation sites by binding to phospho-tyrosine residues through their Src-homology 2 (SH2) or phospho-tyrosine binding (PTB) domains. Signaling pathways activated by ErbBs include the PI3K/Akt, MAPK and protein kinase C (PKC) pathways. Through these pathways the signal is propagated into the nucleus. Transcription factors such as proto-oncogene *fos, jun* and *myc* as well as Sp1, Egr1 and Ets family members are activated, leading to cell division, differentiation, adhesion, migration, or apoptosis (Yarden et al. 2001). The combinatorial interactions of receptors broaden the spectrum of signaling pathways that can be activated and at the same time determines the specificity of the outcome. Signaling is turned off primarily by receptor endocytosis although phophatases

such as brain derived phosphatase1 (BDP1) has also been implicated in attenuation of HER-2 signaling (Gensler et al. 2004).

1.4.4 Oncogenic Properties of HER Family Members

In the early 1980s, the sequence of EGFR was found to be homologous to a previously identified protein encoded by the *erbB* oncogene, originally discovered in the avian erythroblastosis tumor virus (Gandrillon et al. 1987). The v-ErbB oncoprotein is N-terminally truncated and therefore does not bind ligands. However, it is constitutively active. The oncogenic property of ErbB2 was demonstrated in a transfection experiment using genomic DNA isolated from chemically induced murine neuroblastoma. The oncogenic ErbB2 carried a valine to glutamic acid mutation in the transmembrane domain, enhancing the kinase activity of the receptor (Hung et al. 1989). Recently, through much endeavor, improvements in the understanding of the complicated cellular biology of the ErbBs has been made, with the hope of designing better therapeutics to treat diseases such as cancers.

The ErbB/HER family members have become attractive molecular targets for cancer treatment not only because of their expression on the cell surface which makes them readily accessible to the compounds but more importantly, over-expression or mutations of the proteins have been found in various human malignancies, and tumor cells of certain cancers need the receptors for growth and survival. EGFR over-expression was found in cancers of the head and neck, breast, bladder, prostate, kidney, lung and brain where it is often related to poor patient survival (Gorgoulis et al. 1992; Irish et al. 1993). HER-2, which is an important prognostic marker in breast cancer, is also over-expressed in cancers of the lung, pancreas, colon, oesophagus, endometrium and cervix. Over-expression of HER-2 occurs in 15~30% of invasive ductal breast carcinomas and is correlated with tumor size, high grade, spread of the tumor to lymph nodes, increased percentage of S-phase cells and aneuploidy (Slamon et al. 1987). Increased HER-3 levels have been observed in oral squamous cell cancer and is associated with lymph node involvement and poor patient survival (Shintani et al. 1995). The role of HER-4 in breast cancer is less well-defined and its expression is correlated with a differentiated phenotype (Cohen et al. 1998).

Deregulation of the ErbB signaling pathways can also be attributed to ligand over-expression and activating mutations in the receptor. For instance, TGF-α is over-expressed in

prostate (Scher et al. 1995), pancreatic (Yamanaka et al. 1993), lung, ovarian and colon cancers (Saeki et al. 1995). In prostate cancer, TGF-α is initially expressed in the stroma in androgen-dependent cancer but becomes expressed by the tumor itself in late-stage androgen-independent cancer (Scher et al. 1995). Finally, structural rearrangement of the genes could lead to in-frame deletions in the extracellular domain of the receptor as seen in the constitutively-activated EGFRvIII variant in breast, lung, ovarian cancers and gliomas (Moscatello et al. 1995). Aberrant expression or alteration of the receptors enhances signaling through the MAPK and PI3K/AKT pathways required for tumor cell growth and survival.

1.5 HER-Targeted Tyrosine Kinase Inhibitors (TKIs) for Cancer Treatment:

An in-depth understanding of the biology of ErbBs in cancer development has prompted the development of therapeutics that specifically target these receptors. Several strategies have been sought after for this purpose and the main ones currently in clinical trials are the monoclonal antibodies targeting the extracellular domain of the receptors such as Herceptin™ (trastuzumab, Genetech) (Section 2.4), as well as tyrosine kinase inhibitors (TKIs) such as Iressa™ (gefitinib, ZD1839, AstraZeneca), GW572016 (lapatinib, GlaxoSmithKline) and CI-1033 (PD183805, Pfizer).

Gefitinib was the first EGFR kinase inhibitor approved by U.S. Food and Drug Administration (FDA) for cancer therapy. It is a reversible EGFR kinase inhibitor that was shown to suppress ligand-stimulated EGFR phosphorylation and inhibit EGF-dependent cell growth of the tumor cells both *in vitro* and tumor xenografts (Wakeling et al. 2002). Gefitinib was approved in the U.S. for treatment of non-small cell lung cancer (NSCLC) after docetaxel and platinum-based therapy had failed. However, the response rate in unselected patient population was low as only about 10% of the patients respond to the therapy. Since certain subtypes of breast cancer, such as the basal-like breast cancer, also over-express EGFR, a phase II multi-institutional clinical trial has been conducted to examine the efficacy of combining Iressaand docetaxel as a first-line treatment for metastatic breast cancer patients. The combination treatment was reported to be an active regimen in patients with previously untreated metastatic breast cancer with a clinical benefit rate and toxicity profile within the range of that reported for docetaxel alone (Dennison et al. 2007).

Gefitinib as a single agent is not very effective for the response rate is only 11% (Birnham et al. 2005). This could be due to compensatory signaling by other members of the HER family, such as HER-2. Therefore, dual specificity or pan inhibitors have emerged in hope of overcoming this problem. Lapatinib is a dual specificity ErbB inhibitor with potent activity against both EGFR and HER-2. It selectively inhibits growth of cell lines that over-express EGFR or HER-2 by decreasing receptor phosphorylation and MAPK pathway signaling (Xia et al. 2002). Various combination therapies have been tested both *in vitro* and *in vivo*. Synergistic induction of apoptosis was observed when lapatinib was combined with PI3K inhibitor, LY294002. Also, combinations of lapatinib with paclitaxel or doxorubicin have demonstrated improved anti-tumor activity than single agent therapy in xenografts (Rabindran 2004).

Another class of the tyrosine kinase inhibitor is the pan inhibitor exemplified by CI-1033, an irreversible inhibitor that has equivalent activity against EGFR, HER-2 and HER-4 (Allen et al. 2002). This inhibitor binds to a conserved cysteine residue in the ATP binding site in the tyrosine kinase domain thereby inhibiting ligand-stimulated phosphorylation of the receptors (Slichenmyer et al. 2001). Inhibition of this phosphorylation process prevents recruitment of adaptor proteins and other downstream signaling molecules and therefore HER-mediated signaling is inactivated. Like lapatinib, CI-1033 also induces apoptosis in HER-2 over-expressing cells, possibly due to a decrease in AKT signaling and an increased level of p38 MAPK (Nelson et al, 2001). The inhibitor has been investigated in phase I clinical trials in combination with other anti-cancer drugs such as docetaxel for treatment of advanced solid tumors or with paclitaxel and carboplatin for treatment of NSCLC (Garland et al. 2006; Chiappori et al. 2006).

1.6 Herceptin™ and its Mechanism of Action

Herceptin™ (trastuzumab, Genetech), a humanized monocloncal antibody (MAb), was approved in 1998 by U.S. FDA as a front-line treatment for metastatic breast cancer patients as well as breast cancer patients who over-express HER-2. Between the year 2001 and 2004, a large scale international clinical trial named HERA (Herceptin® Adjuvant Trial) was conducted by Roche and BIG (Breast International Group) in 39 countries involving 5,100 HER-2 positive breast cancer patients. The trial aimed to assess improvements in

survival of women with early-stage disease when Herceptin was given after standard chemotherapy. Results from the study indicated that 1 year Herceptin treatment after adjuvant chemotherapy significantly increased disease-free as well as overall survival after a median follow-up of 2 years. To date, the remarkable efficacy of this therapy has benefited a substantial number of patients worldwide as between 25-30% of invasive breast carcinoma features HER-2 amplification or over-expression (Slamon et al. 1987).

The Herceptin monoclonal antibody was produced by inserting the complementary determining regions of a murine antibody (clone 4D5) into the framework of a consensus human IgG1 (Carter et al. 1992). Since Herceptintargets extracellular domain IV (Chopin et al. 2003), which is not involved in receptor-receptor interaction, inhibition of tumor cell growth by the antibody is probably not related to interference of receptor dimerization. How Herceptin functions to suppress tumor growth is not completely understood; however, several mechanisms have been proposed.

Herceptin has been found to down-regulate PI3K/AKT signaling pathway, leading to cell cycle arrest and apoptosis (Dubska et al. 2005). Nagata et al. (2004) proposed that the decrease in PI3K signaling may be a consequence of disrupted interaction between HER-2 and SRC after Herceptin treatment. Inactivation of SRC and subsequent membrane localization and activation of PTEN, the negative regulator of PI3K, may together contribute to inhibition of PI3K/AKT signaling.

Not only does Herceptin have an impact on the survival pathway, it has also been found to reduce proliferation potentially by arresting the cells at G1 phase of the cell cycle. Following Herceptintreatment, expression of cyclin D1 goes down, releasing cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$ that can then bind and inhibit the function of cyclin E/CDK2 complexes that drive cell cycle progression (Sliwkowski et al. 1999; Lane et al. 2000; Lane et al. 2001).

Herceptin monotherapy can drastically reduce the size of HER-2 over-expressing tumors in metastatic breast cancer patients. This was probably not achieved by suppression of proliferation alone but rather with a combination of cytotoxic effects exerted by the MAb. *In vivo*, apoptosis is induced partly by antibody-dependent cellular cytotoxicity (ADCC) mediated by the natural killer (NK) cells. The Fc domain on Herceptin can bind to Fcγ receptor expressed on NK cells and trigger NK-mediated cell lysis. Disruption of this

interaction by eliminating Fcγ receptor abrogates ADCC and the therapeutic effect of Herceptin is largely hampered (Clynes et al. 2000).

Finally, several *in vivo* studies have suggested that in HER-2 over-expressing cells, treatment with Herceptin can lead to a reduction in tumor volume and decrease in microvessel density (Izumi et al. 2002; Klos et al. 2003). Anti-angiogenic effects of Herceptin could be explained by a decreased expression of pro-angiogenic factors and an increased expression of anti-angiogenic factors (Izumi et al. 2002; Klos et al. 2003; Petit et al. 1997).

1.7 The Y-Box Binding Protein-1 (YB-1)

The Y-box binding protein-1 (YB-1) is one of the most evolutionary conserved nucleic acid binding proteins. It carries out a multitude of biological functions including transcriptional and translational regulation, cell proliferation, cellular responses to environmental stress and DNA damage in organisms (Wolffe AP. 1994).

Research carried out in the succeeding 20 years, after first identification of YB-1, has contributed much to the understanding of the physiological functions and oncogenic properties of this transcription/translation factor. The importance of YB-1 in early development was demonstrated through the study of YB-1-null transgenic mice, which displayed defects in neural tube closure, neuroepithelial cell proliferation and are embryonic lethal (Uchiumi et al. 2006). In addition, YB-1 plays an indispensible role in the later stages of murine embryogenesis in regulating cellular stress responses and preventing premature senescence (Lu et al. 2006; Lu et al. 2005). Under normal physiological circumstances, YB-1 promotes growth by the potent proliferative capacity it brings to different tissues and organs. Expression of the protein and its association with the polysome are controlled in an age and a tissue-specific manner, presumably helping regulate gene expression at the translational level (Miwa et al. 2006).

1.7.1 Gene Family and Structure

YB-1 was originally named for its ability to bind to the Y-box of major histocompatilibity complex (MHC) class II promoter (Didier e al. 1988). Around the same time, the protein was found to be one of the DNA binding proteins that interacts with the

EGFR enhancer and HER-2 promoter (Sakura et al. 1988), soon followed by a report suggesting that YB-1, alternatively named DNA binding protein B (DbpB), could bind to CT-rich elements in the *C-myc* promoter (Kolluri and Kinniburgh 1991). The consensus binding sequence for YB-1 is 5'-CTGATTGG-3', or inverted CAATT box, which is found in the promoter of, amongst others, the *MHC class II* gene, *MDR1* and *DNA topoisomerase IIα* genes (Kohno et al. 1994; Ohga et al. 1998). Recently, we have shown by chromatin immunoprecipitation (ChIP), electrophoretic mobility shift assay (EMSA) and reporter assay that YB-1 could directly bind to *egfr* and/*her-2* promoter to transactivate these genes in breast cancer cell lines (Wu et al. 2006; Stratford et al. 2007).

The *yb-1* gene is located on chromosome 1p34 and comprises 8 exons (Toh et al. 1998). The mRNA is approximately 1.5kb long and encodes a 43kDa protein of 324 amino acids (Didier et al. 1988). Structural and functional analysis of the human *yb-1* promoter revealed multiple GC-boxes or E-boxes. The transcriptional regulation of YB-1 expression is not completely understood; however, there is one study suggesting that c-MYC may interact with p73 to transactivate *yb-1* promoter (Uramoto et al. 2002). Another report by Yokoyama et al. demonstrated that GATA-1, a transcription factor, could bind to the 5'-UTR to regulate the *yb-1* promoter in erythroid cells (Yokoyama et al. 2003).

YB-1 is a protein in the cold-shock domain (CSD) superfamily. This highly conserved cold-shock domain, also known as the nucleic- acid binding domain of the verterbrate protein, displays about 40% homology with the 70 amino acid cold-shock proteins found in bacteria where the proteins function as RNA chaperones (Matsumoto et al. 1998). Other members of the CSD superfamily are dbpA cloned in 1995 (Kudo et al 1995) and contrin, or dbp C, which is a germ cell-specific Y-box binding protein in human (Tekur et al. 1999) highly homologous to MSY2 found in mice.

Despite the CSD being well conserved among the family members, each protein (in human) shows distinct expression patterns and may confer tissue-specific functions at discrete developmental stages in the body. For instance, while dbpA is expressed in heart and muscle, dbpC is detected mainly in the germ cells (Tekur et al. 1999). AlthoughYB-1 (dbpB) is found ubiquitously in the body, its expression alters at different developmental stages. YB-1 level is higher in fetal cerebrum, heart, muscle, bone marrow and kidney but undetectable in most adult tissues (Spitkovsky et al. 1992).

1.7.2 Protein Structure and Post-Translational Regulation of YB-1

In vertebrates, all the proteins in the cold shock domain (CSD) superfamily consist of three domains: a variable N-terminal domain (NTD), a highly conserved cold shock domain (CSD) which is also known as nucleic acid-binding domain, and a C-terminal domain (CTD) (Wolffe 1994; Graumann et al. 1998).

The analine and proline rich N-terminal domain is believed to be involved in transactivation (Kohno et al. 2003). The evolutionary conserved CSD gives rise to a five-stranded β-barrel with two conserved RNP motifs (Graumann and Marahuel 1996). This domain is involved in binding to oligonucleotides such as RNA, double and single stranded DNA (Bouvet et al. 1995; Didier et al. 1988; Hasagawa et al. 1991; Kolluri et al. 1992; MacDonald et al. 1995). Finally, the C-terminal tail domain is composed of alternate regions of basic or acidic amino acids, and is known to mediate protein-protein interactions (Bouvet et al. 1995).

Acetylation, ubiquitinylation and phosphorylation are examples of post-translational modifications that could affect protein functions. Like other proteins, the function of YB-1 is also controlled by some of these processes. Lutz et al. (2006) identified F-box protein 33 (FBX33) to be a negative regulator of YB-1 expression. FBX33 was found to interfere with YB-1 mediated functions by targeting the protein for polyubiquitination and proteosomal degradation (Lutz et al. 2006). We previously reported that Akt, when activated, phosphorylated YB-1 at Ser102 in the CSD to promote YB-1 nuclear localization and DNA binding (Wu et al. 2006). PKC and RSK are two other proteins that could potentially phosphorylate YB-1 at Ser102 due to their ability to recognize the consensus phosphorylation sequence (RxRxxS/T) on the protein. Analysis using Motif Scanner identified GSK and ERK1 to be the two kinases that may be able to phosphorylate YB-1 at Ser21 and Ser36, respectively, in the N-terminal domain. Also, Tyr197 located in the nuclear localization signal may be phosphorylated by the p85 subunit of PI3K. More studies need to be performed to characterize the specific functions of these phosphorylation events. However, based on the locations of the phosphorylation sites, it could be postulated that Ser21 and Ser36 might regulate transactivation whereas Tyr197 might be involved in trafficking of the protein. (Wu et al. 2007)

1.7.3 Functions of YB-1

1.7.3.1 Transcription Regulation

YB-1, as a transcription factor, binds to sequence motif CTGATTGG, or Y-box, in the promoter regions of many eukaryotic growth-associated genes to activate or repress gene expression (Didier et al. 1988). Transcriptional control by YB-1 can be classified into 3 groups. 1) YB-1 directly binds to Y-boxes and related sequences by itself or with other transcription factors. 2) It could also interact with other transcription factors and functions as co-activator or co-repressor. 3) YB-1 binds to single-stranded region of the promoters, named S1-sensitive sites, to inhibit or enhance binding of other transcription factors to DNA (Kohno et al. 2003).

To date, most studies have focused on the ability of YB-1 to act as a transcription factor up-regulating expression of target genes. YB-1 is capable of increasing cell growth in part by up-regulating transcription of growth-promoting genes such as *cyclin A, cyclin B* (Jürchott et al. 2003), *topoisomerase IIα* (Shibao et al. 1999) and *DNA polymerase α* (En-Nia et al. 2005). Furthermore, under pathological conditions, YB-1 augments transcription of PTP1B (Fukada and Tonks. 2003), matrix metalloproteinase-2 (MMP-2) (Mertens et al. 2002), matrix metalloproteinase-12 (MMP-12) (Samuel et al. 2005), collagen α1 (Norman et al. 2001) and collagen α2 (Higashi et al. 2003), all of which are involved in cell adhesion, motility, invasion and metastasis. Further, YB-1 was proposed to be a marker for tumor aggressiveness and poor clinical response to chemotherapies, owing to the fact that it could confer drug resistance by increasing expression of multi-drug resistance gene (*mdr1*) (Stein et al. 2001), multi-drug resistance-related protein-1 (*mrp1*) (Stein et al. 2001) and major vault protein (*mvp*) (Stein et al. 2005).

YB-1 can also repress transcription of genes such as class II MHC (Didier et al. 1988; Ting et al. 1994) and FAS (Lasham et al. 2000). Rous sarcoma virus d (RSV-d) (Kandala and Guntaka 1994) and other viruses have also been found to exploit YB-1 to increase production of viral proteins. This YB-1 mediated suppression of class II MHC and FAS and production of viral proteins may together facilitate the evasion of immunosurveillance. Table 1 includes the list of genes that are transcriptionally up or down-regulated by YB-1.

1.7.3.2 Role in Translation

Although YB-1 can exert transcriptional control in the nucleus, the majority of the protein resides in the cytoplasm in complexes with various cellular mRNAs, suggesting that YB-1 may be involved in translational regulation as well (Evdokimova et al. 2006). Several lines of evidence have suggested the involvement of YB-1 in translational repression.

Proteins that are highly homologous to YB-1 were found to be responsible for masking and storage of maternal mRNAs in *Xenopus* oocytes, human and rodent germ cells (Matsumoto K et al.1998; Sommerville et al.1996). YB-1 could actively displace eIF4E and eIF4G from certain mRNAs when it binds in proximity to the mRNA cap structure (Evdokimova et al. 2001; Nekrasov et al. 2003). Subsequent studies have shown that a large number of these YB-1-bound transcripts encode stress and growth-related proteins, transcription factors and metabolic enzymes (Evdokimova et al. 2006). It was proposed that translational inhibition as such helps keep these particular subsets of mRNAs silent and make them available for translation in the presence of the appropriate environmental cues (Evdokimova et al. 2006). In line with this observation, Bader et al. have reported YB-1 to be an anti-oncogenic factor and a repressor of translation in chicken embryonic fibroblasts. The ectopic expression of YB-1 suppresses cap-dependent translation of mRNAs and thus renders the chicken embryonic fibroblasts resistant to PI3K or AKT-mediated transformation (Bader et al. 2005).

A model was proposed by Evdokimova et al. explaining how YB-1 may influence translation by acting as a general cap-dependent mRNA stabilizer protecting mRNA against degradation (Evdokimova et al. 2001; Bouvet et al. 1995). While low concentrations of YB-1 facilitate the binding to mRNA, high concentrations of YB-1 destabilize the interaction of the cap-binding protein and thus enhance mRNA stability. A study by Ashizuka et al. (2002) provided direct evidence for the participation of YB-1 in translational control of ferritin protein. Under low iron conditions, the iron-responsive element-binding protein (IRE-BP/IRP2) binds to iron-responsive element (IRE) at the 5'UTR of ferritin mRNA thereby keeping the mRNA in messenger ribonucleoprotein particles (mRNPs), resulting in translational repression. On the other hand, when iron is abundant, IRP2 binds to YB-1 resulting in the release of IRP2 from IRE, leading to expression of ferritin proteins.

In addition to repressing translation, YB-1 or homologus proteins may also enhance splicing (Stickeler et al. 2001) or function as mRNA chaperones in *Xenopus* germ cells and human germ cells (Meric et al. 1997).

1.7.3.3 Drug Resistance

As mentioned earlier in section 1.2.3.1, YB-1 can up-regulate expression of *mdr-1*, *mrp-1* and *mvp* genes (Stein et al. 2001, 2005), all of which are related to drug resistance. In cancers of the breast (Bargou et al. 1997; Saji et al. 2003), prostate (Gimenez-Bonafe et al. 2004), bone (Oda et al. 1998), ovary (Kamura et al. 1999) and muscle (Oda et al. 2003), cells show drug resistance have elevated nuclear YB-1, which is associated with high p-glycoprotein expression. In addition, over-expression of YB-1 renders cancer cells resistant to etoposide and doxorubicin (Bargou et al. 1997) while disruption of YB-1 function using the antisense approach (Ohga et al. 1996, 1998) or targeted disruption of one allele of the gene ($YB\text{-}1^{+/-}$) (Shibahara et al. 2004) sensitizes the cells to cisplatin and mitomycin C.

YB-1 has been shown to immediately localize to the nucleus upon exposure to UV radiation and anti-cancer drugs such as doxorubicin and 5-fluorouracil (Bargou et al. 1997; Stein et al. 2005). It is probable that genotoxic stress induces translocation of YB-1 into the nucleus where it regulates *mdr-1* promoter. Expression of *mdr-1* gene results in production of p-glycoprotein, an ABC transporter, responsible for efflux of chemotherapeutic compounds from cancer cells, therefore giving cancer cells the drug resistant phenotype (Choudhuri et al. 2006).

1.8 Prognostic Value of YB-1 in Cancer

There is growing evidence indicating YB-1 may be an oncogenic transcription/translation factor in a broad range of human malignancies. YB-1 has been found to be over-expressed in cancers of the breast (Wu et al. 2006), prostate (Gimenez-Bonafe et al. 2004), bone (Oda et al. 1998), lung (Shibahara et al. 2001; Gu et al. 2001), colon (Shibao et al. 1999), muscle (Oda et al. 2003) and pediatric brain tumor (Faury et al. 2007). Importantly, nuclear expression of YB-1 has been implicated in drug resistance in breast (Bargou et al. 1997), ovary (Kamura et al. 1999), prostate (Gimenez-Bonafe et al. 2004), colon (Shibao et al. 1999), lung (Shibahara et al. 2001), muscle (Oda et al. 2003) and

thyroid cancers (Ito et al. 2003). The results that we obtained from a large-scale tumor tissue microarray study have also confirmed the significance of YB-1 as an important prognostic marker in breast cancer. In a multivariate analysis, YB-1 was expressed in 41% of breast cancers (1644/4049) and was associated with poor survival ($p<7.3x10^{-26}$) regardless of tumor subtype ($p<6.7x10^{-7}$, HR=1.45). Importantly, YB-1 was better at predicting poor outcome than HER-2 or ER (manuscript in preparation). Collectively, these studies suggest that YB-1 over-expression and nuclear YB-1 expression may be useful biomarkers for prediction of responsiveness to chemotherapies. Thus, targeted disruption of YB-1 function may be a novel strategy to treat human malignancies

1.9 YB-1 and Breast Cancer

In breast cancer, YB-1 has been found to be associated with tumor aggressiveness, relapse and poor patient survival (Bargou et al. 1997; Wu et al. 2006). Breast cancer patients with high level of YB-1 expression have a 66% relapse rate after post-operative chemotherapy treatment whilst patients with low YB-1 expression have 0% relapse rate (Janz et al. 2002). The correlation between YB-1 expression and tumor aggressiveness could be explained in part by the ability of YB-1 to up-regulate expression of various growth-promoting genes. A tumor tissue microarray study that we performed showed a positive correlation between YB-1 and Ki67, implying that presence of YB-1 may be associated with high proliferation index (Wu et al. 2006). Moreover, relapse and poor clinical outcome after chemotherapeutic treatment may also be attributed to the up-regulation of p-glycoprotein, multidrug resistance-related protein-1 (MRP-1) and major vault protein (MVP) (Stein et al. 2001, 2005).

The oncogenic potential of YB-1 was well demonstrated in a study conducted by Bergmann et al. (2005) who discovered that YB-1 was able to provoke breast carcinomas through induction of genetic instability in a transgenic mouse model (Bergmann et al. 2005). We have previously reported that YB-1, when phosphorylated at Ser102 by activated AKT, translocates into the nucleus where it binds to the promoter of EGFR and HER-2 and up-regulates the expression of these receptor tyrosine kinases (RTKs) (Wu et al. 2006). Consistent with this, over-expression of YB-1 in MCF-7 breast cancer cells increased EGFR,

HER-2 transcript as well as protein level with correlated increase in tumor cell growth in both monolayer and soft agar (Sutherland et al. 2005).

There is mounting evidence suggesting that YB-1 may play a role in the progression of breast cancer, and the fact that YB-1 is not detectable in normal breast ductal epithelial cells but is highly expressed in cancerous breast tissues (Bargou et al. 1997; Rubinstein et al. 2002) further makes it an attractive molecular target for treatment of breast cancer.

1.10 Thesis Objective

Hypothesis: The HER-2 over-expressing breast cancer cells require YB-1 for growth and survival

Aim 1: To examine the biological effects of knocking-down YB-1 *in vitro*

Specific aim 1a: To assess the growth of cells in monolayer and soft agar after YB-1 knock-down

Specific aim 1b: To determine if knocking-down YB-1 leads to morphological changes or induces apoptosis

Significance

We have shown that over-expression of YB-1 enhances growth of MCF-7 cells in monolayer and in anchorage-independent conditions. In MDA-MB-231 cells, a model of ER- breast cancer, knock-down of YB-1 by short interfering RNA (siRNA) resulted in 46% suppression of tumor cell growth in monolayer. Following a large-scale TMA study which suggested YB-1 to be an independent prognostic marker in breast cancer, we have become interested in deciphering the functional importance of this protein in the aggressive subtypes of breast cancer, such as the HER-2 positive tumors. Thus, we proposed to use siRNA to silence YB-1 expression and examine downstream biological effects both *in vitro* and *in vivo* in HER-2 over-expressing breast cancer cells.

Aim 2: To verify if knocking-down YB-1 influences tumor growth *in vivo*

Significance

Although it has been shown that YB-1 provokes breast carcinoma in transgenic mice, there has not been any study examining the effects of knocking-down YB-1 in breast tumor

development. Therefore, results from this study would allow us to understand the importance of this protein in tumorigenesis *in vivo*.

Aim 3: To evaluate the effect of a combination treatment involving siYB-1 and Herceptin *in vitro*

Specific aim 3a: To assess colony formation of BT474-m1 cells (Herceptin-sensitive) treated with Herceptin alone or in combination with YB-1 knock-down.

Specific aim 3b: To find out if YB-1 knock-down renders MDA-MB-453 cells (Herceptin-resistant) responsive to Herceptin.

Significance

A large proportion of patients receiving Herceptin monotherapy develop resistance within one year of treatment. Thus, alternative strategies are sought to improve Herceptin efficacy. We proposed that a combination of Herceptin and YB-1 knock-down (or by YB-1 inhibitor) may be more effective than Herceptin alone. The preliminary study performed in soft agar may provide us with valuable information as to whether this combination therapy would be feasible *in vivo*.

Figure 1

Working Model: The PI3K/AKT/YB-1 Pathway

Upon growth factor stimulation, the PI3K/AKT pathway becomes activated and leads to phosphorylation of YB-1 at Ser102 promoting its nuclear localization. When YB-1 is in the nucleus, it functions as a transcription factor, upregulating the expression of EGFR and HER-2. The increased levels of these receptors further enhance signaling through the PI3K/AKT and the MAPK pathways, resulting in cell growth, survival and proliferation.

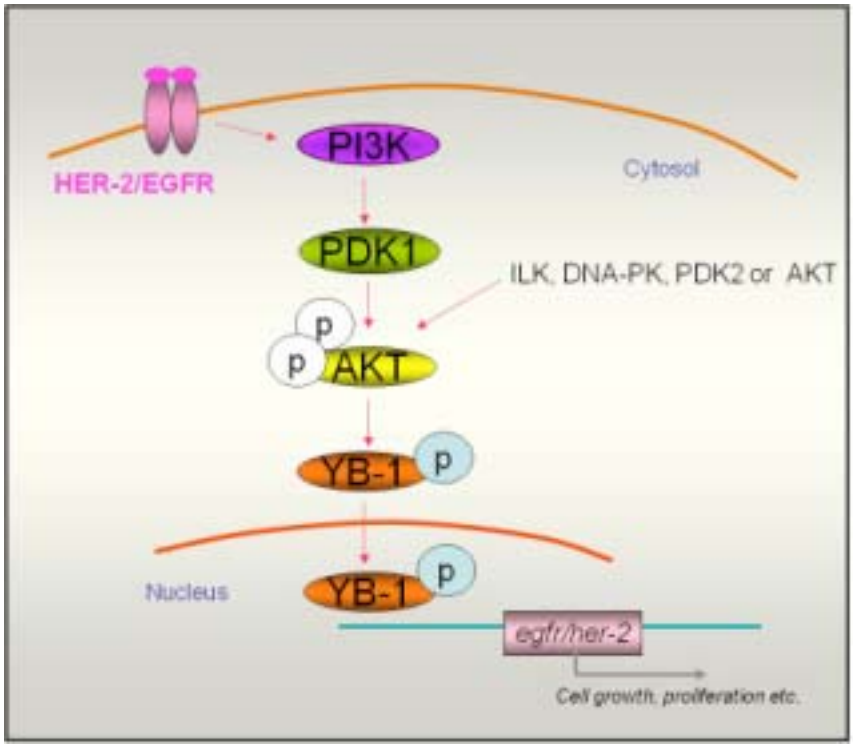


Figure 2

The Basic Structure of the ErbB Family Members

The human epidermal growth factor receptor family is composed of four members: ErbB1 (EGFR, HER-2), ErbB2 (HER-2, *neu*), ErbB3 (HER-3) and ErbB4 (HER-4). The receptors are consisted of an extracellular domain, a single transmembrane domain and an intracellular domain with tyrosine kinase activity. There is no known ligand for ErbB2. ErbB3 does not have intrinsic kinase activity. Therefore, both ErbB2 and ErbB3 need to partner with other receptors within the family to activate downstream signal transduction.

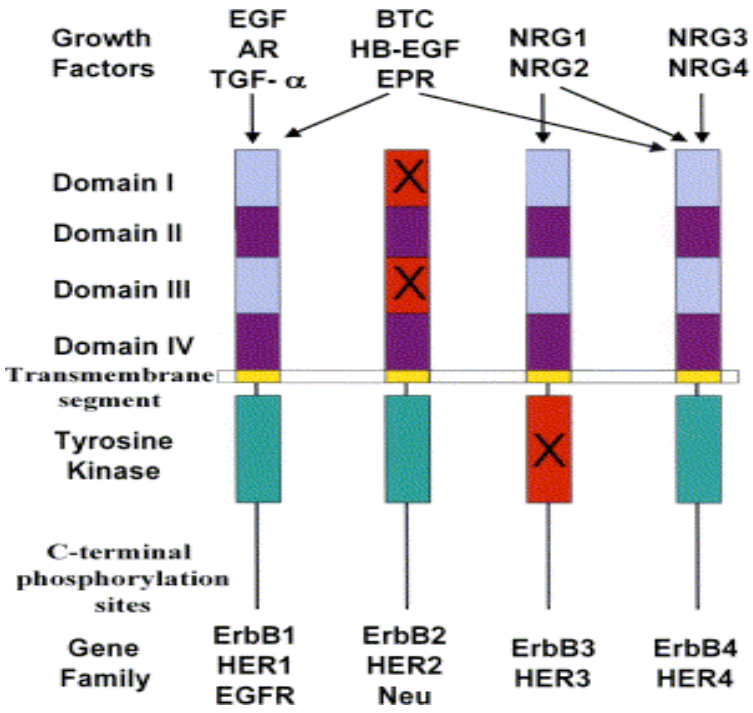


Diagram adopted from Roskoski et al.
Biochem & Biophys Research Communication. 2004

Table 1
Genes that are Transcriptionally Regulated by YB-1

Genes up-regulated by YB-1	Genes down-regulated by YB-1
Cyclin A, cyclin B Topoisomerase IIα DNA polymerase α EGFR HER-2 PTP1B Matrix metalloproteinase-2 Matrix metalloproteinas-12 Collagen α1 Collagen α2 Myosin light chain 2v Human MDR1 GM-CSF p21 HIV-1 Fas/CD95 Rat α1(I) progollagen Rat gelatinase A Jc polyomavirus	Human α2 collagen Human MDR1 Grp78 MHC class II Thyrotropin Collagen α1(I) GM-CSF VEGF FAS

Reference: Kohno et al. BioEssays 2003

CHAPTER 2

MATERIAL AND METHODS

2.1 Maintenance of Cell Culture

BT474-m1 (A gift from Dr. Mien-Chie Hung, University of Texas, MD Anderson Cancer Centre, Texas, USA) and MDA-MB-453 (ATCC®#HTB-131; Manassas, VA, USA) breast cancer cells were cultured in 50% F12 /50% Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Burlington, ON, Canada) and DMEM media respectively, supplemented with 5% fetal bovine serum (FBS) (Invitrogen) and cultured in 37^{o}C, 5% CO_2 incubator. Cells were passaged and subcultured when the confluency reached 80% using 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA) (Invitrogen). BT474-m1 cells are ER-positive, Rb-negative, PTEN-positive and Herceptin sensitive. MDA-MB-4353 cells are ER-negative, Rb-negative, p53-negative, PTEN-positive and Herceptin resistant.

2.2 siRNA Oligos and Transfection

Two validated synthetic YB-1 siRNA oligos (designated siYB-1#1 and siYB-1#2 oligos) targeting the N-terminal or C-terminal region of YB-1 transcripts, respectively, were obtained from Dharmacon (Chicago, IL, USA). Transfection conditions were optimized such that we observed a greater than 70% decrease in YB-1 protein level by western blotting.

BT474-m1 ($3x10^5$ cells) or MDA-MB-453 ($6x10^5$ cells) cells were plated in 6-well culture plates 24 hours prior to transfection. Cells were transfected with either 5nM control (oligonucleotide sequence UUCUCCGAACGUGUCACGU, Qiagen, Maryland, USA) or YB-1 siRNA oligo #1 (oligonucleotide sequence AGAAGGUCAUCGCAACGAA, Dharmacon, Lafayette, CO, USA) or YB-1 siRNA oligo #2 (oligonucleotide sequence CCACGCAAUUACCAGCAAA, Dharmacon) according to manufacturer's protocol. In brief, the appropriate amount of control or YB-1 siRNA stocks were diluted in serum-free media and HiPerfect (Qiagen) transfection reagent was added to the diluted siRNA solution. The mixture was gently vortexed for a few seconds and incubated at room temperature for 5-10 minutes. The complex was added to the cells in each well and the plate was incubated in 37^{o}C, 5% CO_2 incubator until cells were harvested for subsequent studies.

2.3 Protein Extraction and Quantification

2.3.1 Whole Cell Lysis

Harvested cells were washed with cold PBS and pelleted by centrifugation at 5,000rpm for 5 minutes. To lyse the cells, the pellets were resuspended in 4-packed-cell volumes of egg lysis buffer (ELB; Appendix) and incubated on ice for 30 minutes. The lysates were then passed through a 22-gauge needle 7 times to release the cellular contents, and proteins were isolated by centrifuging (microcentrifuge) the samples at 13,000rpm for 10 minutes at 4^{o}C. The supernatant containing the protein was retained for subsequent studies.

2.3.2 Protein Quantification

Proteins isolated from the whole cell lysis procedure were subjected to Bradford assay for protein quantification. To establish standard curves, increasing amounts of bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) (1µg, 2µg, 5µg, 10µg and 15µg) were added in 1ml of Bio-Rad Protein Assay dye reagent (Bio-Rad, Hercules, CA, USA) for 15 minutes at room temperature and absorbance was measured at 595nm with a spectrophotometer. A graph of absorbance for each standard versus protein concentration was plotted to construct a standard curve with calculated linear regression and equation. To measure protein concentration in samples, 1µl of the lysates was added to 1ml of the dye reagent and incubated for 15 minutes at room temperature. Absorbance of the samples was measured at 595nm and protein concentration was determined using standard curve.

2.4 Western Blotting

2.4.1 Electrophoresis

To prepare protein samples for western blotting, 5-50µg of protein was added to 5x sample loading buffer (Appendix), boiled for 5 minutes and loaded on to a SDS-polyacrylamide gel. The gel consists of a stacking gel and a resolving gel (12%). Pre-stained broad range standards (Bio-Rad) were also loaded on each gel, which was subjected to electrophoresis in running buffer (Bio-Rad) at 82 volts for approximately 20 minutes to compress the proteins into single bands, and subsequently at 170 volts for 1.5 hours.

2.4.2 Transfer of Proteins to Nitrocellulose Membrane

After the electrophoresis procedure, a 0.45µm nitrocellulose membrane and the gel were soaked in 1x transfer buffer (Appendix) for 12 minutes. The proteins were transferred to the membrane in chilled 1x transfer buffer overnight at 45 volts, 4^{o}C using Mini Trans-Blot® Electrophoresis Transfer Cell (Bio-Rad). Ponceau-S (0.1% Ponseau-S in 5% acetic acetic acid; Sigma) was used to stain the proteins on the membrane to confirm complete transfer.

2.4.3 Primary Antibody Incubation and Detection

The membrane was incubated in 5% non-fat milk in PBS with 0.1% Tween-20 (PBS-T) (Fischer Scientific, Ottawa, ON, Canada) for 1 hour at room temperature to reduce non-specific binding. After this blocking procedure, the membrane was incubated with appropriate primary antibody overnight at 4^{o}C on a rocking platform. Antibodies used for the studies and their dilutions were as follows: anti-YB-1 (1:10,000; gift from Dr. Colleen Nelson of the Prostate Centre, Jack Bell Research Centre, UBC, Vancouver, Canada), anti-EGFR (1:1000; StressGen, San Diego, CA, USA), anti-HER-2 (1:1000; Abcam, Cambridge, CA, USA), anti-pERK1/2$^{Thr202/Tyr204}$ (1:500; Cell Signaling Technology; Danvers, MA, USA), anti-pAKT^{Ser473} (1:1000. Cell Signaling Technology) and anti-pH2AX^{Ser139} (1:500; Abcam). Anti-Vinculin (1:2000; Sigma clone Vin 11-5, V4505 antibody) or anti- pan-actin (1:1000; Cell Signaling Technology) antibodies, the latter were used as loading controls. The antibodies used were diluted in 5% bovine serum albumin (BSA) (Sigma) in PBS-T. To detect protein-of-interest, the membrane was incubated with appropriate secondary antibody (anti-mouse or anti-rabbit IgG horseradish peroxidase-linked antibody) in 5% non-fat milk in PBS-T for 1 hour at room temperature on a rocking platform, followed by three times, 5 minutes-each washing in PBS-T, enhanced chemiluminescence (ECL) dectection system (GE Healthcare, Bio-Sciences, Piscataway, NJ, USA) and film exposure (Kodak, Burnaby, Canada).

2.5 MTS Assay

Cells were transfected with 5nM control or YB-1 siRNA oligos in 6-well plates. One day after transfection, BT474-m1 cells and MDA-MB-453 cells were seeded in a 96-well plate (5000 and 8000 cells/well respectively). The cells were subjected to the CellTiter 96®

Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) at different time-points to evaluate cell viability. For a 96-well plate, 2ml of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was mixed with 100µl of PMS (phenazine methosulfate) in 9.8ml of serum-free media. 120µl of this mixture was applied to each well. The cells were incubated in the dark at 37°C, 5% CO_2 incubator for 2hrs before the plate was read at 490nm. There were six replicates per treatment and the experiment was carried out on two separate occasions.

2.6 Soft Agar Assay

Two-times concentrated DMEM (2x DMEM) was made according to manufacturer's (Invitrogen) protocol. The feeder layer was prepared by mixing 2x regular growth medium with 1.2% agarose solution in 1:1 ratio. 1ml of the mixture was added to each well of a 6-well plate. The cell layer was made by adding BT474-m1 ($1x10^4$ cells) or MDA-MB-453 ($4x10^4$ cells) to a 1:1 mixture of 2x DMEM and 0.6% agarose solution. For the Herceptin study, varying concentrations of Herceptin (BC Cancer Agency Pharmacy Store, Vancouver, Canada) were added to the cell layer mixture to give final concentrations of 0.2, 2.0 or 2µg/ml. Cells were incubated in 37°C, 5% CO_2 incubator for 28 days. Colony size was measured and colonies that were greater than 300µm (BT474-m1) or 50µm (MDA-MB-453) in diameter were counted under the microscope (Leica DMIL). The experiment was performed in triplicates on three different occasions.

2.7 Flow Cytometry

2.7.1 Propidium Iodide Incorporation

To evaluate the proportion of cells that died after YB-1 siRNA treatments, we used flow cytometry in conjuction with propidium iodide (PI) staining to determine the fraction of cells that incorporated the dye and therefore underwent cell death. PI is a non-permanent dye that selectively penetrates the cell membrane of dying and dead cells and intercalates into the major groove of DNA, thereby allowing the non-viable cells to be detected by fluorescence (Crompton et al. 1992). BT474-m1 cells were transfected with 5nM control or YB-1 siRNA oligos and incubated for 3 days in a 37°C, 5% CO_2 incubator before being stained with propidium iodide (Sigma). Staining was performed as follows; all cells, attached and

floating, were collected and washed once with 1% fetal bovine serum in PBS. The cells were then stained with 30µg/ml of propidium iodide in 1% fetal bovine serum in PBS for 3-5 minutes and then washed in 1% fetal bovine serum in PBS. The cells were centrifuged, supernatant was removed and finally the stained cells were resuspended in 500µl of 1% fetal bovine serum in PBS before flow cytometry (BD FACSCalibur) analysis. The experiment was repeated on two different occasions.

2.7.2 Annexin V-PE/7AAD Staining

During apoptosis, the membrane phospholipid phosphatidylserine, or PS, translocates from the inner to the outer leaflet of the plasma membrane and becomes exposed to the extracellular environment. Annexin V, a 35-36kDa $Ca2^{+}$ dependent protein, has high affinity to PS and therefore binds to the apoptotic cells with exposed PS. Annexin V can be conjugated to Phycoerythrin (PE), a fluorochrome, thereby allowing detection of the apoptotic cells by flow cytometry analysis (van Engeland et al. 1996). To determine the fraction of cells undergoing apoptosis after silencing YB-1, BT474-m1 cells were transfected with 5nM control or YB-1 siRNA oligos and incubated for 3 days in 37^{o}C, 5% CO_2 incubator before being stained with Annexin V-PE (Promega, Madison, WI, USA). Staining was done according to manufacturer's protocol. In brief, the harvested cells were washed twice with cold PBS, followed by a 15 minute incubation, in the dark, in 1x binding buffer containing Annexin V-PE and 7AAD dyes. Following this, 250µl of 1x binding buffer was added to each sample and flow cytometry analysis was performed within 1hr of staining. The experiment was repeated on three separate occasions.

2.8 Hoechst, Propidium Iodide and Phospho-Histone 2AX Staining and Detection by ArrayScan VTI Reader

BT474-m1 cells (10,000 cells/well in a 96-well plate) were plated in collagen I-coated 96-well plate (BD BioSciences) and transfected with 5nM control or YB-1 siRNA oligos for 3 days. Immediately after aspirating the medium, 100µl of 2% paraformaldehyde in PBS was added and the cells were kept at room temperature for 20 min. After washing with PBS three times, the cells were permeabilized in 0.1% Triton X-100 in PBS for 15 min followed by 1% BSA in PBS for 30 min. The cells were stained in Hoechst dye (100µl at

1µg/ml) and PI (100µl at 30µg/ml) for 5 minutes at room temperature, or primary mouse anti-phospho-H2AX antibody (Abcam) diluted 100x for 1hr at room temperature, followed by a secondary donkey anti-mouse antibody (FITC; Jackson ImmunoResearch Laboratories., West Grove, PA) diluted 200x for 1hr at room temperature. The cells were washed with PBS three times after each step mentioned above and were finally kept in PBS (100µl/well) at 4^{o}C. The plates were analyzed and the images were taken on the ArrayScan VTI Reader (Cellomics, Pittsburgh, PA). Six hundred cells were analyzed for each replicate well.

2.9 Pathway Evaluation of Apoptosis Induction

Cells treated with 5nM of control or YB-1 siRNA oligos were immunoblotted for mTOR, pmTOR^{Ser2448}, STAT3, pSTAT3^{Ser727} and MCL-1. From these experiments, it was not clear which pathway could be responsible for phosphorylation of STAT3 at Ser727; therefore, BT474-m1 cells ($5x10^4$ cells/well in a 6-well plate) were plated and treated with 40nM of rapamycin or 20µM of PD98059 for 6 hrs and proteins were extracted for western blotting. The concentrations of rapamycin PD98059 used in the studies were determined from current literature (Cui et al. 2002; Stratford et al. 2007). Rapamycin is a macrolide antibiotic from *Strep. hygroscopicus*. Rapamycin binds FKBP12 forming a FKBP12/rapamycin complex that subsequently interacts with the target of rapamycin (TOR, or mTOR in mammalian cells) to inhibit the protein kinase (Sabers et al. 1995). PD98059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] has been shown to selectively inhibit the MAPK-activating enzyme, MAPK/ERK kinase (MEK) (Dudley et al. 1995). Therefore, PD98059 inhibits phosphorylation of ERK1/2 by MEK. The expression of pSTAT3 ^{Ser727}, pERK1/2$^{Thr202/Tyr204}$ and pS6K^{Ser389} (Cell Signaling Technology) were evaluated after drug treatments at the indicated timepoints.

2.10 Effect of siYB-1 in Xenograft Model

BT474-m1 cells were derived from tumors arising from the parental BT474 cells, which have been shown to be tumorigenic in Amsterdam/IMR rats and nude mice (American Type Culture Collection). For model validation, we first conducted a pilot study to determine the conditions required for tumor formation. The cells were injected subcutaneously into the lower hind region of female Nu/Nu mice (n=12); weight of mice and size of the tumors were

measured twice weekly for 4 weeks. One week after injection, 10/12 of the mice developed palpable tumors that continued to grow until week 4. The size of the tumors varied from 30 mm^3 to greater than 80 mm^3 with an average size of approximately 50 mm^3, calculated using the formula: length x $width^2$ /2. Having confirmed that BT474-m1 cells were tumorigenic in mice with 83% penetrance one week after injection (n=12), we conducted YB-1 knock-down study using 6 mice per treatment group. The treatments included HiPerfect (mock control), control oligo-transfected and siYB-1 oligo #1 and #2-transfected cells (n=6/group).

BT474-m1 cells were transfected with 5nM of control or siRNA oligos. One day after transfection, the cells were harvested by trypsinization (0.25% typsin-EDTA; Invitrogen). Cells were washed with Hanks Balanced Salt Solution (Invitrogen) twice and counted to prepare $1x10^6$ cells, which were subsequently mixed with matrigel (BD Bioscience, MA, USA) in a 1:1 ratio with a total volume of 200µl. The tumor cells/matrigel mixture was injected subcutaneously in to the right lower hind region of female *nu/nu* mice using a 26-gauge needle. Tumor growth was measured using callipers. Mice were terminated, using CO_2, 4 weeks post-injection. Tumors were snap frozen in liquid nitrogen at time of harvest. Measurements of width, length and depth were taken twice weekly and tumor volumes were calculated according to the equation $4/3\pi$ (a/2xb/2xc/2) where a, b and c are width, length and depth of the tumors.

CHAPTER 3

RESULTS

3.1 HER-2 over-expressing breast cancer cells BT474-m1 depend on YB-1 for growth

3.1.1 Knocking-down YB-1 decreased the expression of YB-1 target genes & signaling through the MAPK pathway in BT474-m1 cells

We first characterized the expression of proteins important to the studies in a panel of breast cancer cell lines. We confirmed that HER-2 was indeed over-expressed in BT474-m1 and MDA-MB-453 cells. BT474-m1 cells, which showed a significantly higher level of HER-2 protein than MDA-MB-453 cells (Fig 3), probably due to HER-2 gene amplification (Yakes et al. 2002). HER-2 was reported to be coustitutively activated in BT474, the parental cell line of BT474-m1. The receptor activation was attributed in part by the elevated level of TGF-α secreted by the cells, stimulating EGFR to form heterodimer with HER-2 and activate downstream signaling (Masuda et al. 2003). Unlike BT474 cells in which TGF-α/EGFR autocrine signaling may be sufficient to activate HER-2, MDA-MB-453 cells are not known to produce their own HER family ligands. SUM149 and MDA-MB-468 are two basal-like breast cancer cell lines, and therefore do not over-express HER-2 (Fig 3). We also examined the expression of HER-2 downstream signaling molecules in the PI3K/AKT and MAPK pathways. While $pAKT^{Ser473}$ is expressed at higher levels in BT474-m1 cells than in MDA-MB-453 cells, the opposite is true for $pERK1/2^{Thr202/Tyr204}$ (Fig 3). Although actin was used as a loading control in this study, it would be better to use antibodies against total AKT and ERK1/2 to specifically determine the loading of those proteins.

To determine how long the siRNA oligos could effectively knockdown YB-1, a time-course study was performed to examine protein expression over 14 days. BT474-m1 cells were transfected at a confluency of 50% and harvested 1, 3, 5, 7, 10 and 14 days after transfection. YB-1 protein level was reduced by 50% two days after transfection (data not shown) and the degree of knockdown was even more prominent three days post-transfection (Fig 4). We observed a continued knock-down of >70% up until 10 days post-transfection. While the siYB-1 #2 oligo still showed a minimal effect 14 days post-transfection, it appears

that YB-1 expression returned to the same level as the controls using the siYB-1 #1 oligo at this time point (Fig 4).

Since we were able to show that over-expression of YB-1 increased protein level of HER-2 and EGFR in MCF-7 cells (Sutherland et al. 2005), we questioned whether knocking down YB-1 could reverse this effect in our HER-2 models. Western blots were performed to evaluate the expression of YB-1 target genes such as EGFR and HER-2 after YB-1 was silenced. In BT474-m1 cells, knocking-down YB-1 decreased HER-2 and EGFR protein level as well as signaling through the MAPK pathway, exemplified by a decrease in phosphorylation of ERK1/2 (Fig 5A). In MDA-MB-453 cells, which express very low or undetectable level of EGFR, HER-2 expression was down-regulated after siYB-1 treatment, consistent with that in BT474-m1 cells. However, phosphorylation of ERK1/2 did not seem to be affected (Fig 5B). In both models, $pAKT^{Ser473}$ level was not altered by YB-1 knock-down (Fig 5A and 5B).

Figure 3

Levels of YB-1, EGFR, HER-2, PCNA, pAKT^{Ser473} and pERK1/2$^{Thr202/Tyr204}$ in a panel of breast cancer cell line

The protein expression of YB-1, EGFR, HER-2, PCNA, pAKT^{Ser473} and pERK1/2$^{Thr202/Tyr204}$ were examined in a panel of breast cancer cell lines by western blotting. BT474-m1 and MDA-MB-453 were the cell lines used in subsequent studies. SUM149 and MDA-MB-468 are included for a comparison of protein expression levels. Actin was assessed as a loading control.

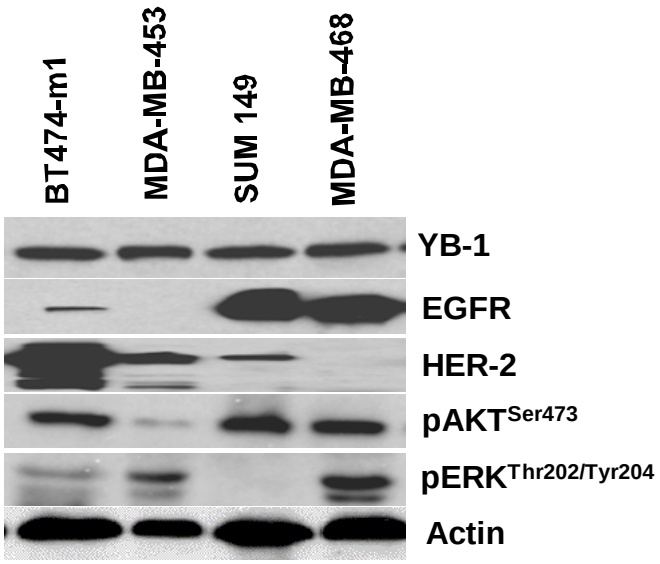


Figure 4

Time-course study examining the reduction of YB-1 protein level by siRNA oligos

Log-growing BT474-m1 cells were transfected with 5nM control or siRNA oligos targeting YB-1 and whole cell extracts were prepared at 1, 3, 5, 7, 10 and 14 days post-transfection. Proteins were separated by SDS-polyacrylamide-gel electrophoresis and immunoblotted with an antibody against YB-1 protein. Vinculin was measured as a loading control.

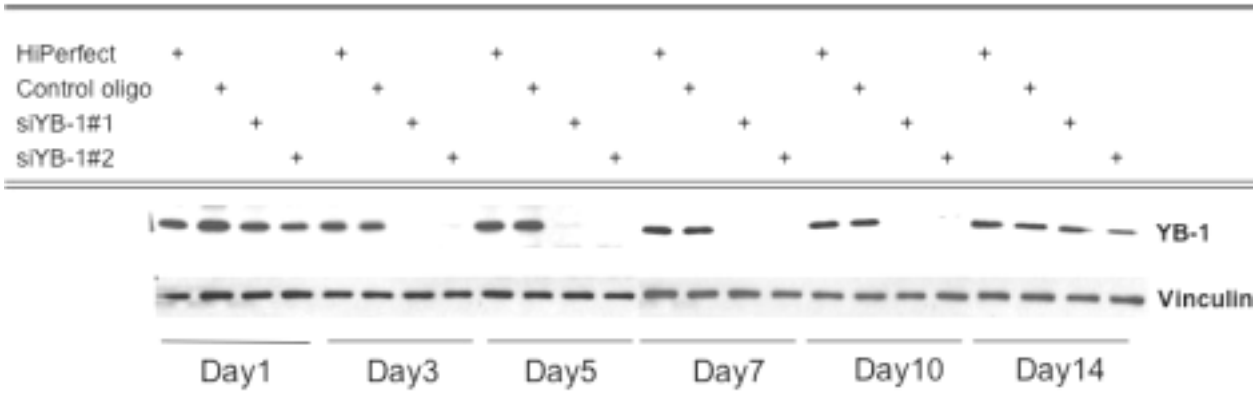
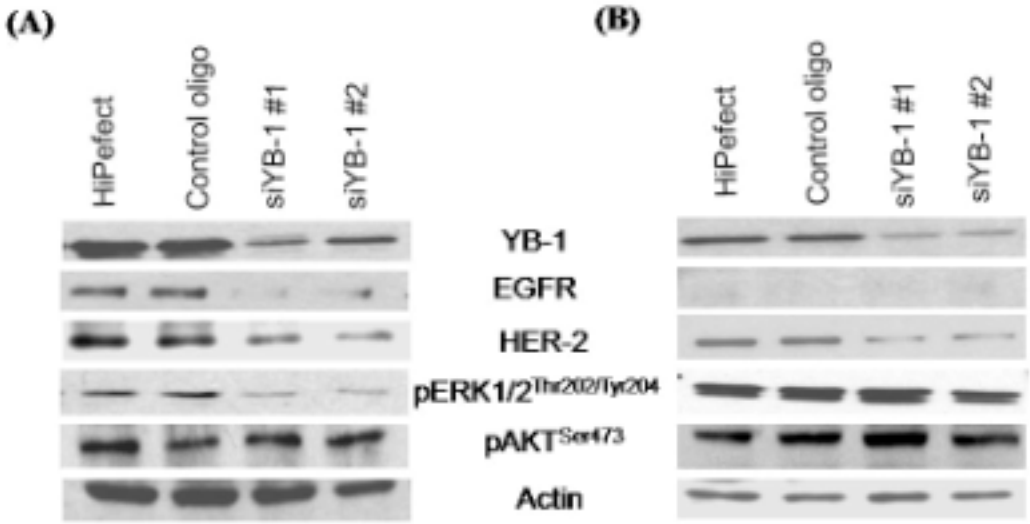


Figure 5
Suppression of YB-1 decreased expression of YB-1 target genes in BT474-m1 and MDA-MB-453 cells

Exponentially proliferating BT474-m1 **(A)** and MDA-MB-453 **(B)** cells were transfected with control or siRNA oligos targeting YB-1. Whole cell protein extracts were prepared 4 days after transfection. Western blots were performed to examine the expressions of *egfr* and *her-2* which are YB-1 target genes. Protein level of $pAKT^{Ser473}$ and $pERK1/2^{Thr202/Tyr204}$ in the PI3K/AKT and MAPK pathways were also evaluated. Pan-actin was examined to ensure that the samples were equally loaded.



3.1.2 Knock-down of YB-1 inhibited the growth of BT474-m1 in monolayer and in anchorage-independent conditions

Having confirmed that the siRNA oligos have specific effects on the expression of YB-1 and its target genes, we questioned if silencing this factor has an impact on growth of the cells. We first examined the effect in monolayer by transfecting BT474-m1 or MDA-MB-453 cells with 5nM control or siRNA oligos in a 6-well plate and re-plating the cells in a 96-well plate 1 day after transfection. Growth of the cells was assessed by MTS cell viability assay 1, 3, 5 and 7 days after re-plating in the 96-well plate. Untreated cells were included to serve as a reference. Percentage cell growth of the treated cells was calculated relative to growth of the untreated cells. Growth of BT474-m1 cells was inhibited by ≥ 40% throughout the 7-day study using siYB-1#2 oligo. Growth suppression was also observed in the last two timepoints (day 5 and 7) using siYB-1#1 oligo (Fig 6A). Conversely, growth of MDA-MB-453 cells was unaffected by either of the siRNA oligo (Fig 6B).

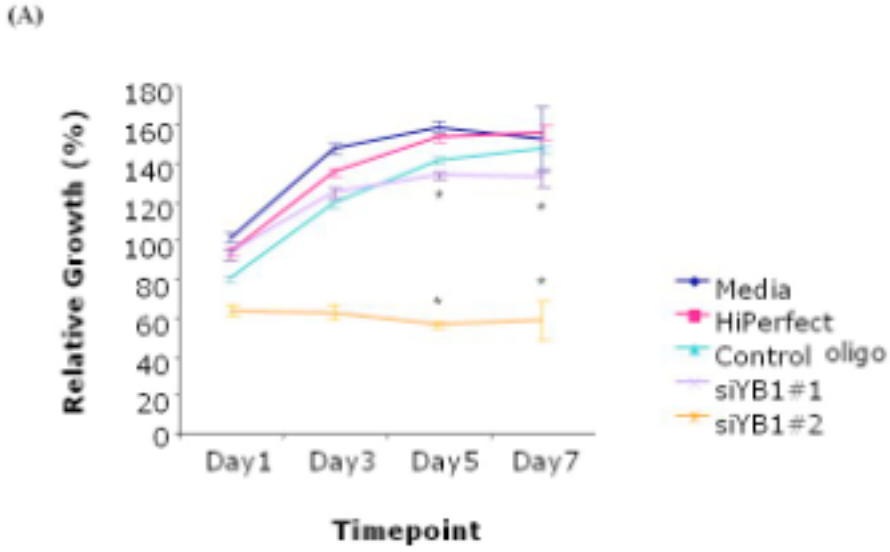
To take the monolayer growth study a step further, we examined growth of the siYB-1 cells in soft agar, which measures anchorage-independent growth of transformed cells. BT474-m1 cells were transfected with 5nM control or siRNA oligos and re-plated in 0.3% soft agar one day after transfection. Seeding density was optimized such that sizeable colonies formed by 28 days in soft agar. The total number of colonies was similar in the controls and the siYB-1 treatment. However, interestingly, big colonies >300 μm were almost exclusively present in the controls but not the siYB-1 group. The colonies that best represent this difference are shown in the photomicrographs in Fig 7.1A. When we used 300μm as the cut-off for colony counting, there were approximately 10 or more colonies greater than 300 μm per well in the controls. In contrast, only 1 or 2 of such colonies were found in the siYB-1 treatment (Fig 7.1B). The average size of colonies in the control groups was approximately 700 μm whereas the colonies from the siYB-1 treatment were around 200 μm. Therefore, the average size difference for the control versus siYB-1 colonies is about 3.5 fold.

A parallel study was performed in MDA-MB-453 cells, which did not form colonies at lower seeding density. Even with an increased seeding density ($1.5x10^5$ cells/well) in a 6-well plate, MDA-MB-453 cells formed much smaller colonies than BT474-m1 cells after 28 days in soft agar. Photomicrographs of colonies from the control and siYB-1 groups were

shown in Fig 7.2A. The average size of colonies from the control groups was around 100 μm, which is 2-fold larger than colonies in the siYB-1 treatment (Fig 7.2A) and there were approximately 2-fold more colonies (≥50μm) in the controls compared to the siYB-1 treatment (Fig 7.2B).

Figure 6
Decreasing YB-1 protein expression perturbed cell growth in monolayer of BT474-m1 but not MDA-MB-453 cells over 7 days

BT474-m1 **(A)** and MDA-MB-453 **(B)** cells were transfected with control or siRNA oligos targeting YB-1 for 1 day before being re-plated in 96-well plates. Cells were subjected to MTS assay 1,3, 5 and 7 after re-plating. Relative percentage of growth of the treated cells was calculated based on growth of the untreated cells (media added only)



(B)

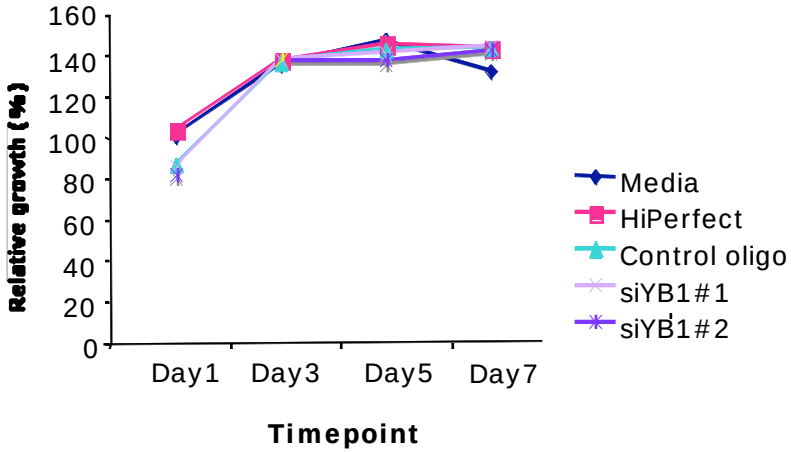


Figure 7.1
Knock-down of YB-1 in BT474-m1 cells resulted in formation of smaller colonies in soft agar

BT474-m1 cells were transfected with 5nM control or siRNA oligos targeting YB-1. One day after transfection, the cells were counted and re-plated in soft agar containing 50% DMEM and 50% 0.6% agarose. Colonies ≥300µm were counted under the microscope after 28 days in soft agar. Representative photomicrographs are shown to illustrate the differences in colony size **(A)**. Quantification of colonies ≥300µm is shown in the bar chart below **(B)**.

(A)

Range of Colony Size (µm)				
709~870	***717~877***	***537~896***	***129~168***	**170~206**

(B)

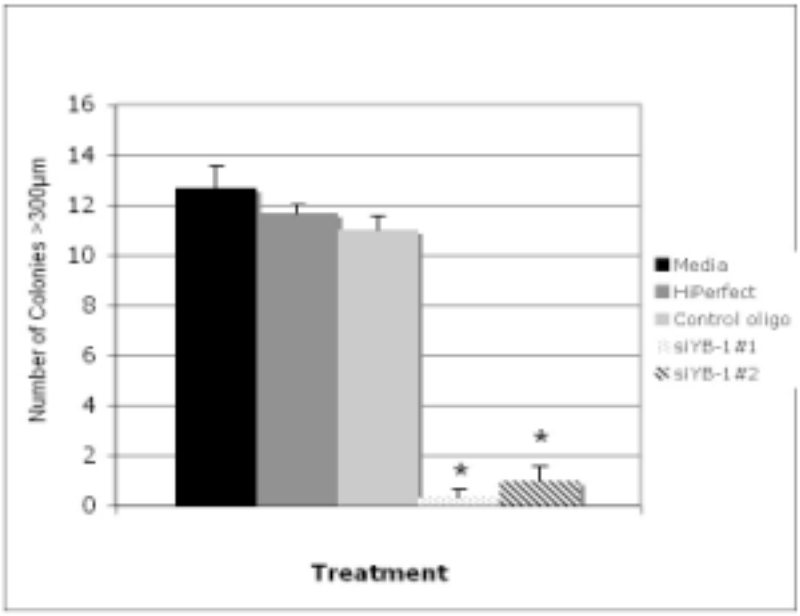
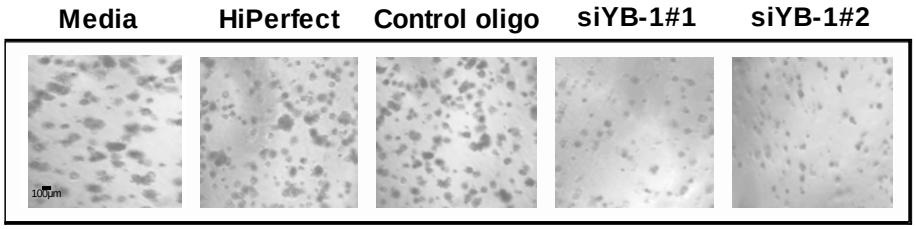


Figure 7.2
Knock-down of YB-1 in MDA-MB-453 resulted in formation of smaller colonies in soft agar

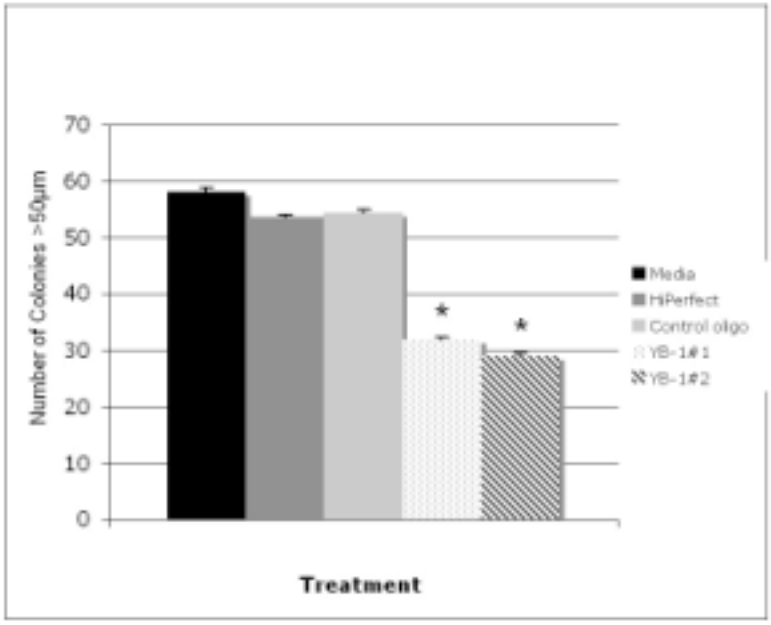
MDA-MB-453 cells were transfected with 5nM control or siRNA oligos targeting YB-1. One day after transfection, the cells were counted and re-plated in soft agar containing 50% regular growth medium and 50% 0.6% agarose. Colonies ≥50μm were counted under the microscope after 28 days in soft agar. Representative photomicrographs are shown to illustrate the differences in colony size **(A)**. Quantification of colonies ≥50μm is shown in the bar chart below **(B)**.

(A)



Range of Colony Size (μm)				
90-120	***82-116***	***85-105***	***58-74***	***55-67***

(B)



3.2 HER-2 over-expressing breast cancer cells BT474-m1 need YB-1 for survival

3.2.1 Induction of cell death was observed after YB-1 siRNA treatment

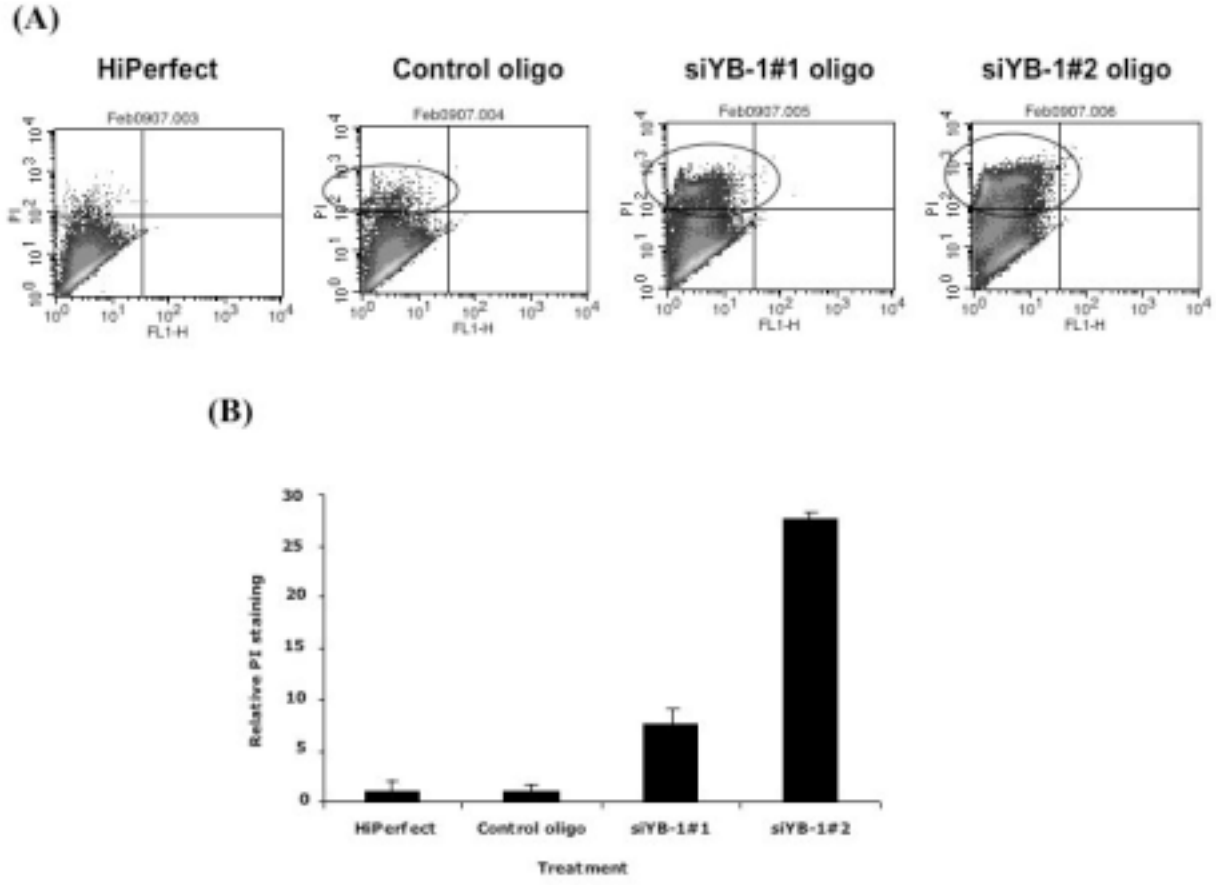
An interesting observation that we made was that BT474-m1 cells began to lift off the plate two days after YB-1 siRNA transfection, a time that correlates with loss of YB-1 expression. However, the control cells including untreated, HiPerfect-treated and the control oligo-transfected cells did not show sign of cell death or morphological changes and remained attached to the wells. An increasing number of floating cells was observed in the siYB-1 treatment starting 2 days post-transfection and by day 4, approximately 60% of the cells lifted leaving only 30-40% of the original cell population still attached to the wells.

Since we observed this notable difference, we compared the viability of the siYB-1 cells with that of the control cells. BT474-m1 cells were transfected with 5nM control or siRNA oligos for 3 days before being harvested for flow cytometry analysis based on propidium iodide incorporation. This time-point was chosen because we could observe substantial cell lifting at this point. Untreated and HiPerfect-treated cells were included as controls to show that the staining procedure itself or the transfection reagent did not cause non-specific cell death. The floating and attached cells in the wells were collected, stained with propidium iodide (PI) and subjected to flow cytometry analysis. The PI-positive cell population is located in the upper left quadrant of each scatter plot and circled (Fig 8A). In this study, the proportion of PI-positive cells is similar in untreated and HiPerfect-treated cells, suggesting that the transfection reagent had minimal toxicity effect on the cells (data not shown). However, there was a striking difference in proportion of PI-positive cells between the siYB-1 cells and control oligo-transfected cells. We quantified the proportion of PI-positive cells in the control oligo-transfected group and siYB-1 treatment and found an 8-fold (siYB-1#1 oligo) or 30-fold (siYB-1#2 oligo) difference in the proportion of PI-positive cells (Fig 8B). The results confirmed our visual observation that knock-down of YB-1 by siRNA oligos decreased the viability of BT474-m1 cells.

Figure 8

siYB-1 treatment triggered cell death in BT474-m1 cells

BT474-m1 cells were transfected with control or siRNA oligos targeting YB-1 for 3 days and stained with propidium iodide to assess cell viability. The cells were washed with PBS containing 1% fetal bovine serum and stained with 30µg/ml of propidium iodide for 3-5 minutes. The cells were subsequently washed and re-suspended in PBS containing 1% fetal bovine serum and subjected to flow cytometry analysis **(A)**. Percentage of propidium iodide positive cells was calculated and compared (in relative fold difference) among different treatments **(B)**.



3.2.2 Apoptosis was induced after YB-1 level was decreased by siRNA

Results from section 3.2.1 suggested that knocking-down YB-1 decreased viability of BT474-m1 cells. Since propidium iodide gets incorporated into all dead cells and does not distinguish necrotic from apoptotic cells, to determine if silencing YB-1 induces apoptosis, we used Annexin V-PE to stain the cells and performed flow cytometry analysis.

The circled population in Fig 9.1A represents the Annexin V-positive cells. On each scatter plot, cells in the lower right quadrant (Fig 9.1A) are Annexin V-positive, 7AAD-negative, which are the early apoptotic cells. The late apoptotic and/or necrotic cells are locate in the upper right quadrant of each scatter plot and are Annexin V-positive, 7AAD-positive (Fig 9.1A). When the results were quantified, we found 3-fold and 6-fold more Annexin V-positive cells in the siYB-1#1 and siYB-1#2 groups, respectively, compared to the control cells (Fig 9.1B).

An increasing number of floating cells was seen in the plate 2 to 4 days post-transfection. We therefore carried out a time-course of Annexin V-PE incorporation to confirm this observation. There was no difference in the degree of Annexin V staining for all the cells at day 1 (Fig 9.2), correlating with our observation that both control and siYB-1 cells remained attached to the wells 1 day after transfection. However, by day 2, siYB-1#1 and siYB-1#2 showed 2-fold and 2.5-fold more Annexin V-positive cells compared to the controls, respectively. The difference in staining between control and siYB-1 treatment was even more evident on day 3 (3-fold for siYB#1 oligo and 4.5-fold for siYB-#2 oligo). The effect of siYB1#1 oligo peaked at day 3 while siYB-1#2 continued to induce apoptosis on day 4 (Fig 9.2). Thus far, we conclude from this result, that knocking-down YB-1 induced apoptosis in BT474-m1 in a time-dependent manner up to 4 days after siRNA oligo transfection.

With the evidence that cell viability was reduced and apoptosis was induced upon siYB-1 treatment, we wished to further examine this topic at the molecular level. Phosphorylation of histone H2AX at Ser139 ($pH2AX^{Ser139}$, also known as γ-H2AX) occurs during apoptosis, prior to the appearance of internucleosomal DNA fragments and externalization of membrane phospholipid phophatidylserine (PS) from inner to the outer leaflet. Phosphorylation of histone H2AX was found to occur at the stage of chromatin modification following initiation of DNA fragmentation during apoptosis (Rogakou et al.

2000). As YB-1 expression decreased from day 1 to day 4, there was a concomitant increase in pH2AX^{Ser139} expression (Fig 10). At 48 hours post-transfection, pH2AX^{Ser139} expression was detectable when YB-1 expression was reduced by ≥50%. The expression of pH2AX^{Ser139} further increased at 72 and 96 hours when there was a more significant loss in YB-1 protein level (Fig 10).

To further substantiate our finding that silencing YB-1 induces apoptosis, we used the ArrayScan VTI high throughput screening instrument for the apoptosis study. This technique allowed us to examine different markers simultaneously in the same cell field on a 96-well plate. BT474-m1 cells were transfected with siRNA in a 96-well plate and incubated for 3 days before being stained and analyzed on the instrument. There were substantially more rounded up cells in the siYB-1 treatment while the control cells did not have signs of morphological changes (data not shown). Staining of the cells and analysis of the results on ArrayScan VTI reader were performed with the help of Dr. Kaiji Hu. First we examined the intensity of Hoechst in the cells. This dye specifically stains the nuclei, which in apoptotic cells, becomes condensed resulting in more intense signal (Fig 11A). For propidium iodide staining, the cells transfected with siYB-1#1 and siYB-1#2 showed 2-fold and 7-fold higher average intensity of staining than the controls, respectively (Fig 11B). The cells transfected with siYB-1#2 oligo displayed a 3-fold increase in average pH2AX^{Ser139} staining intensity relative to the control cells (Fig 11B). Results from the ArrayScan VTI Reader were consistent with those from the previous studies although the difference in percentage of positively stained population appeared to be smaller between control and treatment groups. In the flow cytometry studies, the floating and attached cells were collected for analysis. On the other hand, washing and staining of cells in the 96-well plate for ArrayScan VTI study resulted in loss of the floating cells, which was an inevitable consequence of the experimental procedure itself. The floating cells may have represented a population that was most profoundly affected by the siYB-1 treatment, and loss of this population could probably explain why we saw a smaller difference in the positively-stained cells. Still, we were able to demonstrate that knocking-down YB-1 reduced cell viability and induced apoptosis in this system. Collectively, these data suggest that YB-1 may be an important factor conveying survival advantage to the BT474-m1 cells and silencing expression of this factor leads to cell death.

Figure 9.1

Apoptosis was induced in BT474-m1 cells after knocking-down YB-1 expression

BT474-m1 cells were transfected with control or siRNA oligos targeting YB-1 for 3 days and stained with Annexin V-PE. The cells were washed twice with cold PBS and stained with 1x binding buffer containing Annexin V-PE/7AAD and incubated in the dark for 15 minutes at room temperature. 1x binding buffer was added to the samples, which were analyzed by flow cytometry for Annexin V-PE binding **(A)**. The bar chart below is a quantification of the relative fold difference in Annexin V-PE positive cells among different treatments **(B)**.

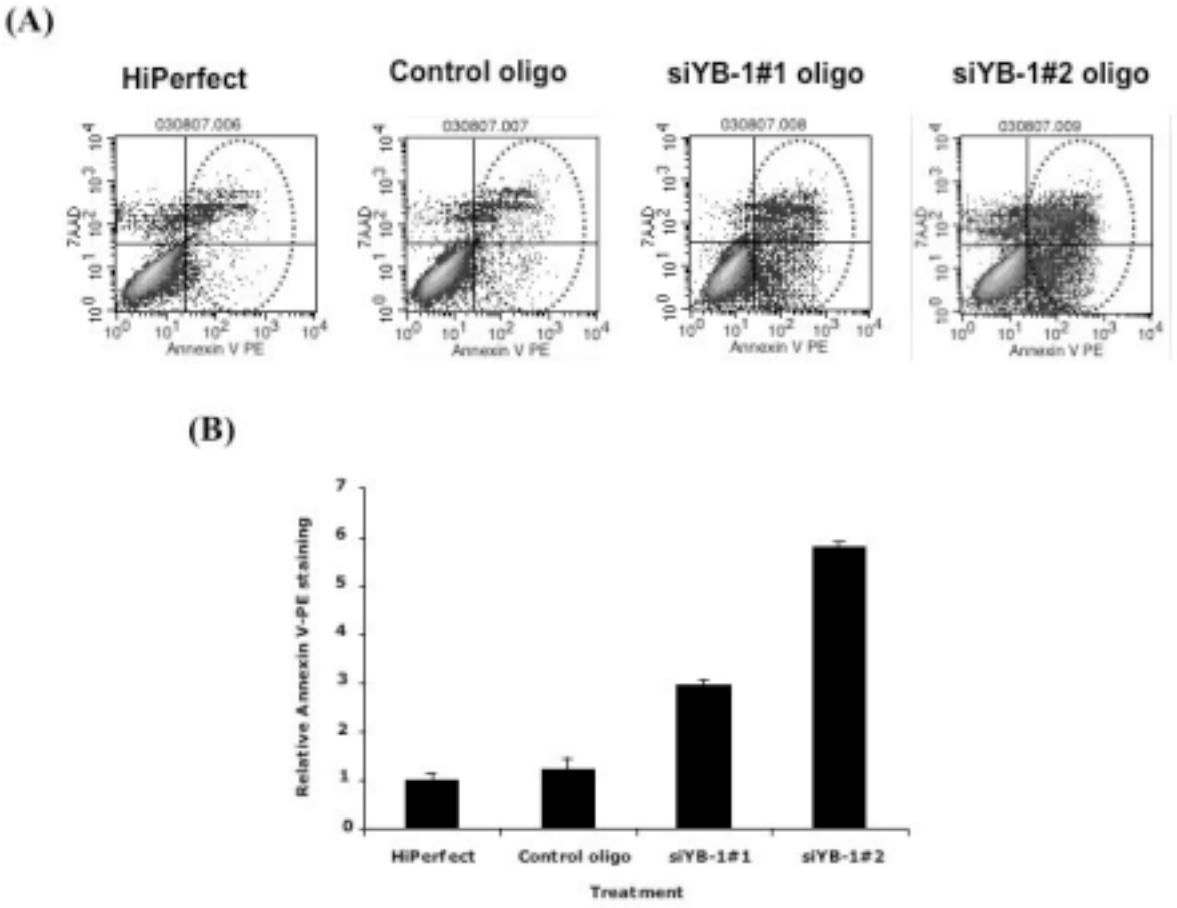


Figure 9.2
Time-course study examining induction of apoptosis in BT474-m1 cells with YB-1 knock-down

BT474-m1 cells were transfected with 5nM of control or siRNA oligos and harvested 1, 2, 3 and 4 days post-transfection and stained with Annexin V-PE. For staining, the cells were washed twice with cold PBS and stained with 1x binding buffer containing Annexin V-PE/7AAD and incubated in the dark for 15 minutes at room temperature. 1x binding buffer was added to the samples, which were analyzed by flow cytometry for Annexin V-PE binding. The bar chart below is a quantification of the relative fold difference in Annexin V-PE positive cells among different treatments.

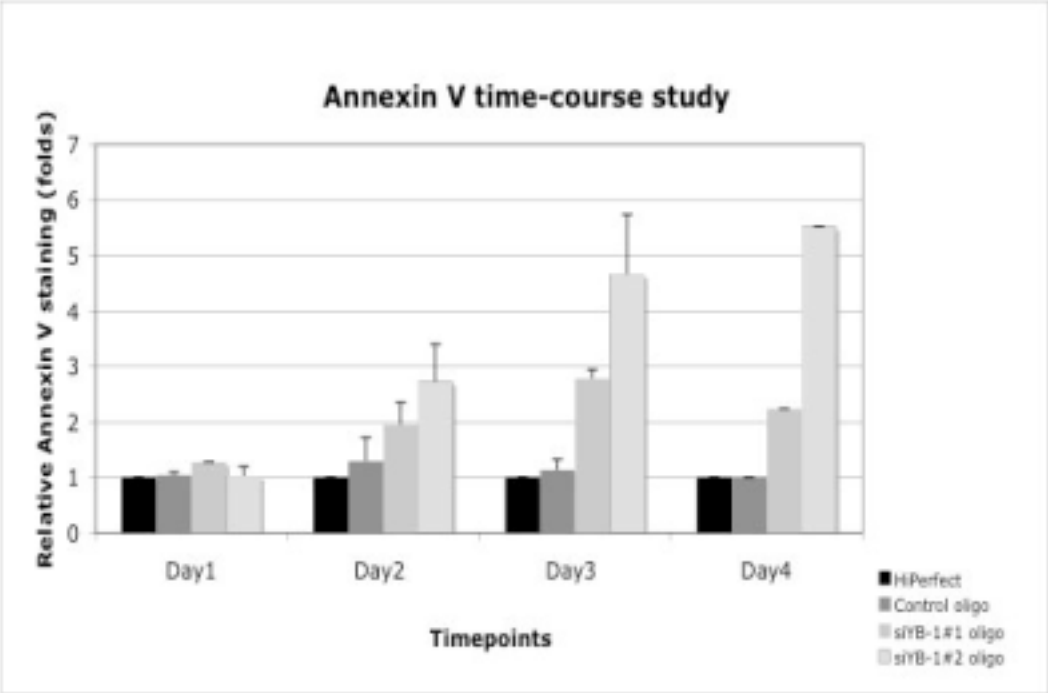


Figure 10

Expression of pH2AX^{Ser139} was elevated after silencing YB-1 in BT474-m1 cells

BT474-m1 cells were transfected with 5nM control or siRNA oligos targeting YB-1. Floating and attached cells were collected 1, 2, 3 and 4 day after transfection. Proteins were isolated from the cells and the lysates were subjected to western blotting to examine the level of histone H2AX phosphorylation (biomarker of apoptosis). Pan-actin was measured as a loading control.

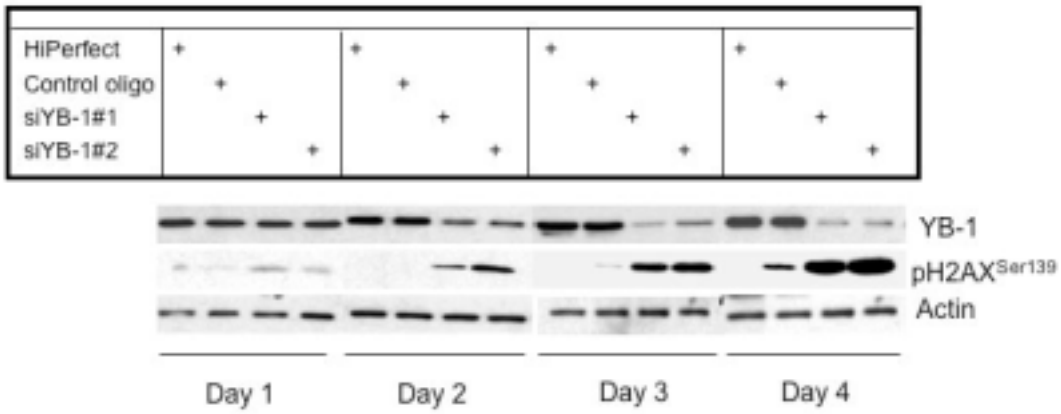
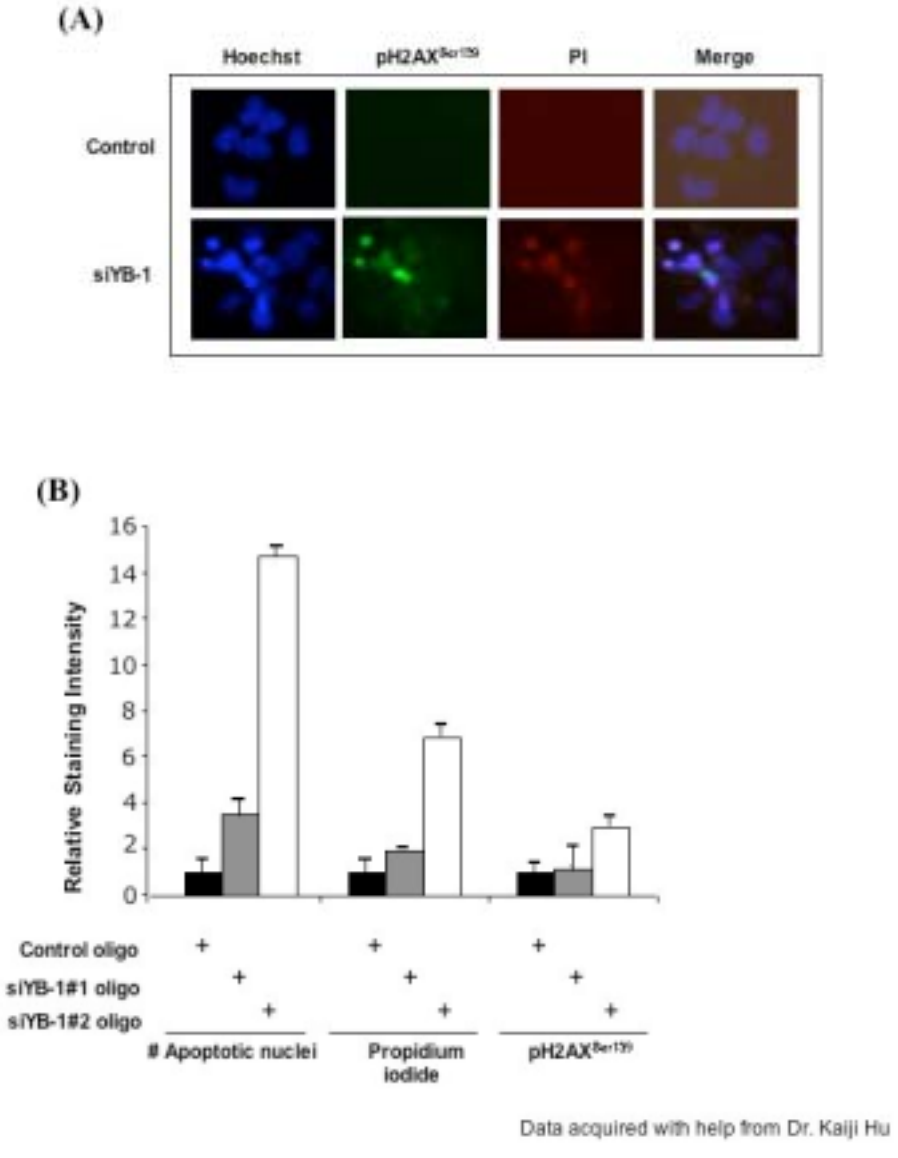


Figure 11
Examining Hoechst, propidium iodide and pH2AX^{Ser139} staining using ArrayScan VTI Reader

BT474-m1 cells were transfected with 5nM control or siYB-1 oligos in 96-well plate and incubated for 3 days. Cells were fixed, permeabilized and stained with Hoechst dye, PI or primary mouse anti-phospho-H2AX^{Ser139} antibody. The plates were analyzed and the images were taken on the ArrayScanVTI Reader. The signal intensities of cells in different treatments were quantified by ArrayScan VTI Reader **(A)**. Relative intensity of Hoechst, propidium iodide and pH2AX^{Ser139} staining was compared between control and treatment groups in the bar chart below **(B)**.



3.2.3 Knocking-down YB-1 decreased signaling through the STAT3 pathway

To this end, we have been able to show that silencing YB-1 decreased viability of BT474-m1 cells. However, how and why apoptosis was induced still remains elusive. Therefore, in the following experiments we investigated a potential mechanism. We previously demonstrated that knocking-down YB-1 led to decreased expression of EGFR and HER-2, which have been proposed to regulate the activation of STAT3 (Signal Transducer and Activator of Transcription 3) (Fernandes et al. 1999; Park et al. 1996). Considerable evidence has suggested the involvement of STAT3 in a multitude of human malignancies such as head and neck cancers (Leeman et al. 2006), mammary carcinomas (Petterino et al. 2006), multiple myeloma (Chatterjee et al. 2007) and certain hematologic malignancies. In these circumstances, STAT3 has been implicated to mediate survival functions of cancer cells by up-regulating expression of anti-apoptotic proteins such as BCL-2, BCL-xL, survivin and MCL-1 (Amin et al. 2004).

Since YB-1 knock-down decreased EGFR and HER-2 expression (Fig 5), we hypothesized that STAT3 activation would be down-regulated after siYB-1 treatment, leading to a decrease in transcription of the aforementioned pro-survival genes. We first examined the phosphorylation of STAT3 at Tyr705. However, we were not able to detect this phospho-protein (Fig 12.1A). Protein extracts of BT474-m1 and MDA-MB-231 cells were subjected to immunoblotting (Fig 12.1B) to confirm that BT474 cells expressed very low or undetectable level of $pSTAT3^{Tyr705}$ (Berishaj et al. 2005). The protein extract from MDA-MB-231 cells served as a positive control in this study. Phosphorylation at the tyrosine residue has been thought to be primarily regulated by cytokines such as IL-6 (Berishaj et al. 2007), which might not be highly expressed in our model. Therefore, we subsequently looked at $pSTAT3^{Ser727}$. Phosphorylation at this site was shown to be controlled by the MAPK pathway (Chung et al. 1997), which was down-regulated after YB-1 knock-down in BT474-m1 cells (Fig 5). Interestingly, we found that the phosphorylation of Ser727 on STAT3 was inhibited when YB-1 was knocked-down (Fig 12.1A). Consistent with this, we observed a decrease in the expression of MCL-1, a target gene of STAT3. However, there was no change in survivin expression, implying that either STAT3 is not the key regulator for survivin expression or that transcription of survivin does not depend on phosphorylation of Ser727 (Fig 12.1A).

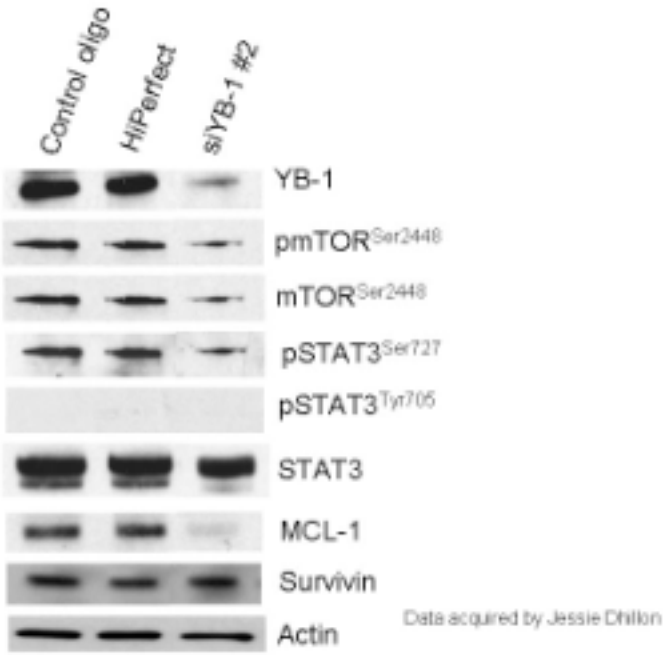
From the literature it became apparent that both mTOR (Yokogami et al. 2000) or ERK1/2 (Chung et al. 1997) could be responsible for the phosphorylation at this site. To determine which pathway is involved, we treated BT474-m1 cells with rapamycin and PD98059, the drugs that inhibit mTOR and MEK, respectively. After 6 hours of treatment, a time-point at which both of the drugs were shown to inhibit their targets, PD98059 (20μM) but not rapamycin (40nM) suppressed the expression of pSTAT3^{Ser727} and MCL-1, implying that STAT3 may be the substrate of ERK1/2 phosphorylation (Fig 12.2). Interestingly, when we conducted the same study for 18 hours using rapamycin, there was a decrease in pSTAT3^{Ser727} level, which was not observed at the 6hr time-point. However, at this time-point, PD98059 did not affect the expression of pSTAT3^{Ser727} due to the loss of drug effect (data not shown). We suspected that the decrease in pSTAT3^{Ser727} was a secondary effect induced after prolonged drug treatment since a related observation was also made after silencing YB-1 for 3 days. We discovered a decrease in phospho-mTOR^{Ser2448} after 72 hours of YB-1 knock-down (Fig 12.1A). Curiously, the total level of mTOR also decreased after YB-1 was silenced (Fig 12.1A). So far, no studies have reported this observation. It would be of interest to investigate this topic in the near future.

Together, these data suggest that the ERK1/2 pathway may be responsible for phosphorylating STAT3 at Ser727. Co-immunoprecipitation studies and *in vitro* kinase assays will be required to ascertain this functional relationship. Collectively, these results indicate that the ERK1/2/STAT3/MCL-1 but not the mTOR pathway may be critical to conveying survival advantage to BT474-m1 cells.

Figure 12.1
Knocking-down YB-1 decreased the expression of mTOR, pmTOR^{Ser2448}, pSTAT^{Ser727} and MCL-1 in BT474-m1 cells

(A) BT474-m1 cells were transfected with 5nM of control or siRNA oligos for 3 days. Both floating and attached cells were collected for protein extraction. The protein lysates were subjected to western blotting to examine the expression of mTOR, pmTOR^{Ser2448}, pSTAT3^{Ser727}, STAT3, MCL-1 and surviving. **(B)** Protein extracts of BT474-m1 and MDA-MB-231 cells were subjected to immunoblotting and examined for pSTAT3^{Tyr705} expression.

(A)



(B)

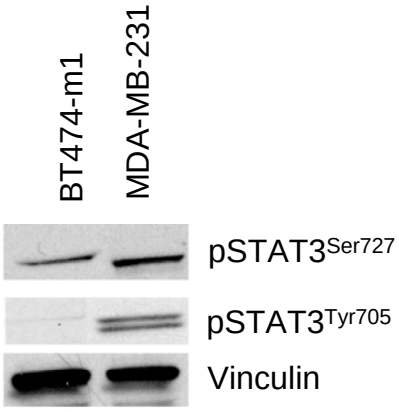
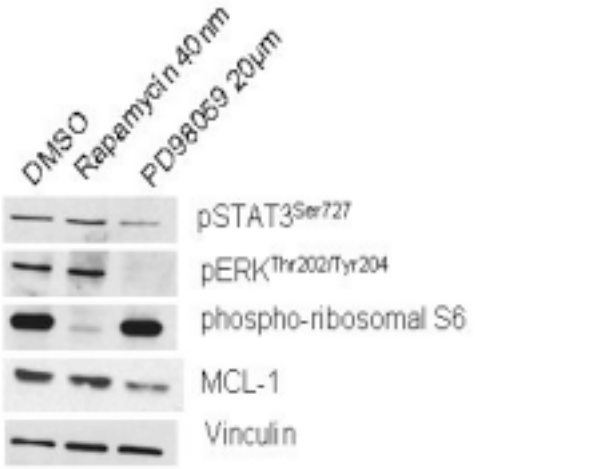


Figure 12.2

Phosphorylation of STAT at Ser727 residue is mediated by the ERK1/2 pathway

BT474-m1 cells were treated with 40nM of rapamycin or 20μM of PD98059 for 6 hrs and harvested for protein extraction. Western blots were performed to examine the level of $pSTAT3^{Ser727}$. In addition, the expression of $pERK1/2^{Thr202/Tyr204}$ and phospho-ribosomal S6 were evaluated to show that the drugs were effective.



Data acquired with help from Jessie Dhillon

3.3 Inhibition of YB-1 function led to decreased tumor incidence and formation of smaller tumors *in vivo*

In carrying out the animal experiment, we took BT474-m1 cells that were either transfected with 5nM of control or YB-1 siRNA oligos and injected $1x10^6$ cells subcutaneously in to the right lower hind region of female Nu/Nu mice. The weight of the mice and size of the tumors were measured twice weekly for 4 weeks. In week 1, 83% of the mice in the control oligo group developed tumors while only 33% and 17% of the mice in the siYB-1#1 and siYB-1#2 group formed tumors, respectively (Fig 13A). It is worth noting that the tumor incidence (83%) of the control oligo group is exactly the same as that from our pilot study (Material and Methods, section 2.10), suggesting the reproducibility of the cell model *in vivo*. All the mice in the control oligo group developed tumors in the second week; however, suppression of tumor growth in the siYB-1 treatment group was still maintained. Three weeks after injection, there were significantly more mice in the siYB-1 group that formed tumors (66% in siYB-1#1 and 83% in siYB-1#2 treatment) (Fig 13A). An explanation for why the siYB-1 group finally "caught up" in tumor formation may be the loss of potency of the siRNA oligos after 2 weeks, as shown in the *in vitro* time-course study (Fig 4A and 4B). Indeed, when we subjected the tumor extracts from the siYB-1 mice to western blotting, YB-1 expression returned 3 week post-injection (Fig 13D). Nevertheless, there was still a great difference in tumor size between the control and siYB-1 treatment. Photomicrographs of tumors representative of the control oligo and siYB-1 groups were shown in Fig 13B. Although at the end of the study, the majority of the mice in the siYB-1 group developed tumors with a surface area similar to that in the control oligo group, we observed a difference in depth of the tumors. The tumors of the siYB-1 group were much flatter and smaller. In Fig 13C, comparison of tumor size was made for the controls and siYB-1 treatment. The tumors in the controls were approximately 4x larger in volume than those in the siYB-1 group (Fig 13C).

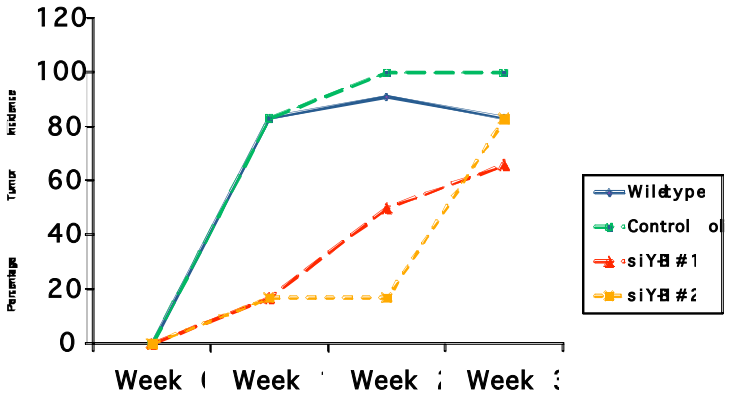
Figure 13

Knocking-down YB-1 delayed onset of tumor formation and resulted in smaller tumors *in vivo*

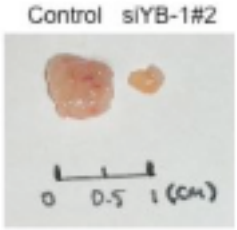
BT474-m1 cells were transfected with control or siRNA oligos targeting YB-1 for 1 day and harvested for injection. $1x10^6$ cells were washed with Hanks balanced Salt Solution and mixed with matrigel in 1:1 ratio; this 200µl mixture was injected to female Nu/Nu mice. Tumor size was monitored twice weekly for 4 weeks. Tumor weight and size were measured after the mice were sacrificed. **(A)** The percentage of tumor baring mice were monitored over 3 weeks and shown in the bar chat. **(B)** Photomicrographs of the tumors from the control and siYB-1 mice. **(C)** Average tumor volume was calculated for the different treatment groups. Percentage tumor volume of control oligo and siYB-1 groups was calculated based on the tumor volume of the HiPerfect group. Protein extracts from the tumors were subjected to immunoblotting to examine YB-1 expression **(D)**.

(A)

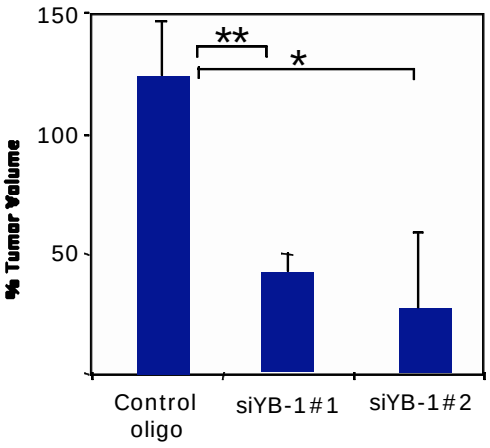
	Week 1	Week 2	Week 3
Wild-type	83% (10/12)	91% (11/12)	83% (10/12)
Control oligo	83% (5/6)	100% (6/6)	100% (6/6)
siYB-1-1	33% (2/6)	50% (3/6)	66% (4/6)
siYB-1-2	17% (1/6)	17% (1/6)	83% (1/6)



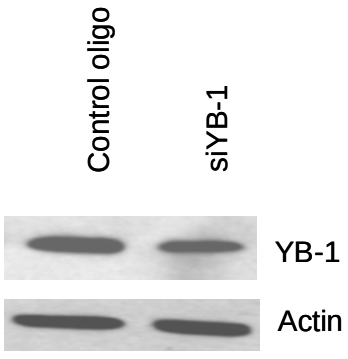
(B)



(C)



(D)



3.4 Knocking-down of YB-1 decreased the concentration of Herceptin needed to inhibit BT474-m1 colony formation in soft agar

One current treatment for HER-2 over-expressing breast cancers is Herceptin, which is a humanized monoclonal antibody targeting HER-2. We reported that YB-1 regulates the expression of HER-2 in MCF-7 and that knocking-down YB-1 led to decrease in HER-2 transcript and protein level (Wu et al. 2006). In this project, we have also demonstrated that silencing YB-1 by siRNA oligos resulted in a down-regulation of HER-2 expression in BT474-m1 and MDA-MB-453 cells. Therefore, we hypothesized that less Herceptin would be needed to inhibit colony formation in soft agar when YB-1 is knocked-down and the growth inhibitory effect may be better with the combination.

We have performed a preliminary study to show that BT474-m1 cells were sensitive to Herceptin, much like its parental cell line BT474 cells while MDA-MB-453 cells were resistant to the antibody in soft agar (Fig 14.1). We therefore questioned whether knocking-down YB-1 would render MDA-MB-453 cells responsive to Herceptin treatment and also if silencing YB-1 would further lower the concentration of Herceptin needed to inhibit colony formation of BT474-m1 cells.

Both BT474-m1 and MDA-MB-453 cells were transfected with 5nM of control or siRNA oligos, incubated for 1 day and re-plated in soft agar containing 0, 0.2, 2.0 and 20μg/ml of Herceptin. Number and size of the colonies were quantified after 28 days in soft agar. We observed a 95% inhibition in colony formation at 20μg/ml of Herceptin for the mock control or control oligo-transfected BT474-m1 cells (Fig 14.2A top two panels). However, when YB-1 was silenced, we could completely suppress colony formation in the presence of 100-fold less Herceptin (0.2 μg/ml) (Fig 14.2A). We focused on the concentration that resulted in remarkable reduction in colony growth and found that the mock control or control oligo-transfected cells treated with 2 or 20μg/ml of Herceptin formed very small colonies but the majority of siYB-1 cells were present only as single cells in soft agar containing 0.2, 2 and 20μg/ml of Herceptin (Fig 14.2A). Knock-down of YB-1 alone resulted in smaller colonies (Fig 14.2A), which was consistent with the result in Fig 7.1A. When we quantified the colonies greater than 300μm, there were 4x more colonies in the controls than siYB-1 treatment in the absence of Herceptin (Fig 14.2B). The difference in colony number was even larger (15x) between the two groups in the presence of 0.2μg/ml of

Herceptin: while the number of colonies in the controls was still high with an average of 15 colonies per well, there was only about 1 colony per well in the siYB-1 treatment (Fig 14.2B). Interestingly, at 2 and 20µg/ml of Herceptin, there was an almost complete absence of colonies in the siYB-1 group (Fig 14.2B). The result of Fig 14.2B was re-drawn on a logarithmic scale to allow IC_{50} determination (Fig 14.2C). However, the YB-1 siRNA oligos alone caused greater than 80% colony growth inhibition control oligo in the absence of Herceptin. Because of the effectives of the siYB-1#2 alone, which caused complete ablation of colony formation, we were unable to ascertain a useful inhibitory concentration.

We performed a parallel study in MDA-MB-453 cells. Although increasing concentration of Herceptin did not appear to affect colony formation either in the controls or the siYB-1 treatment, silencing YB-1 alone reduced the number of colonies (Fig 14.3A and 14.3B).

Figure 14.1
Colony Formation of BT474-m1 and MDA-MB-453 Cells in Soft Agar with Varying Concentrations of Herceptin

BT474-m1 ($1x10^4$ cells) and MDA-MB-453 ($4x10^4$ cells) cells were plated in soft agar containing 50% regular growth medium, 50% 0.6% agarose, and increasing amount of Herceptin (0, 0.2, 2.0 and 20µg/ml). Representative photomicrographs are shown to illustrate the differences in colony size in BT474-m1 and MDA-MB-453.

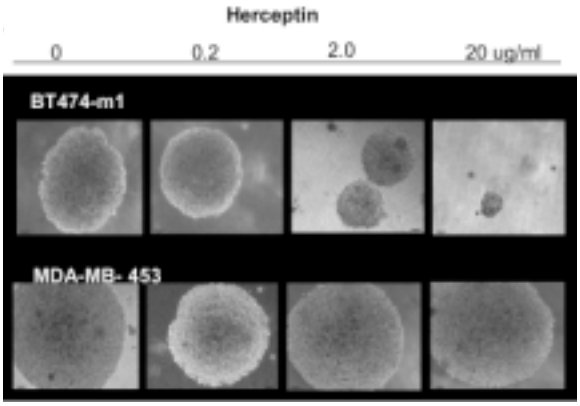
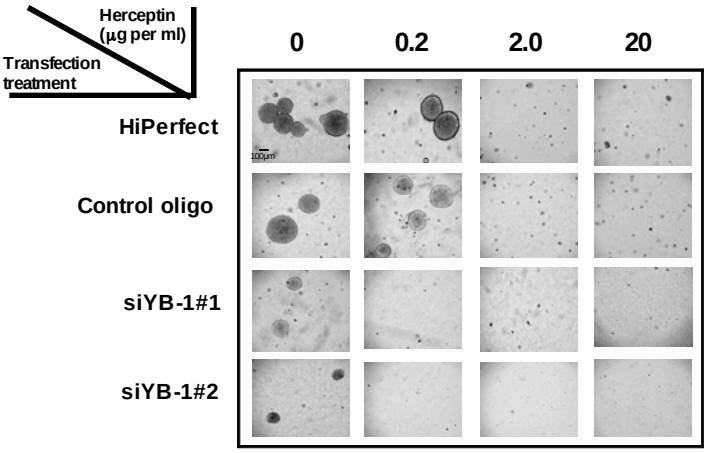


Figure 14.2
Combination treatment of siYB-1 and Herceptin inhibited colony formation of BT474-m1 cells at 0.2 µg/ml of Herceptin

BT474-m1 cells were transfected with control or siRNA oligos targeting YB-1 for 2 days. The cells were re-plated in soft agar containing regular growth medium and 0, 0.2, 2.0 or 20µg/ml of Herceptin and incubated for 28 days. Colonies greater than 300µm were counted under the microscope. Representative photomicrographs are shown to illustrate the differences in colony size **(A)**. The bar chart below is the quantification of colonies greater than 300µm in different treatments **(B)**. The result of 14.3 (B) was also plotted in terms of percentage colony formation in **(C)**.

(A)



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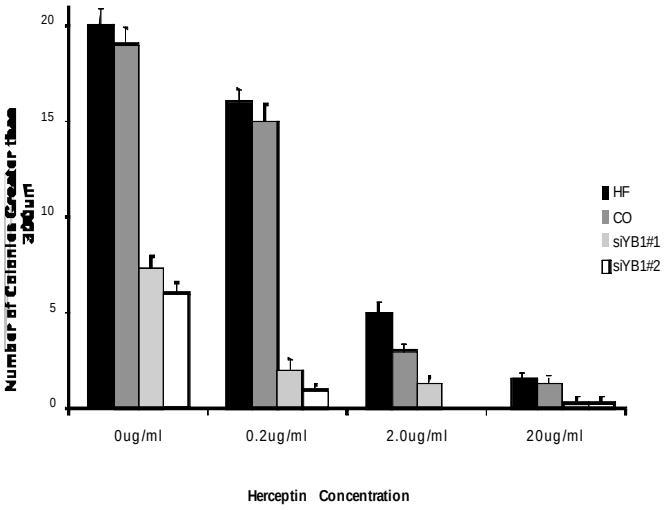


(C)

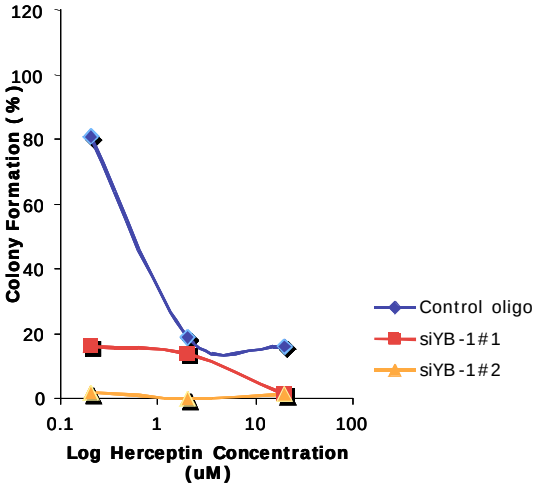
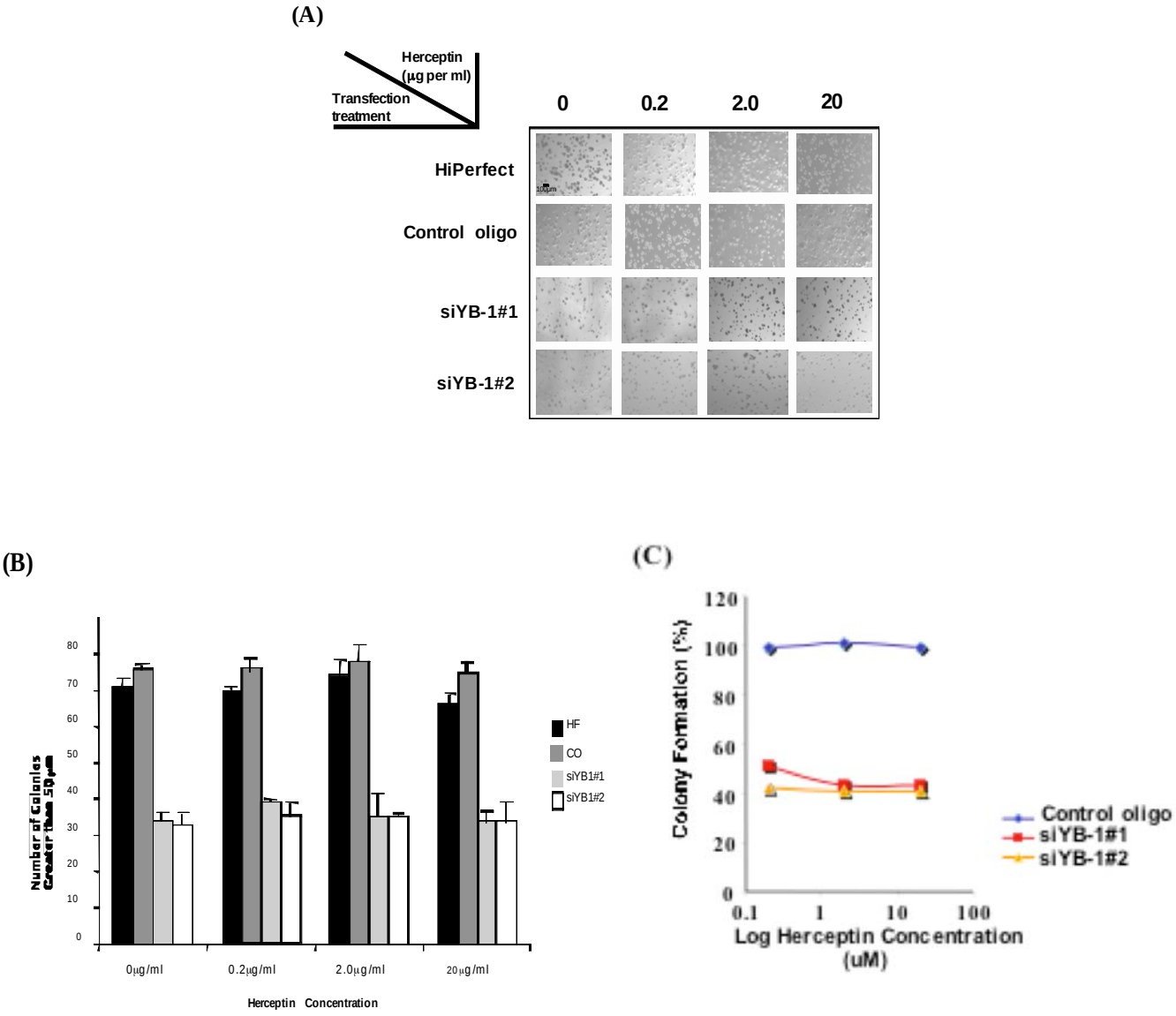


Figure 14.3
Knocking-down YB-1 inhibited growth of the MDA-MB-453 cells in soft agar but the growth suppressive effect was not improved by a combination treatment with Herceptin

MDA-MB-453 cells were transfected with control or siRNA oligos targeting YB-1 for 2 days. The cells were re-plated in soft agar containing regular growth medium and 0, 0.2, 2.0 or 20μg/ml of Herceptin and incubated for 28 days. Colonies greater than 50μm were counted under the microscope. Representative photomicrographs are shown to illustrate the differences in colony size **(A)**. The bar chart below is the quantification of colonies greater than 50μm in different treatments **(B)**. The result of 14.3 (B) was also plotted in terms of percentage colony formation in **(C)**.



CHAPTER 4

DISCUSSION

4.1 The effect of YB-1 knock-down on downstream target genes

The results from these studies indicate that YB-1 is integral to the growth of HER-2 over-expressing breast cancer cells BT474-m1 and MDA-MB-453. Knock-down of YB-1 decreased growth of these cells in monolayer and/or anchorage-independent conditions. Interestingly, apoptosis was induced in BT474-m1 cells treated with YB-1 siRNA oligos, implicating the obligatory role of this protein in survival of these cells.

To gain a deeper understanding of the functional role of YB-1 in cancer development, especially in the aggressive HER-2 over-expressing breast tumors, we proposed to knock-down this protein by siRNA and examine various downstream biological effects. We have previously demonstrated in MDA-MB-453 (HER-2++, EGFR-) and MDA-MB-231 (HER-2-, EGFR++) cells that YB-1 binds to and regulates HER-2 or EGFR promoter (Wu et al. 2006). Similarly, in BT474-m1 cells, we have been able to show a significant decrease in EGFR and HER-2 protein level after YB-1 knock-down 3 and 5 days post-transfection, respectively. The time-points were determined by a 14-day time-course study (data not shown) since we did not know the half-life of EGFR and HER-2 transcripts and proteins. Compared to EGFR, an additional 48 hrs was required for HER-2 to decrease ≥70%. This could be explained in part by the remarkably high level of HER-2 protein in BT474-m1 cells (Fig 5). The result in Fig 7 was selected from a day 4 study, which was able to show a clear difference in EGFR and HER-2 level before and after YB-1 knock-down. Consistent with this, a recent report by Stratford et al. demonstrated the ability of YB-1 to transactivate EGFR promoter in the aggressive basal-like breast cancer cells. Knock-down of YB-1 decreased EGFR expression and inhibited growth of the cells in anchorage-independent conditions (Stratford et al. 2007). On the other hand, pharmacologic inhibition of the PI3K/AKT pathway by PDK-1 inhibitor, OSU-03012, prevented transactivation of EGFR promoter by YB-1 and down-regulated EGFR expression *in vitro* and *in vivo* (To et al. 2007). Interestingly, in another independent study, YB-1 was isolated as factor conferring EGF-independent proliferation in human mammary epithelial cells (HMECs). YB-1

transduced HMECs over-expressed constitutively activated EGFR and were capable of proliferating in the absence of EGF (Berquin et al. 2005). Collectively, these experimental evidence indicate YB-1 as a transcriptional regulator of EGFR and/or HER-2, which could party explain the growth effects.

4.2 YB-1 and growth of the HER-2 over-expressing breast cancer cells in monolayer and in soft agar

In BT474-m1 cells, monolayer growth was suppressed over a week after YB-1 knock-down using siYB-1#2 oligo. This suppression may be attributed to either growth arrest or a balance between cell proliferation and cell death. The latter may be a more plausible explanation since in a separate study, the siYB-1 cells were only 50% confluent (due to cell lifting) when the control cells reached 100% confluency 4 days post-transfection. Therefore, cell death may have offset proliferation leading to zero net growth. Similar observations have been reported in other cell lines. A decrease in cell growth and defects in morphology and cell cycle progression were shown in a chicken pre-B lymphocyte cell line with targeted disruption of one allele of YB-1 (Swamynathan et al. 2002). Matsumoto et al. generated YB-1-null DT40 cells that did not grow at lower temperatures (33^{o}C), although to date, there is still a lack of evidence supporting the role of YB-1 in cold shock response in eukaryotic cells. Even at a higher temperature (39^{o}C), YB-1-null DT40 cells grew much more slowly than the wild type or hemizygous YB-1 ($YB\text{-}1^{+/-}$) mutant cells (Mutsumoto et al. 2005). Finally, an antisense approach targeting YB-1 also inhibited growth of rat mesangial cells up to 50% (En-Nia et al. 2005). Together, these data suggest a role of YB-1 in promoting cell growth.

In addition to monolayer growth studies, we have also shown in colony formation assays that knocking-down YB-1 perturbed anchorage-independent growth of BT474-m1 and MDA-MB-453 cells. The same result was recently reported in the basal-like breast cancer cells SUM149 and MDA-MB-468 (Stratford et al. 2007; Lee et al. in preparation). Interestingly, growth suppression of MDA-MB-453 cells was observed in soft agar assays but not in the monolayer cultures. In fact, there is increasing evidence (Carles-Kinch et al. 2002; Li et al. 1989) suggesting that three-dimensional models allow tumor cells to manifest certain cell behaviours which may not be captured in monolayer culture system. Therefore,

the three-dimensional cultures provide a more sensitive way to study tumor cell growth. In a recent study by Uchiumi et al., YB-1-null mouse embryonic fibroblasts failed to undergo morphological transformation in colony formation assays and remained contact inhibited in culture (Uchiumi et al. 2006). In contrast to their results, small colonies were observed in our soft agar assays despite YB-1 knock-down. The formation of the colonies may be due to the transient effect of the siRNA oligos. Alternatively, oncoproteins other than YB-1 enable the cancer cells to survive under anchorage-independent conditions. The decrease in colony size in our siYB-1 treatment may therefore be a result of reduced cell proliferation. The PI3K/AKT/mTOR pathway has been implicated in promoting anchorage-independent growth since it confers survival advantage, thereby allowing cancer cells to overcome anoikis (Nguyen et al. 2000). In rat intestinal epithelial cells transformed by insulin receptor or IGF-1R, colony formation in semisolid agar medium was significantly inhibited by LY294002 (PI3K inhibitor) and rapamycin (mTOR inhibitor) (Nguyen et al. 2000). A subsequent study by Hermanto et al. further examined this dependency in HER-2 over-expressing cells. Intriguingly, BT-474, MDA-MB-453 and SKBR3 (HER-2 amplified) displayed increased drug sensitivity to LY294002, wortmannin (PI3K inhibitor) and rapamycin compared to other cells that expressed normal levels of the receptors. The suppression in colony formation was accompanied by increased $p21^{cip1}$, $p27^{kip1}$ expression and decreased CDK2 activity (Hermanto et al. 2001). Therefore, HER-2 over-expressing cells have an increased requirement of the PI3K signaling pathway for anchorage-independent growth. This may explain why silencing expression of YB-1 suppressed anchorage-independent growth in our models, since it is a component of the PI3K/AKT signaling pathway (Sutherland et al. 2005; Evdokimova et al. 2006; Oda et al. 2007).

4.3 Apoptosis was induced in BT474-m1 cells after YB-1 knock-down

A recent study by Schittek et al. reported a reduced rate of cell proliferation, increased rate of apoptosis and a corresponding down-regulation in expression of genes related to proliferation and survival after knocking-down YB-1 in melanoma cells. However, they did not propose a mechanism for induction of apoptosis (Schittek et al. 2007). Herein, we have demonstrated the importance of ERK1/2/STAT3/MCL-1 pathway in survival of BT474-m1 cells. The pivotal role of STAT3 or survivin in evasion of apoptosis have been

clearly illustrated by studies showing that persistent activation of STAT3 signaling induces survivin or BCL-2 expression which protects cells from apoptosis (Gritsko et al. 2006; Real et al. 2002) and this could be reversed by dominant-negative forms of STAT3 and survivin, which resulted in decreased survival and enhanced radiosensitization of breast cancer cells (Kim et al. 2006). Interestingly, a group has already developed a cell-penetrating peptide against STAT3 and shown *in vivo* efficacy of this peptide in promoting apoptosis and inhibiting tumor growth specifically in HER-2 over-expressing breast cancer cells (Tan et al. 2006).

In our studies, we were unable to detect BCL-xL and did not observe a decrease in survivin expression after knocking-down YB-1. This implies that survivin expression may be regulated by other factors such as c-MYC (Cosgrave et al. 2006) or proteins in the IGF-1/mTOR pathway (Vaira et al. 2007). However, we have been able to show a decrease in the expression of *mcl-1*, another target gene of STAT3 that encodes a potent anti-apoptotic protein in the BCL-2 family. We hypothesized that the reduction in MCL-1 protein level was a direct consequence of decreased STAT3^{Ser727} activation and was therefore transcriptionally regulated. However, it should be noted that MCL-1 expression can also be negatively controlled by GSK3, which phosphorylates MCL-1, promoting ubiquitination of the protein (Ding et al. 2007). Since we found no change in pAKT^{Ser473} and pGSK^{Ser9} after YB-1 knock-down, the decrease in MCL-1 expression was probably not due to protein degradation through the proteosomal pathway. The result should be confirmed again by examining MCL-1 promoter activity after siYB-1 treatment. MCL-1 has long been implicated in pathogenesis of various human malignancies. There is a recent report by De Biasio et al. who identified a N-terminally truncated form of MCL-1 which was active, decayed less rapidly than wild type MCL-1 and were stabilized in the presence of ERK1/2 activation. They suggested this truncated form of MCL-1 may contribute to the apoptosis-resistance phenotype seen in many cancer cells (De Biasio et al. 2007). We have shown that ERK1/2 phosphorylation was suppressed after YB-1 knock-down; thus, there might exist an association between increased degradation of the N-terminally truncated MCL-1 and decreased survival of the siYB-1 cells.

STAT3 activation has been thought to be through phosphorylation at two sites. While phosphorylation at Tyr705 is an early event required for dimerization and DNA binding, Ser727 phosphorylation at the C-terminal transcriptional activation domain

enhances transcription activity of STAT3 and may involve favourite interactions between STAT3 and co-activator proteins (Bowman et al. 2000). Therefore, phosphorylation at both tyrosine and serine residues is essential for full activation of STAT3 signaling. However, recently, some groups have suggested that STAT3 may be functional in the absence of $pSTAT3^{Tyr705}$ under circumstances such as Notch signaling in stem cells (Androutsellis-Theotokis et al. 2006) and macrophage survival (Liu et al. 2003). Furthermore, over-expression of peptidyl-prolyl cis/trans isomerase 1 (Pin1) promotes recruitment of transcription coactivator p300 and subsequently, transcription of STAT3 target genes in a $pSTAT^{Ser727}$–dependent fashion (Lufei et al. 2007). This raises the possibility that, perhaps under certain conditions, gene transactivation could occur as long as Ser727 is phosphorylated, thereby making Tyr705 phosphorylation dispensible. Finally, the importance of $pSTAT3^{Ser727}$ in cell transformation and oncogenicity is illustrated by over-expression of $STAT^{Ser727Ala}$, which rescued the transformed phenotype of N-Ras-transformed NIH-3T3 cells with elevated levels of pERK1/2 and $pSTAT3^{Ser727}$(Plaza-Menacho et al. 2007). A study by Sasser et al. found that there was a low basal level of activated STAT3 in BT474 cells and that $pSTAT3^{Tyr705}$ could be induced by IL-6 from mesenchymal cells (Sasser et al. 2006), suggesting the intrinsically low level $pSTAT3^{Tyr705}$ of in BT474 cells. In our study, $pSTAT3^{Tyr705}$ in BT474-m1 cells was undetectable, a result confirming that of Berishaj et al. (Fig 12.1B). We are not certain whether STAT3 phosphorylation on the Tyr705 sites is obligatory for STAT3 function in our model. To address this question, a gel shift assay with STAT3 mutant in which tyrosine is mutated to phenylalanine could be created to examine its DNA binding ability.

Bernard Weinstein proposed the concept of "oncogene addiction," which describes an unexplained dependency of cancer cells on particular proteins and signaling pathways for survival and proliferation (Weinstein, 2002). To expand this concept further, Sharma et al. have recently proposed a concept called "oncogenic shock" to explain why "oncogene addiction" may happen in cancer cells. "Oncogenic shock" occurs when the function of an important oncoprotein is inactivated in tumor cells. Under these conditions, prosurvival signals dissipate rapidly whereas proapoptotic signals linger sufficiently long to commit the cells to apoptosis (Sharma et al. 2006). Therefore, it appears that the growth and survival of cancer cells depends heavily on the function of specific key oncoprotein(s). Since YB-1

positively regulates the expression of a multitude of growth-promoting genes such as, *cyclin A, cyclin B1, topoisomerase II α, DNA polymerase α, egfr* and *her-2* (Shibao et al. 1999; Jürchott et al. 2003; En-Nia et al. 2005; Wu et al. 2006; Stratford et al. 2007), it is conceivable that knock-down of this protein may deprive the growth-stimulating signals, therefore exert "oncogenic shock" to susceptible cancer cells. This effect could be further dampened by the inhibition of pro-survival STAT3/MCL-1 pathway (Lee et al. in preparation) and activation of p53 and Fas, whose functions are normally suppressed by YB-1 (Lasham et al. 2003; Lasham et al. 2000).

An important technique central to this project was knocking-down YB-1 using short interfering RNA (siRNA). It is a convenient method to study protein function but failings include the fact that they are short-lived and relatively instable. The concentration of siRNA becomes "diluted" out when cells divide and the effect lasts for approximately one week (Pai et al. 2006). The fact that we could achieve a knock-down 14 days post-transfection was probably due to the slow turn-over of BT474-m1 cells (doubling time: ~4 days). Recognizing the downsides of siRNA, we have made the attempts of establishing stable shYB-1 cell lines by transfecting them with pSUPER-DUPER YB-1 that produces short-hairpin RNA targeting YB-1 transcripts. However, growth of BT474-m1 cells, after transfection with this vector, stagnated for about one month. Curiously, these cells appeared egg-shaped and translucent under the microscope, typical characteristics of cells undergoing senescence (data not shown). This observation may be explained partly by the role of YB-1 in regulating cellular stress response and preventing premature senescence, reported by Lu et al. (2005). Growth arrest of shYB-1 BT474-m1 cells may be resulted from the decrease in cyclin A, cyclin B expression, which are positively regulated by YB-1 (Jurchott et al. 2003). To examine senescence in the YB-1 knock-down cells, it may be useful to evaluate the expression of G(1)-specific CDK inhibitors $p16^{Ink4a}$ and $p21^{cip1}$ since their levels were elevated in senesced YB-1-null mouse embryonic fibroblasts (Lu et al. 2005).

4.4 YB-1 knock-down suppressed xenograft growth in nude mice

We have shown, for the first time, that knocking-down YB-1 delayed onset of tumor formation and inhibited tumor growth *in vivo*. In the siYB-1 group, palpable tumors were not formed until 2 weeks after injection. Since apoptosis was observed after YB-1 knock-down,

one may argue that the lack of tumor formation within the first two weeks was due to apoptosis of the cells. However, some cells certainly survived the YB-1 knock-down since tumors were eventually detected between 2~3 weeks post-injection. Smaller tumors in siYB-1 mice may be explained by two hypotheses: 1) apoptosis was minimal *in vivo* and growth suppression was dominant due to YB-1 knock-down or, 2) apoptosis was induced *in vivo* and therefore, it took more time for the remaining cells to form sizeable tumors. We could not exclude the possibility that both apoptosis and siYB-1 mediated growth suppression together contributes to the formation of smaller tumors.

One suggestion was that some of the YB-1 silenced cells introduced into the nude mice were "destined" to die. Although we were unable to address this question from the animal study, results from colony formation assays can give us some clues as to what may have happened *in vivo*. In the soft agar assays, we found that the majority of the siYB-1 cells (>80%) formed colonies at the end-point (28 days) of the study, suggesting that a large proportion of the cells we cultured in soft agar or inoculated in mice were probably viable. This assumption seems to contradict the observation from the monolayer studies showing that approximately 50% of all cells died 4 days after siRNA transfection. This discrepancy may probably be attributed to differences in monolayer versus three-dimensional (3D) model. It was proposed that the 3D architecture of the cells and extracellular environment can contribute to cell viability in the face of apoptotic stimuli (Reddig et al. 2005). Malignant cells sensitive to intrinsic or extrinsic apoptotic stimuli in 2D cell culture environment could be rendered resistant to the apoptotic stimuli when the cells formed polarized organoid, or acini, in 3D cell culture. Inhibition of apoptosis was conferred in part by integrin α6β4 and NF-κB activity (Weaver et al. 2002).

4.5 YB-1 knock-down improved the sensitivity of BT474-m1 cells to Herceptin treatment

In the last part of the project, we examined the effect of knocking-down YB-1 on Herceptin treatment. Herceptin (trastuzumab, Genetech) was approved in 1998 by the U.S. Food and Drug Administration (FDA) as a front-line treatment for metastatic breast cancer patients as well as breast cancer patients who over-express HER-2. Although Herceptinhas shown clinical efficacy in patients with HER-2 over-expressing breast cancer, the objective

response rates for monotherapy are low as only 12% -34% of the patients respond for a median duration of 9 months (Slamon et al. 1989). Combination of Herceptin with conventional anti-cancer agents such as paclitaxel or docetaxel (Hudziak et al. 1987) have been applied in clinical settings and shown improved response rate and overall survival. However, efficacy of the therapy is still largely hindered by drug resistance that typically develops within one year of treatment. Therefore, a deeper understanding of how resistance occurs and development of novel combination becomes critical to improving patient care.

Having shown that YB-1 is essential for the growth and survival of HER-2 over-expresing cells, the final objective of this project was to find out if targeting YB-1 in the PI3K/AKT pathway in combination with Herceptin was more effective than Herceptin alone *in vitro*. Interestingly, knocking-down YB-1 lowered the concentration needed to inhibit anchorage-independent growth of BT474-m1 cells. The ability of siYB-1 cells to form colonies was nearly completely abolished with 0.2 μg/ml of Herceptin. When the colonies were quantified in all the treatments, we found that 100x less Herceptin (0.2 μg/ml) was required by siYB-1 cells to achieve the same inhibitory effect as Herceptin alone (20 μg/ml). Even more importantly, the majority (>80%) of the cells were single cells rather than colonies when the combination was applied. This finding was exciting to us since the absence of anchorage-independent growth represented loss of viability or tumorigenicity of the cancer cells, which would be the ideal goal to achieve in therapeutic interventions. Relevant animal studies will soon be carried out to assess the efficacy of such combination *in vivo*. Herceptin is an expensive therapy, which costs $50,000/patient/year for treatment. Thus, increasing drug sensitivity in breast cancer cells will not only assist therapeutic efficacy but also reduces the enormous cost of the treatment.

A parallel study was performed in MDA-MB-453 cells, which responded to YB-1 knock-down but the suppression of growth was not improved with increasing concentration of Herceptin. It is not known why there is a difference in response to the combination treatment in the two different cell lines. Since both BT474-m1 and MDA-MB-453 cells are PTEN-wild-type, the status of PTEN (Nagata et al. 2004) was probably not a main contributing factor for Herceptin resistance in MDA-MB-453 cells. Activation of AKT, as exemplified by $pAKT^{Ser473}$ level, is not higher in MDA-MB-453 compared to BT474-m1 cells. We have also compared the expression of IGF-1R, which has been implicated in

Herceptin resistance (Lu et al. 2001), in a panel of breast cancer cells and found no correlation between IGF-1R/pIGF-1R level and Herceptin responsiveness (data not shown) although there is a contradicting report arguing against the involvement of IGF-1R expression in Herceptin resistance (Kostler et al. 2004). Also, since IGF-1R level is comparatively lower in MDA-MB-453 cells than many other breast cancer cell lines we examined, the likelihood of a cross-talk between IGF-1R and HER-2 (Nahta et al. 2005) causing Herceptin resistance is minimal. There is a recent study that correlated the response of 18 different breast cancer cell lines to Herceptin with their pHER-$2^{Tyr1248}$ level. By western blotting, they found that Herceptin-sensitive cell lines such as BT474 express pHER-$2^{Tyr1248}$, which exists at very low level in Herceptin-resistant cell lines such as MDA-MB-453. They subsequently performed IHC analyses examining pHER-$2^{Tyr1248}$ expression in those different cell lines treated with 6hrs and 72hrs with Herceptin. A significant reduction in pHER-$2^{Tyr1248}$ was found in BT474 treated Herceptin for 6hrs or 72hrs. However, pHER-$2^{Tyr1248}$ remained undetectable from 0 to 72hrs of Herceptin treatment in MDA-MB-453 cells (Ginestier et al. 2007). One of the main mechanisms by which Herceptin functions to inhibit cancer cell growth is by down-regulating signaling through the PI3K/AKT or MAPK pathways (Yakes et al. 2002; Wang et al. 2005). If Herceptin does not alter HER-2 activation in MDA-MB-453 cells (Ginestier et al. 2007), it is less likely to have any effect on the down-stream signaling to cause any significant impact on cell growth and survival. Also, oncoproteins downstream of HER-2 and EGFR could be constitutively activated to confer survival advantage. Alternative pathways or molecules other than PI3K/AKT pathway and YB-1 may be involved in Herceptin resistance of MDA-MB-453 cells. We are currently investigating a combination treatment involving treating MDA-MB-453 cells with Herceptin and MAPK inhibitor PD98059 since we have shown that pERK1/$2^{Thr202/Tyr204}$ level is higher in MDA-MB-453 than BT474-m1 cells. Thus, we could not exclude the possibility that activated MAPK pathway may be responsible for the resistance phenotype in MDA-MB-453 cells. Since we have shown that the ERK1/2/STAT3/MCL-1 is important for survival of BT474-m1 cells, it would be of interest to find out if Herceptin resistance is associated with the level of activated STAT3 or other components of the pathway in MDA-MB-453 cells.

Herceptin binds to domain IV that is not involved in receptor-receptor dimerization; therefore signaling through other receptor combinations is not inhibited (Chopin et al. 2003).

Improved drug effects have been demonstrated using EGFR/HER-2 dual kinase inhibitor such as lapatinib (Nahta et al. 2007) or combination treatment with Herceptin and gefitinib (Ritter et al. 2007). Since EGFR level was undetectable in MDA-MB-453 cells, the aforementioned strategies are not expected to render the cells sensitive to Herceptin. Austin et al. studied the role of TGF-α in induction of Herceptin resistance and found that introduction of TGF-α could caused Herceptin-sensitive cells to become less responsive to growth inhibition mediated by the antibody (Austin et al. 2004). Whether or not TGF-α over-expression accounts for Herceptin resistance in our model needs prospective validations.

SUMMARY & FUTURE DIRECTIONS

In this study, we have determined the functional role of YB-1 in HER-2 over-expressing breast cancer BT474-m1 and MDA-MB-453 cells, both of which depend on expression of this protein for growth. Knocking-down YB-1 decreased the protein level of EGFR and/or HER-2 in both the cell lines. However, the decrease in MAPK signaling was only observed in BT474-m1 cells. The PI3K/AKT signaling, on the other hand, was not affected in either of the cell line after YB-1 knock-down. Consistent with the loss of EGFR and HER-2, growth of the siYB-1 cells was suppressed in monolayer and/or soft agar and formation of tumors was delayed *in vivo*. Intriguingly, apoptosis was induced in BT474-m1 cells after YB-1 knock-down, suggesting the critical role of this protein in cell survival. Finally, we investigated the possibility of combining siYB-1 with Herceptin in BT474-m1 and MDA-MB-453 cells. Although MDA-MB-453 cells failed to respond to the combination treatment, a remarkable improvement in inhibition of colony formation was observed in BT474-m1 cells.

We have recently obtained a pRevTet-Off-IN vector that will be used to establish shYB-1 cell lines for future animal studies. Production of shRNA could be controlled by adding or removing doxycycline in the growth media, allowing a precise regulation of YB-1 expression. With the pRevTet-Off-IN system, which allows a better control of YB-1 expression, we will be able to perform the combination study *in vivo*. Furthermore, whether or not over-expression of YB-1 could render BT474-m1 cells resistant to Herceptin is a topic that requires a close examination. It would also be motivating to expand this combination study on other HER-2 amplified models.

By using ChIP (chromatin immunoprecipitation), EMSA (electrophoretic mobility shift assay) and reporter assays, we have demonstrated the ability of YB-1 to bind and transactivate *her-2* and *egfr* promoter in MCF-7, MDA-MB-453 and SUM-149 cells (Wu et al. 2006; Stratford et al. 2007). However, whether or not YB-1 is a contributing factor for HER-2 over-expression in breast cancer remains elusive. Further studies by array CGH (comparative genome hybridization) with FISH (fluorescence *in situ* hybridization) may allow us to perform a positional isolation of the highly amplified regions of the genes. From that, potential YB-1 responsive elements (YRE) can be identified and examined (Barland et al. 1997). There are studies indicating a co-amplification of *her-2* adjacent genes, such as *topoisomerase II α* (*top2a*) (Jarvinen et al. 2006), *grb7* and *stard3* (Kao et al. 2006), the expression of which are integral to tumor cell growth and survival. Therefore, it would be of interest to study genes that are co-amplified with *her-2* in our models.

We have recently developed peptidomimetics targeting YB-1. The inhibitors were designed to interfere with YB-1 function by blocking the Ser102 phosphorylation site important for YB-1 nuclear translocation and DNA binding. The efficacy of the inhibitors has undergone rigorous evaluations in a panel of cancer cell lines using various monolayer and soft assays. Relevant *in vivo* studies will also be performed for further inspections of these compounds. We will correlate the results obtained from the siRNA studies with those from the inhibitor studies. Molecular and phenotypic changes observed in the siYB-1 cells are expected in BT474-m1 and MDA-MB-453 cells treated with the peptide or small molecule inhibitors.

With the improved understanding of YB-1 structure, function and its role in pathogenesis of a broad range of human malignances, novel therapeutic approaches developed to target YB-1 are expected to provide a new and promising avenue in treatment of cancer.

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APPENDIX

BUFFERS AND REAGENTS

Egg Lysis Buffer (ELB)

100mM HEPES (pH7.4)
500mM NaCl
10mM EDTA (pH8.0)
0.2% NP-40
1mM DTT

3% Stacking Gel (SDS-PAGE)

4% bis-acrylamide
0.1% SDS
0.125M Tris (pH6.8)
0.1% TEMED
0.05% ammonium persulfate

RIPA Buffer

150mM NaCl
1% NP-40
0.5% sodium deoxycholate
0.1% SDS
50mM Tris (pH7.2)
Adjusted pH to 8.0

12% Seperating Gel (SDS-PAGE)

12% bis-acrylamide
0.1% SDS
0.375M Tris (pH8.8)
0.1% TEMED
0.05% ammonium persulfate

Phosphate Buffered Saline (PBS)

8g NaCl
0.2g KCl
1.44g Na_2HPO4
0.24g KH_2PO_4
Make up to 1L with distilled water
Adjust pH to 7.4

Western Blot Sample Loading Buffer

0.5M Tris-HCl (pH6.8)
0.8ml glycerol
10% SDS
1% bromophenol blue
3.8ml distilled water
0.4ml of 14.3M 2-β-mercaptoethanol

10x Transfer Buffer

140g glycine
30.3g Tris

*1x transfer buffer is made by 100ml of 10x transfer buffer, 200ml of methanol and