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BIOLOGICAL AND THERAPEUTIC ASPECTS OF BREAST CANCER PROGRESSION

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Biological and therapeutic aspects of breast cancer progression

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family,

″நோய்நாடி நோய்முதல் நாடி அதுதணிக்கும்

வாய்நாடி வாய்ப்பச் செயல்″ - (திருக்குறள் ≈ 4 B.C)

Diagnosing the disease, detecting its root cause,

discerning its cure and then act aptly (Thirukkural ≈ 4 B.C)

ABSTRACT

Breast cancer is the second leading cause of cancer related death in women worldwide[1]. Although majority of primary breast cancers are curable with current treatment strategies, treatment outcome of metastatic breast cancer is dismal. The main focus of my doctoral studies is to investigate the causes of breast cancer recurrences and to eventually improve the survival outcome of metastatic breast cancer. Several factors has been attributed for the recurrence of breast cancer such as presence of cancer stem cells (CSCs) and the ongoing genomic evolution of cancer cells leading to intra tumor heterogeneity, which can in turn give rise to therapy resistant subclones. In this thesis we sought to investigate these two factors using breast cancer specimens.

Firstly, in paper I, we optimized the method called "superficial scraping from tumor". Using this method we were able to isolate epithelial breast cancer cells from which we can generate CSCs with ultra-low attachment and serum free conditions. Mammospheres generated from scraping material phenotypically resemble CSCs with ALDH1+, CD44+, and CD24- expression. Apart from CSC generation, scraping method can be used to biobank small tumors for future research purposes, without compromising routine histopathological analysis of patient samples. Next, we evaluated the expression of second estrogen receptor ERβ and its role in patient derived CSCs (Paper II), using the method optimized from paper I. We found that ERβ was predominantly expressed in both normal mammary stem cells (MSC) and CSCs. ERβ was found to be crucial for cancer stem cell phenotype and stimulation of ERβ using specific agonist increased mammosphere formation. Microarray analysis on ERβ stimulated MCF7 derived mammospheres, identified enhanced glycolysis metabolism pathway. Antagonizing ERβ in cell lines and in patient derived xenografts (PDX) demonstrated that ERβ is a therapeutical target in breast cancer and can be utilized to specifically target the CSC population.

Tamoxifen is an important therapy for ERα positive breast cancers, however around 30-40% of patients relapse during endocrine therapy[2]. To investigate the endocrine resistance from a cancer stem cell perspective (Paper III), we treated adherent breast cancer cells (ER+) and CSCs with tamoxifen. Interestingly, CSCs where found to be resistant to tamoxifen treatment, while tamoxifen inhibited the adherent cancer cell population. To understand the mechanism behind the CSC induced endocrine resistance, we performed microarray analysis on patient derived CSCs treated with tamoxifen. Interestingly, mTOR signaling related pathways were found to be induced by tamoxifen in CSCs. This induction of mTOR effector downstream targets were observed only in CSCs but not in adherent cancer cells. Further, mTOR signaling was also found to be elevated in CSCs compared to the adherent cancer cell population. mTOR inhibitors such as rapamycin and everolimus were found to be effective in reducing the mammosphere formation. Therefore, combined tamoxifen and mTOR inhibitors can effectively target both differentiated cancer cells and the CSC population.

Next, we explored the genomic landscape of metastases, patterns of metastatic spread and the role of axillary lymph node metastasis in seeding distant metastasis (Paper IV). We performed whole exome sequencing on 99 tumor samples from 20 breast cancer patients with matched primary and metastatic lesions. We observed both linear progression (i.e. metastasis seeding successive distant metastasis) and parallel progression (i.e. different distant metastasis were

seeded from primary tumor directly rather than seeded by other distant metastasis) model during breast cancer progression. Majority of the distant metastasis where polyclonally seeded. We observed lack of axillary lymph node involvement in seeding distant metastasis. This indicates that, the majority of cancer cells are seeded hematogenously rather than utilizing the lymphatic system for cancer spreading. On average, only half of primary mutations were retained in the distant metastatic lesions with considerable disparity between individual patients ranging from 9 to 88%. Several putative driver alterations occurred late, privately in distant metastasis, highlighting the need to characterize the genomic alterations of metastatic lesions for making better informed clinical decision at metastatic setting. Further, we also observed specific mutational signatures such as APOBEC-associated signature, were significantly higher in distant metastasis compared to their respective primary tumors. Finally, in paper V, we profiled (RNA sequencing) multiple regions of the same tumor from 12 breast cancers. Molecular subtypes and transcriptomic grades for each tumor piece was determined. Primary breast cancers exhibited substantial intra-tumor genomic heterogeneity, but limited transcriptomic heterogeneity at macroscopic level. Our data suggested that, intra-tumoural heterogeneity is unlikely to have an impact on transcription based molecular diagnostics for most patients.

In conclusion, we have identified potential therapeutic targets such as ERβ and mTOR pathway for inhibiting CSCs. Drugs targeting both CSCs and differentiated cancer cells are promising strategies to eradicate cancer recurrences. More clinical trials involving cancer stem cell targeting agents along with traditional therapies are required to investigate their clinical efficacy. Further, genomic characterization of both primary tumors and metastatic lesions are crucial for improving the treatment outcome for advanced breast cancer patients.

LIST OF SCIENTIFIC PAPERS

I. **Superficial scrapings from breast tumors is a source for biobanking and research purposes.**
Ran Ma, Irma Fredriksson, **Govindasamy-Muralidharan Karthik**, Gregory Winn, Eva Darai-Ramqvist, Jonas Bergh and Johan Hartman.
Laboratory Investigation. 2014 Jul;94(7):796-805

II. **Estrogen receptor β as a therapeutic target in breast cancer stem cells.**
Ran Ma, **Govindasamy-Muralidharan Karthik**, John Lövrot, Felix Haglund, Gustaf Rosin, Anne Katchy, Xiaonan Zhang X, Lisa Viberg, Jan Frisell, Cecillia Williams, Stig Linder, Irma Fredriksson and Johan Hartman.
Journal of the National Cancer Institute, 2017 Feb;109 (3): djw236

III. **mTOR inhibitors counteract tamoxifen-induced activation of breast cancer stem cells.**
Govindasamy-Muralidharan Karthik*, Ran Ma*, John Lövrot, Lorand Levente Kis, Claes Lindh, Lennart Blomquist, Irma Fredriksson, Jonas Bergh and Johan Hartman.
Cancer letters. 2015 July;367:76-87

IV. **Genomic analyses of primary breast cancer and matched metastases reveal both linear and parallel progression with minimal seeding from axillary lymph node metastasis.**
Ikram Ullah*, **Govindasamy-Muralidharan Karthik***, Amjad Alkodsi*, Una Kjällquist, Gustav Stålhammar, John Lövrot, Nelson-Fuentes Martinez, Jens Lagergren, Sampsa Hautaniemi, Johan Hartman# and Jonas Bergh#.
Manuscript Submitted

V. **Intra-tumor heterogeneity in breast cancer has limited impact on transcriptomic-based molecular profiling.**
Govindasamy-Muralidharan Karthik*, Mattias Rantalainen*, Gustav Stålhammar, John Lövrot , Ikram Ullah, Ran Ma, Lena Wedlund, Johan Lindberg, Jonas Bergh and Johan Hartman.
Manuscript Submitted

*Equal Contributions- First Author.
# Equal Contributions- Last Author.

PAPERS NOT INCLUDED IN THE THESIS

I. **Oestrogen receptors β1 and βcx have divergent roles in breast cancer survival and lymph node metastasis.**
Gustaf Rosin, Jana De Boniface, **Govindasamy Muralidharan Karthik**, Jan Frisell, Jonas Bergh, Johan Hartman.
British Journal of Cancer (2014) 111, 918–926.

CONTENTS

LIST OF ABBREVIATIONS

AI	Aromatase inhibitor
ALDH1	Aldehyde dehydrogenase 1
APOBEC	Apolipoprotein B mRNA editing enzyme
BSCs	Breast cancer stem cells
CSCs	Cancer stem cells
ctDNA	Circulating tumor DNA
CTC	Circulating tumor cells
DC	Dendritic cells
DCIS	Ductal carcinoma in situ
DPN	Diarylpropionitrile (ERβ receptor agonist)
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERE	Estrogen response element
E2	Estradiol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FACS	Fluorescence-activated cell sorting
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
GPER	G protein-coupled estrogen receptor 1
GSEA	Gene set enrichment analysis
HER2	Human epidermal growth factor receptor 2
Hh	Hedgehog signaling
HR	Homologous-recombination
HT	Hormone therapy
IF	Immunofluorescence
IHC	Immunohistochemistry
IGFR	Insulin-like growth factor receptor
LBD	Ligand binding domain

LCIS	Lobular carcinoma in situ
MSC	Mammary stem cell
mTOR	Mammalian target of rapamycin
MET	Mesenchymal to epithelial transition
NGS	Next generation sequencing
NK	Nature killer cells
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
OXPHOS	Oxidative phosphorylation
PARP	Poly (ADP-ribose) polymerase
PDX	Patient derived xenografts
PHTPP	4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (ERβ receptor antagonist)
PI3K	Phosphatidylinositol-3-kinases
PPT	4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (Estrogen receptor α agonist)
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
REDOX	Reduction-oxidation reaction
ROS	Reactive oxygen species
RT	Radiotherapy
RTPCR	Reverse transcription polymerase chain reaction
SERM	Selective estrogen receptor modulator
TN	Triple negative

1 INTRODUCTION

1.1 BREAST CANCER ORIGIN

Breast cancer is the most common cancer with more than 255,000 new cases expected in 2017 in the United States, followed by lung cancer and prostate cancer[1]. It is the second leading cause of cancer related death in women and its incidence rates are further increasing[1]. Breast cancer has been in prevalence since ancient times, it has been reported by Egyptians, more than 3500 years ago as "tumors or ulcers of the breast". The normal breast consists of fat, connective tissue and mammary tissues with lobes and glands. These lobes produce milk during lactation and form a network of milk ducts connecting to the nipple. During breast cancer, epithelial cells in the breast tissue grow in an uncontrollable fashion, leading to formation of a lump that can either be benign (non-cancerous) or malignant (cancerous). Breast cancers can arise from different parts of the breasts: ducts, lobules and in their connective tissues. Malignant cancers has the ability to invade and spread to distant organs of the body, a process called metastasis, which is the ultimate cause of the death. The exact mechanism by which breast cancer develop, is still not well understood. Breast tumors are considered malignant when they start to invade and pass through myoepithelial cell layer and basement membrane[3]. Traditionally, a stepwise progression cancer model i.e., from non-to pre- to malignant stages has been proposed, based on histopathological examinations in morphological studies[4]. Many pre-malignant stages has been reported in breast cancer development such as hyperplasia, atypical hyperplasia and cancer in situ[3]. However, it has been demonstrated in experimental studies, that the development of breast cancer is much more complex than this proposed classical model[4]. Not all pre-malignant lesions leads to breast cancer and many of these lesions are not mandatory for breast cancer development[4]. However, presence of certain pre malignant lesions increases the risk of developing invasive breast cancer in later stages of life[3]. Breast cancer is a complex disease featuring multiple clinical, morphological and molecular distinct subgroups[5-7]. Currently breast cancers are broadly divided into non-invasive and invasive cancers based on their morphological features[8].

1.1.1 Non-invasive breast cancer

These type of cancers are also called as carcinoma in situ, of which the most common type is called ductal carcinoma in situ (DCIS). DCIS origins from the epithelial cell lining of the milk ducts and is referred to as premalignant lesion, since the cancer cells has not yet invaded through the basement membrane. Based on cellular morphology, architecture and nuclear pleomorphism, DCIS is classified into low, intermediate or high grade and each of them are associated with different clinical outcomes[9]. The developmental mechanisms responsible for low and intermediate grade DCIS are proposed to be different to that of high grade DCIS[10]. Genetic studies has identified different alterations between different grades of DCIS[11]. Lower grade DCIS exhibit loss of 16q chromosome, while high grade DCIS often exhibits 17q gain[10]. Consequently, low grade DCIS often advances to low grade invasive cancers, while high grade DCIS advances in to high grade invasive cancers. In fact, numerous data indicates that the cytonuclear grade of DCIS remains consistent from cancer in situ to invasive cancer and even in metastatic disease[12]. Several genetic aberrations and gene expression patterns that are found in invasive breast cancer can be seen already in DCIS[4]. DCIS itself is not life threatening, but

a higher pathological or nuclear grade is often associated with higher probability of invasive cancer recurrence[13]. The second most common type of non-invasive breast cancer is called lobular carcinoma in situ (LCIS). LCIS is often considered as risk indicator for invasive lobular cancers[4]. Both DCIS and LCIS are referred as precursor lesions of the breast by WHO-classifications[8].

1.1.2 Invasive breast cancer

Invasive breast cancers has the ability to spread to the surrounding normal tissues. The most common type of invasive breast cancer is called invasive carcinoma of no special type (NST) previously referred as invasive ductal carcinomas not otherwise specified (IDC-NOS)[8]. Almost 80% of all breast cancer cases accounts under this morphological category. Invasive carcinoma (NST) as well as other subtypes has the ability to spread to nearby lymph nodes and potentially to other organs of the body. Invasive lobular carcinoma (ILC) is the second most common type of invasive cancers. About 10% of the all invasive cancers are ILC. Together these two types of invasive breast cancer accounts for about 95% of all breast cancers in this category. Apart from these two invasive breast cancer types, WHO also classifies multiple other entities such as tubular carcinoma, medullary carcinoma, invasive papillary carcinoma etc. However their incidence rates are much lower compared to the two previously discussed cancer types[14].

1.2 IMMUNOHISTOCHEMICAL ASSESSMENT OF PREDICTIVE AND PROGNOSTIC BIOMARKERS

Apart from the stratifications based on pathological features, breast cancers are also stratified based on their expression of estrogen receptor (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). These biomarkers are routinely used in the diagnosis and treatment of breast cancer patients. With the available targeted therapies for breast cancer, these biomarkers are crucial in identifying patients who will be benefited from such treatments.

1.2.1 Estrogen receptor alpha status

Hormone receptor status (ERα and PR) is a main factor for management of breast cancer patients. Hormones such as estrogen, androgen and progesterone have been shown to stimulate cancer cell growth[15-17]. The hormone stimulatory effect depends on hormone receptor expression in breast cancer cells. ERα protein expression in breast cancers are predictive biomarker of endocrine therapy response and also associated with good response[18]. Around 70% of all primary breast cancers are ERα positive. Currently, protein expression of ERα in breast tumors is investigated in the routine pathology setting and predicts response to endocrine treatments. In 2010, the American Society of Clinical Oncology (ASCO) and College of American pathologists (CAP) set guidelines, with cut off of 1% positive cells to distinguish ERα positive from ERα negative tumors, traditionally the cut off was set at 10% of positive cells[19]. In Swedish medical society, 10% cut off is still applied[20]. Patients with very less ERα expression were also shown to be benefited from endocrine therapy[21] and ERα negative tumors do not respond to tamoxifen treatment at all[22]. Hormone receptor positive cancers can be targeted using three different groups of hormonal therapies, including tamoxifen, aromatase inhibitors (AI) and fulvestrant. Each class of endocrine therapy has a different mode of action.

Tamoxifen is a selective estrogen receptor modulator (SERM), which blocks the effect of estrogen via estrogen receptor (ERα) in breast tissues. Aromatase inhibitors (AI) on the other hand reduce the plasma estrogen levels in postmenopausal women by inhibiting or inactivating aromatase. Aromatase is an enzyme responsible for the production of estrogens from androgenic substrates such as testosterone[23]. Aromatase inhibitors starves the hormone receptor positive cancers, as they are deprived of hormones to grow [24].

1.2.2 Progesterone receptor status

Progesterone receptor (PR) is one of the target genes of ERα[25]. ERα transcriptionally activate PR, with the help of estrogen response elements (ERE) present upstream of PR gene[25]. Estrogen treatment of breast cancer cell lines stimulate PR expression and this is often noted as a marker for functional ERα signaling[25]. PR expression strongly correlates with ERα expression and therefore believed to predict endocrine therapy response[19,22]. Prognostic values of PR expression has been demonstrated in numerous studies, independent of ERα expression and other biomarkers[26,27]. However, till date, there is no specific approved therapy targeting PR in breast cancer.

1.2.3 HER2 (human epidermal growth factor receptor 2) status

HER2/neu (*ERBB2*) is a growth and survival promoting protein expressed on the surface of the breast cancer cells[28]. Traditionally, over expression of HER2 was considered as poor prognostic marker, until the first targeted therapy against HER2 was established[29,30]. HER2 as a biomarker has now evolved from being a poor prognostic factor to a therapy predictive biomarker[29,30]. In the absence of targeted therapy against HER2, patients have increased mortality and recurrence rate[31]. About 15% of breast cancers are HER2+ overexpressing through gene-amplification[32]. HER2 status can be determined using immunohistochemistry staining or fluorescent in situ hybridization (FisH) technique[33]. In current scenario, targeted HER2 therapies improve patient survival significantly[34]. However, anti-HER2 therapies has been shown to benefit patients only with HER2 overexpression or gene amplification[35]. Drugs such as trastuzumab (Herceptin) and lapatinib are anti-HER2/neu therapies. These therapies are not provided to HER2 negative patients.

1.2.4 Proliferation rate (Ki-67)

Another important biomarker that pathologists quantify during breast cancer diagnosis is proliferation rate. This is measured by manual scoring of mitoses but also Ki-67 using immunohistochemistry (IHC) staining[36]. Although, the function of Ki-67 is unknown, its expression is associated with proliferation and ribosomal RNA transcription[37]. Ki-67 is expressed throughout the active cell cycle phase (G1, S, G2 and M-mitosis) and absent in resting phase of cell cycle (G0), this makes it a perfect marker for proliferation[38]. Number of cells positive for Ki-67 directly proportionate to higher degree of proliferation[39]. Ki-67 has been identified as an independent prognostic parameter for disease free survival and overall survival in breast cancer patients[40]. Ki-67 score is used to stratify patients with high or low risk of recurrence and as a surrogate marker for differentiating luminal A subtype versus luminal B subtype cancers (described in later part), in addition to ER, PR and HER2 statuses[41]. Variability between laboratories, in assessing Ki-67 scores are reported in several studies[42]. Due to this,

no general consensus has been established with regards to the cut-off for Ki-67 for classifying patients based on proliferation[43]. In 2015, the St. Gallen International Expert Consensus recommended a cut off of 20% for Ki-67 to classify tumors between luminal-A and luminal-B[44].

1.3 HISTOLOGICAL GRADE AND STAGE

Histological grade is determined based on the differentiation levels of individual tumors and it is used as an important prognostic factor in breast cancer management. One of the most widely validated and used method to determine the differentiation grade of the tumors is the Nottingham histological grading system (also referred as Elston-Ellis grade)[45]. Based on the macroscopic examination of morphological and cytological features of cancer cells, three main factors are analyzed; degree of tubule formation, nuclear pleomorphism and mitotic count. All three factors are given a score of 1-3 and the scores from all these factors are then combined to determine the grade[46]. Based on the total scores, tumors are then classified into three grades; grade 1 (well differentiated, slow growing, total score of 3-5), grade 2 (moderately differentiated, total score of 6-7) and grade3 (poorly differentiated, highly proliferative, total score of 8-9)[46]. Grade 1 tumors have good prognosis, while grade 3 tumors have worst prognosis[46,47]. Grade 2 tumors are considered as intermediate group, however their existence has been questioned, and some researchers argue that the grade 2 tumors might be a blend of grade 1 and grade 3 tumors. Further, grade 2 tumors doesn't add much of clinically actionable information with regards to the therapeutic planning[48]. The prognostic significance of grade 1 and grade 3 tumors are clinically relevant while grade 2 tumors which comprises of approximately 50% of all breast cancers are not well defined[49]. Gene expression and RNA sequencing technologies are reported to be better in classifying these grade 2 tumors for improving their clinical relevance[50,51].

Staging of breast cancers also provides valuable prognostic information for patients. It is determined by TNM classification method[52]. Primary tumor size (T), spreading of cancer to local lymph nodes (N) and distant metastasis (M) are considered in determining the stage of the cancer. Broadly breast cancers are classified into stage 0-4, depending on the tumor progression state[52]. Non-invasive tumors such as DCIS and LCIS are stage 0 tumors. Stage 1-3 tumors are tumors with no distant metastasis and stage 4 tumors are with distant metastatic disease. Detailed description of TNM classifications is described well in 7th edition of TNM classification manual[52,53].

1.4 MOLECULAR INTRINSIC SUBTYPING OF BREAST CANCERS

Characterization of breast cancer molecular subtypes is considered as the major breakthrough of last decade, in the field of breast cancer classification. Technological advancements in the field of high throughput gene expression analysis has made it possible to classify breast cancer based on global gene expression. In the seminal work by Perou *et al,* breast cancers were initially classified into five intrinsic subtypes: luminal A, luminal B, HER2 enriched, basal-like and normal like[5,54]. Each of these molecular subtypes was associated with different prognosis. These data illustrate that breast cancer exhibit substantial molecular heterogeneity, however genetic alterations corresponding to each molecular subtype and their origin is still unknown. Molecular subtyping of breast cancer highlighted the need to identify new therapeutic targets

for each and every specific subtype. Currently, intrinsic subtype classifications of breast cancers is mainly used for research purposes and has a great potential in planning treatment and developing new therapies for breast cancer. Below are the most common intrinsic subtypes explained briefly:

1.4.1 Luminal A

Luminal A is the most common intrinsic subtype, representing 50-60% of total breast cancers. At protein level these cancers express ER and PR and are characterized by expressing genes involved in estrogen receptor (ER) transcription factor. They also have low expression of proliferation genes[5,54]. At protein level they do not overexpress HER2 and express very low Ki-67. Prognosis for this group of cancers is good. The 15-year distant relapse rate (27.8%) is significantly less than other subtypes of breast cancer[55]. Treatment for this group of patient involves selective estrogen receptor modulators (SERM) tamoxifen, fulvestrant or hormonal aromatase inhibitors (AI)[56].

1.4.2 Luminal B

Luminal B cancers make up to 10-20% of all breast cancers. This group of cancers are more aggressive than Luminal A cancers. They have high histological grade and proliferation rate in turn correlating to worse prognosis. Although luminal A and the majority of luminal B express ERα, luminal B prognosis is very different from luminal A. This is because luminal B cancers have higher expression of proliferation associated genes such as Ki-67, cyclin B1 and growth factor genes such as EGFR and HER2[5]. Identification of biomarker specific to luminal B is crucial to understand the biology of these cancers and eventually design targeted therapy against it. Ki-67 is been currently used as a biomarker to differentiate luminal A from luminal B cancers[57]. The cut-off level of Ki-67 to divide the Luminal tumors into distinct groups is not yet standardized worldwide, leading to variability while assessing Ki-67 as biomarker. IHC of biomarkers (ER/PR/HER2/Ki-67) has been used as surrogates for determining molecular subtypes. Luminal A has been defined as ER+/and/or PR+/HER2-/Ki67<20%, while luminal B tumors as ER+/and/or PR+/HER2-/Ki67>20% or ER+/and/or PR+/HER2+/Ki67 any[44]. Luminal B cancers are also treated with tamoxifen or AI, however their response rate is poor[58]. Hence, they also receive chemotherapy. They often respond to chemotherapy (17%), however this response rate is probably lower than for HER2-enriched and basal-like cancers (36% and 46% respectively)[59], for this reason treatment for luminal B cancers are challenging and much effort needed to find new pathways involved to target them.

1.4.3 HER2-enriched

This group of cancers are characterized by having high expression of the ERBB2 (HER2) gene located in the 17q12 chromosome. HER2-enriched cancers constitute about 15-20% of all breast cancers. These cancers express low levels of luminal genes and their IHC profile are defined as ER-/HER2+. Only 70% of tumors classified as HER2-enriched subtype by gene expression have an over expressed HER2 protein[59,60]. Moreover, not all HER2 amplified or overexpressing tumors falls under the HER2-enriched intrinsic category. Depending on the ER status, if IHC profile is ER+/HER2+ the tumor is classified as luminal B. Based on the pathological characteristics (usually high histological grade), these tumors are highly

proliferative and have poor prognosis. However, targeted anti-HER2 therapies such as trastuzumab has been substantially developed during last decade, improving the survival of patients with both metastatic and primary cancers[61].

1.4.4 Basal-like

About 10-20% of all breast cancers are basal-like. Basal like cancers are characterized by high histological grade, high frequency of lymph node infiltration, large tumor size at diagnosis, high rate of p53 mutations and occurs frequently in women of African origin[54,62]. The IHC profile of these cancers is defined as ER-/PR-/HER2-; therefore in clinical terms it is also referred as triple negative (TN) cancers. However, not all triple negative cancers are basal-like cancers and there is a discordance of 30% described between these two groups[63]. Basal-like cancers have poor prognosis compared to luminal groups[54,64] and the metastatic relapse sites are predominantly in visceral organs such as lung and central nervous system[55,65]. Although basal-like cancers have higher response rates to chemotherapy than luminals[66], basal-like subtype have higher relapse rate during the first 3 years[67]. Germline mutation in BRCA1 gene give rises to sporadic breast cancers, which falls under basal-like subtype[64]. The BRCA1 gene is involved in DNA damage repair mechanism, which inspired the need to target this mechanism for future therapies for basal-like cancers. The poly-ADP ribose- polymerase-1 (PARP-1) is a key molecule in single strand DNA break repair system. PARP-1 inhibitors are becoming a promising strategy in BRCA1 mutated patients, leading to accumulation of double stranded DNA breaks causing cell death[68].

1.4.5 Normal-like

Normal-like intrinsic subtype of breast cancer occurs rarely, about 5-10% of all breast cancers are believed to come under this intrinsic group. Their gene expression profile groups under fibroadenomas and normal breast samples[5], hence the term normal-like. These cancers are also ER-/PR-/HER2- (TN) with intermediate prognosis, placed between luminal and basal-like. The difference between normal-like and basal-like cancers is that normal-like cancers do not express cytokeratins and EGFR[69,70]. Some researchers are skeptical about their real existence, as they believe it might be a technical artifact in microarray technology, which includes normal breast tissue contamination in cancer samples which contributes to this intrinsic subtype[71]. Since this intrinsic subtype is rare, very few studies are reported about this group and the clinical significance of this subtype is yet to be determined.

1.4.6 Claudin-low

Claudin-low intrinsic subtype has been identified in later studies[72]. The term claudin-low was coined due to its characteristic low expression of genes involved in intercellular adhesion and tight junctions such as claudin-3, 4, and 7 and E-cadherin. This subtype of cancers clusters together with basal-like subtype, with low HER2 and luminal genes, however claudin-low subtype over expresses a set of immune response genes (40 genes) suggesting an increased immune infiltration in this type of cancers[59,73]. Although this subtype has low expression of proliferation associated genes, they have poor long-term prognosis[73]. This might be because they overexpress another set of genes involved in mesenchymal differentiation and epithelial-mesenchymal transition (EMT). These features are related to acquiring cancer stem cell (CSC)

phenotype which are implicated in tumor progression and metastasis[74]. IHC profile of claudin-low subtype is normally TN, however 20% of them are hormone receptor positives[60].

Certain expert panels like The St. Gallen International Expert Consensus for Early Breast Cancer 2015 has recommended molecular profiling of breast cancers to be included in treatment planning process for breast cancer. This expert panel states five clinico-pathological defined groups; luminal A (ER+/and/or PR+/HER2-/Ki67<20%), luminal B (ER+/and/or PR+/HER2-/Ki67>20%) or (ER+/and/or PR+/HER2+/Ki67 any), HER2 enriched (ER-/PR-/HER2+) and triple negative (ER-/PR-/HER2-)[44]. Uncertainty still persist with regards to the intrinsic subtype classification between luminal A and luminal B for intermediate Ki-67 expression group (Ki-67 levels of 10-35%)[44]. In short, luminal A cancers are treated only with endocrine therapy. Endocrine therapy combined with chemotherapy is recommended for luminal B. Anti-HER2 therapy combined with chemotherapy for HER2 enriched and only chemotherapy for Triple negative cancers[75].

<table>
<tr><th></th><th>Luminal A</th><th>Luminal B</th><th>HER2 +</th><th>Triple negative
(Basal like)</th></tr>
<tr><td>Percentage at diagnosis</td><td>40-50%</td><td>15-20%</td><td>10-15%</td><td>15-20%</td></tr>
<tr><td rowspan="2">Receptor expression</td><td colspan="2">Estrogen (ERα) and/or progesterone (PR)</td><td></td><td rowspan="2"></td></tr>
<tr><td></td><td colspan="2">HER2 +</td></tr>
<tr><td>Proliferative index</td><td>Low Ki67</td><td>High Ki67</td><td>Usually high Ki67</td><td>Usually high Ki67</td></tr>
<tr><td rowspan="4">Treatment strategies</td><td colspan="4">Chemotherapy</td></tr>
<tr><td></td><td colspan="2">HER2 targeted therapies</td><td></td></tr>
<tr><td colspan="2">Hormonal therapies</td><td></td><td></td></tr>
<tr><td colspan="4">Novel targeted therapies</td></tr>
</table>

Table 1. Breast cancer subtypes defined by histology and immunohistochemistry. Different tumor characteristics for ER, PR, HER2 and Ki67 within established intrinsic subtypes. The normal like and claudin low have been omitted. Adapted from Norum et al.2014[76]

1.5 INTRA-TUMOR HETEROGENEITY IN BREAST CANCER

Intra-tumor heterogeneity signifies the existence of subpopulation of cancer cells that differ in their genetic, epigenetic and biological make up within a tumor. Massive parallel sequencing studies of primary breast cancers have demonstrated that both spatial and temporal heterogeneity are common within a tumor[77-80]. Specific driver gene aberrations such as HER2 amplifications, TP53 and P13KCA somatic mutations are reported to be heterogeneous with in

neoplastic cells in primary tumors[81,82]. Furthermore, it has been demonstrated that individual breast cancers have distinct subpopulation of cancer cells across different region of the same tumor (spatial heterogeneity)[77,83] or cancer cells genetically evolve over time between primary tumors and subsequent recurrences (temporal heterogeneity)[79].

Currently, there are two theories to explain why we observe intra tumor heterogeneity in cancers 1) the cancer stem cell hypothesis and 2) the clonal evolution model[84]. In recent years it has been perceived that these two models are complimenting each other and they might not be mutually exclusive as previously thought[85]. Both models propose that cancer originate from a single cell with abnormal genomic aberrations leading to indefinite proliferative phenotype and tumor microenvironment can impact cellular composition of tumors. However, the main difference between these two models is the support for existence of cellular hierarchical organization by CSC model. According to CSC model, intra tumor heterogeneity is attributed to deregulation of the differentiation process of CSC's and proposes the existence of a hierarchical organization of cancer cells, where cancer stem cell (CSC) are at the apex of the hierarchy. These CSCs are considered as a minor subset of cells in a tumor which are lineage committed progenitor cells, which can give rise to more differentiated cancer cells[86]. This model also proposes that the tumor growth and disease progression is governed by this small subset of cells with stem cell features (CSC), while the rest of the bulk tumor cells do not contribute to tumor growth[87]. This hypothesis however, being disputed, since evidence illustrating the existence of a dynamic interconversion between differentiated cells and CSCs via epithelial mesenchymal transition (EMT) process has been reported[88]. Therefore, in some cancers CSC phenotype might represent only a '"state of stemness" which, cancer cells within a tumor can attain rather than being a distinct hierarchical based subset of cells. The clonal evolution model on the other hand attribute the observed intra-tumor heterogeneity as a Darwinian evolution of cancer cells, where the cancer clones that would survive tumor micro-environmental conditions and treatment pressure propagates further leading to therapy resistant clones[89,90]. Unarguably, it is evident that breast cancers exhibit high degree of phenotypic and genetic intra-tumor heterogeneity, this might have direct impact on both diagnosis and disease management.

1.6 BREAST CANCER THERAPEUTICS TARGETING MAJOR SIGNALING PATHWAYS

With our previous knowledge of molecular intrinsic subtypes of breast cancer, we can clearly understand that breast cancer is a heterogeneous disease. Each molecular subtype harnesses a distinct growth stimulatory advantage[91]. Recent technological and experimental developments have provided more insights on cellular process and pathways involved in the development of breast cancer. Several signaling pathways are implicated in breast cancer development affecting cellular process such as cell survival, proliferation, migration, differentiation and apoptosis[92-94]. These signal transduction pathways frequently cross-talk between each other to ensure breast cancer cells responds appropriately to the extracellular growth factors. Slow and gradual disruptions of these signaling pathways provided the growth advantage to the cells leading to cancer later on. In this thesis, we will discuss the most common signaling pathways targeted in breast cancer treatment.

1.6.1 Estrogen receptor signaling

Estrogen receptors (ERs) are ligand regulated transcriptional factors which transduce hormonal signals in various organs for variety of physiological responses[95]. There are two estrogen receptors; ERα and ERβ, products of two different genes on two chromosomes and are structurally similar but differentially expressed in tissues. ERs regulate cell proliferation and differentiation in normal mammary gland in an estrogen dependent fashion by both canonical genomic and non-genomic transcriptional mechanisms[96]. In response to estradiol binding, ERα undergo conformational change which controls its interactions with other co regulators, leading to its binding to the estrogen response element (ERE) within the promoter of target genes. ER-dimerization upon E2 stimulation, recruits other co-regulators involving chromatin remodeling, which enhances the transcriptional activity[97]. E2 bound ERα complex alter the transcription of genes involved in proliferation, differentiation, survival and pathways crucial for cancer such as invasion, metastasis and angiogenesis[98]. Estrogen also signals through non-genomic pathways via membrane ER[99] and trans-membrane G-protein-coupled receptor complexes (GPCR)[100]. ER mediated transcription is a complex process involving many co-regulators and cross-communication between different signaling pathways and has been well described in detail in other review articles[101]. In short, growth of ER+ cancer cells are E2 dependent and the removal of E2 can reduce their growth, therefore ERα is a well-established predictive marker of hormone sensitivity. Currently luminal BCs are treated with SERMs and also with aromatase inhibitors which can improve the overall survival by almost 50% during the period of first 5 years[22]. Extending this endocrine therapy to 10 years has also shown to be more beneficial[22]. However, the response is often not permanent and certain patient become resistant to endocrine therapy[102].

Another important signaling axis, which is closely linked to estrogen signaling is cyclin D1-CDK4/6 (cyclin dependent kinases)-Rb (retinoblastoma protein) pathway. Hyper phosphorylation of Rb by CDK/cyclin D1 is crucial for G1-S transition during cell cycle[103,104]. Estrogen stimulated MCF7 cells induce cyclin D1 gene activity followed by CDK4 activation and Rb phosphorylation[105,106]. Overexpression of cyclin D1 renders breast cancer cells to be endocrine resistant[107]. Inhibiting cyclin D1-CDK 4/6-Rb pathway is a novel potential therapeutic target in luminal breast cancer[108]. Palbociclib, a CDK4/6 inhibitor was recently approved by FDA for use in combination with endocrine based (in combination of letrozole-aromatase inhibitor) therapy for metastatic disease in post-menopausal women with ER+, HER2- advanced breast cancer[109]. Palbociclib in combination with letrozole, reported an improvement in progression free survival (PFS) from 10.2 months (only letrozole treatment group) to 20.2 months (combination treatment group) in a large phase 2 study[109].

1.6.2 EGFR (Epidermal growth factor receptor) and HER-2

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase glycoprotein (170kDa) belonging to the ErbB family. This family of proteins are activated aberrantly in various human cancers including breast cancers[110]. The ErbB family contains four proteins: Epidermal growth factor receptor (EGFR/HER-1/ErbB-1), Human epidermal growth receptor HER-2 (ErbB-2), HER-3 (ErbB-3) and HER-4(ErbB-4)[111]. There are three main functional domains in these receptors: a ligand binding, a hydrophobic transmembrane and a cytoplasmic tyrosine-kinase domain. Ligand activation of EGFR, leads to homo or hetro-

dimerization (with other family member receptors) and subsequent auto phosphorylation of tyrosine kinase domain which in-turn activates downstream signaling pathways such as MAPK/ERK, PI3K/AKT, STAT (signal transducer and activator of transcription) pathways[112-114].

HER-2 appears on the surface of some of the breast cancers[115]. Overexpression of this protein and activation of its downstream signaling pathway has in fact lead to separate molecular intrinsic subtype of breast cancers namely "HER-2 enriched" which has been described earlier in this report highlighting the importance of this molecule in breast cancer. Around 15% of breast cancers overexpress HER-2 protein by genomic amplification, which makes cancers more aggressive[28], and has the potential to spread to other body parts[116]. Over expression of HER2 leads to homo or hetro-dimerization (with other family members), eventually phosphorylating tyrosine residues on each other[117]. HER-2 transduces its function mainly via PI3K/AKT[118] and Ras/MAPK signaling pathway [119]. Activation of HER-2 upregulates expression of anti-apoptotic proteins such as survivin and Bcl-2 in cancer cells[120]. Targeting HER-2 Signaling is beneficial to patients with aberrant HER-2 activation. Drugs like tratuzumab, pertuzumab and lapatinib are specifically targeted against HER-2[121].

1.6.3 PI3K/AKT pathway

Another important signal transduction pathway, which is often deregulated in many human cancers, including breast cancer is PI3K/AKT pathway[118]. The PI3Ks are lipid kinases, whose function is to phosphorylate phosphoinositides[122]. Class IA PI3Ks comprise of two components; a regulatory subunit (p85) and a catalytic subunit (p110). Growth factors or ligands binding to their respective receptor tyrosine kinases (RTK) such as EGFR, HER-2 and IGF-1R initiates P13Ks. Upon receptor activation, p85 subunit of PI3K interact with the RTK's intercellular domain, which activates p110 catalytic subunit of P13K. Activated P13K phosphorylates phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol trisphosphate (PIP3). AKT, a serine/threonine kinase docks to PIP3, which is the central effector of this pathway. Phosphorylated AKT stimulates protein synthesis and cell growth by inducing mammalian target of rapamycin (mTOR)[122]. AKT increases anti-apoptotic proteins such as Bad, and promotes cell cycle proteins such as c-Myc and cyclin D1 and decreases cell cycle inhibitors such as p27 and p21, resulting in boosting cell survival. This pathway is crucial for many cellular process such as cellular metabolism, cell survival, proliferation, differentiation, cancer progression and motility[123]. mTOR is a key mediator of cellular response to multiple stimuli such as cellular nutrients and growth factors. In response to these stimuli, mTOR activates the translational machinery leading to the enhanced mRNA translation of genes involved in cell growth and cell progression[124]. Phosphatase and tensin homologue (PTEN), is an intrinsic negative regulator of PI3K/AKT pathway. PTEN converts PIP3 back to PIP2, thereby inhibiting the further signaling cascade of PI3Ks. Uncontrolled activation of PI3K/AKT/mTOR pathway (can be either genomic or epigenetic alterations) contributes to the establishment and progression of many human cancers including breast cancer[125]. In breast cancers, majority of activating mutations occur at the catalytic subunit (p110 α) of PI3Ks, which increases the enzymatic activity of PI3K in a ligand independent manner thereby contributing to the oncogenic transformation. About 20%-25% of all breast cancers, depending on their intrinsic subtype have PI3K mutations. It's interesting to note that, luminal breast cancers have higher frequency (>30%) of these mutations in PI3K than triple negative breast

cancers (5%)[126]. Further, loss of PTEN is also reported in breast cancer but with much lower frequency. Activating mutations in PI3Ks and loss of PTEN confers resistance to the anti-receptor therapies such as trastuzumab against HER-2[127]. Therefore, this pathway is a potent target for anticancer agents. Several clinical trials are currently ongoing targeting mTOR (ClinicalTrials.gov: NCT00876395, NCT01698918, NCT01783444 etc.,) and PI3Ks/ AKT inhibitors such as buparlisib is in phase 3 clinical trials (ClinicalTrials.gov; NCT01610284, NCT01082068 etc.). Everolimus (mTOR inhibitor) is now approved for post-menopausal women with advanced metastatic breast cancer (ER+ and HER2-) in combination with endocrine therapy (exemestane) as this combination significantly improves the progression free survival in these patients[128].

1.6.4 PARP signaling pathway

Poly (ADP-ribose) polymerase 1 (PARP-1) is a critical molecule involved in DNA repair and apoptosis process [129]. PARP is responsible for recognizing single strand DNA breaks and repairs it via base excision pathway[130] and also bind to double stranded breaks preventing accidental recombination of homologous DNA[131]. In wild type cells, the double stranded breaks often repaired via homologous recombination with the help of BRCA1 and BRCA2 proteins[132]. However, cells deficient with BRCA1 and BRCA2 are unable to repair the double stranded DNA breaks leading to chromosomal instability, cell cycle arrest and subsequent apoptosis[133]. Hence PARP inhibitors have shown efficacy in breast cancers with BRCA1 or BRCA2 inherited mutations[68]. PARP inhibition is a recently developed strategy which exploits the DNA damage response pathway in cancer cells[134]. Enhanced PARP-1 expression has also been observed in triple negative breast cancers[133]. PARP inhibitors such as olaparib, veliparib and iniparib have shown promising anti-cancer response for breast cancer and are currently validated in clinical trials. PARP inhibitors in combination with inhibitors of AKT or mTOR are also under clinical trials (ClinicalTrials.gov; NCT02338622 and NCT02576444).

1.6.5 Angiogenesis

Angiogenesis is a complex and dynamic process involving formation of new blood vessels. It has been widely accepted that, cancer cell growth and proliferation is dependent on angiogenesis for tumor development and progression. Angiogenesis plays a crucial role in both primary breast cancer development and in metastasis[135]. Hypoxia is a key switch for the induction of the angiogenesis process. Under hypoxic conditions, HIF1- α (transcription factor) is stabilized and transcribes genes involved in the angiogenesis process[136]. Six different pro-angiogenic factors have been identified to be commonly expressed in invasive breast cancers, with vascular endothelial growth factor (VEGF) being the predominant one[137]. The VEGF and PDGF (platelet-derived growth factor) family of proteins and their receptors (VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α and PDGFR-β) seems to be the central players of angiogenesis process[138]. Signal transduction via VEGFRs and PDGFRs initiate many cellular process such as survival, mitogenesis, migration and differentiation[138,139]. Numerous studies have found an inverse correlation between VEGF expression and overall survival (OS) in both lymph node-positive and negative breast cancers[140,141]. Advanced breast cancers expressing higher VEGF are less responsive to chemotherapy and endocrine therapy[142]. A fraction of invasive breast cancers which over–expresses PDGFR-α have been associated with more aggressive phenotype with increased metastatic potential[143]. Due to their important role in

tumor angiogenesis process, VEGF and PDGF are the key targets for experimental breast cancer treatment. Although the implication of angiogenesis is prominent in tumor biology, targeting angiogenesis is often challenging as it requires inhibition of more than one receptors to block angiogenesis. It has been proposed that, anti-VEGF therapy can perform two important functions; first, it can block the development of new tumor vasculature resulting in tumor regression, second, it can normalize the existing inefficient tumor vessels thereby supporting drug delivery into the tumor[144]. For this reason, anti-angiogenic therapy has potential when combined with chemotherapy[144,145]. Several anti-angiogenic therapies has been developed in recent years. For example: monoclonal antibodies such as bevacizumab (avastin) which binds to VEGF, thereby reducing the VEGF content from the circulation, subsequently preventing the activation of VEGFRs[146]. This drug was approved by FDA for the treatment of multiple different cancers such as non-small-cell lung, colorectal, renal cell, glioblastoma and breast[147]. In breast cancer however, data from multiple clinical trials have shown that, the effect of bevacizumab in improving progression free survival was modest with some adverse side effects[147,148]. Consequently, FDA has removed bevacizumab's approval for treatment of HER2 negative metastatic breast cancer[147]. However, it is still approved by European medicine agency (EMA). More development and validations of biomarkers are necessary to identify patients who will benefit from angiogenesis based therapies.

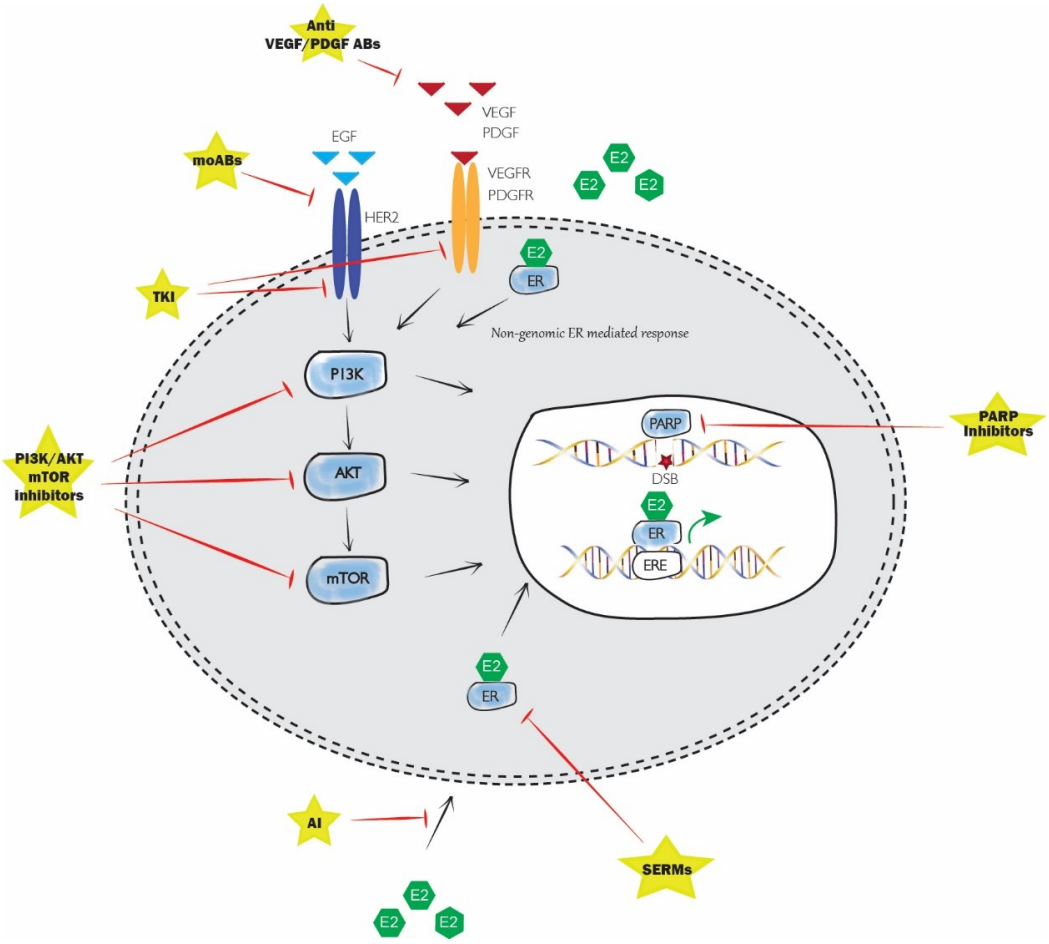

Figure 1: Key signaling pathways and their potential inhibitors involved in breast cancer. (E2 estradiol, TKI-Tyrosine kinase inhibitor, moABS-monoclonal antibodies, AI-Aromatase

inhibitor, SERMs-Selective estrogen receptor modulators, ER-estrogen receptor, DSB-double stranded DNA break.

1.7 BREAST CANCER DRUG RESISTANCE

Drug resistance is one of the main challenges in the treatment of breast cancer. Breast cancer cells acquire resistance to both chemotherapeutics (taxanes and anthracyclines) as well as targeted therapies (anti-HER2, tamoxifen and anti-VEGF). Some patients possess cancers, with innate resistance to chemotherapy and do not respond, other patients have tumors with partial response, in such cases only a fraction of tumor cells are killed during chemotherapy[149,150]. The remaining fraction of cells continues to grow and establish recurrent tumors. Gain of *de novo* resistance during chemotherapy (also referred as "acquired resistance") were also reported in breast cancers[151,152]. Many biochemical and cellular mechanism have been proposed for drug resistance. In this thesis, we will discuss only about endocrine resistance in breast cancer cells.

1.7.1 Endocrine resistance in breast cancer

Majority of the breast cancers are ERα positive (70-75%). Tamoxifen, a SERM is the mostly widely used endocrine therapy against ER positive breast cancers for both pre-menopausal and post-menopausal patients[153]. Tamoxifen is a partial antagonist of ERα, as it competes with estrogen for ERα receptor binding and impairs its function[154]. Tamoxifen as adjuvant therapy in early breast cancer patients improves overall survival of patients and has significantly lowered the breast cancer mortality over the last decade in luminal breast cancer patients[155,156]. Further tamoxifen has been reported as a potential preventive agent against hormone dependent breast cancer[157]. Despite the good response rate of tamoxifen, around 40% of patients receiving tamoxifen as adjuvant therapy eventually relapse with tumors[158]. Loss of ERα expression in recurrent tumors might be one of the reasons for the poor endocrine response[2,159]. Aromatase inhibitors (AI) are another class of endocrine drugs which prevents the conversion of androgens to estrogens, thereby reducing the plasma estrogen levels in the body[23]. It has been reported that, AI are more efficient than tamoxifen in reducing cancer recurrences[160], however they are provided only to post-menopausal women since, ovaries in pre-menopausal women can produce estrogen and neutralize the effect of AI[161]. Further, use of AI is associated with higher risk for osteoporosis compared to tamoxifen, however it has reduced risk of thromboembolic events[162]. Despite the efficacy of AI therapy, around 20% of patients relapse later in their life[22]. Intrinsic resistance and acquired resistance to endocrine therapies is a major challenge in treating hormone dependent breast cancers. Understanding the biological mechanisms behind this resistance will hopefully provide us with novel biomarkers associated with resistance and improved breast cancer treatments.

ERα expression is the routine prognosticator of tamoxifen treatment[155]. PR expression is also used to increase the accuracy of predicting endocrine therapy response, as it represents functional ER pathway and estrogen dependence in luminal cancers[159]. Loss of ERα expression and its function has been reported as the main mechanism for *de novo* resistance to tamoxifen[163]. Few patients with ERα-positive cancers will relapse with a metastatic disease, which do not express ERα[164]. However, majority of resistant patients still do express ERα during disease progression. Acquired mutations in ERα may lead to functionally negative ERα phenotype in spite of ERα expression[165]. In fact up to 20% of patients with tamoxifen resistant

recurrent cancers, still respond to aromatase inhibitor or fulvestrant, suggesting functional ER regulation in the tumor growth of these tamoxifen resistant patients[166,167]. Alterations in pharmacological tolerance of tamoxifen has also been reported to cause tamoxifen resistance[168]. Transcriptional co-activators and co-repressors play an important role in transcriptional activation of ER. Alterations in co-regulatory proteins such as AIBI has also been reported to cause a tamoxifen resistant phenotype in breast cancer[169]. A number of studies demonstrate that, cross-talk between ER signaling and other growth factor receptor signaling pathways, such as insulin-like growth factor receptor (IGFR) and EGFR/HER2, as an important endocrine resistance mechanism. Ligand bound ER can activate IGFR directly at the cell membrane leading to the downstream ERK1/2 MAPK signaling cascade[170]. Membrane bound ERα is also reported to interact directly with HER2 and also activates EGFR by phosphorylation[171]. Cell-lines under the constant hormonal therapy such as tamoxifen and fulvestrant, acquire resistance to these drugs by overexpressing HER2/EGFR signaling pathways and these resistant cells are sensitive to EGFR inhibitors[172]. Induction of growth factor receptor signaling during tamoxifen treatment may cause loss of hormone dependence and in turn lead to reduced sensitivity to tamoxifen in breast cancers. ER signaling also seems to cross-talk with P13K/AKT signaling pathway which is involved in proliferation and anti-apoptotic responses. ERα can bind to p85 subunit of P13K in a ligand dependent manner, thereby activating P13K/AKT downstream effectors[99]. Relationship of ERα with P13K/AKT pathway is reciprocal, activated P13K, activates AKT, which phosphorylates ERα at serine-167, resulting in ligand independent activation[173]. Another growing field of research in therapeutic resistance, focuses on cancer stem cells, however their role in endocrine resistance is not yet well understood.

1.8 BREAST CANCER STEM CELLS (BSCS)

The idea of small population of cancer cells with self-renewal capabilities are referred as cancer stem cell (CSC) or cancer initiating cells (CIC), which is believed to be responsible for cancer initiation and maintenance. CSC hypothesis was postulated long time back, however conclusive evidence of their existence was obtained relatively recently in human leukemic developmental process[174]. Majority of the breast cancer treatment fails due to the tumor evolution leading to metastasis and chemotherapy resistant disease. This highlights the possibility of a fraction of cancer cells with stem cell-like characteristics, which are resistant to chemotherapy and radiotherapy[175], could be the cause of cancer recurrence and progression[176]. The definitive existence and characterization of CSC are not yet fully validated in majority of human malignancies. Currently, CSCs are identified using three main characteristics: 1) expression of cell surface markers associated with stemness; 2) the capability to grow in non-adherent conditions, resistant to anoikis (apoptosis induced during loss of cell-matrix detachment) and without serum; 3) the ability to self-renew and can rebuild the heterogeneity of the original tumor via differentiation process in xenograft models[177,178].

In breast cancer, CSCs were initially identified using cell surface markers characterized by $CD24^{-/low}/44^{+/high}$. They were highly tumorigenic and a few hundred cells were enough to form an heterogeneous tumor, representing the parental tumor when inoculated in NOD/SCID mice[179]. Since, breast cancer stem cells (BSC) are resistant to anoikis (apoptosis induced during loss of cell-matrix detachment), they can be isolated and propagated as mammospheres in non-adherent culture conditions (without serum and supplemented with growth factors)[178]. CSCs

can be isolated from patient derived tumors[178] as well as from breast cancer cell lines[180]. Although the combination of $CD24^{-}/44^{+}$ are important markers for BSC isolation, only a fraction of these cells were highly tumorigenic, highlighting the need for more markers to represent true BSC. The enzymatic activity of aldehyde dehydrogenase (ALDH1) was reported to be associated with tumor initiating breast cancer cells[181]. ALDH1 has been previously reported as stem cell marker in hematopoietic, lung and colon cancers[182,183]. There is only a partial overlap between the $ALDH1^{+}$ and $CD24^{-}/44^{+}$ cells, the small population of cells which are identified by combing all these markers $ALDH1^{+}$ and $CD24^{-}/44^{+}$ are proven to be even more tumorigenic than other sub-populations, only 20 cells were sufficient to form tumors when xenografted to NOD/SCID mice[181]. Gene expression analysis identified that $ALDH1^{High}$ cells were linked with more epithelial genes, while $CD24^{-}/44^{+}$ cells were associated with mesenchymal-related genes. This suggests that BSC population can be composed of two different subsets of cells; 1) CSCs with mesenchymal-like phenotype (for invasion and metastasis) and 2) CSCs with epithelial-like characteristics (for tumor growth). Numerous publications has reported that BSCs are capable of switching between epithelial-like state to mesenchymal-like state and *vice-versa* which drives them to colonize distant site and form metastasis[184]. Interestingly, it has been shown that BCSs share similar DNA alterations compared to the bulk tumor cells highlighting the importance of differentiation and de-differentiation between CSCs and tumor cell population during cancer progression[185]. BSCs are shown to express gene signatures similar to cells subjected to epithelial to mesenchymal transition (EMT) process[186]. This process is crucial for cancer invasion, as the percentage of BCSs are higher in metastatic lesions[187]. Pathways active in CSC maintenance and self-renewal processes such as Notch, Wnt and Hedgehog signaling pathways are induced in EMT process as well[188,189].

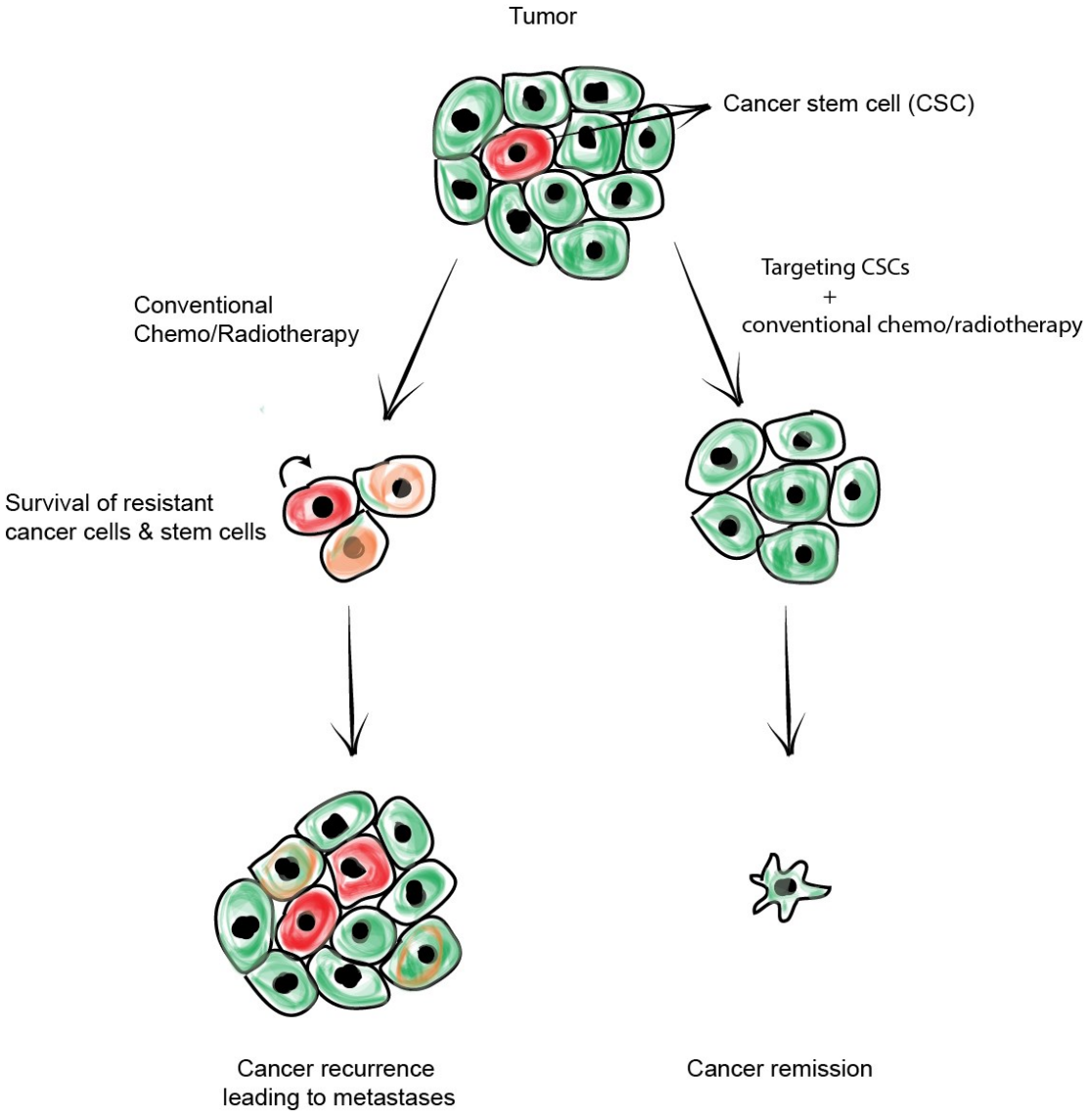

Figure 2: Schematic representation of cancer recurrences using conventional therapy versus combination therapy (targeting CSCs + conventional therapy).

1.8.1 CSC resistance to anti-cancer therapeutics

Current cytotoxic anti-cancer drugs target rapidly proliferating cells and spares the quiescent cells with stem cell characteristics. This has been shown by the increased number of $CD24^{-}/44^{+}$ stem-like cells in residual tumor after chemotherapy[190]. In another study, researchers have demonstrated that letrozole and docetaxel treatment on different molecular subtypes of breast cancer induced $CD24^{-}/44^{+}$ molecular signatures[191]. BSCs were also shown to be resistant and increased in numbers after radiotherapy[192] and this effect can be attributed to two explanations; First, BSC's ability to generate reactive oxygen species is impaired[193] and second, Wnt pathway signaling in BSCs may mediate this response[194]. A few suggested mechanisms by which CSCs can intrinsically resist chemotherapy are summarized below: 1) CSCs are in quiescent state and maintain low proliferation rate[195], 2) CSC over express anti-apoptotic proteins such as bcl-2 and other growth factor signaling essential for the stemness phenotype[196], 3) CSCs express high amounts of drug efflux mechanism related proteins, which helps to decrease the amount of drugs retained in the cells[197], 4) increased efficiency of DNA repair and

alterations in cell cycle regulators, thereby rendering CSCs resistant to traditional radiation and cytotoxic compounds[198].

1.8.2 Current strategies to target CSCs

1.8.2.1 CSC's self-renewal pathway inhibition

Self-renewal is one of the most important properties of CSCs to maintain their proliferative capability. Self-renewal pathways play a crucial role in normal stem cells during early developmental stages to determine cell proliferation, differentiation, cellular fate and polarity. These self-renewal pathways are therefore strictly regulated. In CSCs however, self-renewal pathways are deregulated leading to increased cell proliferation and this is considered as early stages of tumorigenesis[199]. Notch, Wnt and Hedgehog (Hh) pathways are implicated in maintaining self-renewal capabilities of both normal and cancer stem cells in the breast[200]. Evidences suggest that these pathways are deregulated in many human breast cancers[201,202] and deregulation of these pathways in transgenic mice models leads to the onset of breast cancers[203,204].

Four transmembrane Notch receptor proteins (Notch 1-4) are present in mammals. Ligands binding to these Notch receptors can activate Notch signaling[205], which can further downstream induce expression of Hey, cyclin D1, c-Myc and Hes[206]. Activated Notch signaling increase the mammosphere forming efficiency of normal mammary stem cells and can be inhibited by using specific notch inhibitors[207]. In another study, Notch-4 activity was found to be increased significantly in BSC population and it can be inhibited using Notch-4 specific inhibitor in breast cancer cell lines[208]. Antibodies against Notch-1 and Notch-4 can decrease mammosphere forming efficiency from patient derived BSCs and PDX models[209,210]. Currently, Notch inhibitors such as γ secretase have entered clinical trials, where these compounds are combined with existing cytotoxic agents[211,212].

Hedgehog pathway (Hh) is involved in developmental of normal mammary gland. Hh pathway via paracrine signaling can induce progenitor cell proliferation in the mouse mammary gland[213]. Other similar studies, also reported that activation of Hh signaling can promote mammosphere forming capacity of normal mammary stem cells, while treatment with Hh inhibitor (cyclopamine) can inhibit this process[214,215]. In patient derived BSCs, Hh signaling was found to highly activated[214] and activation of Hh signaling induced mammosphere formation in cells derived from mouse (p53-null) mammary tumors[216]. In a recent study, Hh pathway was found to be associated with poor prognosis in breast cancer patients with the $CD24^{-}/44^{+}$ phenotype[217]. In addition, Gli1, which is one of the main components of Hh signaling was found to initiate triple negative breast cancer[218]. These findings suggest that, targeting Hh pathway is one of the ways to target BSCs. Several compounds are currently being tested for utilizing Hh signaling for targeting CSCs[212]. In addition to Notch and Hh pathways, Wnt/β catenin signaling pathway was also demonstrated to be crucial of BSC survival and its inhibition suppresses breast cancer metastasis by inhibiting CSC-like phenotype[219].

1.8.2.2 HER2/P13K signaling in CSCs

Apart from stemness specific self-renewal pathways, HER2 signaling, PI3K/AKT/mTOR and JAK2/STAT3 pathways are also involved in BSCs maintenance and drug resistance[220-222]. In

recent years, anti-HER2 therapy in adjuvant setting reduced the tumor recurrence significantly[223]. It could be possible that, the remarkable clinical efficacy of trastuzumab (Anti-HER2 therapy) might be due to the fact that it targets BSCs as well. For instance, use of traditional chemotherapy increased the CSC population, while combination of anti-HER2 agent such as lapatinib and chemotherapy reduced CSC population[220]. Further, this study demonstrated a positive correlation of ALDH1 expression (a stem cell marker) and HER2 amplification in breast cancers. Cells over expressing HER2 also increased CSC population with high invasive potential[220]. Unfortunately, 50% of the patients who initially respond to HER2 therapy, develop resistance and relapse[127]. The mechanism behind resistance to HER2 therapy is still not clear, however few mechanisms such as hyper-activation of the downstream pathway such as P13K/AKT and loss of PTEN (tumor suppressor gene) are partially held responsible for this[127]. Therefore, inhibiting pathways downstream of HER2 signaling can also potentially target the CSC population. One such AKT targeting agent called perifosine has been shown to effectively reduce BSCs in xenografts[224]. In another study, it was demonstrated that targeting both Notch and HER2 signaling was crucial for prevention of HER2 positive recurrences[225].

1.8.2.3 CSC microenvironment inhibition

Apart from the intracellular self-renewal signaling pathways, tumor microenvironment also influences BSCs. Tumor microenvironment of BSCs also referred as "CSC niche" often consist of differentiated tumor cells, inflammatory cells, fibroblasts, endothelial cells and mesenchymal stem cells[226]. The interaction of these different stromal cells with the CSC is governed via paracrine signaling. This paracrine signaling often results in activation of previously described self-renewal pathways of CSCs such as Wnt, Notch and Hedgehog signaling. Inflammatory cytokines such as IL-6 and IL-8 are noted to be crucial regulators of self-renewal capacity of CSCs, both in *in-vitro* and xenograft models[224,227]. These inflammatory cytokines induce the STAT3/NF-kB pathways in both tumor and stromal cells to further increase cytokine production (positive feedback cytokine loop), which drives CSC self-renewal mechanisms[228]. Serum levels of IL-6 and IL-8 in advanced breast cancer patients were positively correlated to metastatic occurrence and poor survival[229,230]. Further, studies have demonstrated that, cellular cytotoxicity induced by conventional chemotherapy leads to increased production of local IL-8 which may increase CSC population after chemotherapy, which can in turn result in cancer recurrences/metastasis[224]. Therefore, interfering this inflammatory cytokine loop in CSC niche provides a novel strategy to target CSC population. Antibodies/small molecule inhibitory compounds against IL-8 receptor CXCR1 have selectively depleted BSC population in *in-vitro* cell line models[231]. Similarly, anti-IL-6 antibody shown to inhibit CSCs by suppressing JAK1/STAT3 pathway[232]. Monoclonal antibodies/inhibitors against IL-6 or its receptors have entered clinical trials for treating multiple myeloma[233].

1.8.2.4 Immunological approach to target CSCs

In recent years, advances in immunotherapies for cancer therapeutics are promising and produced good clinical response with lower toxicities[234]. However, these therapies are currently focused to eradicate the bulk tumor, which might remise due to the presence of CSCs. Therefore, immunologic strategies specifically designed to target CSCs are probably required

to eradicate cancer recurrences. CSCs are phenotypically different from their differentiated bulk tumor cells and they are also heterogeneous due to their plasticity, which is regulated by genetic and epigenetic mechanisms. Therefore, it is crucial to find multiple novel antigens which are CSC specific in order to utilize immune based therapies. CSC markers such as ALDH1, CD44, CD133 etc., are currently used to isolate CSCs, however they are also potential targets for immune based therapies. One of the immunological approaches is to use dentritic cells (a professional antigen presenting cell) to produce tumor specific T cells. In one study, human $CD8^+$ T cells were stimulated with ALDH peptide-pulsed autologous DCs (dentritic cells-antigen presenting cells) which significantly reduced $ALDH^+$ tumor cells *in vitro*, preclinical patient derived xenograft growth and metastasis formation in immune deficient mice[235]. Another study, investigated DC primed with ALDH+ CSC in xenograft models, and showed that these vaccine can significantly reduce lung metastasis of melanoma cells compared to the vaccine (DC) primed with bulk tumor cells[236]. CSC primed DC vaccine combined with radiotherapy (RT) had significantly lower lung metastases compared to the RT treated group alone in mouse models[237]. It has been suggested that CSC primed DC vaccine can be used in an adjuvant setting to target microscopic residual cancer cells rather than targeting the bulk tumor as the ratio of CSCs are much lower compared to the entire tumor mass[238]. These data clearly highlight the importance of ALDH as a potential target for T cell based immunotherapy against CSCs. Apart from ALDH, CD133 and HER2 receptors are also under investigation for priming different immune cells such as NK (Natural killer cells), T cells and DCs against CSCs[239,240]. Immunological approaches against CSCs are promising cancer therapeutics which can be combined with conventional therapies for preventing cancer recurrences.

1.8.2.5 CSC metabolism inhibition

Cellular metabolism is an important biological process for cell survival and proliferation. Different cells utilize different metabolic pathways such as oxidative phosphorylation (OXPHOS) or anaerobic glycolysis for their energy (ATP) production. Cancer cells tends to utilize anaerobic glycolysis to support their rapid proliferation rate even under normoxic conditions was first reported by Otto Warburg in 1920s[241]. This phenomenon is referred as "Warburg effect". Similarly, several recent studies have demonstrated that embryonic and adult stem cells depend on glycolysis for their ATP production[242]. Even in the case of induced pluripotent stem cells (iPSCs), metabolic shift towards glycolysis has been reported[243] and the expression of glycolytic genes was increased before the actual increase of stemness markers[244]. This suggests that metabolic reprogramming is crucial to acquire stemness phenotype. CSCs derived from breast cancers exhibit higher glycolytic metabolism[245], while CSCs obtained from glioma where relying mainly on OXPHOS for their energy production[246]. This suggests that, CSCs can adapt to different oxidative stress situations depending on their microenvironment. Reduction oxidation (redox) homeostasis is another crucial factor for maintaining self-renewal and pluripotent capabilities[193]. Normal mammary epithelial stem cell have lower levels of ROS when compared to differentiated cells[247], increase in ROS level can lead to lineage specific differentiation[248]. Similar to normal mammary stem cells, BSCs also exhibit lower ROS levels when compared with non-cancerous cells[193]. Warburg effect is also shown to be associated with mitochondrial activity and the redox levels in cancer cells[249]. Increased glycolysis and limited mitochondrial function may hinder mitochondrial depended ROS production, thereby

maintaining a CSC phenotype[250]. Warburg effect is often considered as a cellular response to hypoxic (lower oxygen) conditions occurring in tumor microenvironment, however in the case of BSCs, HIF1-α was found to be expressed in high levels even in normoxic conditions[251]. Lactate and pyruvate which are produced during glycolysis are found to be in higher levels in BSCs and they further induce hypoxia inducible genes independent of hypoxic conditions eventually leading to accumulation of H1F-1α even in normoxic conditions[252]. Alterations in metabolic pathways has been reported to induce different phenotypic changes in cancer cells, for instance silencing gluconeogenic enzyme fructose-1,6-biphosphate, which activates fermentative glycolysis resulting in a stemness phenotype[250]. CSCs isolated from different solid cancers exhibit higher glycolytic metabolism compared to their differentiated cancer cells[253,254]. Therefore, it is highly important to target CSC specific metabolism in order to eliminate them. A recent study demonstrates that, BSCs heavily rely on fermentative glucose metabolism and were found to be sensitive to 2-deoxyglucose (2-DG) treatment (a glycolysis inhibitor). Further research on CSC specific metabolomics can identify more CSC specific targets.

1.8.2.6 CSC differentiation

Another interesting way to target CSCs is to differentiate them, so that they become sensitive to the traditional therapies. This approach is very useful in hematological cancers such as acute promyelocytic leukemia, where treatment with retinoids substantially improved patient survival [255]. In a recent study, all-trans-retinoic acid (ATRA) treatment in breast cancer cells inhibited ALDH1 activity and restored sensitivity to both chemotherapy and radiotherapy[256]. The possibility of differentiation therapy applied on CSCs is a promising approach to eradicate CSC population in tumor. In summary, it is widely accepted that a subpopulation of breast cancer cells possessing stem cell-like capabilities, with high plasticity, intrinsically able to escape from current clinical therapies plays a major role in tumor initiation and progression. It is vital to target BSCs along with the bulk tumor for improved breast cancer treatment response.

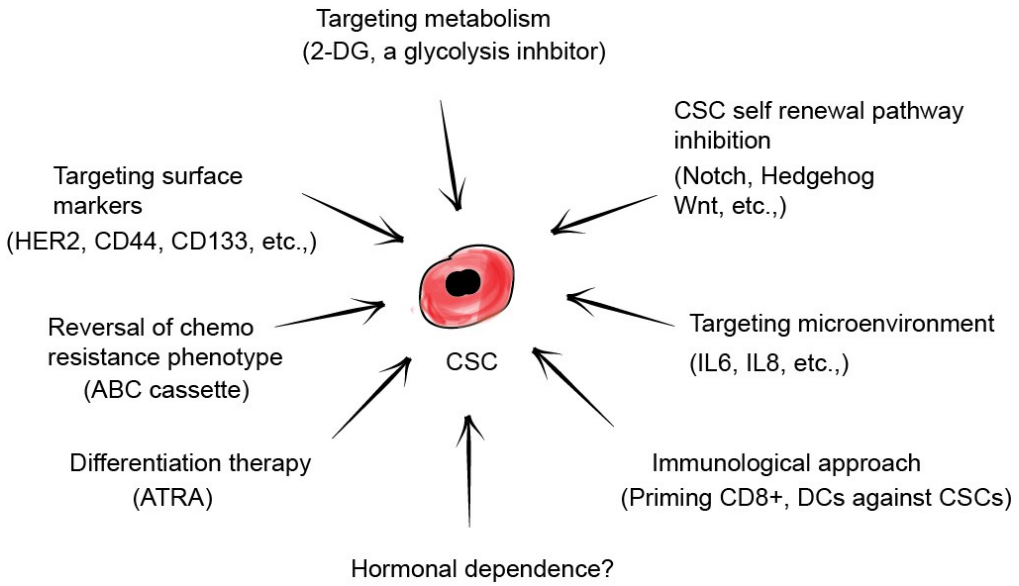

Figure 3: Summary of various mechanisms to target cancer stem cells (CSCs)

1.8.3 Estrogen receptors and BSCs

Estrogen is crucial for development of normal mammary tissue, however adult mammary stem cell population are known to be ERα-negative[257]. Residual cancer cells from neo-adjuvant endocrine therapy (aromatase inhibitor) treatment express mesenchymal and tumor initiating gene signatures[191]. This suggests that, hormone responsive breast cancers may contain hormone resistant BSCs. Isolated BSCs from breast cancer tissues and cell lines are reported to have reduced or no ERα expression[257]. Investigating the normal mouse and human breast development supports the cellular hierarchy pattern of ERα expression, where ERα negative stem cells give rise to ER positive luminal cells[258]. Although BSCs are ERα negative, estrogen stimulates BSCs mammosphere formation efficiency by induction of EGF, Notch receptor signaling pathways via paracrine regulation[259]. Earlier in this thesis, endocrine resistant mechanisms caused by other growth factor signaling where discussed, however the role of BSCs in endocrine resistance and the underlying mechanism is not well understood. One of the aims of my doctoral thesis is to study this mechanism and to elucidate the effect of tamoxifen on BSCs versus the adherent cells. In a recent study, a novel variant of ERα, called ERα-36 found to be highly expressed in ER-positive breast progenitor cells and meditate antiestrogen resistance through induction of P13K/AKT in BSCs[260].

Apart from ERα, there is a second estrogen receptor called ERβ. These two ER subtypes are coded by different chromosomes. ERβ also binds with high affinity to estradiol-17β (E2) with its ligand binding domain (LBD), as there is a 58% homology in the protein sequences of the LBDs between the two Estrogen receptors[261]. Interestingly, they both share high homology (96% in human) in their DNA binding domains, which can bind to specific DNA sequences such as ERE (Estrogen Regulating Elements) and transcriptionally regulate the ER target genes. ERβ has been identified as the major form of ER expressed in the normal breast tissues using immunohistochemical (IHC) analyses, located in the luminal and myoepithelial cells of the healthy breast. ERβ is also present in various other cell types such as fibroblasts and lymphocytes in the tumor microenvironment[262]. ERβ expression has been studied in ERα-positive and negative breast cancers. Around 58% of the cancers express both ERα and ERβ; while 14% express only ERα and 18% express only ERβ[263-265]. Studies determining the ERβ expression at the protein level using IHC techniques on the breast cancers were not always in consensus. In some studies, high levels of ERβ expression, irrespective of their ERα status correlated with a better response to hormonal therapy and a longer disease free survival[266-269]. In contrast, ERβ expression in ERα-negative breast cancers correlated with a poor prognostic phenotype such as increased proliferation has also been reported[270,271]. One possible reason for such variation in the expression studies of ERβ using IHC is due to the co-existence of multiple ERβ isoforms. Until now, the expression levels and the role of ERβ in BSCs are unknown. One of my paper in this thesis addresses this issue to evaluate the ERβ's role in cancer stem cells and if we can potentially target them for endocrine treatment using ERβ specific antagonist.

1.9 METASTATIC BREAST CANCER

Breast cancer mortality is solely a consequence of the spread of primary cancers to distant sites known as metastasis[272]. Disseminated cancer cells from primary breast cancer can spread to multiple different organs and most preferably to bone, lung and liver[272]. Metastasis formation

is a multi-step process, where cancer cells acquire the ability to invade surrounding tissues, penetrate into lymphatic or blood vessels (a process referred as "Intravasation"), survive in circulatory system and eventually extravasate through the endothelium of a new distant organ, where the cancer cells attach and proliferate to form secondary cancer lesions[273]. Understanding the biological mechanisms behind the formation of metastasis are of utmost importance to prevent and eradicate metastatic disease. In this thesis, one of the most important mechanisms involved in the process of metastasis formation named "Epithelial to Mesenchymal transition- EMT" is discussed in detail.

1.9.1 Epithelial to mesenchymal Transition (EMT)

Epithelial to mesenchymal transition (EMT) process is involved in organ developmental process during embryogenesis[274]. As the name suggests, this process involves in changing the phenotype of an epithelial cell to a mesenchymal state and this process is exploited by cancer cell during cancer progression[275]. By EMT, cancer cells disrupt the cell adhesion molecules (CAMs)[276], degrades neighboring extra cellular matrix (ECM)[277] and the basement membrane, thereby increasing the motility and invasiveness of cancer cells[278]. Additionally, cells that have gone through EMT process acquire resistance to senescence and apoptosis[274]. Therefore, the EMT process is crucial during early stages of cancer cell dissemination from primary cancers.

1.9.1.1 Regulators of EMT

EMT processes can be induced by a plethora of signaling pathways such as transforming growth factor-β (TGF-β), Wnt, Notch, tumor necrosis factor-α (TNF-α/NF-kB) and P13K/AKT pathways[279]. Several transcription factors, including the snail/slug family, twist, EF1/ZEB1, SIP1/ZEB2, and E12/E47, respond to different micro environmental stimuli and function as master regulators of the EMT program[279]. In addition, snail family proteins collaborate with other transcription factors, such as twist and ZEB1, to orchestrate the EMT regulation[280]. One of the main characteristics of EMT induction is loss of E-cadherin (a key cell-cell adhesion molecule) expression or activity [278]. Twist, snail and slug represses E-cadherin expression by directly regulating the E-box elements present in promoter region of this gene by recruiting co-factors and histone deacetylases[281]. Apart from the downregulation of E-cadherin, β-and γ-catenin expression, mesenchymal genes such as vimentin are upregulated. This results in alterations of morphological features (spindle-like) and increased migration in cells undergoing EMT[282]. High expression levels of snail have been observed in some invasive breast cancer[283] and is linked to tumor grade, metastasis, recurrence and poor prognosis[281,284,285].

Apart from the signaling pathways, miRNAs are also a major player in regulating EMT program. When EMT program is induced, the expression of several miRNAs (miR-200 family and miR-205) are drastically reduced[286]. miRNA-200 family directly regulates the expression of ZEB1 and ZEB2 mRNA, thereby increasing the E-cadherin expression leading to epithelial phenotype[286]. Loss of miR-200 is reported in invasive breast cancer cell lines with mesenchymal phenotype. On the other hand, miR-10b expression is increased during EMT process, induced by twist and limiting the expression of HOXD10, which in turn facilitates the metastasis of breast cancer cells[287]. Other miRNAs such as miR-155, miR-29a and miR-21 are reported upregulated by TGFβ induced EMT. Also, their expression levels are higher in

mesenchymal like cell lines compared to epithelial like cells[288,289]. Overall, the differentially regulated miRNAs might be critical for EMT and cancer metastasis. Finally, EMT can also be regulated at genetic and epigenetic level. For example, a gene mutation and hyper methylation at the promoter region of E-cadherin can inactivate this gene[290]. Molecular mechanism leading to promoter methylation of E-cadherin in breast cancer is not well understood.

1.9.1.2 EMT and stemness

Induction of EMT is closely associated with "stemness" in development process and carcinogenesis. During the gastrulation process, embryonic stem (ES) cells in the inner mass of the blastocyst have epithelial phenotype[291] which ingresses to form the primary mesoderm[292] via induction of EMT process, illustrating the importance of EMT during the early differentiation process. The association of EMT and stemness also extends to carcinomas. Expression of snail and twist in mammary epithelial cells induce EMT, leading to a $CD24^{-}/44^{+}$ phenotype[186], which is associated with breast cancer stem cells (as described before under breast cancer stem cell section). TGFβ signaling seems to be associated with EMT and CSC formation in cancer. Mammary CSCs express high amounts of TGFβ1 and TβRII than the more differentiated epithelial counterparts, and inhibiting TGFβ signaling in CSCs can re-establish the epithelial phenotype[293]. Apart from TGFβ signaling, Notch and Wnt Signaling also contributes in CSC generation in colon and pancreatic cancers, which is also known to induce EMT process[294,295]. Recently, a core EMT gene signature was identified and it correlated with claudin low and metaplastic breast cancer subtypes[296].These evidences suggests that induction of EMT and the gain of CSC-like properties are closely linked, which may be crucial for metastasis. Changing phenotype from epithelial to mesenchymal state might be crucial for acquiring invasive abilities and survival benefits during systemic circulation for metastatic seeding.

1.9.1.3 EMT and therapeutic resistance

Considering the relationship between CSCs and EMT process, intrinsic drug resistance of CSCs can be partially explained by EMT mediated drug resistance. Indeed there are several reports suggesting that, EMT induction can cause therapeutic resistance. EGFR induced EMT is associated with increased tamoxifen resistance and increased invasiveness in the MCF7 cell line[297]. In another study, doxorubicin treatment increased the fraction of cells with EMT phenotype and they were resistant to vincristine and pacilitaxel[298]. Increased expression of twist was observed in highly invasive breast cancer cell lines, and the upregulated twist provided survival benefits and paclitaxel resistance[299]. Snail and slug over expression renders cellular resistance to apoptosis in MCF7 cells induced by the DNA damaging agent doxorubicin and increases invasive properties[300]. Basal-like tumors are often associated with poor clinical outcomes and it is interesting to note that, a subset of basal-like breast cancer cell lines were found to be clustered together with mesenchymal transcriptomic profile[301]. Further investigation on these specific cell lines demonstrated that they were associated with an EMT phenotype, such as reduced expression of E-cadherin and increased expression of vimentin[302]. Underlying mechanisms between EMT process and drug resistance are not well understood. It is important to determine whether therapeutic drugs enrich for cells with EMT phenotype or these drugs induce EMT and in turn makes them therapy resistant. Identification of these

molecular mechanisms contributing the EMT and CSC induced drug resistance are crucial for the development of novel therapeutics to treat metastatic disease.

1.9.1.4 EMT and Immunosuppression

Immune surveillance is the host protection system against microbes and infections, and also for early cancer prevention. Cancer immune surveillance is critical for inhibiting tumorigenesis and to maintain cellular homeostasis. Unfortunately cancer cells evade immune surveillance by suppressing immune cells in the host[303]. It has been reported that cancer cells undergoing EMT acquire immune suppressive properties[275], suggesting that cancer cells can utilize the EMT process for survival during cancer initiation and cancer cell dissemination to escape immune system. It has been reported that snail-induced EMT increase metastasis through induction of immunosuppressive cytokines and regulatory T-cells (Treg), as well as impairing the dendritic cells (DC) and cytotoxic T-cell functions[304]. Further, snail knock-down significantly reduces tumor growth and metastasis formation by increasing tumor-infiltrating lymphocytes and systemic immune responses[304]. Another signaling pathway regulating the EMT process is Wnt/β-catenin, which is also known to generate regulatory DCs and increase regulatory T-cell survival, thereby compromising the cancer immune surveillance[305]. Therefore, therapies targeting EMT process could be both anti-metastatic and anti-immunosuppressive in cancer patients[274].

1.9.2 Mesenchymal to epithelial Transition (MET)

Substantial amount of research is currently focused on identifying the biological processes involved in metastasis formation such as, EMT. However less is known regarding, how disseminated tumor cells are colonizing distant sites. Mesenchymal to epithelial transition (MET), opposite of EMT process has been proposed as the main mechanism for the successful seeding and outgrowth of metastatic lesions at distant sites such as bone, lung, liver, brain etc., Intravasated cancer cells from primary tumor are capable of surviving in systematic circulation, this may be due to their EMT phenotype[274]. Extravasation of disseminated cancer cells (also referred as circulating tumor cells – CTCs) to secondary distant site requires recognition and adhesion to vascular endothelial cells and invade distant organ by matrix degradation[273]. All these processes demands a highly plastic and motile phenotype similar to "mesenchymal like" cells[306]. MET process is a relatively under investigated mechanism which might be crucial for colonization of cancer cells in distant organs[307].

1.9.2.1 MET during metastasis formation

Importance of EMT process in early stages of metastasis were discussed earlier, emphasizing that disseminated cancer cells from primary tumors are in "mesenchymal-like" phenotype. However distant metastatic lesions have been reported as of "epithelial-like" breast cancer phenotype in the ectopic tissues[308,309]. Researchers have reported that E-cadherin expression (loss of E-cadherin is the hallmark for EMT process) is equal or higher in distant metastases when compared with their respective primary cancers[308]. In two other studies, E-cadherin expression was observed to be higher in metastatic lesions, originating from E-cadherin- low or negative and poorly differentiated primary cancers[310,311]. More than 50% of liver, brain and lung metastases express high levels of E-cadherin compared to the infiltrating primary breast

ductal carcinoma[309]. Using *In-vitro* xenograft mice models, injection of invasive, metastatic mesenchymal like MDA-MB231 breast cancer cells in mice produced spontaneous lung metastases expressing higher E-cadherin than the parental tumor cells[310]. Mice xenograft experiments using MDA-MB-468 breast cancer cells revealed a gradual transition of invasive cancer cells with mesenchymal phenotype to epithelial phenotype in lymph vasculature[312]. These data suggests that, MET process can be a plausible explanation for re-expression of E-cadherin and importance of MET at metastatic sites. Micro environmental factors in the distant organs also contribute to the re expression of E-cadherin and the subsequent MET process. In another study, E-cadherin negative MDA-MB231 cells re-gained E-cadherin expression when co-cultured with hepatocytes in a metastatic xenograft model, indicating the significance of micro environmental ques on the MET process[313].

1.9.2.2 MET is crucial for active cell proliferation

Several reports have demonstrated that invading cancer cells undergoing EMT proliferate less compared to their non-migratory primary cancer cells[314,315]. Invasive front of primary colorectal adenocarcinoma are reported to have low Ki-67 expression, while the center of the tumor expresses high Ki-67[314]. They observed low E-cadherin expression and nuclear localized β-catenin in these Ki-67 negative cells. Another study reported higher expression of cell cycle inhibitor, $p16^{INK4A}$ (inhibitor of kinase 4) in invasive front of colorectal cancers compared to the tumor center, suggesting the inverse correlation of EMT and proliferation[314]. EMT induced reduction in cell proliferation is believed to be caused by EMT regulators such as β-catenin, snail and ZEBs. For instance, over expression of snail and ZEB2 induced EMT and demised cyclin D1 in kidney (MDCK) and epidermoid carcinoma cells[316]. In colon cancer cells, ZEB1 expressed in invasive fronts and is associated with lower proliferative markers[317]. Therefore, for a successful outgrowth of cancer cells to colonize the secondary site, it seems the transition from mesenchymal phenotype to epithelial phenotype is required to provide growth advantages.

Factors secreted by active stromal compartment such as bone marrow-derived myeloid progenitor cells, present in the secondary site microenvironment induces MET process on MDA-MB231 cells and provide a favorable pre-metastatic niche[318]. This factor (chondroitin sulphate proteoglycan versican) increased the cell proliferation by suppressing snail expression in MDA-MB231 cells thereby aid in formation of metastases in a xenograft model. In a recent study, Lawson et al, demonstrated that metastatic cells exhibit EMT and stemness characteristics in low-metastatic burden sites compared to high-metastatic burden tumors in PDX models, using FACS (Fluorescent activated cell sorting) based single cell analysis[319]. High metastatic burden tumors were similar to primary tumor, which were more heterogeneous and expressed high levels of luminal differentiated genes with increased proliferation[319]. Therefore, initiation of metastatic process by EMT and stem-cell like cells followed by MET during re-colonization of cancer cells at the secondary site is crucial for establishing advanced metastatic disease. Although this alteration of EMT to MET are demonstrated using cell lines and xenograft models, no direct evidences of MET in actual human derived metastatic lesions compared to their respective parental primary cancers are studied until now. Furthermore, it is unclear whether this MET in metastatic lesions are stable phenotype or these cells can further undergo another round of EMT process to seed successive metastases. In summary tumor progression can be conceived as a highly dynamic process with change of different phenotypes

rather than a stable process where cancer cell progress with higher and higher degree of dedifferentiation and proliferation.

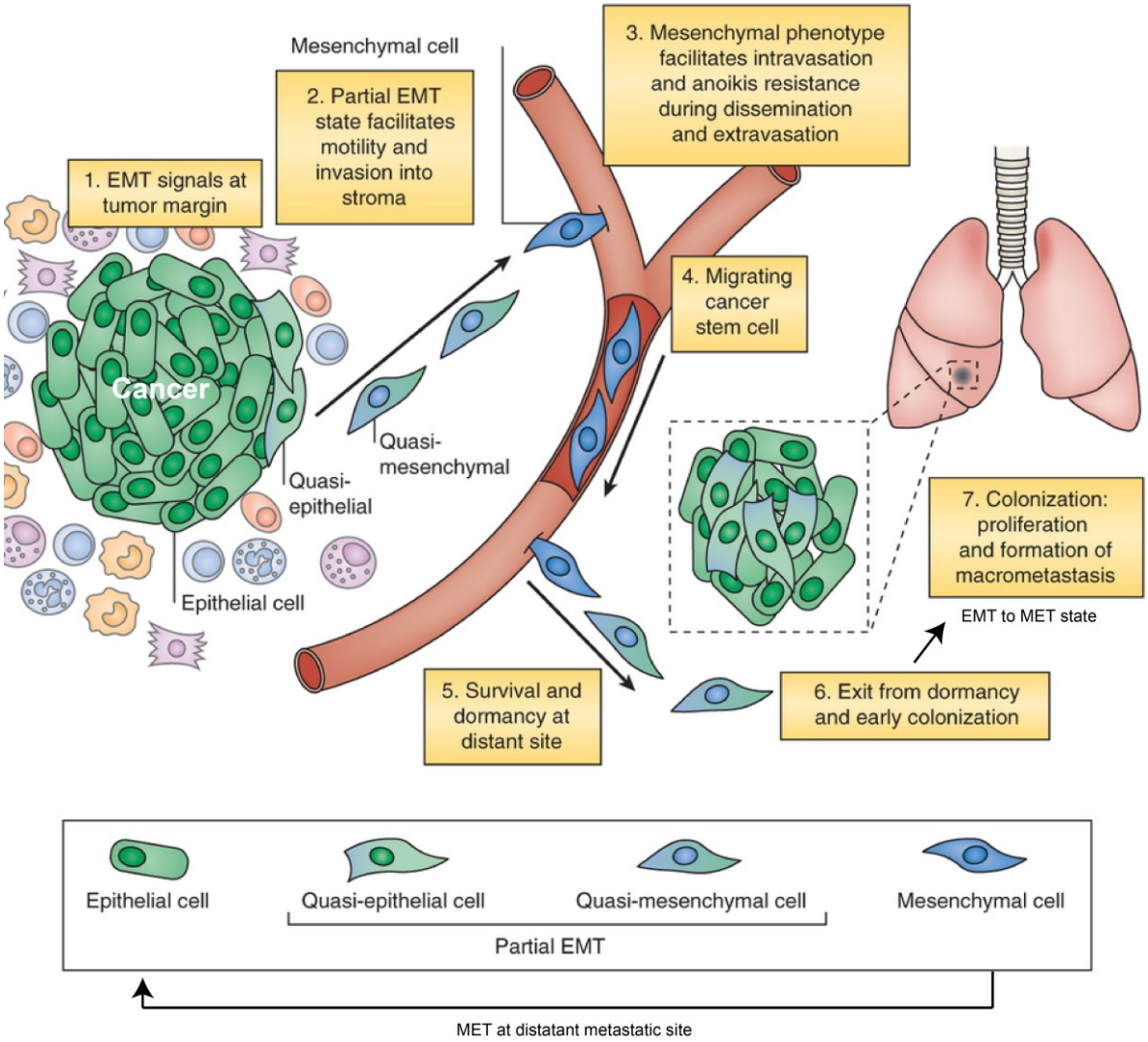

Figure 4: Schematic representation of biological processes involved during metastasis. Modified from Wai Leong Tam and Robert A Weinberg (2013)[320]. Reprinted with permission from Macmillan Publishers Ltd: Nature Medicine, copyright (2013)

1.10 MODES OF BREAST CANCER METASTASIS AND SEEDING PATTERNS

In breast cancer biology, it is not clear how distant metastases arise from primary cancer. Much uncertainty still persist on when cancer cells dissemination occur, either at early or at late stages of cancer development, and whether multiple distant metastases arise directly from primary tumors (in parallel fashion) or they give rise to each other (linear or cascading fashion) remains to be answered. Genetic alterations during cancer progression acts as forensic evidence, to study the phylogenetic relationships of metastases and primary cancer. Most genetic alterations may or may not be crucial for actual metastases formation process, however they can provide valuable information regarding past dissemination events and their origin. Four models of metastatic progression has been proposed; linear and metastatic cascade progression, parallel progression, tumor self-seeding and dormancy[321].

1.10.1 Linear progression model

"Linear progression model" is one of the traditional models suggested for metastatic progression in cancer. This models assumes that only genetically advanced cells can leave the primary tumor and successfully colonize a distant site. Multiple clonal expansions during primary cancer development leads to cancer cells with disseminating phenotype in a stepwise manner[322]. Therefore, in this model metastasis is assumed to occur shortly after a tumor becomes clinically detected, that is during later stages of primary cancer development. Further linear progression model predicts a smaller evolutionary distance between primary and metastatic lesions thereby suggesting that primary tumors can be a good surrogate for the molecular phenotypes of metastases. However, there is another model similar to linear progression called "metastatic cascades", during later stages of cancer progression[323]. Highly vascularized central organs such as lung and liver are reported to give rise to successive metastases in a cascading manner also referred as "shower of metastases"[324]. Metastatic cascade model predicts aggressive and high growth rate of metastases[323]. According to this model once, a distant organ is colonized with a metastatic cancer cell population, they in turn give rise to successive metastases which are more-closely related to each other than to the primary tumor.

1.10.2 Parallel progression model

Parallel progression model assumes that the cancer dissemination occurs early in the tumor progression[325] and that primary cancer and distant metastases evolve independently[323]. Parallel progression is opposite to linear progression model as it suggests that disseminated cancer cell doesn't have to be in a genetically advanced state (high mutational load) and few mutations can be enough to trigger the dissemination process. According to this model, distant metastases can evolve separately and extensively adapt itself to the local microenvironment, thereby substantial genetic diversity between metastases and its primary tumors, as well as between metastases at different anatomical sites is observed. Under this model, using primary cancer characteristics for treating metastatic disease becomes invalid.

1.10.3 Tumor self-seeding / bi-directional seeding model

"Tumor self-seeding" model is a recently coined hypothesis which proposes that cancer cell dissemination can be bidirectional, with dynamic cell exchange between synchronous tumor lesions[326], while both linear and parallel models regard metastasis progression as unidirectional process that begins from primary tumor and ends in metastases. Primary cancers are proposed to shed cancer cells into systemic circulation, where highly selective circulating tumor cells (CTCs) can return to the same primary tumor to drive progression. Similarly, metastases at a distant site can shed cancer cells into the systemic blood circulation and then back to the primary tumor. This process can hinder the genomic evidences of independent tumor evolution at different anatomical sites as in the case of parallel progression.

1.10.4 Dormancy model

Dormant cancer cells may be one of the reasons for really late recurrences that is, after more than 10 years of disease free survival[327]. Dormancy model proposes that disseminated cancer cells can be dormant for many years by being in a "senescence-like" state at a potential distant

site or colonized tumor mass with very low proliferation ("tumor mass dormancy") at distant sites. Since dormant cells do not divide, they do not accumulate mutations, therefore genomic evolutionary analyses doesn't reveal whether a metastatic lesions arose at late stages of cancer progression or they underwent a period of dormancy at the distant site. Until now, tumor mass dormancy cannot be demonstrated in human cancers, as it is difficult to find a tumor mass at distant metastasis which is not dividing, metachronous, and it should ideally be genetically identical or very similar to the primary cancer without any specific accumulation of mutations.

Recently, patterns of metastatic spread in prostate cancer was revealed by sequencing multiple metastatic lesions along with their respective primary tumors in ten patients. This study demonstrated that prostate cancer cells can spread in both monoclonal or in a polyclonal fashion and metastasis can seed successive metastasis (metastatic cascading)[328]. Metastatic cascading model was also evident in pancreas[329] and renal cancer[330]. However it is still unknown whether metastases can give rise to successive metastasis in breast cancer. In breast cancer studies are limited to multi region DNA sequencing of primary breast cancers that revealed high intratumoral heterogeneity and subclonal evolution during breast cancer progression[83]. In addition, DNA sequencing have been performed in the primary tumor and one metastasis of a single patient[78-80]. In order to investigate progression models, more than one distant metastatic lesion and primary tumor must be sequenced, and acquiring such multiple metastatic lesions from the same individual is difficult. In a recent study, multi colored lineage tracing xenograft experiments demonstrated that breast cancer metastases arise from multiple subclones present in the primary cancer[331]. More extended validation of this polyclonal seeding phenomenon has to be validated in clinical samples.

1.10.5 Involvement of axillary lymph node metastasis in distant metastatic spread

It is still unclear if distant metastases are seeded via the lymphatic or haematogenous route in breast cancer. Local regional and distant lymph nodes are the most common sites of metastatic engagement and are two-fold more frequent than lesions in the second most common site of metastasis i.e., liver[332]. Although positive axillary lymph nodal status has high negative prognostic value in breast cancer[333], its role in seeding distant metastasis has not been validated in clinical studies. Due to its negative prognostic value, it has been assumed that axillary lymph node metastases may be the precursor of distant metastatic lesions. This motivated extensive surgical and radiotherapy interventions to eradicate local regional disease[334]. However some researchers question the benefit of such interventions[335,336]and even argued that lymphatic lesions are unlikely to seed distant metastases[337]. One study also reported that positive axillary lymph nodes do not metastasize[338]. Evident role of ipsilateral axillary lymph node metastasis in seeding distant metastasis is not yet proven. Inferring this, will provide more insights about the routes of metastatic spreading and the importance of axillary lymph node metastasis during cancer progression. One approach to investigate this issue would be to sequence the axillary lymph node metastases along with the subsequent distant metastases and primary tumor, and performing phylogenetic analyses to understand their genetic relationship between them can be the way forward to answer this question.

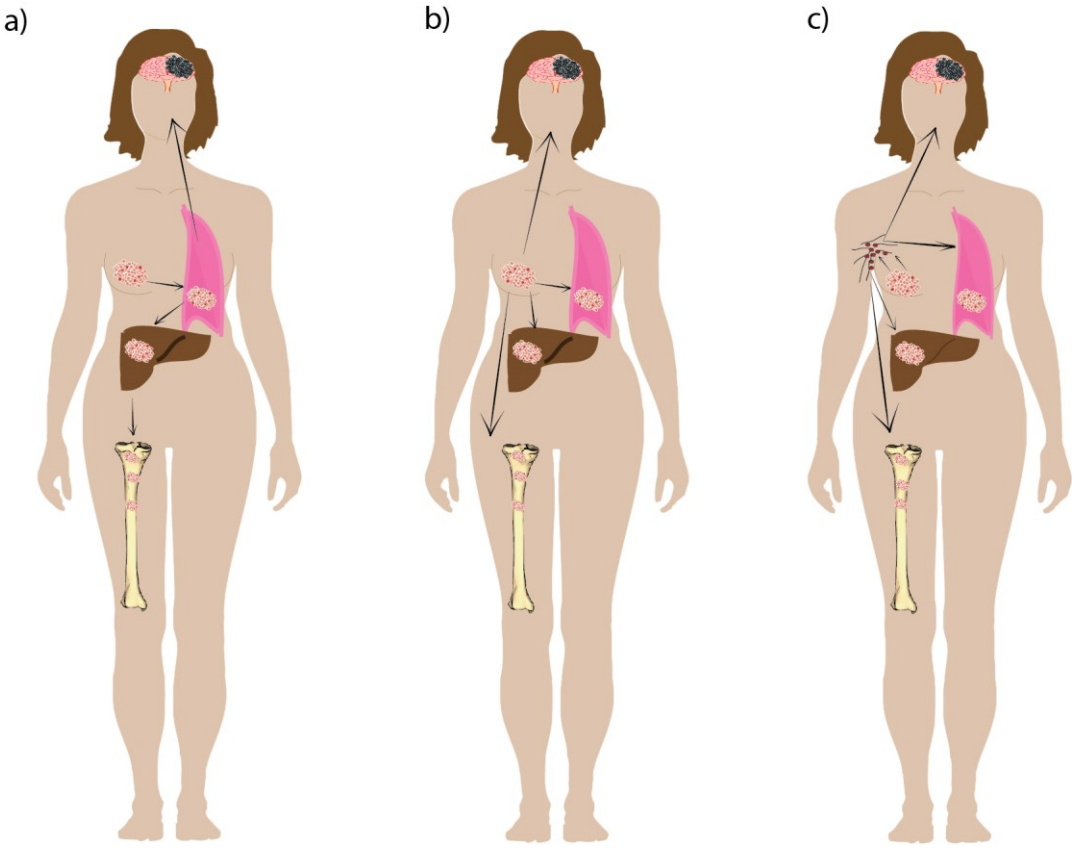

Figure 5: Schematic representation of different cancer progression models. a) Linear progression model b) parallel progression model c) axillary lymph node seeding distant metastasis

1.11 ALTERED CANCER BIOLOGY IN METASTASIS

Primary breast cancers are curable to about 50% [339] with present treatment regimes. However, distant metastasis are rarely curable with the same treatment strategies. The difference in the treatment response between primary cancer and distant relapses are attributed to an ongoing evolution of cancer cells leading to treatment resistant cancer cell populations[84]. In our group, we have previously shown that clinically used markers such as ER, PR and HER2 are altered in recurrent breast cancers when compared to their respective primary cancer. ER, PR and HER2 were altered in 33.6%, 32.0% and 15.7% of patients respectively when assessed with multiple consecutive relapses to their primary cancers[164]. Further, patients with ER-positive primary tumors that changed to ER-negative in metastatic lesions had a significant increased risk (48%) of death[164]. From this study, it is evident that breast cancer evolves during cancer progression leading to change in clinical biomarker expression and in turn affecting the outcome. Alterations in therapy predictive biomarkers in metastasis also lead to change in clinical management in 1 out of 6 to 7 patients[340,341]. Although these studies are based on IHC based analysis, alterations of molecular intrinsic subtype based on PAM50 analysis during cancer progression using matched primary-metastasis will provide more additional validation of the existing data.

Alterations in the tumor characteristics during cancer progression can be attributed to one or more following reasons; First, primary breast cancer are known to exhibit high intra tumor

heterogeneity with varying metastatic capabilities[273]. Second, inherent host and cancer biology can induce clonal expansion of specific subclones in primary tumor, leading to the spread of these sub populations with distinct characteristics to colonize distant sites thereby affecting patient's survival outcomes[342]. Third, biological process such as EMT-MET during metastasis, and microenvironment at systemic circulation and at distant organs has the potential to enrich certain subclones[273]. Finally, adjuvant therapies can also induce enrichment of treatment resistant clones which can metastasize with different characteristics from those of primary cancers[343].

Treatment in the metastatic setting is often based on the characteristics of the primary tumor. However, it is evident that standard biomarkers can change during cancer progression. Therefore, investigating the tumor characteristics of relapses are crucial to improve the patient survival. Many breast cancer clinical management consortiums such as Conference on Advanced Breast Cancer (ABC1), American Society of Clinical Oncology (ASCO), European Society for Medical Oncology (ESMO) and Swedish Breast Cancer Group (SweBCG) now recommends to examine the relapse tumor characteristics with regards to the expression of ER, PR and HER2[344-346]. Before we begin to personalize the clinical management in a metastatic setting, we must validate the relapse tumor characteristics with patient survival. For this purpose a study from our group previously reported that intrinsic molecular subtype of relapses significantly influences post-relapse survival based on relapse tumors from 111 patients, suggesting that molecular characterization of distant lesions does provide prognostic and clinically relevant information[347]. Moreover, this study also identifies key signaling pathways such as high AKT-MTOR, RAS and BETA-C are significantly associated with poor post-relapse survival. Patients with poor-post relapse survival were also found to express high basal-like, cell cycle and mesenchymal related genes, and express low amounts of luminal and apoptotic pathway genes[347].

Analyzing multiple metastatic lesions for therapy management is difficult as it requires complicated invasive procedures and can be confounded by intra-tumor heterogeneity[330], therefore noninvasive liquid biopsies would be ideal to monitor the tumor progression. Recently it has been shown that, blood in systemic circulation contains DNA derived from different metastatic lesions referred as "circulating tumor DNA" (ctDNA) and tumor cells which are shed by metastatic tumors known as "circulating tumor cells" (CTC)[348]. Latest advancement in the field of genomics has made it possible to use ctDNA for determining genomic alterations such as rearrangements[349], amplifications[350] and to identify therapy induced resistant mutations[351]. Investigating them would provide a more comprehensive overview of the status of genomic alterations across different metastatic lesions than analyzing a single metastatic lesion[350]. Several researchers have been able to confirm that mutations present in ctDNA are concordant with that of the matched primary tumor[351,352]. Comparative genomic analysis on plasma derived ctDNA and synchronous metastatic lesions in two patients revealed a good overall correlation between them, but also reported an increase of certain mutations allele frequencies[350] as it represents the bulk of tumor burden from different metastatic sites, which can be an advantage for such a method. Currently less is known regarding the mechanism of DNA release from the tumor cells. It is highly important to know what type of cancer cells release DNA; whether it is highly proliferative or dying cancer cells. If it is dying cancer cells, then it is not as clinical important as it has limited potential to target

cancer with that acquired information from ctDNA. On the other hand, CTCs are intact cancer cells from which functional studies can be performed to reveal novel therapeutic targets and identify drug resistance mechanisms during cancer progression. Thus, CTCs are also crucial to investigate which can provide additional information regarding the metastatic disease, complementing ctDNA. Intra and Inter metastatic tumor heterogeneity might complicate the interpretation of the data, but in principle ultra-deep sequencing of ctDNA can provide additional information on tumor heterogeneity and clonal evolution at distant metastatic lesions as well[353], although this is yet to be proven. Further, sensitivity, specificity, predictive and prognostic value of ctDNA in clinical setting has to be evaluated. If clinically proven, ctDNA has the potential to strengthen the idea of personalized medicine in clinic for monitoring and managing advanced breast cancer disease.

2 AIMS OF THE THESIS

The aim of the thesis is divided in to two parts; first we optimized a working protocol to isolate breast cancer stem cells from breast cancer patients, after which we investigated estrogen signaling and anti-estrogen therapy resistance in breast cancer stem cells (BSCs). In the second part, we investigated the extent of genomic alterations in metastatic lesions with respect to their corresponding primary tumors and the patterns of metastatic spread in humans.

The individual aims of each paper included in this thesis are described below:

Paper I: The aim of this study was to optimize a methodology called "superficial scraping from tumor surface" to biobank small breast tumors for future research purposes, without compromising routine histopathological assessments. We further investigated if this method could be used to isolate and propagate breast cancer stem cells (BSCs) from tumors.

Paper II: In this study, we aimed to investigate the expression and the function of second estrogen receptor; ERβ in BSCs, which can be a potential target for endocrine therapy against BSCs.

Paper III: The aim of this paper was to understand the mechanism of tamoxifen resistance in both cell line and patient derived BSCs.

Paper IV: The aim of this study was to examine the patterns of metastatic breast cancer spread and the role of ipsilateral axillary lymph node metastasis in seeding distant metastasis, using exome sequencing data from matched primary breast cancer and metastatic lesions.

Paper V: In this study, we aimed to investigate to what extent transcriptomic profiling of therapy predictive biomarkers and prediction of molecular subtypes are displaying intra-tumor heterogeneity in breast cancers.

3 MATERIALS AND METHODS

3.1 PATIENT SAMPLES AND ETHICS

All the fresh breast cancer samples were obtained from Karolinska University Hospital, according to the standardized surgical procedures. For the first three papers (Paper I, II and III) included in this thesis, utilized the "superficial scraping technique" to isolate cancer stem cells from fresh tumor samples. Detailed methodology of superficial scraping technique is discussed in Paper I. Paper II and Paper III included around 88 and 23 fresh cancer samples patients respectively. Normal breast samples were acquired from reduction mammoplasties surgeries at Capio St Göran's Hospital, Stockholm, Sweden. For Paper IV, we assembled a retrospective cohort of 20 patients with metastatic disease. These patients were identified through the IT-support system using the digitalized patient medical records. The search term was "metastatic adenocarcinoma" and the years included were 2000 through 2011 (N=3823). We retrieved the FFPE tumor blocks from primary tumors, local recurrences, ipsilateral axillary metastases and distant metastases for these patients. We isolated RNA and DNA, from all the FFPE tissue blocks, to perform gene expression and whole exome sequencing. Tissue microarray (TMA) blocks were made to analysis standard breast cancer biomarkers using IHC. For the last Paper (Paper V), fresh tissue biopsies were isolated from 12 breast cancer patients. From each one of these patients at least two spatially separated tumor pieces were collected and snap-frozen. In total, 43 pieces were collected (2-6 pieces per tumor). For all studies, permits were acquired from the Stockholm medical biobank and the experimental procedures were approved by the regional ethics review board (Etikprövningsnämnden) in Stockholm, Sweden. All the patient IDs were coded for study purposes and anonymised for publications.

3.2 CANCER STEM CELL ISOLATION

Cancer stem cell isolation from primary breast cancer samples and cell lines is a crucial methodology deployed in most of my papers. We used selective medium containing necessary growth factors such as EGF, FGF and insulin, and lacks serum. This media promotes cells to grow in ultra-low attachment conditions and aids self-renewal and a stemness phenotype. Detailed methodology of cancer stem isolation is described in Paper I. We have characterized the cancer stem cells isolated using this method using FACS and immunofluorescence (IF) staining techniques. FACS identified around 30% of mammospheres as ALDH1^{high} and 40-50% of mammospheres as CD44+/CD24- (Paper I, Figure 3c). IF staining illustrated that cultured mammospheres were CD44+/CD24-, ALDH1^{high} and EpCAM+.

3.3 MOLECULAR TECHNIQUES

In this thesis, a number of different molecular techniques have been used. Some of them are stated below. Effects of different compounds on the growth of cancer stem cells are measured using mammosphere formation and proliferation assays. Next, in order to study the protein of interest in mammospheres, immunofluorescence (IF) technique was used after cytospin technique. This technique requires very low amount of cells, thereby it is perfect for staining valuable patient materials. Western blot analysis were performed to study the panel of proteins involved in a signaling pathway (e.g., mTOR pathway), but it requires large amount of sample, therefore, cell line derived spheres were mostly used for western blot analysis. For adherent

cancer cell lines, WST-1 assay was used to measure the cell viability after treatment with drug of interest. Quantitative real time PCR is also a major technique, used throughout my thesis to measure mRNA levels at different experimental conditions. Detailed methods are described in the "materials and methods" section of each paper.

3.4 BIOINFORMATICS

A wide range of bioinformatics were performed in this thesis. Analysis and interpretation of the raw data acquired from different experiments was performed by myself, with the support of bioinformaticians in our group. In paper II and paper III, microarray based gene set enrichment analysis (GSEA) were performed to identify pathways, which are significantly affected during specific drug treatment in cell line and patient derived CSCs. In paper IV, whole exome sequencing was performed on 99 FFPE tumor samples from 20 breast cancer patients, to investigate cancer progression. We investigated the evolutionary history of cancer cells across different tumor sites of the same individual by a method called "Dollo parsimony". Further, subclonal analysis was performed using a method called "Pyclone". Our collaborators at the University of Helsinki, provided bioinformatics support on mutational signature analysis during breast cancer progression. In paper V, RNA sequencing and microarray analysis were performed on multiple regions of same primary tumor. Intrinsic molecular subtypes were determined based on the gene expression profile to demonstrate the transcriptional intra tumor heterogeneity. Detailed description of the methods are provided in the "materials and methods" section of each paper.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Superficial scrapings from breast tumors is a source for biobanking and research purposes.

Apart from the routine pathological assessments many cancer hospitals store the valuable tumor specimens for future research projects or for future clinical investigation[354]. Currently there are two ways of bio banking tumor samples; 1. Resected breast cancer tissues are preserved in formalin fixed paraffin embedded blocks (FFPE). These blocks are ideal for investigating predictive IHC markers and for any protein of interests in future. DNA isolated from sections of FFPE blocks are now used for whole genome sequencing analysis[355]. RNA and protein isolation from FFPE is still not well optimized. 2. Surgically removed breast cancer tissue pieces are snap frozen and stored in a low-temperature freezers. This storage method is good for preserving DNA, RNA and protein for later research purposes[356,357]. For downstream molecular analysis such as DNA/RNA sequencing, fresh frozen tissues are preferred over FFPE blocks. But there are problems with biobanking small breast tumors. With the latest advancement in mammography, increased number of patients are surgically treated at early stage with very small tumors. Moreover, increased usage of neo-adjuvant therapies leads to reduced tumor sizes at the time of surgery. Tumors less than 10 mm are currently difficult to biobank because all the resected tissue is needed for routine histopathological analysis. This might lead to storage of increased number of large tumors and reduced number of small tumor, thereby creating an inherent bias for later research activities. Therefore, there is a need for an efficient and elegant method to biobank small breast tissues for research purposes.

In this study we reported a simple and robust technique called “superficial scraping method” for isolating epithelial breast cancer cells. First, we investigated the tumor cell percentage present in the smears after scraping the tumor surface. Cytological assessment of the smears was investigated by a board-certified cytologist. Microscopic evaluation of smears from eight patients revealed that all eight patients had more than 95% cancer cells in their smears, while corresponding hematoxylin-eosin stained tissue sections contained 75-80% cancer cells.

One of the most important part of the study was to generate CSCs by the scraping method. It has been previously reported that CSCs can be isolated by culturing breast cancer cells *in vitro* in ultra-low attachment plates by letting them form multicellular spheres[180,358]. Normal adherent cancer cells tend to undergo apoptosis under these conditions leading to enrichment of cancer cells with CSC phenotype in the form of mammospheres[180,358]. Tumor cells are isolated by scraping three-ten times by scalpels on the surface of the tumor and then directly transferred into conditional stem cell media (serum free) and cultured in ultra-low attachment plates. After 3 days of culturing, tumor cells tended to form clumps that are enzymatically dissociated into single cells and continued to be culture in conditional stem cell media for 5 more days, leading to mammosphere formation. We could able to produce mammospheres from all four major molecular subtypes of breast cancer. In addition, we could also able to propagate the cultures for three more generations. Immunocytochemistry revealed the presence of CSC markers CD44+, CD24-, ALDH1+ and PKH26+ in patient derived mammospheres. Further, using FACS, we determined that around 30% of cells in mammospheres were

ALDH1+ and 40-50% of cells were CD44+/CD24-. These experiments suggested that the mammospheres isolated using scraping method are phenotypically similar to CSCs. Adherent patient derived cancer cells could also be cultured by dedifferentiating mammospheres in serum rich medium.

Next, we analyzed the DNA/RNA isolated from fresh frozen scrapping material and compared it with the fresh frozen bulk tumor. Data revealed that DNA/RNA isolated from both materials are of similar quality and meet the quality assurance standards for next generation sequencing (NGS). Further, RNA isolated from scrapings were of good quality for downstream RTPCR analyses. We also showed that DNA from tumor scrapings retained the methylation status of genes, after observing higher ERα promoter methylation in an ERα negative patient.

In summary, we show that superficial scraping technique can be used for biobanking small breast tumors and could potentially increase the number of breast cancers biobanked at our hospitals. Approximately 60% of all breast cancers operated at Karolinska University Hospital are currently stored in the form of fresh frozen tissue. By including scraping material we can also include the small tumors in the biobank. Microarray analysis and next generation sequencing are at the verge of entering breast cancer clinics and it is highly important to find novel methods to include majority of the operated breast cancers for this purpose. In this study, we show that the quality of DNA and RNA isolated from scrapings are good for NGS and gene expression studies respectively. Further, we demonstrate that CSCs can be isolated from these scraping materials and clinicians can provide this valuable clinical samples to researchers without hampering their regular pathological assessments. Currently CSCs are isolated from fresh biopsies which are seldom available to researchers. We have used this method to isolate CSCs from patients for two of the following studies included in this thesis.

4.2 PAPER II

Estrogen receptor β as a therapeutic target in breast cancer stem cells.

A subpopulation of breast cancer cells with stem cell phenotype referred as “cancer stem cells” (CSCs) are highly tumorigenic and possess tumor initiating capabilities[179]. Interestingly, a few hundreds of these cells are enough to reestablish tumors in mouse xenograft models[177,179]. Although breast cancer stem cells (BSCs) are ERα negative, these cells can be expanded by incubation with estradiol[359]. This motivated us to investigate the second estrogen receptor; ERβ in BSCs.

Firstly, dual immunohistochemical staining of ERβ and CD44 in a cohort of 187 patients revealed that ERβ was enriched within the CD44+ cell population without any specific correlation to molecular subtype. Mammospheres isolated from patients where characterized for phenotypic stem cell markers. Similar to previous reports we found that CSCs are ERα negative, however they also expressed ERβ. Similar to BSCs, mammary stem cells (MSCs) were also ERβ-positive and ERα-negative. Also, cell-line derived mammospheres expressed 5-11 times higher levels of *ERβ* and other stem cell genes such as *SOX2*, *NANOG* and *OCT4* when compared against adherent counterparts. These results indicate that ERβ is predominantly expressed in MSCs and BSCs.

Next, to investigate if ERβ plays any role in maintaining the stem cell phenotype, shRNA-mediated knock-down of ERβ (MCF7 and MDA-MB231) was performed. ERβ knock down reduced the mammospheres forming efficiency by 41% and 27% in MCF7 and MDA-MB231 cell lines respectively. This phenomenon was also verified in patient derived CSCs. In contrast, overexpression of exogenous ERβ increased the sphere-forming capacity in successive generations of mammospheres. Further, forced differentiation of patient-derived spheres also reduced ERβ expression and ALDH1 expression indicating a tight connection between ERβ and the stem cell phenotype.

To investigate if ERβ can affect the proliferation of CSCs, the ERβ-selective agonist Diarylproprionitrile (DPN-70-fold selectivity over ERα) was used. DPN significantly increased the number of mammospheres from cell-lines compared to the untreated controls. This finding was also confirmed in patient-derived primary tumor cells. Further, DPN treatment of MCF7 spheres induced embryonic stem cell gene expression and induction of the ER-target genes *PR* and *PS2*, which are biomarkers for functional ER-signalling. To further explore ERβ mechanism of action on CSCs, we performed whole-transcriptome analysis of mammospheres. Treatment with DPN revealed significant enrichment of "Glycolysis metabolism pathway". Hence, L-lactate assay was performed on after DPN treatment to validate the effects on the glycolytic process. DPN treatment of both cell line and patient derived mammospheres significantly stimulated L-lactate secretion and the ERβ antagonist 4-[2-Phenyl-5,7*bis*(trifluoromethyl) pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP) neutralized the stimulation by DPN, confirming the specificity of DPN. However, DPN failed to induce L-lactate secretion after ERβ knockdown in MCF7 spheres. Additionally, DPN impaired the oxygen consumption rate of mammospheres, thereby shifting the cells towards glycolysis.

Using *in-vivo* xenograft models from MCF7 and MDA-MB231 cells, we confirmed that ERβ induces tumor growth and can be targeted by PHTPP. We also found similar effects in two patient-derived PDX models (triple negative breast cancers) indicating that targeting ERβ is a potential therapeutic strategy for triple negative cancers. For luminal breast cancer, we sought to investigate whether a combination of tamoxifen and ERβ-modulator would be more efficient to block tumor growth as a consequence of ERβ expression in CSCs. Combining tamoxifen with PHTPP caused a gradual decrease of tumor size with increasing concentration of PHTPP in MCF7 derived xenograft, indicating that PHTPP in combination with tamoxifen can synergistically reduce tumor growth.

This study identifies the predominant ERβ expression in BSCs and the role of ERβ in maintaining stem cell phenotype. ERβ stimulation increase mammosphere proliferation by regulating glycolytic metabolism which is in turn is crucial for the cancer stem cell phenotype[360]. We propose a novel concept of breast cancer stem cells which are estrogen sensitive through the expression of ERβ, and a novel target for endocrine therapy for targeting CSCs.

4.3 PAPER III

mTOR inhibitors counteract tamoxifen-induced activation of breast cancer stem cells

Tamoxifen is a selective estrogen receptor modulator (SERM) and has remained as the major focus in endocrine therapy of ERα-positive breast cancer during the last few decades[22].

Tamoxifen is the first line therapy for hormone receptor positive breast cancers for premenopausal women and even suggested for many post-menopausal women because of its side effects. However, 30-40% of patients with metastatic disease initiated from hormone responsive primary tumors do not respond to tamoxifen[2]. Many mechanisms have been proposed for this effect such as lost ERα expression[164], acquaintance of ERα mutation during endocrine therapy[165], cross reactivity with other growth factor signalling[361], presence of the second estrogen receptor (ERβ)[362] and deregulated PI3K/AKT/mTOR signalling[363] can affect the effectiveness of tamoxifen leading to tamoxifen resistance. In previous work, we observed that tamoxifen doesn't affect the proliferation of patient derived mammospheres. This motivated us to further investigate the tamoxifen resistant behaviour of breast cancer stem cells, which might provide more insights on CSC induced tamoxifen resistance.

First, we characterised the mammospheres derived from patients as described before. Mammospheres showed high expression of ALDH1, CD44, cytokeratin and EpCAM, and low expression of CD24. CSCs were shown to express lower levels of ERα[257]. To study the effect of tamoxifen on mammosphere forming capacity, patient derived mammospheres (n=5) were dissociated and treated with 4-OHT (4-hydroxy tamoxifen - a metabolite of the antiestrogen, tamoxifen, in humans and other mammals) 100 nM for 12 days. Tamoxifen did not affect the viability and mammosphere forming ability of CSCs. Additionally, we tested the effect of tamoxifen on three different ERα positive adherent cell lines (MCF-7, T47D and ZR-75-1) and corresponding mammospheres. Mammospheres were treated with varying concentrations of tamoxifen (100nm-10μM) for 5 days. Similar to patient derived spheres, cell viability of cell line derived mammospheres were not affected by tamoxifen, in fact, tamoxifen increased the mammosphere formation at higher concentrations from MCF7 and ZR-75-1 cells. Further, treatment with tamoxifen significantly increased the expression of stem cell genes such as Oct4, Sox2, Nanog and Notch1 on MCF7 spheres compared to vehicle controls, indicating its potential stem cell enhancing phenotypic effect.

In order to investigate the biological process involved in the tamoxifen resistant phenotype of CSCs, we performed whole transcriptome analysis on tamoxifen treated mammospheres derived from seven patients. Gene set enrichment analysis revealed "ribosome synthesis" and "mRNA translation" processes up regulated by tamoxifen. Further, we observed that these processes were induced only in tamoxifen treated CSC population but not in tamoxifen treated adherent MCF7 cells using publically available datasets. mTOR is the central regulator of ribosome synthesis and mRNA translation process, therefore we hypothesized that tamoxifen could activate mTOR pathway and thereby rendering CSCs tamoxifen resistant. Key mTOR downstream effectors such as phosphorylated S6K1 (Thr389), S6RP (Ser235/236), 4E-BP1 (Thr37/46) and mTOR (Ser2448) were investigated in tamoxifen treated patient derived mammospheres using both immunofluorescences (n=4 patients) and western blot (n=2 patients). Tamoxifen treatment induced phosphorylation of mTOR effectors in CSCs, indicating the activation of mTOR pathway. Further this induction was observed only in CSC population but not in the differentiated adherent cells derived from patients. Inter-individual patient differences were observed in tamoxifen's effect to induce mTOR pathway, as we observed hyper activation of mTOR signalling already present in CSCs derived from some patients. We confirmed the activation of mTOR pathway in CSCs by comparing MCF7 adherent cells and mammospheres generated from MCF7 cells.

We used two mTOR inhibitors (rapamycin and everolimus) and one dual mTOR/PI3K inhibitor (PF04691502) to check if these inhibitors can counteract the mTOR activation induced by tamoxifen on CSCs. In both patient (n=2) and cell line derived mammospheres (MCF7 and T47D), we were able to demonstrate that these compounds antagonized the effect of tamoxifen. Due to hyper activation of mTOR pathway in CSCs derived from cell lines, we used restrictive media (without insulin, EGF and FGF) to reduce the endogenous mTOR stimulation. Combination treatments (tamoxifen + mTOR inhibitors) revealed that tamoxifen induced downstream mTOR effectors when compared to mTOR inhibitors treatment alone on CSCs. Further, mammosphere formation assay on three patient and two cell line (MCF7 and T47D) derived CSCs demonstrated that all the investigated mTOR inhibitors reduced mammosphere formation significantly and the dual mTOR/PI3K inhibitor being the most effective one irrespective of tamoxifen treatment.

Tamoxifen is the most prescribed drug for endocrine therapy as it has profound effects on cell proliferation of bulk tumor cells. However, the effect of tamoxifen on CSCs are not well established. There are reports suggesting that tamoxifen resistant MCF7 cells enrich CSCs formation[364] and these studies have most often been based on ERα-positive MCF7 cells. Therefore, it is important to study the effect of tamoxifen on patient derived CSCs. In this study, using microarray analysis on tamoxifen treated CSCs revealed activation of "ribosome synthesis" and "mRNA translation" which are regulated by mTOR. We show that, tamoxifen has a non-expected side effect by inducing mTOR pathway in CSCs. We believe this effect can contribute to tumour recurrence and therapeutic resistance. Although, the exact mechanism of how tamoxifen induces mTOR pathway is not yet investigated, there are few possible mechanism we can speculate. Since CSCs are known to have lower ERα expression and the effect of tamoxifen stimulation was observed even on CSCs derived from ERα negative patients in our study, the effect could be meditated via ERβ or any other estrogen binding receptors such as GPER (G-protein coupled receptor 30). Few reports support this hypothesis as tamoxifen acts as agonist via GPER in various cancer cell lines[365,366]. More investigation is needed to fully understand this mechanism.

In summary, we report an increased activity of mTOR pathway in CSCs and it is further induced by tamoxifen therapy. However, this induction could be antagonized by adding mTOR inhibitors. Combined treatment with tamoxifen and mTOR inhibitors can be a potential strategy to eradicate both bulk tumour and CSC population in parallel to avoid cancer recurrences.

4.4 PAPER IV

Genomic analyses of primary breast cancers and multiple matched metastatic lesions reveal both linear and parallel progression with minimal role of axillary lymph node metastasis

Metastasis is the main reasons for breast cancer mortality[272]. Currently, metastatic disease are not curable as it is treated based on the primary tumor characteristics. We and others have shown that prognostic and therapy predictive markers alter during breast cancer progression [164,367] which significantly influences their survival. These alterations highlights that breast cancer progression is a dynamic process where an ongoing evolution of heterogeneous cancer

cell populations give rise to treatment resistant clones under therapeutic pressure[84]. Therefore, it is important to investigate the genomic landscape of metastatic breast cancer with respective to their corresponding primary tumors to identify novel therapeutic targets and biological processes involved in metastasis formation. Further little is known regarding the patterns of breast cancer spreading and the involvement of ipsilateral axillary lymph node metastasis in breast cancer progression. To address these issues, we have performed whole exome sequencing on 99 tumor samples from 20 breast cancer patients with multiple spatially and temporally distributed primary tumors, local recurrent, axillary lymph node and distant metastasis.

We observed high inter-individual differences in the number of mutations shared between primary cancers and metastasis, indicating varying points of divergence from primary tumor to distant metastasis. On average 55% of primary mutations were retained in the distant metastatic lesions with considerable disparity between individual patients ranging from 9 to 88%. In order to investigate the timing of putative driver events during cancer progression, we used a set of putative driver genes in breast cancer compiled by Yates et al [83]. Driver alterations such as *TP53*, *PIK3CA*, *PTEN* and *GATA3* mutations, and *MYC* and *ERBB2* amplifications were predominantly early events, however, a few were late events occurring privately in distant or lymph node metastasis. These included *NOTCH2* and *MED12* mutations, as well as *AKT2* and *EGFR* amplifications. These data suggests that distant metastatic lesions exhibit high inter-individual differences in genomic disparity when compared to its respective primary tumors and late driver event alterations are common during cancer progression.

In order to determine the patterns of metastatic spread, we used Dollo Parsimony[368] to investigate the evolutionary history of cancer cells across different sites from the same individual. We used the separating property in phylogenetic trees to infer the progression patterns in metastatic breast cancer. In addition to this, we used a Bayesian clustering method called PyClone[369] to infer the subclonal composition of the same set of data. To determine the progression pattern, we need sequencing data from primary cancer and more than one distant metastases from the same patient. Five patients (patients 1, 4, 5, 8 and 19) were suitable for this analysis. Using the phylogenetic tree re-construction we inferred that four out of the five patients (patients 1, 5, 8 and 19) followed a linear progression model that is successive metastasis-to-metastasis spreading of tumor cells. Additionally, subclonal analysis revealed that, in all of the four patients, one or more subclones were shared specifically between different metastases, but not with their corresponding primary cancers. However, one patient (patient 4) followed parallel progression model [321], that is distant metastases seeded directly from primary tumor rather from other metastases. No subclones were shared exclusively either among all or between any pair of distant metastases in this patient. Subclonal analysis revealed both monoclonal and polyclonal seeding from primary breast cancer to distant metastases in our cohort. Four out of fifteen patients (patients 8, 15, 17 and 19) followed monoclonal seeding (27%) and eleven out of fifteen patients (patients 1, 2, 3,4,5,9,10,11,14, 18 and 20) followed polyclonal seeding (73%). Next, we analyzed eight patients (patients 2, 3, 8, 10, 14, 15, 17 and 18) having primary cancer, ipsilateral axillary lymph node metastasis and distant metastasis using the separating property in phylogenetic trees. This analysis revealed very low support for ipsilateral axillary lymph node-based seeding to distant organ metastases. Subclonal analysis revealed that, except for one patient (patient 3), no subclones were shared exclusively between

axillary lymph node metastases and any distant metastases thus complementing the phylogenetic results.

Apart from clonal evolution, we also investigated the activity of different mutational process involved in breast cancer progression. At least four signatures were found operative in our cohort and three of them were mapped to known mutational processes such as "age at diagnosis", "APOBEC" and "deficient homologous recombination (HR)" [370]. We evaluated signature contributions across site-specific categories in patients to assess their signature contribution separately. We observed increased activity of mutational processes of APOBEC signature (p-value < 0.01), unknown etiology signature (p-value < 0.05) and HR signature (p-value < 0.05) in metastasis-specific relative to the primary-specific category.

One of the most important observations from our study is the lack of axillary lymph node metastasis involvement in seeding distant metastasis. Even though nodal status have an unquestionably high prognostic value [333,371], we demonstrate that they are not responsible for further seeding of cancer cells. Nodal status might reflect only the tumor biology of the breast cancer with acquired capability of cancer to survive, migrate and proliferate in other organs thereby having high prognostic value. Hence, dissection of positive axillary lymph nodes will not reverse this acquired capability since spreading to distant sites occurs in a different path and most likely directly from the primary tumor. Our data suggests that breast cancer spreading is a complex process, where we observe both linear and parallel progression with frequent polyclonal seeding and the existence of an ongoing evolution of mutational signatures during breast cancer progression. Together these observations relate to several factors challenging the prevention and treatment of metastatic breast cancer.

4.5 PAPER V

Intra-tumor heterogeneity in breast cancer has limited impact on transcriptomic-based molecular profiling

In breast cancer, determining the tumor grade and molecular subtype by IHC surrogate classification is highly sensitive to the cut-off of Ki67 as well as the region of the tumor investigated[372,373]. Further, inter-individual variability between pathologists also accounts for variations in tumor classification[374,375]. Therefore, next generation technologies such as automatic pathology image processing, gene expression based molecular profiling and genetic testing are considered as the future of cancer diagnostics. In order to translate such technologies to the clinic, they should be sufficiently robust and consistent in providing therapy predictive and prognostic information without being affected by intra-tumor heterogeneity. Transcriptomic profiling of breast tumors provides opportunity for subtyping and molecular-based patient stratification for prognostic and therapy prediction purposes. For reliable transcription based molecular profiling as well as for biobanking, it is essential that the region of the tumor profiled represents the whole tumor and is not influenced by intra-tumor heterogeneity. However, breast cancers commonly exhibit intra-tumor heterogeneity at both a molecular and morphological level, which can arise during tumor evolution. Currently it is not established to what extent a random sampling approach may influence molecular breast cancer diagnostics.

In this study we applied RNA-sequencing to quantify gene expression in 43 pieces (2-5 pieces per tumor) from 12 breast tumors. We determined molecular subtype and transcriptomic grade for all tumor pieces and analyzed to what extent pieces originating from the same tumors are concordant or discordant with each other. Molecular subtyping was consistent in 11 out of 12 12 tumors. Similarly, transcriptomic grade assignments were consistent in 11 out of 12 tumors as well. Additionally, we validated our finding in an independent retrospective cohort consisting of 19 pieces (2-6 pieces per tumor) from 6 breast tumors profiled using microarray technique. Molecular subtype predictions revealed consistent subtypes in four out of six patients in this cohort. Further, we also performed whole exome sequencing on these 19 tumor pieces (from 6 patients) to investigate intra-tumor genomic heterogeneity in breast cancer. Interestingly, we observed extensive intra-tumor genomic heterogeneity (based on putative driver gene variant allele frequencies and subclonal analysis) in these tumor pieces but not in their molecular subtype classifications.

Our data suggest that the average expression profile collected from any part of the tumor in most cases is representative for the entire tumor, at least with respect to transcriptomic grade and molecular subtype with existing microarray technologies. Further, the variability introduced by random sampling of material from the tumor is not expected to have a major impact for most patients for transcription based molecular diagnostics, even though these intra-tumor pieces demonstrates substantial intra-tumor genomic heterogeneity.

5 FUTURE PERSPECTIVES

Future perspectives for all the studies included in this thesis are individually summarized below. In the first study, we have optimized the protocol for bio-banking small breast tumors for future research purposes, where we have demonstrated that, DNA and RNA isolated form the scraping material are in sufficient quality to perform next generation sequencing and microarray analysis. In future, apart from bio-banking tumor samples we aim to establish patient derived *ex-vivo* tumor cultures for therapy prediction and drug discovery purposes. We are currently optimizing protocols to establish whole tumor cultures (WTC) and organotypic cultures (fresh tissue sections) from patient derived tumor samples. Preliminary data from these *ex-vivo* cultures suggest that they retain key therapy predictive biomarkers such as ER/PR/HER2 and Ki67. One of the advantages with organotypic culturing is that it retains the tumor micro environment i.e., the cultured tumor sections retains fibroblasts, infiltrated immune cells and tumor cells. Using these ex-vivo cultures, the aim is to predict if a patient will respond to a particular therapy or not *ex-vivo*. The specific panel of biomarkers to verify therapy response is currently investigated.

Results from paper II reveals that BSCs express ERβ, which can be used as a potential therapeutic interventions against BSCs and can be used in combination with present chemotherapeutics and endocrine therapies for targeting both differentiated cancer cells and BSCs in parallel. Further, in this study we identified the potential mechanism by which ERβ is maintaining breast cancer stem cell phenotype. Stimulation of ERβ in BSCs aid the shift towards glycolytic metabolism which in turn maintains cancer stem cell phenotype. Further, breast cancer stem cells were found to be relying on fermentative glycolysis and are sensitive to glycolytic inhibitors[360]. These studies highlight the importance of glycolytic metabolism for BSC survival. Glycolytic inhibitors are reported to be efficient in targeting cancer cells with mitochondrial defects or under hypoxic environment, which are often associated with conventional therapy resistant phenotype, similar to CSCs[74]. There are many ongoing pre-clinical/phase I-III clinical trials with glycolytic inhibitors as anti-cancer therapeutics[376]. Therefore, it is highly interesting to investigate the combination of glycolytic inhibitors and traditional chemo/ endocrine therapies against cancer as it targets both CSC and more differentiated cancer cells using patient derived CSCs, xenograft (PDX) and *ex-vivo* models.

In our next study, we demonstrated that tamoxifen treatment of BSCs induce mTOR signaling pathway, thereby conferring endocrine resistance. However, the upstream biding partners and key regulators involved in tamoxifen induced mTOR activation in BSCs are yet to be identified. Since, BSCs are ERα negative, it is tempting to speculate the second estrogen receptor ERβ could potentially mediate the mTOR activation during tamoxifen treatment in BSCs. More mechanistic studies are required to address this hypothesis. Apart from ERα and ERβ, there is another novel estrogen receptor named G-protein coupled receptor 30, which is also referred as GPER, structurally different from the classical ERs[377]. Interestingly, anti-estrogen therapies such as tamoxifen and fulvestrant acts as a GPER agonist and induce cell proliferation in endometrial and thyroid carcinomas[366,378,379]. Cell viability and motility was induced in triple negative breast cancers when treated with 17β-estradiol (E2) and tamoxifen via GPER[380]. These data suggests that, GPER might be another potential mediator of tamoxifen induced

mTOR activation in BSCs. Therefore, GPER expression and its role in BSCs can be a potential future project to expand our knowledge on estrogen signaling in BSCs.

In our next study, we focused on the genomic relationship of matched primary tumors and distant metastatic lesions from twenty breast cancer patients. This study demonstrated the complex patterns of metastatic spreading with lack of axillary lymph node metastasis involvement in seeding distant organ metastasis. This conclusion could have important clinical implication and must be confirmed in a larger cohort. Apart from exome sequencing, we have also performed microarray analysis on all the tumor samples (including both primary and distant metastases). Using this gene expression profile we have reported PAM50 molecular subtype switching in four patients in total out of fifteen patients for which we have PAM50 subtype information. In future, we are planning to perform gene set enrichment analysis (GSEA) by grouping all primary tumors versus distant metastases to identify biological process which are enriched and down-regulated in metastases. Further, it is still unclear whether mesenchymal epithelial transition (MET) process in crucial for metastatic cells at distant sites to gain growth advantage in breast cancer patients. Therefore, we aimed to investigate the epithelial mesenchymal transition (EMT) process regulation in actual distant metastases with respective to its primary tumors using both gene expression data and immunohistochemical (IHC) staining of EMT biomarkers. In our final study, we have reported that spatial intratumor heterogeneity has limited impact on transcriptomic analysis in breast cancer. In the future, we would like to increase the cohort size with more representation of different molecular subtypes, to validate if intrinsic molecular subtypes can influence the extent of intra tumor heterogeneity which in turn can affect current transcription based diagnostic technologies.

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