ABSTRACT

Treatment with aromatase inhibitors (AIs) has proven to be effective against estrogen receptor positive (ER^+) breast cancer in postmenopausal women. However, resistance to AIs remains a cause of disease relapse and mortality in a significant number of breast cancer patients. Recently it has been shown by a number of studies that a rare and biologically distinct undifferentiate (TICs) are involved in drug resistance and tumor relapse. However, the role of TICs in hormone therapy resistance is still unclear. Several lines of evidence suggest that human epidermal growth factor receptor-2 (HER-2) regulates TICs. Previous studies from our lab indicate that resistance to AIs is mediated by up-regulation of HER-2 signaling. The purpose of the current study was to first characterize hormone resistant breast cancer cells for tumor initiating characteristics, second to determine the role of HER-2 in regulation of the TIC phenotype in hormone resistant breast cancer and finally to determine the effect of a HER-2 inhibitor and a differentiating agent alone and in combination in targeting TIC population.

Our results demonstrate that resistance to AIs is associated with an increase in TIC characteristics. In addition, HER-2 regulation of hypoxia inducible factor (HIF-1α) likely plays a role in activating genes involved in AI resistance and TIC characteristics, such as breast cancer resistance protein (BCRP), which confers survival benefit to TIC cells. Lastly we provide evidence that TICs in hormone resistant breast cancer can be targeted either by inhibiting HER-2 signaling or by inducing differentiation in them.

Role of Lapatinib and Retinoids In Overcoming Intrinsic Resistance to Hormonal Therapy In Breast Cancer

By

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Dissertation submitted to the faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2011

Dedication

Dedicated to my Husband and Son

AKNOWLEDGMENTS

This dissertation would not have been possible without the mentorship of Dr. Angela Brodie. Her guidance, support and optimism, to my ideas have proved invaluable. I am grateful for all that I have learned from her. My gratitude is also extended to the members of my thesis committee: Drs. Anil K Jaiswal, Katherine S Squibb, Anne W Hamburger, Yun Qiu and Laundette. Jones their insight and suggestions have been immensely helpful in shaping the course of my project. I am thankful to all the members of Dr. Brodies lab, Dr Armina Kazi, Dr Guari Sabnis, Dr Sarayana Chumsri and Amanda Schech. My sincere gratitude goes to Dr Armina for her help with the HIF-1 studies.

I want to thank my lovely son Rayyan Shah for his patience and love. His cute drawings were a source of inspiration for me. I want to thank my husband, Dr. Zahoor A Shah whose unwavering support and love made it possible for me to successfully accomplish our dream. Also, I want to thank the most important person in my life my brother Dr. Imtiyaz Gilani who is a constant source of inspiration and determination for me. Lastly, I thank my mom and dad and my sister Yasmeen and my brother Dr. Ishtiyaq Gilani for their constant love, prayers and support. Without it, all of my achievements would not have been Possible.

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LIST OF ABBREVATIONS

ABCG2	ATP-binding cassette, sub-family G
AI	Aromatse inhibitors
ANAR	Anastrazole resistant aromatase transfected cells.
ATRA	All trans retinoic acid
BCRP	Breast cancer resistance protein
CSC	Cancer Stem Cell
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
ER	Estrogen receptor
FGF	Fibroblast growth factor
FTC	Fumitremorgin C
HER-2	Human epidermal growth factor receptor-2
IGF	Insulin like Growth Factor
LTLT-Ca	Long term letrozole treated - aromatase transfected cells
MAPK	Mitogen-activated protein kinase
MCF-7	Human breast adenocarcenoma cell line
MCF-7 Ca	Human breast adenocarcinoma cell line Aromatase transfected MCF-7 Ca cell line
MDR	Multiple drug resistance
MEGM	Mammary epithelial growth media
Pg-R	Progesterone receptor
RA	Retinoic acid
SP	Side population
TIC	Tumor initiating cells

INTRODUCTION

A. The mammary glands

The mammary glands are exocrine glands whose main function is to produce milk after a full term pregnancy (Tortora 2000; Ali and Coombes 2002). The basic components of a mammary gland are the lobules and the ducts. Each lobule is composed of a group of alveoli (Figure 1.1). The lobules join up to form a lactiferous duct that drains into openings in the nipple. The branching tubular system of mammary glands is composed of an inner layer of luminal epithelial cells lining the ducts and an outer layer of myoepithelial cells. The luminal epithelial cells are functionally active milk-producing cells and are the most common target cells for carcinogenesis (Gudjonsson, Adriance et al. 2005).

The myoepithelium constitutes the basal cell layer of the epithelium that harbors the progenitor/stem cells (Petersen and Polyak 2010). These progenitor stem cells play an active part in branching morphogenesis. Collectively the breast is made up of lobules, ducts, fatty connective tissue and lymphatic tissue (Williams and Daniel 1983). Unlike other tissues, the mammary glands undergo continuous morphological changes with the greatest changes occurring during puberty, pregnancy and lactation. Steroid hormones predominantly regulate developmental changes occurring within the mammary glands (Kastner, Krust et al. 1990).

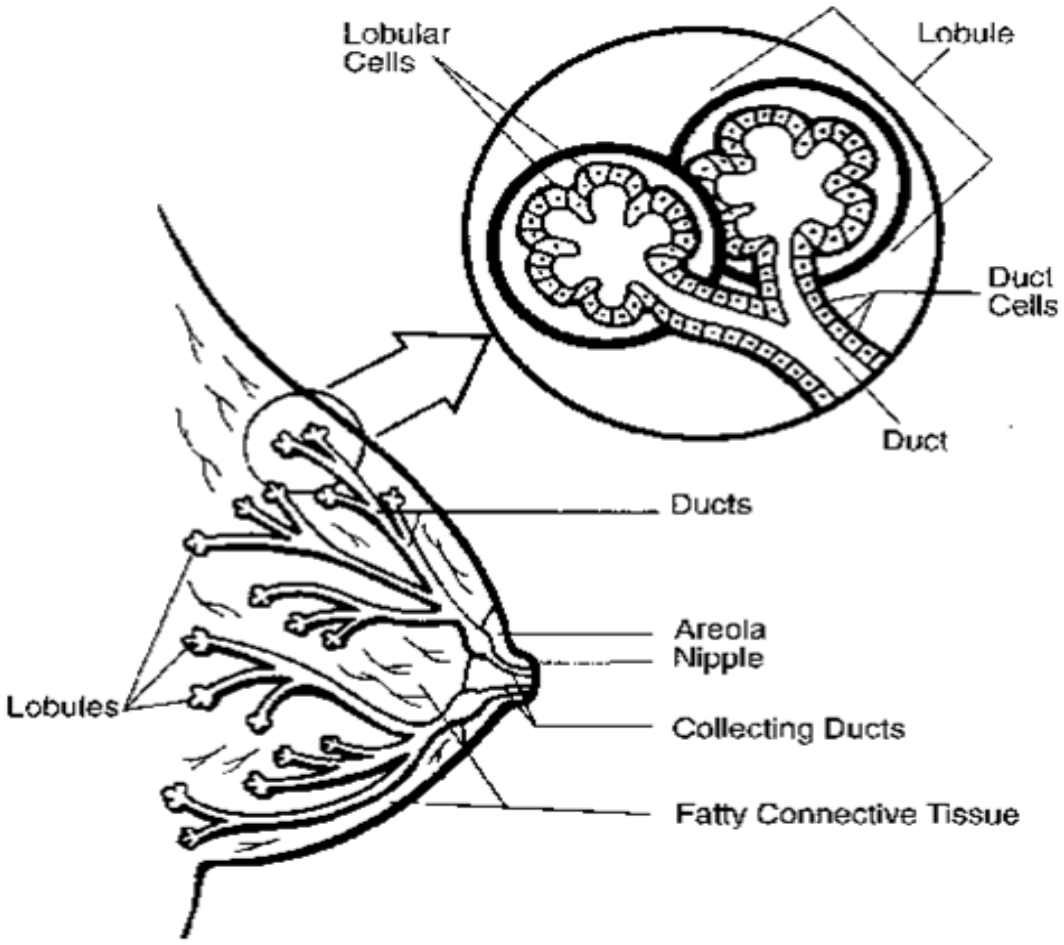

Figure 1.1: Diagram and histology of a normal breast. Section illustrates the structure of a normal mammary gland. Morphological features are annotated in the given diagram. Adapted from *http://www.benignbreastdisease.us/content/about-bbd/normal-breast-structure.html*

During early developmental stages both males and females form rudimentary mammary ducts. These mammary ducts are further developed at the onset of puberty in females, due to activated estrogen signaling. Estrogen signaling is mainly regulated by the estrogen receptor, which is present on the epithelial cells of the mammary glands.

Progesterone receptor and epidermal growth factor receptor are two other major receptors responsible for regulating the normal growth and function of mammary glands. An excessive unregulated stimulation of the receptor signaling in the mammary gland is believed to increase the incidence of breast cancer.

B. Breast cancer

In the United States, breast cancer is the most common cancer and the second leading cause of deaths among women. Each year a large number of women are diagnosed with breast cancer. According to the American Cancer Society, around one in nine women who live up to the age 70 will develop breast cancer at some time in their life (2008; Jemal, Siegel et al. 2010). Around 192,370 women were diagnosed with breast cancer in the year 2009 (Figure 1.2), though mortalities due to breast cancer are far less than the estimated diagnosed cases. Death occurs when tumors metastasize or become resistant to therapy. While there is no known cause of breast cancer, there are several contributing factors such as family history, early age at menarche, genetics and obesity, which may increase the risk of breast cancer.

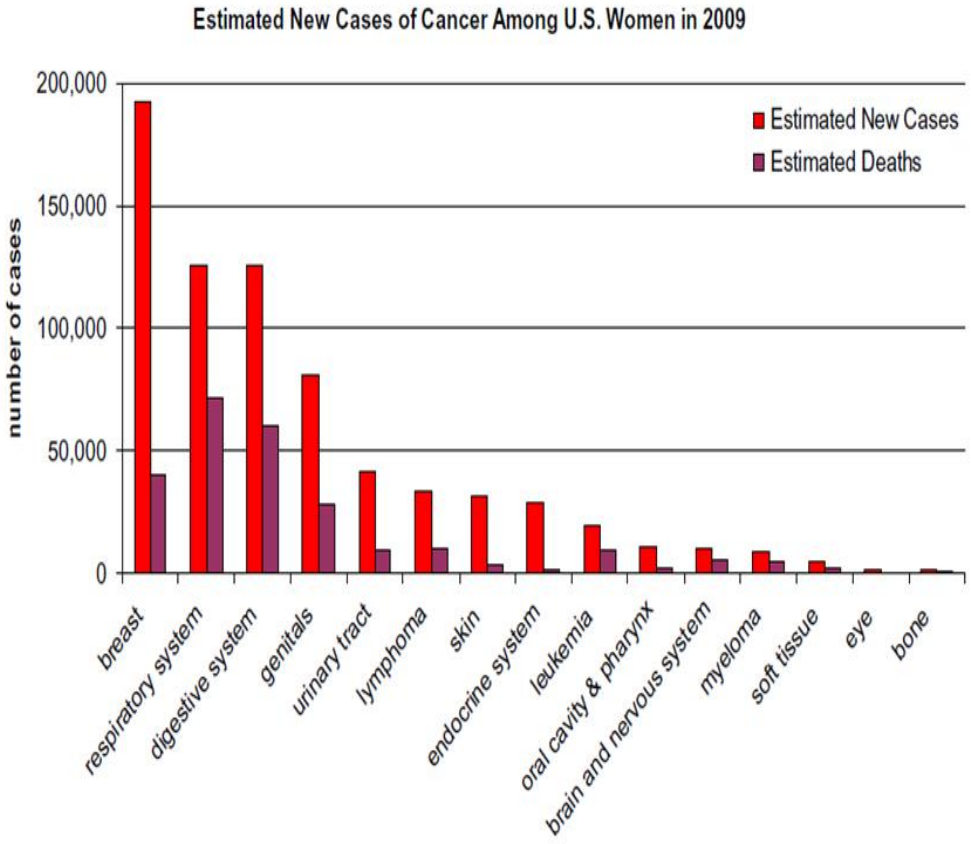

Figure1.2: Estimated new cases of different cancers among women in year 2009. Adapted from ACS Cancer Facts and Figures 2009.

C. Breast cancer diagnosis and treatment

Early detection of breast cancer is critical for both effective treatment and long-term survival. The aggressiveness of the breast tumor is predominantly based on the morphological and immunohistological characteristics of the tumor tissue. Recent evidence has revealed the benefit of classifying breast cancers based on gene expression profile. Gene expression profiling has generated a classification system, which allows breast cancer to be divided into five main subtypes. The five subtypes include luminal A, luminal B, human epidermal growth factor receptor (HER-2) type, basal and normal type.

These classifications are based on the expression profile of molecular markers in different breast cancers, including the estrogen receptor (ER), the progesterone receptor (PgR) and HER-2. Luminal A and Luminal B subtypes consist of breast cancers, which are derived from the epithelial cells that line the ductal or lobular lumen of the mammary gland. Both luminal A and B cancer types express ER and PgR. Differences between the two types are found in the luminal B subtype which also express HER-2 and is more aggressive then luminal A subtype. HER-2 subtypes of breast cancers do not express ER and PgR but overexpress HER-2. The basal type breast cancer is derived from the myoepithelial cells in the breast. This subtype is also known as triple-negative breast cancer because of its lack of expression of ER, PgG and HER-2. The basal type breast cancers are often aggressive and difficult to manage due to their lack of molecular targets. The normal breast-like tumors show morphological characteristics of a normal breast (Sorlie, Perou et al. 2001; Polyak 2007). The selection of breast cancer therapy and outcome of the therapy largely depend on these classifications.

D. Therapies for the treatment of breast cancer

Breast cancer therapies are broadly classified into two main categories: local and systemic. Local therapy includes surgery and radiation, and is currently offered as a standard treatment for all localized, well-differentiated breast cancer tumors. Surgical removal of tumors is ineffective if the tumor has spread from the site of primary tumor, which is known as metastasis. Approximately 40% of breast cancer patients relapse with metastatic disease (Colditz 1993). Systemic therapy includes chemotherapy, hormone therapy, or other targeted therapies. Use of these therapies largely depends on the type, stage and expression profile of molecular targets, which include ER, PgR and HER-2.

Basal breast carcinomas that lack expression of any of these receptors, as well as metastatic disease are treated with chemotherapy. Drugs used in chemotherapy are cytotoxic and include anti-mitotic agents, such as taxanes, DNA cross-linking drugs, topoisomerase inhibitors or antimetabolites. These drugs target dividing cells. Some of the most common chemotherapy drugs used to treat breast cancer are listed below.

i. **Anthracyclines:** This class of drugs includes doxorubicin (Adriamycin), epirubicin (Ellence), and liposomal doxorubicin (Doxil).

ii. **Taxanes:** This class of drugs includes docetaxel (Taxotere), paclitaxel (Taxol) and protein-bound paclitaxel (Abraxane).

iii. **Cyclophosphamid**e (Cytoxan), Capecitabine (Xeloda) and 5 fluorouracil (5 FU) Vinorelbine (Navelbine), Gemcitabine (Gemzar). Chemotherapy is often given as combination of one or two drugs.

Overexpression or amplification of HER-2 is associated with aggressive tumors and poor prognosis in breast cancer. Targeted systemic therapies against HER-2 include trastuzumab (Herceptin) and lapatinib (Tykerb)**.** Trastuzumab is a monoclonal antibody used to target the extracellular domain of HER-2. Lapatinib is a dual tyrosine kinase inhibitor which interferes with both the epidermal growth factor receptor (EGFR) and the HER-2 signaling pathway (Higa and Abraham 2007) by inhibiting HER-2 and HER 2 kinase activity and prevents the activation of downstream cellular signals that promote tumor cell survival and proliferation (Figure 1.5). It has been approved as first-line of treatment for patients, which show progression on trastuzumab.

E. Targeting Estrogen Signaling in Breast Cancer

The majority of breast cancer patients are ER positive. These tumors largely depend on estrogen for their survival and proliferation. The first evidence for a connection between steroid hormones and breast cancer growth was presented in 1896 by British physician Beatson, who discovered that removing the ovaries of premenopausal women could cause regression of advanced breast tumors (GW1896). The discovery of the receptor for estrogen in 1958 by Elwood Jenson opened up the possibility of targeting ER for the treatment of breast cancer (Green, Walter et al. 1986 ; Green and Chambon 1986). In premenopausal women, the ovaries mainly produce estrogen. However, most breast cancers occur in postmenopausal women where ovaries no longer produce estrogen. Even though, the plasma levels of estrogen are dramatically reduced after menopause, the circulating levels of estrogen detected in breast cancer patients are produced by non-ovarian sources such as adipose tissue, muscle, liver, bone, bone marrow, fibroblasts and hair roots (Russo and Russo 2006). Progression of breast cancer in the presence of estrogen makes the ER an important therapeutic target. Estrogen on binding to the ER acts as a transcription factor that regulates the expression of the genes which have estrogen receptor element on their promoter region (Green and Chambon 1986; Greene, Gilna et al. 1986; Arts, Kuiper et al. 1997; Kuiper, Carlsson et al. 1997). The most common genes regulated by estrogen signaling include genes associated with cell cycle progression and cell survival include as PgR, cyclin D1, c-myc and Bcl-2. These genes are the main players in maintaining cancer cell progression and survival. Measurement of the ER and PgR in breast tumors determines whether patients are suitable candidates for hormonal therapy. Hormonal therapy consists of two classes of

drugs, antiestrogens and aromatase inhibitors, which block estrogen signaling. This is done in two ways: either by blocking estrogen from binding to the estrogen receptor using antiestrogens, or reducing the production of estrogen in peripheral tissues and within the tumor using aromatase inhibitors (AIs).

1. Antiestrogens

The introduction of hormonal therapy played an important role in improving breast cancer treatment. Tamoxifen has been used for its antiestogenic activity for over 25 years for the treatment of hormone responsive breast cancer. It acts as an estrogen receptor antagonist and competitively inhibits estrogen binding, thus preventing estrogen receptor activation and transcription of estrogen responsive genes. The antiestrogenic activity of tamoxifen has established it as a standard treatment for all stages of breast cancer in premenopausal women (Fisher, Costantino et al. 1998). Tamoxifen is usually well tolerated by most breast cancer patients. However the most serious side effect of the tamoxifen is linked to its agonistic action in the endometrium. The estrogenic activities of tamoxifen increase the risk of mortality from endometrial cancer especially with prolonged treatment and also increased risk of stroke (Buzdar 2002; Altundag and Altundag 2003; Baum, Buzdar et al. 2003; Gerken 2004). Due to the serious side effects of the antoestrogens a series of clinical data documented that those patients who relapse after responding to tamoxifen can achieve further response with aromatase inhibitors over tomoxifen (Coombes, Hall et al. 2004; Goss, Ingle et al. 2005; Howell, Cuzick et al. 2005).

2. Aromatase inhibitors:

Aromatase inhibitors (AI) are a class of drugs which inhibit the aromatase enzyme, the key enzyme in the biosynthesis of estrogen (Brodie, Lu et al. 1997). The aromatase enzyme is responsible for the conversion of estradiol to estrone in the body (Figure 1.3).

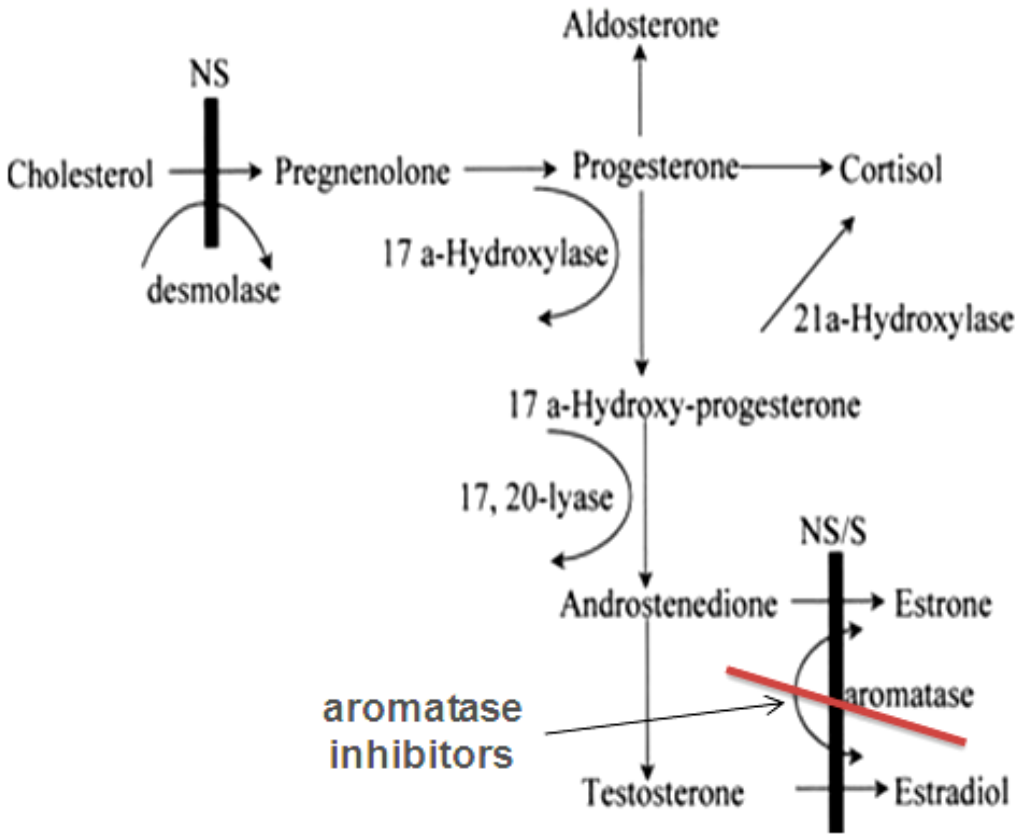

Figure 1.3: Formation of estradiol from cholesterol. Aromatase inhibitors block the activity of aromatase by binding to the enzyme to inhibit the production of estrogen. (Adapted from website, www.medscape.com).

AIs bind to the aromatase enzyme reversibly or irreversibly. The irreversible steroidal aromatase inhibitor binds to the aromatase enzyme permanently e.g exemestane

and the reversible non-steroidal inhibitors bind reversibly (letrozole and anastrazole). (Brodie 1985). The three FDA-approved AIs, two non-steroidal derivatives anastrazole (Arimidex) and letrozole (Femara) and one steroidal derivative exemestane (Aromasin) are now widely used as first-line drugs in the endocrine treatment of estrogen-dependent breast cancer in postmenopausal patients.

F. Resistance to endocrine therapy

Even though treatment with AIs in hormone-dependent breast cancers in postmenopausal women has proven to be effective in the clinic, resistance to these endocrine therapies may eventually develop. Resistance to therapy in general is broadly classified either as an intrinsic resistance, where the tumor does not respond to the therapy from the beginning and acquired resistance, where the tumor respond to therapy initially but eventually resistance to the therapy develops. In breast cancer despite the recognition of the estrogen receptor as a predictive marker for response to endocrine therapy, a significant number of patients do not benefit from endocrine therapy. That is believed to be due to the intrinsic resistance of the tumor to any therapy. Alternatively, a large number of estrogen responsive ER positive patients respond initially to endocrine therapy but eventually develop resistance. The mechanism of resistance to AIs is not completely understood. Studies trying to understand and divulge the mechanism of resistance have been an important scientific question and a main focus of research in our lab. Studies from our lab and others suggest that continuous deprivation of estrogen by AIs in breast cancer results in a switch from dependence on ER signaling to dependence on growth factor-mediated pathways, such as epidermal growth factor receptor (EGFR)/HER-2 and insulin-like growth factor receptor (IGFR) for cell proliferation and

survival (Sabnis, Jelovac et al. 2005). The activation of mitogenic signaling such as HER-2 and MAPK on acquiring resistance to hormonal therapy results in non-genomic activation of ER and its co-activators (Figure 1.4).

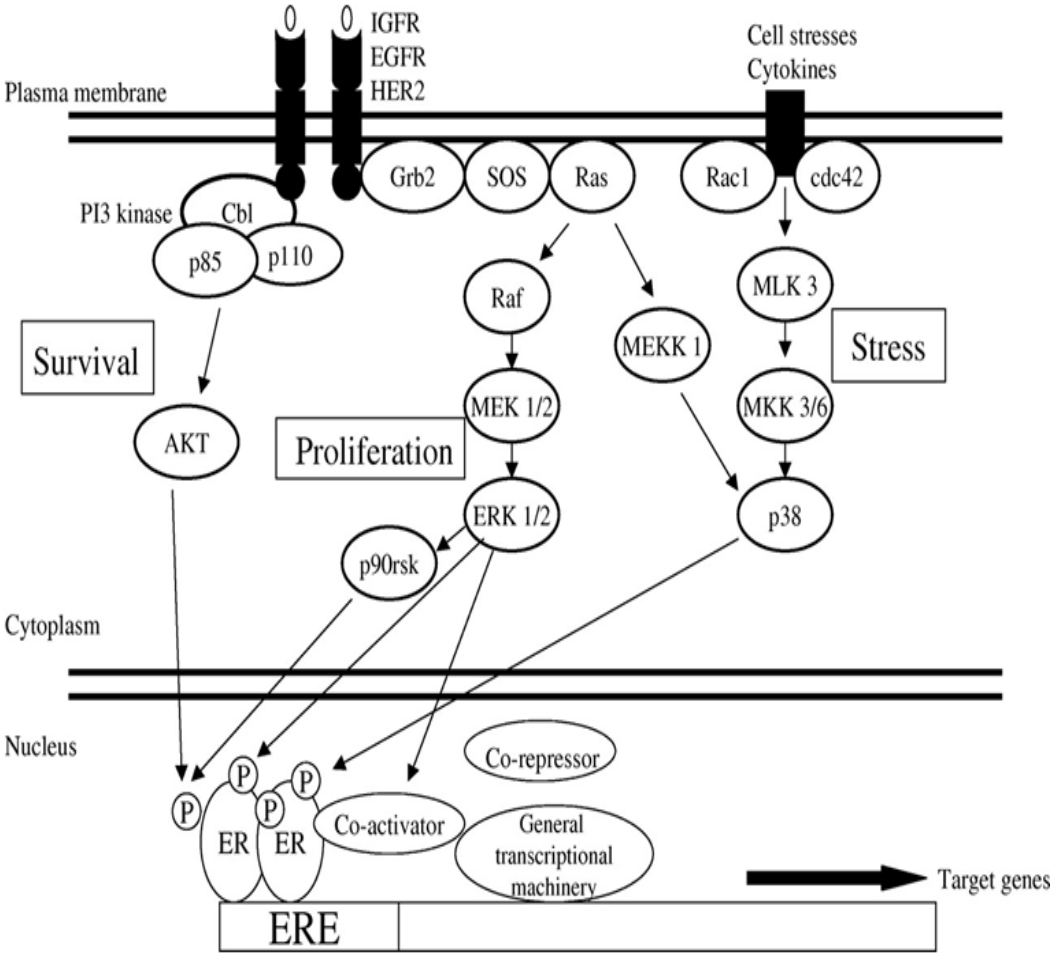

Figure 1.4: Activation of ER by activation of EGFR signaling molecules. (Adapted from Endocrine Related Cancer 11(4) 643-658)

The in vitro studies are supported by the clinical findings showing that tumors that start out expressing low levels of EGFR and HER-2 that undergo prolonged treatment with hormonal therapy (tamoxifen) are associated with increased expression of

EGFR and HER-2 (Meng, Tripathy et al. 2004; Gutierrez, Detre et al. 2005). Several independent studies have demonstrated that in AI resistant tumors adapt to estrogen depletion by activating MAPK, EGFR and signaling through the HER-2 pathway (Jeng, Yue et al. 2000; Martin, Reiter et al. 2003; Sabnis, Jelovac et al. 2005). These observations have been suggested to have a central role in resistance to hormonal therapies.

To better understand the mechanism of resistance to endocrine therapy, our lab has generated an AI resistant breast cancer cell line. This cell line was derived from MCF-7 cells transfected with the aromatase enzyme (MCF-7Ca) that were inoculated into nude mice to form tumors and then treated with letrozole until the tumors became resistant to the treatment. The resistant tumors were then removed from the mice and cells isolated, herein denoted as LTLT-Ca. LTLT-Ca cells were then analyzed for the effects of AI resistance. Our results indicate that prolonged depletion of estradiol with letrozole in ER positive HER-2 negative MCF-7Ca tumors resulted in an increase in the HER-2/MAPK signaling pathway and a down regulation of ER (Brodie, Sabnis et al. 2006). Consequently, HER-2 inhibitors trastuzumab and lapatinib have been explored as treatments for AI-resistant breast cancer (Sabnis, Schayowitz et al. 2009). The mechanism by which HER-2 is involved in AI resistance remains unclear. It is, therefore, important to further elucidate the HER-2-mediated pathway that contributes to AI resistance. In particular it is important to identify the characteristics associated with AI resistant breast cancer cells and to identify other potential factors involved that may also be biomarkers and targets for therapy.

1. HER-2 in breast cancer

The mammalian epidermal growth factor receptor (EGFR) family comprises of four receptors, EGFR, ErbB2, ErbB3, and ErbB4. HER-2 is a second member of the epidermal growth factor receptor family ErbB2. The erbB2 (also known as HER-2 or neu) gene encodes a 185-kDa transmembrane glyco-protein. ErbB2 is a receptor tyrosine kinase with intrinsic kinase activity. Overexpression of HER-2 is associated with increased proliferation and is an oncogene in breast cancer (Bargmann, Hung et al. 1986; Yamamoto, Ikawa et al. 1986). HER-2 signaling is involved in the regulation of normal breast growth and development (Carraway KL 3rd and J 1997) and its overexpression or amplification is associated with malignancy and a poor prognosis in breast cancer (Winer March 2007). HER-2 is overexpressed in 20% of breast cancers and is also indicated as a predictive marker in endocrine resistance (Slamon, Godolphin et al. 1989; Yu and Hung 2000). HER-2 has been implicated in promoting cell survival, cell cycle progression and tumor metastasis by activating major signaling pathways such as phosphatidylinositol-3-kinase (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways (Figure 1.5) (Yarden and Sliwkowski 2001; Hynes and Lane 2005).

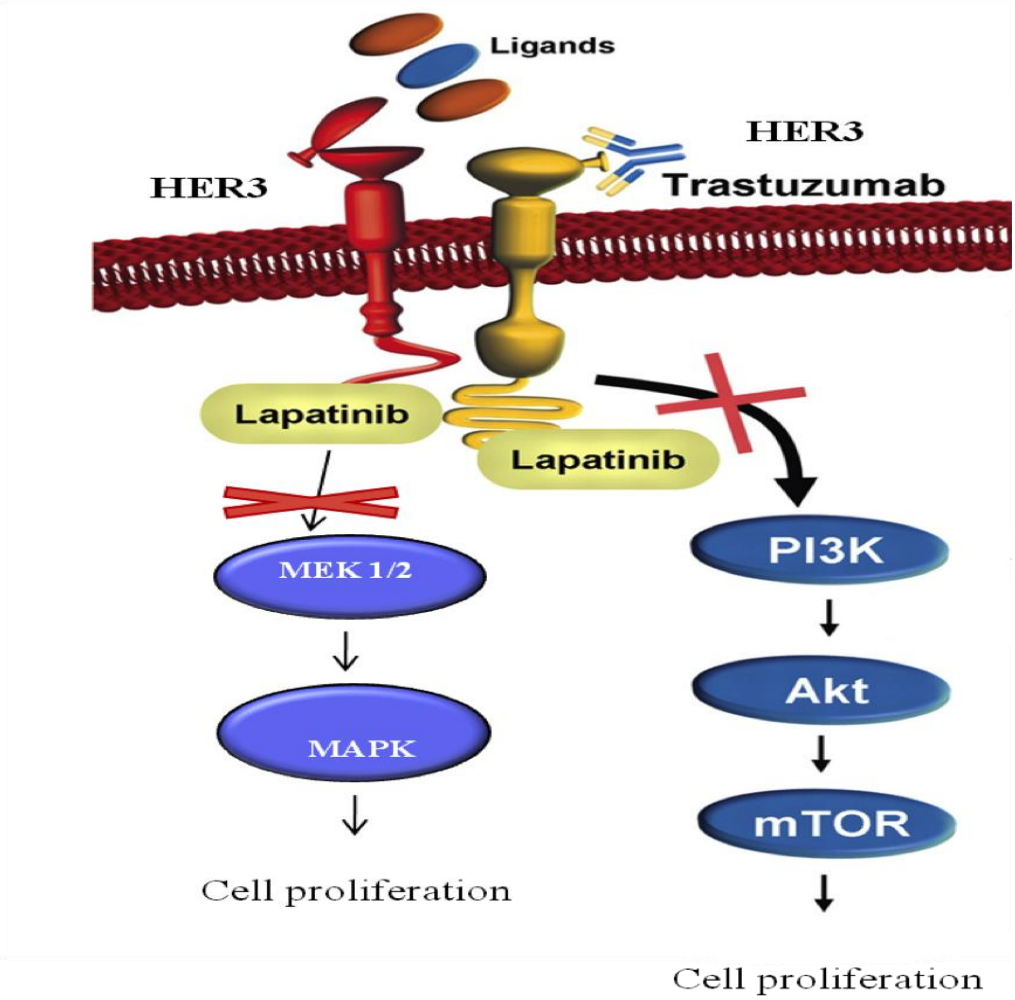

Figure 1.5: The HER-2 signaling pathways and inhibitors of the signaling. HER-2 receptor dimerizations cause activation of signaling through MAPK and PI3/Akt tyrosine kinase pathways, involved in proliferation, cell survival and tumor progression. Trastuzumab is a monoclonal antibody against HER-2 extracellular domain. Lapatinib is a dual tyrosine kinase inhibitor of both MAPK and PI3/Akt signaling from HER-2. Adapted from Vogel C et al. Jpn. J. Clin. Oncol. 2010 (Vogel, Chan et al. 2010).

Recent studies provide evidence that HER-2-mediated carcinogenesis and tumorigenesis might be due to the effect of HER-2 signaling on cancer stem cell/tumor-initiating cells (Magnifico, Albano et al. 2009). The cancer stem cell hypothesis, which is a new paradigm in cancer research, proposes that tumors are initiated and maintained by a subset of cells within a tumor, known as tumor initiating cells (TIC) or cancer stem cells (Chumsri, Phatak et al. 2007; Korkaya and Wicha 2007; Chumsri and Burger 2008). The first evidence for the existence of tumor-initiating cells (TICs) in breast cancer was demonstrated by the Al Hajj group, which showed that as few as 100 cancer cells with a $CD44^{high}/CD24^{low}$ phenotype isolated from human breast tumors could initiate breast cancer in Nonobese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice (Al-Hajj, Wicha et al. 2003). Their study demonstrated the heterogeneity and self-renewal capability of a subset of breast cancer cells within the tumor. In addition to the $CD44^{high}/CD24^{low}$ phenotype, cells with high drug efflux capacity, or breast cancer cells, which express aldehyde, dehydrogenase (ALDH) is known to have TIC properties. The expression of molecular metabolic mediatorALDH1was reported to be amplified in leukemic TIC and has implications for the resistance to cyclophosphamide. (Magni, Shammah et al. 1996). ALDH1 activity in breast TICs was later identified to be associated with poor prognosis and is known as a putative stem cell marker (Ginestier, Hur et al. 2007). Later on cells capable of growing in suspension as mammospheres were also reported to have characteristic of TIC properties in breast cancer (Dontu and Wicha 2005; Ginestier, Hur et al. 2007).

G. HER-2 and Tumor initiating cells in breast cancer:

HER-2 overexpression is commonly associated with an increase in cell proliferation, increased angiogenesis and increased metastatic potential (Korkaya, Paulson et al. 2008; Magnifico, Albano et al. 2009). However, increasing evidence suggest that HER-2-mediated carcinogenesis and tumorigenesis may be due to the effect of HER-2 signaling on tumor-initiating cells (Korkaya and Wicha 2007; Magnifico, Albano et al. 2009). The overexpression of HER-2 protein was implicated in regulating the self-renewal pathway, which is a hallmark of TICs(Magnifico, Albano et al. 2009). Apart from its role on self renewal pathway ectopic overexpression of HER-2 in human mammary carcinoma cells in breast cancer results in amplification of TIC characteristics, such as an increase in mammosphere formation (suspension culture for maintaining TIC in undifferentiated state) and increase in ALDH as reported by (Dontu and Wicha 2005; Korkaya, Paulson et al. 2008). Due to the differences in the breast cancer subtypes the amplification in the $CD44^{+}/CD24^{-/low}$ population with increased HER-2 was mainly demonstrated in only basal breast cancer cells (Charafe-Jauffret, Ginestier et al. 2009; Ginestier, Wicinski et al. 2009). Overall these studies suggest that HER-2 may be one the important molecules regulating TIC fraction.

H. Resistance due to tumor initiating cells:

The major challenge for current treatment in breast cancer does not generally lie in the lack of initial response of tumors or induction of remission in response to drug treatment, but rather often lies in resistance and recurrence of the tumor after the treatment. If TICs have an intrinsic ability to initiate and sustain tumor growth then

practically these cells become an attractive target to overcome resistance and attain a long-term survival. One of the ubiquitous features of TIC is to survive in presence of high concentration of drugs, due to expression of drug efflux pumps. One of the efflux pumps particularly Breast cancer resistance protein (BCRP) is known to play a functional role in the maintenance of TIC fraction by not only extruding toxins out but also maintaining these cells in undifferentiated state (Dontu, Al-Hajj et al. 2003).

1. Breast cancer resistance protein (BCRP)

BCRP is a typical drug efflux pump that mediates multidrug resistance in various cancers. It consists of two repeated halves; the 72-kDa BCRP is a half-transporter consisting of only one nucleotide binding domain followed by one membrane-spanning domain (Figure 1.6).

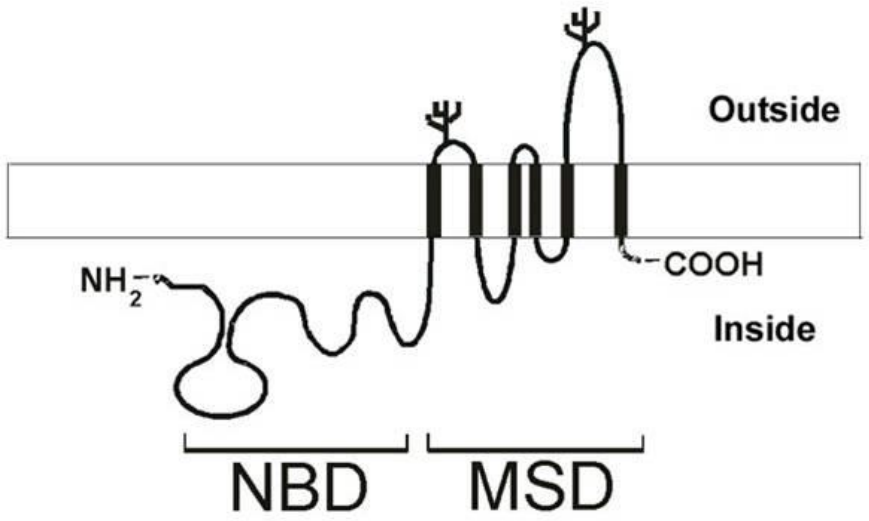

Figure 1.6: Membrane topology model of BCRP. BCRP contains one nucleotide-binding domain (NBD) followed by one membrane-spanning domain (MSD) with 6 -helices. Adapted from AAPS Journal. 2005; 07(01): E118-E133.

It may function as a homodimer or homotetramer. Overexpression of BCRP in cancer cells is often associated with poor response to anticancer drugs including anthracyclines, mitoxantrone and camptothecins by enhancing drug efflux (Ross, Karp et al. 2000; Bailey 2001; Steinbach, Sell et al. 2002). In addition to its role of conferring resistance against chemotherapeutic agents, BCRP actively transports structurally varied organic molecules, conjugated or unconjugated, such as estrone-3-sulfate, 17β-estradiol 17-(β-D-glucuronide) and methotrexate. BCRP is highly expressed in normal tissue such as the placental syncytiotrophoblasts, in the apical membrane of the epithelium in the small intestine, and in the liver canalicular membrane. The apical localization of BCRP may limit the bioavailability of orally administered anticancer agents that are BCRP-substrates, such as topotecan (and its metabolite SN-38), irinotecan, camptotecin derivatives, methotrexate (Sarkadi, Homolya et al. 2006), as well as endocrine therapies, particularly tamoxifen (Konecny, Pauletti et al. 2003). Structural and functional studies of BCRP have provided important insight into the molecular mechanisms essential for BCRP-mediated multidrug resistance. Several mutational studies in the transmembrane domain elucidated the molecular mechanism involved in the selectivity and specificity of substrates for BCRP (Gottesman, Fojo et al. 2002; Ambudkar, Kimchi-Sarfaty et al. 2003). For most ABC transporters, the binding and subsequent hydrolysis of ATP at their nucleotide binding domains (NBDs) is required to provide energy for the movement of their substrates across membranes. The ATPase activity closely correlates with the respective transport activity of these proteins. The ATPase activity is also stimulated by the presence of various hydrophobic compounds (Traxler, Furet et al. 1996). Two

sequence motifs located 100 ï200 amino acids apart in each nuclear binding domain, designated Walker A and Walker B, are conserved among all ABC transporter super family members, as well as numerous other ATP-binding proteins (Walker, Saraste et al. 1982). Even though significant progress has been made in understanding drug resistance associated with overexpression of BCRP, the clear mechanism of how BCRP acts, as a drug transporter is still poorly understood. Several investigators have studied the relationship between expression of BCRP and clinical response to a number of drugs (Robey, Polgar et al. 2007). Higher expression of BCRP was found in many types of cancer, including leukemia and other solid cancers. Burger and colleagues found a correlation between MDR1 expression and poor prognosis in breast cancer patients receiving chemotherapy (Burger, Foekens et al. 2003). Drugs such as anthracyclines and taxanes used as first-line therapy in breast cancer up-regulate MDR1 expression following chemotherapy administration. In metastatic breast carcinoma, the majority of the tumors show an increase in MRP1 expression associated both with shorter progression free survival and decreased overall survival (Rudas, Filipits et al. 2003). Several other studies predicted that high expression of drug resistance genes in primary breast tumors may be related to altered biological behavior of the tumor cells, including a more aggressive phenotype resulting in drug resistance.

2. BCRP Inhibitors

Experimental evidence shows a clear involvement of BCRP expression with drug resistance in various cancers. Hence, development of inhibitors to BCRP would be of interest for improving the clinical outcome of chemotherapeutic drugs in multidrug resistance (MDR) phenotype expressing cancers. In breast cancer many cytotoxic agents,

such as anthracyclines and taxanes, are disposed to MDR-mediated loss of sensitivity. Several strategies have been adapted to overcome MDR, mostly through inhibition or modulation of these pumps. Despite successful proof of concept in the laboratory, to date none of these agents has had a major impact in the clinic. A range of BCRP inhibitors have been developed, but their use in the clinic is precluded due to high toxicity. Additionally, expressions of these pumps in other anatomic locations (such as the brush border of the gastrointestinal epithelium and blood-brain barrier) increase the difficulty of developing therapeutics against them.

3. Role of BCRP and tumor initiating cells:

Self-protection or damage tolerance due to the expression of efflux transporters is an important characteristic of TIC. This ability of cancer cells to overcome therapeutic stress may have profound implications for cancer treatment. BCRP is one of the most studied efflux pumps in relation to expression of a stem cell phenotype (Doyle and Ross 2003). Enrichment and maintenance of high BCRP expression in undifferentiated cells elucidate their role in the stem cells (Zhou, Schuetz et al. 2001; Scharenberg, Harkey et al. 2002).. BCRP is a drug transporter protein capable of effluxing a variety of substrates, including cytotoxic drugs, by using ATP energy (Zhou, Schuetz et al. 2001). High expression of these transporters may help protect cancer stem cells from cytotoxic agents used for cancer treatment. The BCRP transporter has been shown to specifically pump out the Hoechst 33342 dye that intercalates DNA (Zhou, Schuetz et al. 2001). The cells that effluxes out the Hoechst dye are identified as a side population (SP) cells by flow cytometric analysis. SP as a functional assay for BCRP has been used to isolate TIC from

mammary glands. TIC isolated using SP assay were demonstrated to give rise to the whole breast tissue when transplanted into cleared mammary fat pads (Welm, Tepera et al. 2002; Alvi, Clayton et al. 2003). In addition, characterization of cells within the SP demonstrates that they are immature, poorly differentiated, and highly tumorigenic (Decraene, Benchaouir et al. 2005). Studies by vesho wn ghat in ô s group luminal breast cancer, SP analysis is a predominant method of identification of cells with TIC characteristics (Nakanishi, Chumsri et al.). Their studies also suggest the role of HER-2 signaling in regulation of SP cells in breast cancer.

4. Regulation of BCRP in breast cancer:

BCRP has largely been studied in context of chemoresistance and HER-2 is known to be a predictive marker for response to drugs that are effluxed through BCRP and Pgp. Effluxed drugs include docetaxel (Xie, Xu et al. 2008) as well as endocrine therapies, particularly tamoxifen (Konecny, Pauletti et al. 2003). Studies from head and neck cell carcinoma suggest the role of Her-2 signaling in regulation of SP (Chen, Pardo et al. 2006). In addition, reports from Schwabedissen *et al* (Meyer zu Schwabedissen, Grube et al. 2006) have demonstrated the stimulatory effect of epidermal growth factor on BCRP gene transcription in breast and ovarian cancer. Studies by D. Ross groups have shown that the efflux ability of BCRP has an essential role in the survival of hematopoietic stem cells under hypoxic conditions. There group also demonstrated that hypoxia inducible factor (HIF-1α) acts as a transcription factor resulting in activation of BCRP gene (Krishnamurthy, Ross et al. 2004). More recent studies by (Martin, Ferdous et al. 2008) further supports the role of HIF-1α protein in regulation of BCRP in cardiac cells.

5. The hypoxia inducible factors (HIFs) and TIC:

HIFs are a helix loop helix family of transcription factors known to mediate response to hypoxia by regulating the expression of genes required for regulating angiogenesis, glycolysis and erythropoiesis, such as vascular endothelial growth factor (VEGF) and many others (Wang and Semenza 1995; Forsythe, Jiang et al. 1996; Gleadle and Ratcliffe 1997; Sandner, Wolf et al. 1997; Krieg, Marti et al. 1998). HIF-1α exists as a heterodimer, consisting of an alpha and beta subunit. There are three isoforms of the alpha subunit: HIF1 Ŭ HIF2 Ŭand HIF3 Ŭ HIF1 and HIF2 have several shared transcriptional sites. The role of HIF-1α has become important in understanding tumor growth and survival. Even though low oxygen tension is a known stimulatory factor in initiating HIF-1αresponse, studies have shown that HIF-1α expression and activation can also be regulated by growth factors, hormones, and cytokines independent of O_2 levels (Laughner, Taghavi et al. 2001; Kondo, Kim et al. 2003; Peng, Karna et al. 2006). Experimental evidence has emphasized the role of hypoxia in cancer initiating cells by demonstrating the increased importance of the HIF proteins in maintaining an undifferentiated phenotype in neuroblastoma stem cells (Jogi, Ora et al. 2002) and in ductal breast carcinoma (Helczynska, Kronblad et al. 2003). Furthermore, several hypoxia regulated genes such as Glut1, Serpin B9, and VEGF were shown to have higher expression in the cancer stem cell sub-population (Bao, Wu et al. 2006; Li, Bao et al. 2009). Using *in vivo* knockdown experiments, studies have demonstrated that HIF- 1 Ŭ h a s a potential role in promoting the tumor initiating cell like phenotype and driving tumor

growth. In general HIF-1 Ŭhas been implicated in the survival mechanism. In hematopoietic stem cells, survival due to HIF-1 Ŭis manifested by inducing BCRP-mediated transport essential for the protection of cells against the accumulation of deleterious porphyrins (Krishnamurthy, Ross et al. 2004). However, the nonhypoxic (i.e., normal cell culture conditions of 95% air which is ~20% O_2, 5% CO_2) regulation of HIF-1 Ŭand its potential role in regulation of BCRP is not yet known.

Resistance to chemotherapeutic agents is often associated with the presence of efflux pumps. However, it is not known if hormone resistance SP TICs in breast cancer is in any way related to the presence these efflux pumps. Although, HER-2 has been shown to regulate BCRP expression (Chen, Pardo et al. 2006), the precise mechanism of HER-2 regulation of SP in hormone resistant breast cancer is not clear as yet. Therefore, understanding the mechanism of activation of drug-resistant SP cells in hormone resistant cancers could provide an explanation for the poor response of HER-2 positive and luminal-type tumors to cytotoxic chemotherapy.

I. Tumor initiating cells and resistance to anticancer therapy:

In the past two decades, more than 30 new anticancer drugs have been introduced, but survival rates have improved only marginally. Most anti-cancer therapies are effective at debulking the tumor mass but treatment effects are transient, resulting in tumor relapse and metastasis. Recent work in several different cancers support that TIC population is the root source of resistance to chemotherapy, radiation and endocrine therapy (Nicolini, Ferrari et al. ; Phillips, McBride et al. 2006; Chumsri and Burger 2008; Li, Lewis et al. 2008; Creighton, Li et al. 2009). Tumor initiating cells are slow-dividing, have a lowered ability to undergo apoptosis and a higher ability for DNA repair, making

them more resistant to traditional methods of cancer treatment such as radiation chemotherapy and hormonal therapy (Bao, Wu et al. 2006; Ma, Lee et al. 2008; Nicolini, Ferrari et al. 2011). In addition as already mentioned expression of ABC drug efflux transporters impart an additive advantage for TICs to survive in presence of chemotherapeutic and hormone therapies (Konecny, Pauletti et al. 2003; Dean, Fojo et al. 2005)..In fact in breast cancer, treatment with chemotherapy has been shown to cause an increase in the percentage of $CD44^{high}CD24^{low}$ TICs in patients (Li, Lewis et al. 2008). Several *In vitro* studies show that chemo and radiation therapy resistant cancers often contain a higher proportion of TICs compared to drug sensitive cancers (Al-Hajj, Becker et al. 2004; Singh, Clarke et al. 2004). One recent study by the Creighton group has shown that tumors from breast cancer patients treated with letrozole exhibited a marked increase in TIC (Creighton, Li et al. 2009).

The inability to eradicate this subpopulation of cancer cells may result in disease recurrence and subsequent progression after an initial shrinkage of the tumors. For therapy to be more effective, cancer treatment should be targeted against quiescent, tumor initiating cells. This might be achieved by either exploiting the basic characteristics of TICs such as their undifferentiated phenotype or by targeting pathways that regulate them. This new approach of targeting TIC could have a major impact on the treatment of disease. Future therapies will need to effectively target the TIC to induce clinically significant remission of disease. New treatments specifically targeted against TIC may not be fully optimal by themselves and will need to be studied in detail and placed in combination therapy with existin be employed after debulking of the differentiated tumor tissue. Induction of

differentiation in TICs results in the loss of their ability to self-renew, an important characteristic of the TIC phenotype. RA has been effectively employed as a differentiation agent in the clinic in AML (Massard, Deutsch et al. 2006).

1. Retinoids

The retinoids are a class of drugs that are structurally and chemically similar to vitamin A (retinol) and related derivatives (Chomienne, Balitrand et al. 1986). The retinoid family comprises of vitamin A (retinol) and its natural derivatives such as retinaldehyde, retinoic acid, and retinyl esters. The synthetic derivatives of retinoid include ATRA and its isomers ï9-cis-retinoic acid (9-CRA), 13-cis-retinoic acid (13-CRA) and 11-cis-retinoic acid (11-CRA) (Antille, Tran et al. 2004). RA is a metabolic product of vitamin A (retinol). Retinol first binds to the cytoplasmic retinol binding protein. In the cytoplasm retinol is converted to retinoic acid by the activity of aldehyde dehydrogenase. The expression of genes necessary for cellular growth and differentiation are activated by the binding of retinoid nuclear receptors that exist as either retinoic acid receptors (RAR) or retinoid X receptors (RXR). These receptors bind promoter consensus sequences retinoic acid receptor element (RAREs) as either RXR iRAR heterodimers or RXR iRXR homodimers. RA restores transcription by inducing conformational changes in the receptors, thereby promoting the release of corepressors and allowing for recruitment of coactivators with histone acetyltransferase (HAT) activity (Evans 1988) (Figure 1.7). RA-based therapy started its use in acute promyeloctyic leukaemia (APL) followed by several clinical and preclinical studies to employ it as a therapeutic drug for other cancer types including breast cancer (Ohno, Asou et al. 2003).

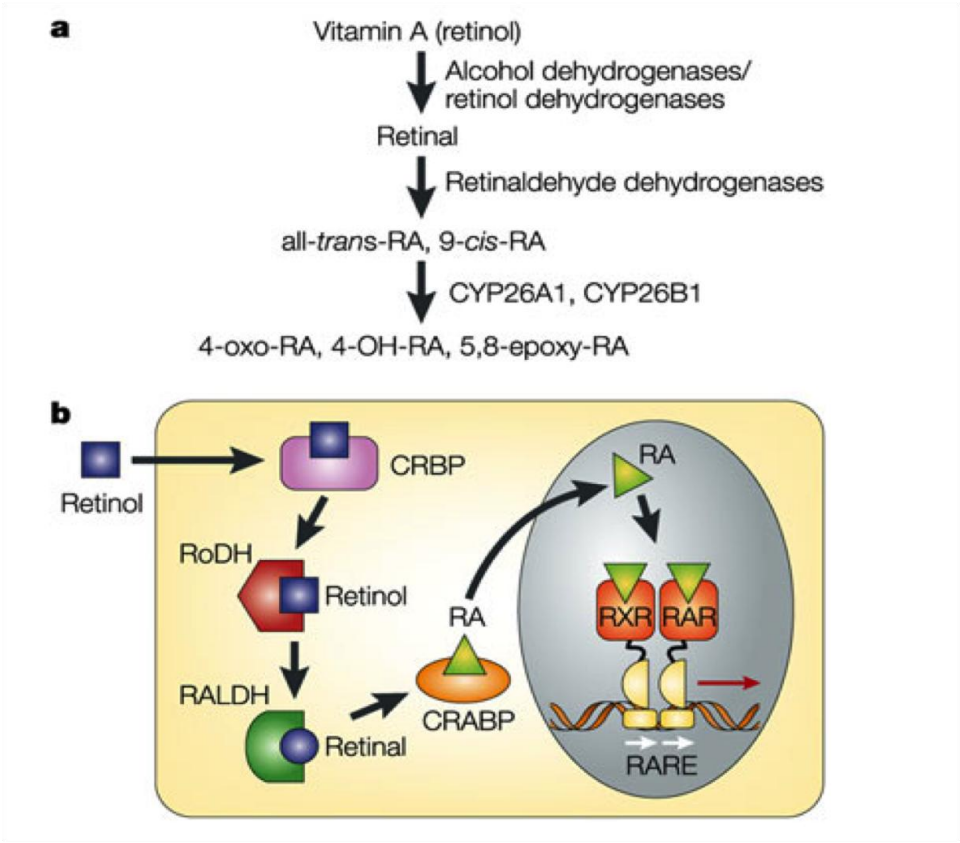

Figure 1.7: The retinoid-signaling pathway. a, RA is a metabolic product of vitamin A (retinol). **b,** Retinol binds to cytoplasmic retinol binding protein. Retinol is being converted to retinoic acid by the activity of aldehyde dehydrogenase. The expression of genes necessary for cellular growth and differentiation are activated by the binding of retinoid nuclear receptors, which exist as either retinoic acid receptors (RAR) or retinoid X receptors (RXR). These receptors bind promoter consensus sequences retinoic acid receptor element (RAREs) as either RXR iRAR heterodimers or RXR iRXR homodimers. Adapted from (Maden 2002), Nature review.

2. Retinoid Therapy:

The most well-known use of retinoids is in APL (Huang, Ye et al. 1988). APL is caused by the translocation of the retinoic acid receptor gene and its fusion with N-terminus of promyelocytic leukemia (Borrow, Goddard et al. 1990; de The, Chomienne et al. 1990). The resulting protein product of the fused gene interferes with normal retinoid signaling. Treatment with ATRA allows DNA transcription and differentiation of the immature leukemic promyelocytes into differentiated granulocytes. Unlike other chemotherapies, ATRA does not directly kill malignant cells. ATRA reduces proliferation by inducing the terminal differentiation of the leukemic promyelocytes, differentiated malignant cells either undergo spontaneous apoptosis or lose their uncontrolled dividing potential. (Breitman, Collins et al. 1981; Sidell 1982; Brand, Petkovich et al. 1988). Retinoids, such as fenretinide [N (4-hydroxyphenyl)-retinamide (4-HPR)], and its isomers are effectively used as differentiating agents in AML (Dragnev, Rigas et al. 2000). The phenomenon of induction of differentiation by RA was also studied in several other cancers such as neuroblastoma (Sidell, Altman et al. 1983) teratocarcinoma (Sidell and Horn 1985); melanoma (Lotan and Lotan 1980; Meyskens and Fuller 1980). Various clinical and preclinical studies have suggested the role of retinoids in cancer prevention (Dragnev, Rigas et al. 2000; Niles 2000). Based on experimental evidence that RAR expression is lost in many cancers, including breast cancer, led to the conclusion that RAR may act as a tumor repressor (Castillo, Milano et al. 1997; Widschwendter, Berger et al. 1997). In more recent clinical studies, chemopreventive efficiency of tazarotene, which is a form of retinoid against anti-basal cell carcinoma, was reported (So, Fujimoto et al. 2008). Another study on tazarotene in

combination with retinoids was proposed to have antineoplastic properties (Yen, Fenning et al. 2004). In lung and breast cancer a novel retinoid NRX194204 (NuRx Pharmaceuticals, Irvine, CA) with anti-inhibitory and anti-inflammatory properties was shown to reduce the number and size of adenocarcinomas (Liby, Royce et al. 2007). In a mouse model baxarotene was shown to be effective in both estrogen receptor positive and estrogen receptor negative tumors (Wu, Kim et al. 2002; Wu, Zhang et al. 2002).

A major focus of clinical attention for breast cancer-related retinoid use has been chemoprevention. Several recent studies are investigating the antitumor effects of RA in combination with current therapeutic drugs group has shown that differentiation promoted by retinoids in combination with inhibition of HER-2 signaling by trastuzmab have synergistic antiproliferative effects on both ER-positive and ER-negative human breast cancer cells (Koay, Zerillo et al. 2010).

Despite success in preclinical studies, in the clinic retinoids did not raise the same enthusiasm because of their limited activity. The effect of retinoids on TIC in breast cancer has not been reported as yet. We believe that conflicting outcomes of preclinical and clinical studies may lie in deciphering the downstream effects of retinoid actions and not only studying their actions as differentiating agents on TIC. Evaluating the effects of retinoids may potentially lead to the development of these agents for clinical use in combination with current therapies. The development of retinoid as differentiating agents against TIC is a novel idea. The concept of using ATRA has been reported by Wicha and colleagues demonstrating the role of retinoid signaling in the regulation of TIC self renewal and differentiation genes (Ginestier, Wicinski et al. 2009). Their group utilized gene set enrichment analysis to verify genes, which are differentially altered by

modulation of retinoid signaling. They showed that treatment with ATRA cause a down-regulation of various sets of genes normally shown to be up-regulated in TIC cells. Drug resistance and self-renewal genes are among those important genes modulated by retinoid signaling. Hence, Wichaôs study of retinoi the effect of activation of retinoid pathway in resistant breast cancers.

Characteristically TIC have an amplified ability to pump chemotherapeutic drugs out of cells, suggesting that increased drug removal ability may serve as a mechanisms to protect them from chemotherapy (Chaudhary and Roninson 1991; Johnstone, Cretney et al. 1999; Pallis and Russell 2000; Zhou, Schuetz et al. 2001). In breast cancer a large percentage of patients are hormone receptor positive and respond to hormone therapy, however resistance to hormone therapy eventually occurs resulting in hormone resistance and tumor relapse. The role of TIC in hormone resistance is not yet clear. Our group has previously shown that in estrogen receptor positive hormone responsive breast cancers proliferation is suppressed by blocking estrogen signaling with aromatase inhibitors. However, compensatory signaling pathways are upregulated by prolonged use of AIôs offset the effects of inhibition of estrogen signaling. HER-2 is one of the important signaling pathways more frequently upregulated in hormone deprived breast cancers (Brodie, Sabnis et al. 2006). The mechanism by which HER-2 is involved in AI resistance largely remains unclear. It is, therefore, important to 1) further elucidate the HER-2-mediated pathway that contributes to AI resistance; and 2) identify other potential factors involved that may serve as novel molecular markers and therapeutic targets. Since role of TIC in resistance to anticancer drug has received a prominence in breast cancer

research field, which prompted us to study the role of TIC in hormone resistance in detail in this project.

Hypothesis and specific Aims

We hypothesize that the acquisition of enhanced HER-2 signaling in hormone resistant breast cancer results in the amplification of undifferentiated TICs population. These cells can be targeted either by inhibiting HER-2 signaling or by inducing differentiation.

In order to test our hypothesis we followed:

Specific Aim 1: Characterize hormone resistant and hormone sensitive breast cancer cells for tumor initiating characteristics.

Specific Aim 2: Study the role of HER-2 in regulation of TIC phenotype in hormone resistant breast cancer.

Specific Aim 3: Determine the effect of HER-2 and differentiating agent alone and combination of differentiating agent with HER-2 inhibitor in targeting TIC population.

MATERIALS AND METHODS

To investigate the impact of hormonal therapy, particularly hormone resistance on BC T-ICs, SP cells were analyzed; Mammosphere formation and expression of efflux pumps and stem cell markers were analyzed in acquired aromatase-overexpressing MCF-7Ca aromatase inhibitor and anastrazole-resistant lines. LTLT-Ca cells are grown in a phenol red-free IMEM supplemented with 5% CSS, 1% penicillin/streptomycin, 600 εg/ml, geneticin, Ca 1 Bba (Invitrogen) and ANA 1 εM let are grown in the same media as above suppl anastrazole and 1 εM-7Ca aromatase transfected cells are inoculated in mice and the mice are treated with letrozole for 56 weeks till they become resistant to letrozole. Tumors are extracted and a cell line formed from resistant tumors was analyzed for the impact of hormonal therapy.

A. Cell lines and Reagents

Letrozole was supplied by Dr. D. Evans, Novartis, Pharma, Basel, Switzerland). IMEM, RPMI, T-medium, penicillin/streptomycin solution (10,000 IU each), 0.25% trypsin-EDTA (1 mM/L) solution, phosphate buffered saline, Dulbecco's p obtained from Invitrogen (Carlsbad, CA). Regular and charcoal stripped fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Tween 20 were obtained from Sigma Chemical Company (St. Louis, MO). ECL che

nitrocellulose membranes were purchased from Amersham Biosciences (Piscataway, NJ). Antibodies against ER, HIF-1, p-Akt (Ser-473 and Thr-308) and Beta Actin were purchased from Cell Signaling Technology (Beverly, MA). Antibody for HER-2 was purchased from Millipore. Antibody for BCRP, CD24and CD44antibody was purchased from BD Biosciences (Franklin Lakes, NJ USA). The Aldeflour kit and mammmosphere kit were purchased from Stem Cell technologies, (BC, Canada). Cell line: MCF-7Ca cells (supplied by Dr. Chen, City of Hope, Duarte, CA) are MCF-7 cells stably transfected with the human aromatase gene. MCF-7Ca cells were routinely cultured in DMEM supplemented with 5% fetal bovine serum, 1% penicillin/ streptomycin and 700 µg/mL G_{418}. LTLT-Ca cells were developed fromMCF-7Ca cells from tumors of mice treatedwith letrozole for 56 wk. LTLT-Ca cells were maintained in steroid-depletedmedium, which consisted of phenol red ïfree IMEM supplemented with 5% dextran-coated charcoal-treated serum, 1% penicillin/streptomycin, and 750 µg/mL G_{418} and 1 µM/L letrozole. Lapatinib was supplied by Ms. Jane Oakley. ATRA is available commercially from Sigma.

B. Cell viability Assay

An MTT assay was carried out to determine the inhibitory concentration (IC), which inhibits cell viability at 20, 40, and 50%. To determine the effect of retinoids and lapatinib on cell proliferation, each cell type was transferred into steroid-free medium 3 days before the start of the experiments (steroid-free medium consisted of phenol red ï free RPMI supplemented with 5% dextran-coated, charcoal-treated serum and 1% penicillin/streptomycin solution). Growth studies were then done by plating cells (1.5×10^4 cells per well) in 24-well multi well dishes (Corning, Inc. NY). After a 24-h

attachment period, the medium was aspirated and replaced with steroid-free medium containing vehicle or the indicated concentrations of the compounds (1 nmol/L ï10 ε M / L) . C o n t r o l w e l l s w e r e t r e a t e d w i t h v e h changed every 3 days and the numbers of viable cells were compared by MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT; LNCaP) assay on the 7th day. For the MTT procedure, following incubation of cells for the above-mentioned time, 0.5 mg/mL MTT was added to each well and incubated at 37°C for 3 h. Following incubation, the medium was aspirated completely, with care t a k e n n o t t o d i s t u r b t h e f o r m a z a n c r y s t a l s crystals. After slight shaking, the plates were read at 540 nm with a Victor 1420 scanning multi well spectrophotometer (city state). All results represent the average of a minimum of three wells. An additional control consisted of medium alone with no cells.

C. Side population and fluorescence-activated cell sorting analyses

Side population analysis was performed as per the method of Goodell (Goodell 2002). Briefly, $1x10^6$ cells/ml were incubated in prewarmed Dulbecco's Modified Eagle's Medium containing 5% fetal bovine serum, 10/ m M H E P E S , / ml aï1 n H d e c h s 5 ε g 33342 (H33342, from Sigma-Aldrich, St Louis, MO, USA). Hoechst 33342 was prepared fresh by suspending in sterilized water at a concentration of 1 mg/ml and 5 µl of dye was added to the cell suspension for 90 minutes in a 37^{o}Cwater bath. Cells were also incubated concurrently with the BCRP inhibitor Ko143 (1/ ε M , a g i f t f r o m D r

Netherlands Cancer Institute). After incubation, cells were centrifuged at 4°C and resuspended in ice-cold Hank's Buffered Salt Solution containing 2% fetal calf serum and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Samples were analyzed with a BD LSR II four-laser flow cytometer (BD Biosciences San Jose, CA, USA) at a dilution of 10µl/1×10^6 cell/ml. CD24 and CD44 cell surface expression was analyzed using Allophycocyanin and phycoerythrin labelled primary antibodies (from BD Biosciences, NJ, USA). Propidium iodide (2/ ε/gml ï1; Sigma-Aldrich) was added to discriminate dead cells. Data was processes using flow-jo (Tree star) provided by the core facility.

D. Immunoblotting

HER-2, E-cadherin, N cadherin and ER expression were determined by western blot analysis. Untreated breast cancer cells or cells treated with HER-2 inhibitors were washed with ice-cold PBS and homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.5; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM sodium orthovanadate; 1 mM NaF; 10 mM sodium pyrophosphate; 1 µg/mL leupeptin; 1 µg/mL aprotinin; and 2 mM Pefabloc SC) by sonication. Debris was removed after centrifugation. The protein concentration of the resultant supernatant was determined by the Bradford method and 30-40 µg of cellular lysate was subjected to 10% SDS ï polyacrylamide gel electrophoresis and then electrotransferred onto a polyvinylidene difluoride membrane. Membranes were blocked in 5% milk in TBS with 0.1% Tween 20 for 1hr at room temperature followed by an overnight incubation at 4ºC in rabbit polyclonal anti HER-2 (Chemicon International,CA;; 1:1000), E-Cadherin (Sigma;

1:1000), rabbit polyclonal N-cadherin (sigma,1:1000), or ER (HC-20 Santa Cruz Biotechnology; 1:500). Secondary detection was performed with IgG conjugated horseradish peroxidase (1:5000; GE Healthcare) and blots were visualized with ECL plus chemiluminescent, detection kit. (Amersham Pharmacia Biotech, Piscataway, NJ).

E. Mammosphere Assay

TheMammosphere assay was performed as per the method by Dontu *et al.*(Dontu, Abdallah et al. 2003). Single cells were suspended in Dulbecco's Modified Eagle's Medium/F-12 containing 5 mg/mL insulin, 0.5 mg/mL hydrocortisone, 2% B27, and 20 ng/mL epidermal growth factor (Invitrogen Ltd., Paisley, Scotland) and plated in ultra low attachment well plates (Corning) at a density of 20,000 cells/ml. Fresh media was added weekly. The cultured mammospheres were passaged every 2 weeks by incubating with trypsin for 3 minutes at 37^{o} C then dispersed by repeated pipetting with a 23-gauge needle.

F. Aldehyde dehydrogenase activity

Aldefluor kit (stem cell technologies) was used for the isolation of Aldefluor positive cells. Aldehyde dehydrogenase activity is used for the identification of stem cells (Ginestier, Hur et al. 2007). The aldehyde dehydrogenase activity was measured using Aldefluor kit (Stem Cell Technologies, Vancouver, BC, Canada) according to manufactures instructions. Briefly the 60-70% confluent cells were trypsinized and centrifuged at 2000x for 5 minutes. Approximately one million cells were used for the assay. Cells were suspended in aldefluor assay buffer containing uncharged ALDH1-substrate, BODIPY-aminoacetaldehyde (BAAA), and incubated for 30-40 min at 37°C.

Uncharged BAAA is taken up by live cells through passive diffusion and then converted into a negatively charged reaction product BODIPY-aminoacetate by the activity of Aldehyde dehydrogenase enzyme. The negatively charged BODIPY-aminoacetaldehyde (BAAA) is retained inside cells expressing high levels of ALDH1, causing the cells to become brightly fluorescent. The brightly fluorescent ALDH1-expressing cells (ALDH1-positive cells) were detected in the green fluorescence channel (520-540 nm) of a FACScan instrument (BD Biosciences). A set of cells stained using the identical conditions with the specific ALDH inhibitor, diethylaminobenzaldehyde (Sigma), to serve as a negative control for each experiment. Because only cells with an intact cellular membrane could retain the aldefluor reaction product, only viable ALDH1-positive cells were identified. Cells incubated with BAAA and diethylaminobenzaldehyde were used to establish the baseline fluorescence of these cells (R1) and to define the (ALDH1)-positive region (R2). Incubation of cells with the substrate in the absence of diethylaminobenzaldehyde induced a shift in BAAA fluorescence defining the aldefluor-positive population. Data were analyzed by using flow jo software (BD Biosciences). Each experiment was repeated three times.

G. RNA Extraction and Reverse Transcription (RT)

1. RNA EXTRACTION

RNA was extracted and purified using the RNeasy Mini Kit (QIAGEN).Total RNA concentration and purity were determined from 260 nm and 280 nm absorbances. RNA was diluted with water to 0.08 µg/µl and reverse transcribed. The RT reaction mixture consisted of 6 µl diluted RNA (0.48 µg), 4 µl 5x Reverse Transcriptase buffer, 4 µl deoxynucleotide triphosphate mix (2.5 mM each of deoxy-ATP, deoxy-CTP, deoxy-

GTP, deoxy-TTP), 2 µl 0.1 M dithiothreitol, 2 µl of 1 mg/ml BSA, 1 µl of 0.5 µg/µl random primers, and 1 µl (200 U) of Moloney murine leukemia virus reverse transcriptase (Invitrogen, San Diego, CA). The mixture was incubated for 1 h at 37^{o} C.

2. REVERSE TRANSCRIPTION (RT)

Total RNA concentration and purity were determined from 260 nm and 280 nm absorbance. RNA was diluted with steam water and transcribed into cDNA. The RT reaction mixture consist 4 µl dNTP mix (dATP, dCTP, dGTP, dTTP; Invitrogen), 2 µl 0.1M DTT (Invitrogen), 1 µl (200 U) MLV reverse transcriptase (Invitrogen). The mixture was incubated for 1 h at 37 C, after which the RT sample (complementary DNA, cDNA) was stored in freezer.

3. Real-Time PCR

Analysis of BCRP, HER-2, ER, VEGF and HIF-1α mRNA expression was carried out by real-time PCR. Real-time PCR analysis was done using a DNA Opticon system (MJ Research, Boston, MA). Each 30-µl reaction mixture included 3 µl cDNA, 15 µl DyNAmo SYBR green qPCR mix (MJResearch), 1.2 µl primer mix (5 µM each primer), 10.8 µl sterile water. Each sample was assayed in duplicate. A standard curve was generated by serially diluting uterine cDNA. The yield of product for each unknown sample was calculated by applying its threshold cycle, or C (T), value (the cycle at which the sample's fluorescence rises above background noise and begins to increase exponentially) to the standard curve using the opticon monitor analysis software (version 1.01, MJ

Research). Values were normalized to corresponding 18S rRNA values and expressed as the fold increase relative to 0 h. human 18S rRNA +364 to +647: forward 5'-CAACTTTCGATGGTAGTCGC-3' and reverse 5'-CGCTATTGGAGCTGGAATTAC-3' (GenBank accession nos. X01117 and h01593). BCRP1: 5'-CCA TAG CCA CAG GCC AAA GT-3' and 5'-GGG CCA CAT GAT TCT TCC AC-3', Both the VEGF and HIF-1α primer pair êexon borders. PCR products were visualized on 8% polyacrylamide gels, and the relative yield of product per sample was determined by densitometry using a GeneWizard capture and analysis system (Syngene, Cambridge, UK). Primers that encompassed the BCRP promoter region 194 to -115 HRE-containing region were used for PCR. These were forward 5`-TACTGATCAGCCAATGAGC-3` and reverse 5`-CTGAAAGCGCACACGTGTC-3`(Genbank accession No.U22373).

H. Chromatin Immunoprecipitation (ChIP) Assay: In Vivo ChIP

The Chromatin immunoprecipitation (ChIP) assay was carried out using Millipore's Magna-ChIP A kit (Millipore, Lake Placid, NY) following manufacturer's protocol. In brief, freshly prepared 18.5% formaldehyde was added to 60-70% confluent cells at a final concentration of 1%. Cells were incubated at 37°C for 10 min, then after washing twice with cold PBS the cells were scraped into 1 ml cold PBS containing 1× protease inhibitor cocktail II. Briefly, pellets were resuspended in 600 µl lysis buffer [50 mM Tris-HCl (pH 8.1); 5 mM EDTA; 1% sodium dodecyl sulfate; and protease inhibitors (as above)] and incubated on ice for 15 min. Samples were sonicated on ice for 10 x 10 sec cycles, with 20-sec pauses between each cycle, using a Microson Ultrasonic Cell Disruptor (Misonix, Farmingdale, NY) at power level 2.5. Sonicated samples

were then divided into 100-µl aliquots and stored at −80° C. Sonicated sample aliquots were thawed on ice and diluted 1:1000 with dilution buffer [20 mM Tris-HCl (pH. 8.1), 150 mM NaCl, 2 mM EDTA, 1% triton X-100, and protease inhibitors (as above)] before being immunocleared in a solution containing 45 µl of a 50% slurry of either Protein A or Protein G Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ) in Tris-EDTA (TE) buffer, pH 8, 2 µg salmon sperm DNA (Invitrogen), and 20 µl normal serum (Sigma) for 2 h at 4° C; use of protein A or protein G was determined based on which had stronger affinity for each particular antibody (determined based on supplier recommendations). Supernatants were collected and incubated overnight at 4° C with one of the following antibodies: 1 µg mouse monoclonal human ERα antibody (Ab-10; NeoMarkers/Lab Vision, Fremont, CA), 2 µg mouse monoclonal human HIF-1α antibody (BD Biosciences, Palo Alto, CA), 5 µg mouse monoclonal human HIF-1ß antibody (BD Biosciences), 5 µg anti-p300 (N-15) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 4 µg rabbit polyclonal Sp1 (H-225 or PEP 2) antibody (no. sc-14027 or sc-59; Santa Cruz Biotechnology), and 5 µg rabbit polyclonal Sp3 (H-225) antibody (no. sc-13018; Santa Cruz Biotechnology). For the "no antibody" control, an equal volume of normal mouse or rabbit serum was substituted for the specific mouse or rabbit antibody. Protein A or protein G sepharose beads (45 µl of a 50% slurry in TE buffer) and salmon sperm DNA (2 µg) were then added and incubated for 1 h at 4° C. The beads were then washed sequentially with 1 ml each of TSE I, TSE II, and buffer III, and 2 x 1 ml of TE buffer (pH 8.0); each wash was for 10 min each at 4° C. The protein-DNA complexes were then eluted by twice incubating beads in 100 µl of elution buffer (1% sodium dodecyl sulfate, 0.1M $NaHCO_3$) for 10 min at room temperature with vigorous

mixing. To separate immunoprecipitated protein and DNA, the pooled elutes were incubated at 65° C overnight. The DNA was purified using the Qiaquick PCR Purification kit (QIAGEN, Valencia, CA). The final volume was 50 µl (10 mM Tris-HCl, pH 8.5). The yield of target region DNA in each sample after ChIP was analyzed by both conventional and real-time PCR, as described previously. In both cases, 3µl of each 50-µl sample was amplified. For real-time PCR, standard curves were generated by serially diluting an input chromatin sample. The following primers were used for ChIP PCR analysis (30 cycles at 60° C annealing temperature).

I. Statistical analysis

All experiments were carried out for at least three times in replicates of five. Statistical analyses were performed using Stu samples) using Graph Pad Prism software. *P*-values of $p \leq 0.05$ were considered statistically significant. For western blots, the results are expressed as a single representative of three different experiments.

Specific Aim 1

Treatment with aromatase inhibitors has proven to be effective against breast cancer in postmenopausal women. However, some patients may eventually become resistant. This acquired resistance remains a significant cause of mortality and disease relapse in ER positive breast cancer patients. Recent studies have established that a rare and biologically distinct population exists in breast cancer and other solid tumors. There is also increasing evidence that over expression of HER-2 is associated with an increase in the TIC population in breast cancer. Previous studies from our lab indicate that resistance to aromatase inhibitors is mediated by up-regulation of HER-2/MAPK signaling. In hormone sensitive ER positive and HER-2 negative tumors, prolonged treatment with letrozole results in activation of the HER-2/MAPK signaling pathways. The acquisition of enhanced HER-2/MAPK signaling with letrozole resistance results in amplification of cells with tumor initiating properties. Based on the literature and preliminary results from our lab, we hypothesize that activation of HER-2/MAPK with letrozole may result in more TIC like characteristics and hence may have a role in hormone resistance. In our aim I in order to investigate the impact of hormonal therapy, particularly hormone resistance we characterized both hormone resistant and hormone sensitive breast cancer cells for tumor initiating characteristics.

Results of Aim 1

Hormone resistant breast cancer cells are enriched in a side population

We investigated whether hormone sensitive cells subjected to long-term continuous exposure to letrozole contain an expanded cancer stem cell ìlike population. Hoechst side population (SP) analysis has proven to be an important technique for identifying stem and early progenitor cells in several cancer types including breast cancer(Nakanishi, Chumsri et al.). In this method, the cell population of interest is incubated with DNA binding dye Hoechst 33342.Hoechst 33342 is a fluorescent dye that has an absorption wavelength of 350nm in the UV range, and two emission wavelengths of 450nm and 675 nm, in the blue and red region respectively. 298, 299 The dye is commonly taken up by all cells, but certain cells have an enhanced ability to efflux the dye; such cells are r e f e r r e d t o a s ñ s i d e p o p u l aof i o n ò (S the bulk of the positively stained cells in FACS analysis plots(Arndt-Jovin and Jovin 1977; Lalande and Miller 1979). The SP cells are known to possess high self-renewal capacity and have an undifferentiated phenotype (Patrawala, Calhoun et al. 2005; Christgen, Ballmaier et al. 2007). Owings to the relevance of SP in identification of cells with TIC phenotype, we subjected both hormone sensitive and hormone resistant cells to SP analysis. SP cells were counted by fluorescence-activated cell sorting method already described in detail in materials and methods section. Both MCF-7Ca and LTLT-Ca cells were stained with the Hoechst 33342 DNA staining dye followed by SP analysis using fluorescence-activated cell sorting method. The SP cells exhibited a low blue (440- to

460-nm) and low red (>675-nm) fluorescent staining pattern. This pattern is created by efflux of the Hoechst 33342 dye from the SP cells. We observed a 9.17%±2.0% increase in the SP in letrozole resistant cells compared to 0.02%±0.01% *p <0.01 SP in letrozole sensitive MCF7-Ca cells. As a positive control for both Mcf-7Ca and LTLT-Ca a parallel sample was incubated with BCRP blocker ko143 was also analyzed. Since Ko143 blocks BCRP activity, no SP fraction was observed in Ko143 treated sample, which signifies that the SP pattern observed is specific to BCRP activity (Figure 1.8A).

Hoechst is preferentially effluxed by BCRP and is associated with SP phenotype (Hirschmann-Jax et al, 2004;(Zhou, Schuetz et al. 2001). We analyzed both letrozole sensitive and letrozole resistant cells for BCRP expression. We compared the BCRP protein and mRNA expression levels between MCF-7Ca and LTLT-Ca using western blot and real time RT-PCR. We also compared the membrane expression levels of BCRP using FITC conjugated antibody against BCRP protein. We observed show significantly higher levels of BCRP both at the protein and mRNA level in LTLT-Ca compared to hormone sensitive Mcf-7Ca cells (Figure 1.8 C&D). Higher membrane expression levels of BCRP were observed in LTLT-Ca then Mcf-7Ca cells (Figure 1.8 B). These results indicate that a significant fraction of cells in letrozole resistant cells acquire sp phenotype due to upregulation of BCRP.

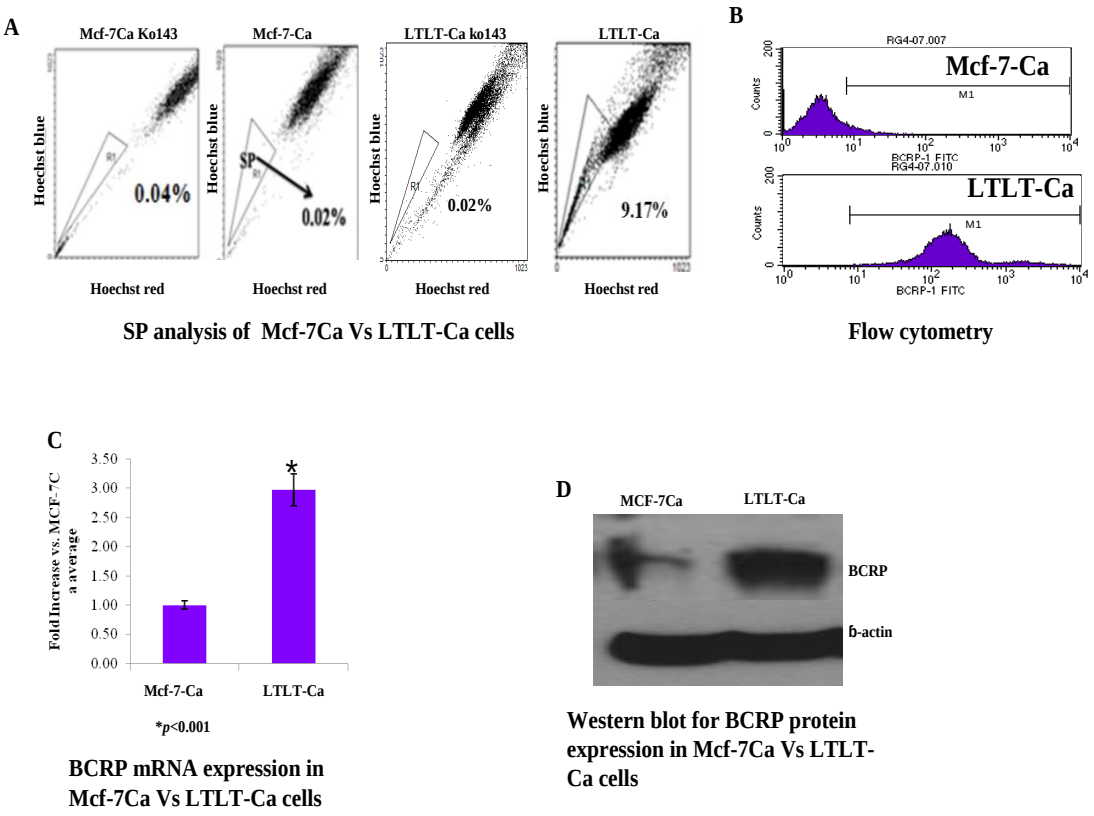

Figure 1.8: Side population analysis of letrozole resistant cells compared to letrozole sensitive cells. A. LTLT-Ca cells were incubated with 5 εC for 90 min. As a negative control, ko143 an inhibitor of ABC transporter was also added to a final concentration of 1 εmol/L staining, the LTLT-Ca were analyzed on fluorescence activated cell sorter. Analysis was performed on a dual laser flow cytometer (Becton Dickinson FACS Vantage SE cell sorter, San Jose, CA). The 355-nm ultraviolet laser excited the Hoechst dye, and its fluorescence was measured at 2 wavelengths using a 424/44 (Hoechst blue) band-pass filter and a 585/42 (Hoechst red) band-pass filter. Each plot is representative of at least three independent experiments n = 3 independent cell samples *p< 0.001. **B.** Expression of BCRP in MCF-Ca and LTLT-Ca cell was studied by flow cytometry with the FITC antibody against BCRP labeled **C,** Total RNA was extracted and BCRP mRNA and 18S rRNA were analyzed by real-time RT-PCR analysis. Results are expressed as the fold-change in mRNA levels compared with MCF-7Ca cells after normalization to 18S rRNA

(n = 3 samples/group; c vs. d, *p< 0.001). **D**, LTLT-Ca and parental MCF-7Ca cells were plated and cultured in their respective passage media. Total protein was extracted and BCRP and was analyzed by Western blot analysis. β-actin was used as a loading control

SP was analyzed in anastrazole resistant cells and compared to anastrazole sensitive AC1 cells. Anastrazole resistant cells also show an increase in SP from 0.81% to 6.08% *p<0.001(Figure 1.9).

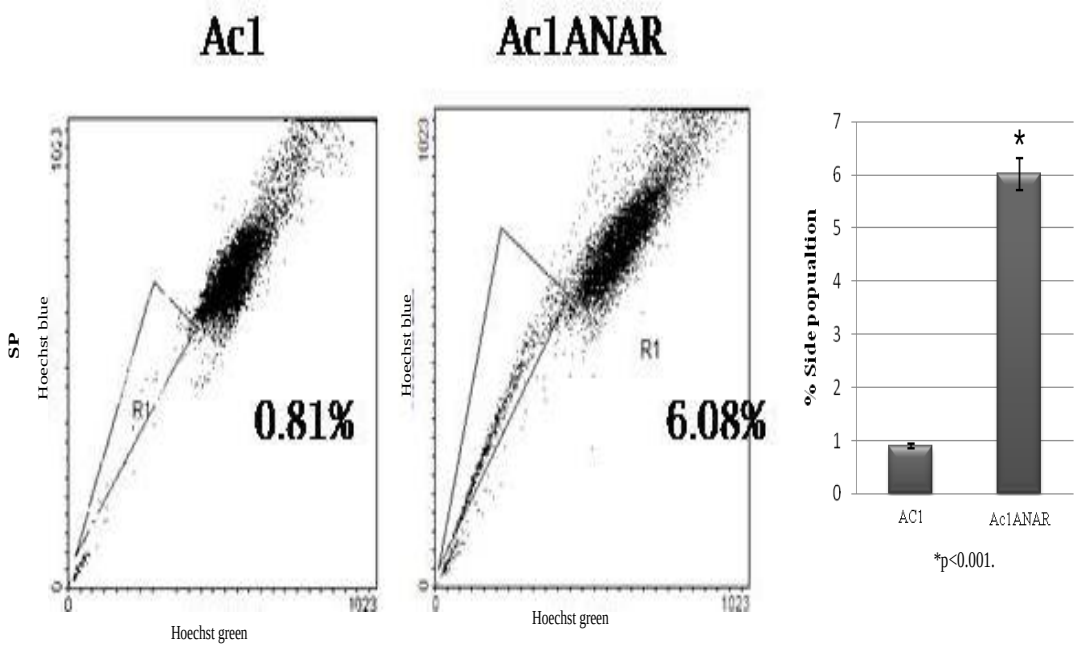

Figure 1.9: Side population analysis in anastrazole sensitive compared to anastrazole resistant cells. Anastrazole resistant and anastrazole sensitive cells were incubated with 5 εg/°mL for 90 min. As a negative control, 2 dye a ko143 an inhibitor of ABC transporter was also added to a final concentration of 1 εmol/L with Hoechst 33342. A-Ca were analyzed Hoechst 3 on Fluorescence activated cell sorter. Analysis was performed on a dual laser flow cytometer (Becton Dickinson FACS Vantage SE cell sorter, San Jose, CA). The 355-nm ultraviolet laser excited the Hoechst dye, and its fluorescence was measured at 2 wavelengths using a 424/44 (Hoechst blue) band-pass filter and a 585/42 (Hoechst red)

band-pass filter. Each plot is representative of at least three independent experiments n = 3 independent cell samples *p< 0.001.

CD44^{high}/CD24^{low} and ALDH analysis of AI resistant cells:

These cells were also analyzed for CD44^{high}/CD24^{low} positivity as breast cancer showing CD44^{high}/CD24^{low}expression are known to have TIC characteristics (Al-Hajj, Becker et al. 2004) Differences in breast cancer subtypes results in difference in TIC population and CD24^{low}/CD44^{high}expression is more significantly observed in highly metastatic basal breast cancer cells. We were interested to analyze whether resistance to aromatase imparts basal TIC characteristics in otherwise luminal hormone sensitive MCF7-Ca cells. Interestingly, there was an increase from 0.02% to 1.67% *p<0.05 in CD44^{high}/CD24^{low} population in LTLT-Ca compared to hormone sensitive Mcf-7Ca cells (Figure 1.10). Interestingly we did not observe any significant increase in CD44^{high}/CD24^{low} cell fraction in AC1-ANAR cells. Indicating that mechanism of resistance may differ in different hormones.

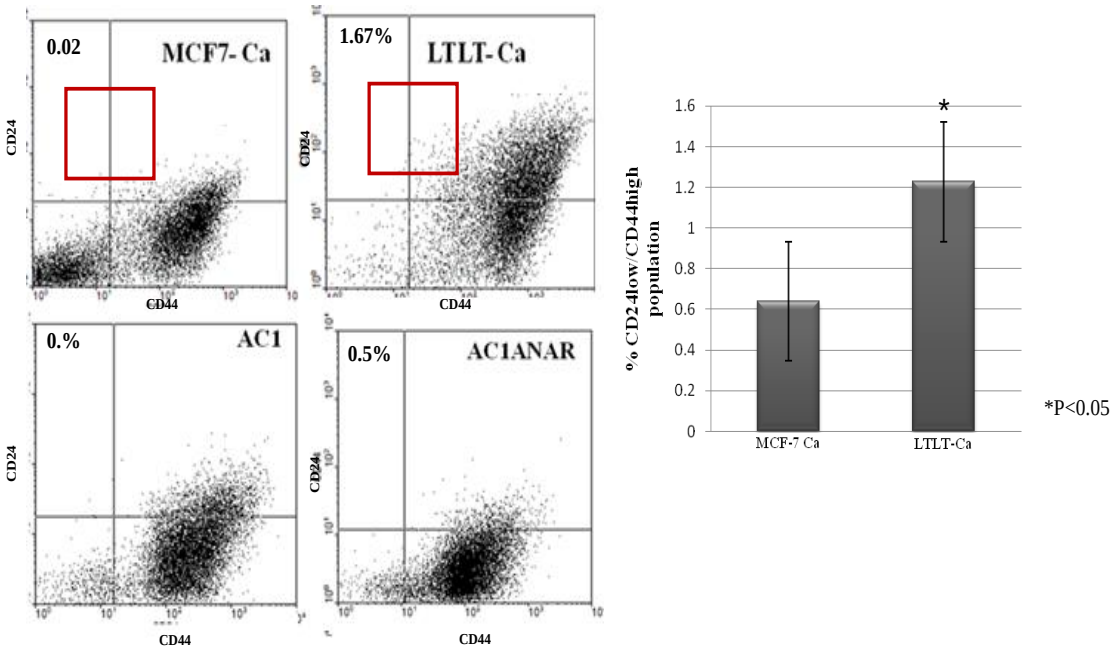

Figure 1.10:CD44^{high}/CD24^{low} analysis of hormone sensitive MCF7-Ca and Ac1 was compared to CD44^{high}/CD24^{low} expression of hormone resistant LTLT-Ca and Ac1/ANAR cells. To determine CD44^{high}/CD24^{low}expression, cells were co-incubated with anti-CD44 (conjugated with allophycocyanin (APC) and anti-CD24 (conjugated with fluorescein isothiocyanate (FITC). CD44^{high}/CD24^{low} expression of hormone sensitive MCF7-Ca and Ac1 was compared to CD44^{high}/CD24^{low} expression of hormone resistant LTLT-Ca and Ac1/ANAR cells.

Another significant stem cell marker used to study TIC biology in breast cancer is aldehyde dehydrogenase (ALDH) expression. ALDH is a cytosolic isoenzyme responsible for oxidizing retinoic acid to retinol, which in turn activates the differentiation genes in stem cells (Yoshida, Hsu et al. 1992; Yen, Fenning et al. 2004). HER-2 signaling is known to be involved in increasing ALDH activity (Korkaya, Paulson et al. 2008). Since, hormone resistance is associated with HER-2 up-regulation, we analyzed AI resistant cells for ALDH activity. Interestingly, LTLT-Ca cells show a

significant increase of 3.08% in ALDH activity compared to 0.53% *p<0.05 activities in hormone sensitive MCF7-Ca cells (Figure 1.11). However, there was no significant difference in the ALHD activity in anastrazole resistant cells compared to anastrazole sensitive cells. Again these results signify the difference in resistance mechanism to different hormonal therapies. Taken together these results suggest that prolonged continuous selection to hormone resistance appears to increase the population of cells with tumor initiating cell characteristics.

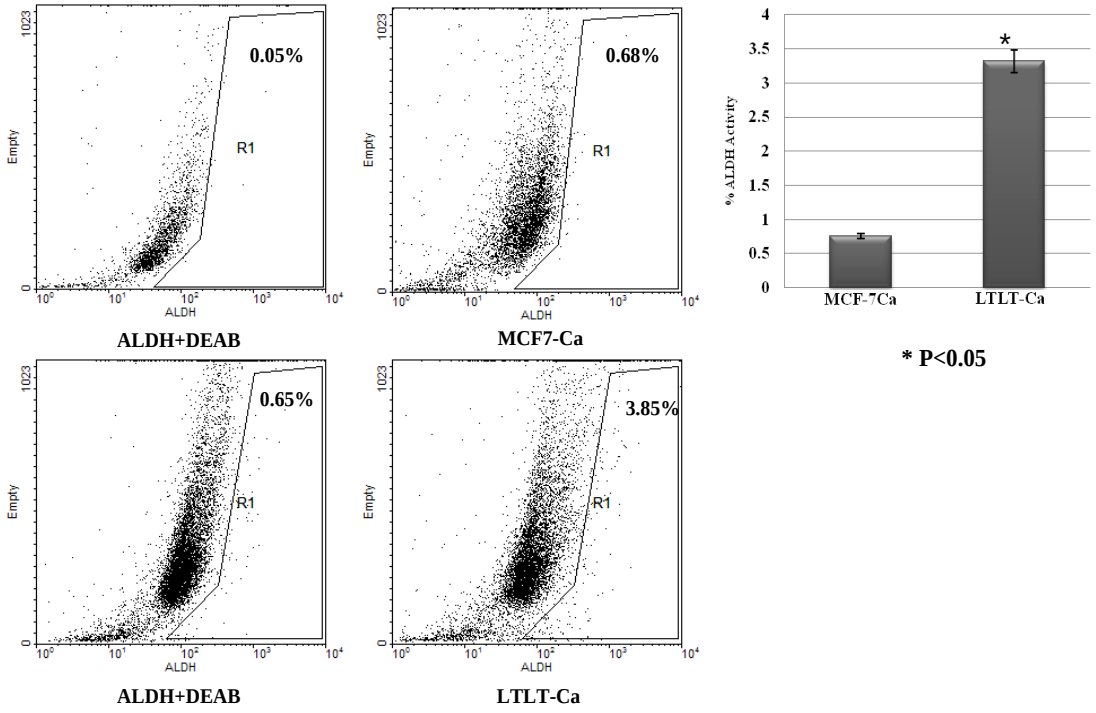

Figure 1.11: Aldehyde dehydrogenase activity of letrozole and anastrazole resistant cells compared with hormone sensitive Ac1 and MCf-7Ca breast cancer cell line. Aldehyde dehydrogenase 1 (ALDH1) was analyzed by measuring cellular fluorescence of BODIPY-aminoacetate (BAAA) in the presence or absence of DEAB, a specific inhibitor for ALDH1. The data shown represent the mean of three independent experiments (* denotes significant changes relative to vehicle control) P<0.05.

Mammosphere cells enriched in $CD44^{high}/CD24^{low}$ population:

Use of improved *In-Vitro* culturing conditions, the cells which proliferate in serum free media and in a suspension culture were found to be enriched in TIC population (Dontu and Wicha 2005). These cells in suspension form spheroids known as mammospheres. We analyzed the mammosphere forming capacity of both hormone resistant and hormone sensitive cells. Both LTLT-Ca and Ac1-ANAR cells were cultured in ultra low attachment plates in mammosphere media supplemented with heparin and hydrocortisone as described in detail in materials and methods section. Mammospheres formed after 7 days were counted manually. Each cell line was cultured in triplicate and the average was taken for analysis. Each experiment was repeated at least three times. We observed that hormone resistant cells showed enhanced mammosphere formation compared to hormone sensitive cells (Figure 1.12) even though we did not observe a significant increase in $CD44^{high}/CD24^{low}$ and ALDH in AC1 ANAR. These cells did form significant more mammosphere from 100±45 to 300± 25 $*p<0.05$, indicating that resistant cells confer enhanced ability to form mammosphere which is independent of $CD44^{high}/CD24^{low}$ and ALDH activity in case of AC1 ANAR cells. Next cells grown in both mammosphere and differentiating conditions were also analyzed for $CD44^{high}/CD24^{low}$. Although, the resistant cells in regular culture an increase from 0.02% to 1.67% in $CD44^{high}/CD24^{low}$ population, the same cells grown in mammosphere culture show an increase from 0.68% to 10.2% $CD44^{high}/CD24^{low}$ indicating that mammosphere culture condition maintain the propagation of progenitor cells in cultural conditions these

results are consistent with what is already known, that TICs are maintained in undifferentiated state in mammosphere (Figure 1.13**)** (Dontu and Wicha 2005).

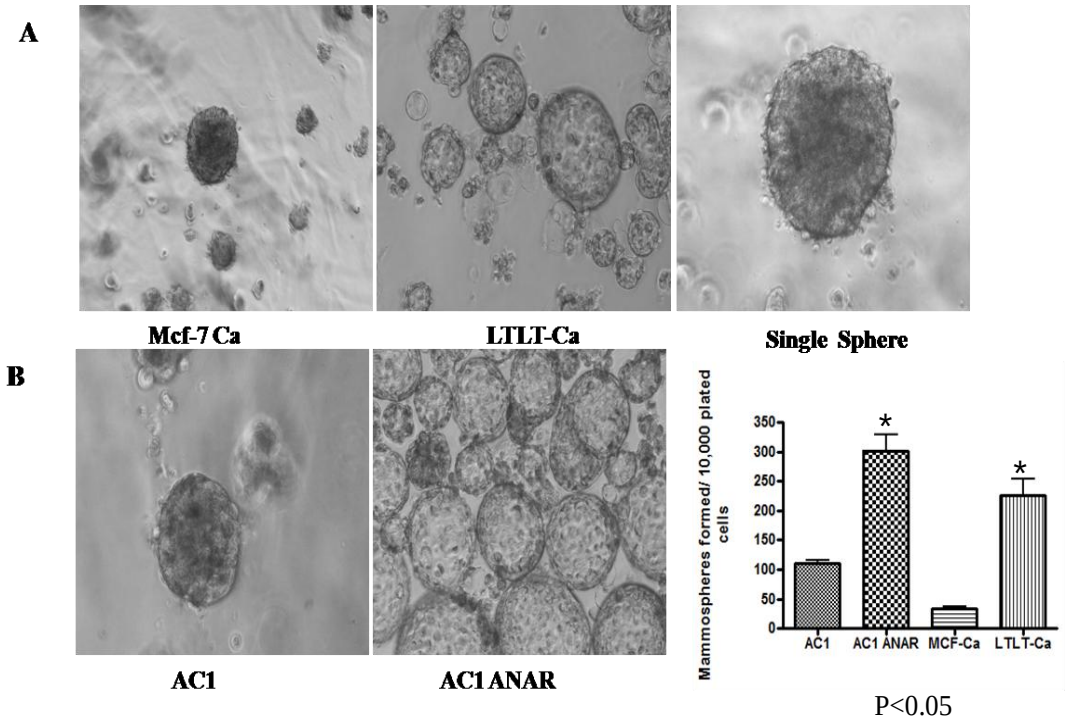

Figure 1.12: Mammosphere formation in letrozole and anastrazole resistant cell lines. (A) Shows mammosphere formation in resistant cell lines compared to parental cell line. MCF-Ca letrozole sensitive and letrozole resistant LTLT-Ca cells were plated in low attachment culture plates supplemented with mammosphere media. **(B)** Anastrazole resistant and anastrazole sensitive Ac1-ANAR and there parental sensitive cell line Ac1 were grown under similar conditions at a density of 20,000 viable cells/mL in primary culture and 1000 cells/mL in passages. Mammospheres were counted manually and pictures were taken after 7 days *p<0.05.

Figure 1.13: $CD44^{high}/CD24^{low}$ analysis of Letrozole resistant cells from mammosphere and regular culture. Flow cytometry analysis of cells derived from primary mammosphere and cells from regular cultures stained differentially for $CD44^{high}/CD24^{low}$. Top panel shows analysis of LTLT-Ca cells grown in IMEM containing growth factors seeded in a 24-well plate and stained with anti CD24-FITC and anti CD44-PE antibodies. Bottom panel shows same cells grown in a serum free mammosphere culture seeded in a 24-well Ultra low attachment plate, and stained with anti CD24-FITC and anti CD44-PE antibodies.