DEPARTMENT OF ONCOLOGY-PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

STUDIES OF STEM CELL AND ADHESION PROTEINS IN BREAST CANCER

Salah-Eldin Gadalla

Karolinska Institutet

Stockholm 2014

Published by Karolinska Institutet
Printed by Åtta.45

ISBN 978-91-7549-684-9

Studies of stem cell and adhesion proteins in breast cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Salah-Eldin Gadalla

Principal Supervisors:
Monica Nistér, MD, PhD
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor:
Christer Ericsson, PhD
Karolinska Institutet
Department of Oncology-Pathology

Johanna Sandgren, PhD
Karolinska Institutet
Department of Oncology-Pathology

Daniel Hägerstrand, PhD
Karolinska Institutet
Department of Oncology-Pathology

Lambert Skoog, MD, PhD
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Lars-Arne Haldosen, MD, PhD
Karolinska Institutet, Huddinge
Department of Biosciences and Nutrition

Examination Board:
Paraskevi Heldin, PhD
Uppsala University
Department of Medical Biochemistry and Microbiology

Lui Weng-Onn, PhD
Karolinska Institutet
Department of Oncology-Pathology

Khalil Helou, PhD
University of Gothenburg
Department of Oncology
Institute of Clinical Sciences

Dedicated to the sole of Prophet Mohammad peace be up on him!

ABSTRACT

Breast cancer is the commonest cancer and second leading cause of cancer death in women. It has been shown that breast cancer tumorigenic/stem cell like cells are $CD24^{-/low}$ $CD44^{+}EpCAM^{+}$. These cells constitute less than 5% of the cells within a cancer and are probably responsible for recurrence and metastasis. In the first paper of the thesis we show that there is uncoupling of the ERα regulated morphological phenotype from the cancer stem cell phenotype in human breast cancer cells. Experimental silencing of ERα resulted in a reduced epithelial appearance and partial reduction of CD24 mRNA, while levels of CD44 and EpCAM were unaltered. Moreover, knockdown of ERα led to a change in the morphology of the cells similar to the epithelial to mesenchymal transition phenotype and was associated with decreased E-cadherin level. Our findings offer new insights into the regulation of the breast cancer cell phenotype by ERα.

In the second and third papers we chose to immunoprecipitate the stem cell and cell adhesion protein EpCAM to identify new EpCAM interacting proteins. We have found a candidate EpCAM associated protein named Endoplasmic Reticulum Aminopeptidase 2 (ERAP2). ERAP2 was co-precipitated and colocalized with EpCAM in breast cancer cells both in the cytoplasm/ER and the plasma membrane. We expressed the two proteins in vitro in presence of dog pancreas rough microsomes (ER vesicles) and confirmed N-linked glycosylation of both proteins and the size of EpCAM. We conclude that the association between ERAP2 and EpCAM is a unique and novel finding, providing new ideas on how antigen presentation may be regulated.

In the third paper we continue to search for EpCAM associated proteins using co-immunoprecipitation (IP) and mass spectrometry. We found that Annexin A2 co-precipitated with EpCAM. IP, Western blotting and reverse co-IP confirmed the finding. Furthermore both

EpCAM and Annexin A2 colocalized in the cytoplasm and cell membrane in EpCAM+ cells. This association requires more studies to show the role of Annexin A2 in breast cancer.

In the fourth paper we have assembled a list of genes potentially associated with the breast cancer stem cells and genes that are involved in epithelial-mesenchymal transition (EMT). We performed a gene expression clustering analysis of breast cancer cell lines using cancer cell lines encyclopedia and GenePattern. We found three clusters, one epithelial (cluster alpha), one mesenchymal (beta) and a third (gamma). Both cluster beta and gamma were characterized by relatively low levels of ESR1 (ERα) as compared to cluster alpha. Clustering analysis performed on clinical samples also generated two distinct groups with low ESR1 levels. Further analysis of these three clusters will show whether there are unique gene expression patterns or overlap between them, especially between cluster beta and gamma. Subsequently we have used the same gene list and analyzed different breast cancer datasets present in the Oncomine® platform to study relationship between EMT and stem cell phenotypes expressing these genes and their correlation with molecular subtypes, and clinical outcome.

LIST OF SCIENTIFIC PAPERS

I. **Salah-Eldin Gadalla**, Anna Alexandraki, Mikael S. Lindström, Monica Nistér, Christer Ericsson. Uncoupling of the ERα regulated morphological phenotype from the cancer stem cell phenotype in human breast cancer cell lines; Biochemical and Biophysical Research Communications (2011; 405, 581–587)

II. **Salah-Eldin Gadalla**, Karin Öjemalm, Patricia Lara Vasquez, IngMarie Nilsson, Christer Ericsson, Jian Zhao, Monica Nistér. EpCAM associates with endoplasmic reticulum aminopeptidase 2 (ERAP2) in breast cancer cells; Biochemical and Biophysical Research Communications (2013; 439, 203–208)

III. **Salah-Eldin Gadalla**, Christer Ericsson, Daniel Hägerstrand, Monica Nistér. The Association between Annexin A2 and EpCAM in Breast Cancer Cells. Manuscript.

IV. **Salah-Eldin Gadalla**, Johanna Sandgren, Philip Jonsson, Christer Ericsson, Cecilia Williams, Daniel Hägerstrand, Monica Nistér. EMT and stem cell genes show distinct and overlapping gene expression patterns in breast cancer cell lines and breast cancer tissues. Manuscript.

CONTENTS

AIM OF THESIS

The aim was to find answers to the following questions:

1. Do the breast cancer stem cell markers CD24, CD44 and EpCAM exist in human breast cancer cell lines?
2. Is there a relationship between the breast cancer stem cell phenotype and well known adhesion proteins such as EpCAM, CD44 and others?
3. What is the effect of ERα knockdown on the stem cell markers CD24, CD44 and EpCAM?
4. Is there any relationship between ERα knockdown and epithelial mesenchymal transition?
5. What are the proteins that interact with the stem cell protein EpCAM?
6. What is the relationship between EpCAM and endoplasmic reticulum aminopeptidase 2 (ERAP2) and Annexin A2 (ANXA2)?
7. Is there any overlap in gene expression profile of breast cancer stem cells and EMT transformed breast cancer cells?

SUMMARY AND FUTURE PERSPECTIVES

The aim of this work is to study the role of adhesion proteins and stem cell genes in breast cancer. The second major task is to figure out the relationship and possible overlap between stem cells and their signature and epithelial-mesenchymal transition in breast cancer. Expression of both CD24 and CD44 is correlated to EpCAM and ESR1 (ERα) status in breast cancer. ERα silencing in breast cancer cell lines led to morphological changes similar to that of EMT with decrease in E-cadherin level and increase of the intermediate filament protein, vimentin. ESR1 (ERα) silencing also down regulated CD24 by 30% but did not affect gene expression either of CD44 or of EpCAM. ERα silencing did not change the fraction of cells expressing the breast cancer stem cell composite phenotype $CD24^{-/low}$ $CD44^{+/high}$ $EpCAM^{+/high}$. EpCAM expression is correlated to ESR1 expression pattern. When MDA-MB-231 cells were forced to express ESR1 (ERα) by permanent transfection (MC2 cell line) the latter cell line expressed both EpCAM and ESR1. It turned out that ERα silencing led to EMT but did not alter the stem cell status in studied breast cancer cell lines.

EpCAM immunoprecipitation in the ZR-75-1 cell line discovered ERAP2 as an EpCAM associated protein. Consistently ERAP2 is expressed in all tested cell lines where MDA-MB-231 was considered as an EpCAM negative control cell line. The result of EpCAM IP and mass spectrometry was validated by reverse co-IP followed by Western blotting. ERAP2 and EpCAM colocalized in both cytoplasm and plasma lemma in $EpCAM^{+}$ cells while ERAP2 was mainly localized in the cytoplasm of the $ERα^{-}$ MDA-MB-231 and HS-578T cells. The pattern of the cytoplasmic ERAP2 staining is consistent with ER localization with a typical reticulate pattern and enhancement of ERAP2 close to nuclear membrane sparing the nucleus with a peri-nuclear enhancement. EpCAM staining was enhanced in mitotic or actively dividing cells. Tunicamycin treatment of MCF-7 and ZR-75-1 cell lines abolished the post-translationally modified glycosylated mature EpCAM protein. Tunicamycin treatment

resulted in the appearance of an additional double band at 30 kDa of EpCAM. Expression of EpCAM and ERAP2 in vitro in pancreatic dog microsomes confirmed N-linked glycosylation, signal peptidase cleavage and the size of EpCAM. This association between EpCAM and ERAP2 is very important, it points to the role of antigen processing by ERAP2 during breast cancer progression.

Another EpCAM co-immunoprecipitated protein in ZR-75-1 cells is Annexin A2, the result was validated by reciprocal co-IP followed by Western blotting. Annexin A2 showed cytoplasmic as well as nucleolar staining patterns in all tested breast cancer cell lines. EpCAM showed membranous localization in MCF-7, ZR-75-1 and MC2 cells but EpCAM was lacking in the $ER\alpha^-$ cells of MDA-MB-231 and HS-578T cells. Annexin A2 and EpCAM co-localized together, mainly at the plasma membrane while in MC2, where only a subset of MC2 cells express EpCAM, Annexin A2 tended to be localized only in the cytoplasm not in the cell membrane of $EpCAM^-$ cells. These findings demand functional assays by EpCAM RNAi knock down and transfection of both proteins in vitro to address the functional significance of EpCAM-Annexin A2 colocalization in epithelial breast cancer cells.

To address if EMT and stem cell gene signatures in breast cancer overlap, we made hierarchical clustering analysis of breast cancer cell lines and tissues based on expression of a list of genes that had been assembled from reviewing the literature as potentially related to EMT and stem cell phenotype. These genes serve in breast cancer progression and they are clinical predictors of survival. We observed three different and distinct clusters, namely, epithelial (cluster alpha), mesenchymal (cluster beta) and CSC (cluster gamma) clusters. Using Ivshina dataset [1], which includes 289 breast cancer samples, two distinct clusters of low ESR1 levels were observed indicating significance of the clusters observed in cell lines. A subset of the genes was used to inquire Oncomine database using histological, molecular and clinical filters and we found many promising novel trends. Furthermore silencing of

ESR1 specifically down regulated MYC and ZEB1 while E-cadherin and NANOG were upregulated. So it looks like ERα negative status has two pathways to give rise to either EMT and/or stem cell phenotype with a gene expression that can overlap partially.

All these findings are very helpful to understand the molecular mechanisms by which breast cancer tumorigenesis proceed and accordingly its behavior that predicts clinical outcome, this will be helpful in re-evaluation of breast cancer in the clinic and may be involved in designing new therapeutic modalities.

LIST OF ABBREVIATIONS

ERα Estrogen receptor alpha

ERβ Estrogen receptor beta

PR Progesterone receptor

CSCs Cancer stem cells

EpCAM Epithelial cell adhesion molecule

ERAP2 Endoplasmic reticulum aminopeptidase 2

NOD-SCID Obese nondiabetic severe combined immunodeficient mice

DCIS Ductal carcinoma in situ

EGFR Epidermal growth factor receptor

CK Cytokeratin

RB Retinoblastoma protein

CDK2 Cyclin dependent kinase 2

SERM Selective estrogen receptor modulator

FISH Fluorescence in situ hybridization

IDC Invasive duct carcinoma

BMI Body mass index

FNAC Fine needle aspiration cytology

MRI Magnetic resonance imaging

PET Positron emission tomography

IGS Invasive gene signature

HH Hedgehog

SP Side population

DNMT1 DNA methyl transferase 1

DNMT1α DNA methyl transferase 1α

DNMT1β DNA methyl transferase 1β

HDAC Histone deacetylase

APC Adenomatous polyposis coli

GSK3β Glycogen synthase kinas-3 beta

MMP7 Matrix metalloproteinase 7

TCF1-alpha /LEF1 T cell-specific transcription factor 1 alpha/lymphoid enhancer-binding factor 1

WIF1 Wnt1 inhibitory factor 1

DKK1 Dickkopf WNT signaling pathway inhibitor 1

EMT Epithelial mesenchymal transition

CTC Circulating tumor cells

CHK1 Cell cycle checkpoint kinase 1

CHK2 Cell cycle checkpoint kinase 2

MHC Major Histocompatibility complex

ER Endoplasmic reticulum

L-RAP Leukocyte-derived arginine aminopeptidase

AS Ankylosing spondylitis

ASMA Alpha smooth muscle actin

OAS Overall survival

RFS Recurrence free survival

AIs Aromatase inhibitors

DRMs Detergent-resistant membrane domains

GEMs Glycolipid-enriched micro domains

1. INTRODUCTION

1.1 NORMAL AND CANCEROUS MAMMARY GLAND

Breasts present as two skin appendages, with their underneath structures and glands they constitute human mammary glands, which are located in front of the anterior chest wall. In women the normal breast is composed of epithelial and mesenchymal elements. The epithelial is element made up of lobules and ducts (luminal cells) that form and deliver milk [2] and the myoepithelial cells (basal cells). Ducts and lobular glands are surrounded by fibrovascular mesenchymal tissues formed from fibroblasts, adipocytes and blood vessels (figure 1). Mammary glands undergo crucial changes in pregnancy and lactation. The breast is affected by many types of neoplastic and non-neoplastic lesions as shown in figure 1.

Cancer is a genetic and multifactorial disease, evolving when single cells develop multiple mutations driving cells from normal to malignant state [3]. Each cancer is unique from other types in its own genomic and proteomic constitution. However for cancer to evolve it should acquire the so called MUST HALLMARKS including: self efficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless potential for replication, sustained angiogenesis, tissue invasion and metastasis [4]. Other hallmarks that contribute to tumorigenesis and cancer progression are avoiding immune destruction, enabling replicative immortality, tumor promoting inflammation, genome instability and mutation and deregulating cellular energetics [5].

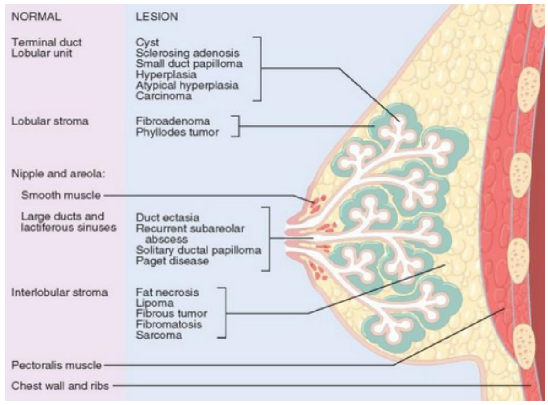

Figure 1. ROBBINS AND COTRAN PATHOLOGIC BASIS OF DISEASE, 8th edition 2010, p: 1261

The human breast contains six to ten major ductal systems. Branching of the large ducts eventually leads to the terminal duct lobular unit, which branches into a grapelike cluster of small acini to form a lobule. Here are the lesions that arise from different structure of the breast.

2. BREAST CANCER

2.1 EPIDEMIOLOGY

Breast cancer is a significant cause of morbidity and mortality in women. It represents a heavy health burden with a high incidence of recurrence and treatment failure. Breast cancer is the most common cancer in women and second most common cause of cancer associated death after lung cancer in women [6]. Most breast cancer is sporadic but there is a small proportion occurring in a hereditary or familial form.

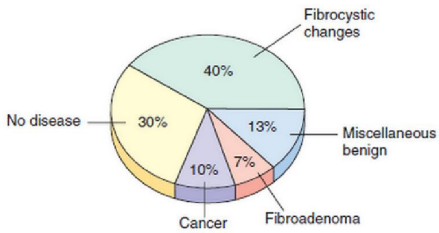

Figure 2, Elsevier. Kumar. et al: Robbins basic pathology, 9e, page 704. Percent and prevalence of different breast lesions.

In many developed countries like Sweden, Canada and USA 1 out of 8 women above 45 years of age will develop a form of breast cancer in their life [7]. Breast cancer incidence has doubled since 1960; at the time of diagnosis many women will have already loco-regional infiltration and local lymph node metastasis. In Sweden the incidence is 138 per 6569 women with a death of 1487 per 100000 women, which gives a mortality rate of 29% of all new breast cancers in women [8, 9], but Swedish women have the highest incidence of five year survival in Europe because of regular screening and early diagnosis [10, 11]. There have been documented cases of recurrence and metastasis 20 years after diagnosis denoting that we are dealing with a fatal heterogeneous disease rather than a single disease entity, a disease with diverse genetic characteristics [12-14].

Male breast cancer is rare. In the US, it accounts for approximately 1700 new cases of breast cancer every year.

Breast cancer is more common in western and well developed countries, such as northern European countries, USA, Canada, Australia, New Zealand, etc. and less common in the Middle East, Africa and much less common in Asia like Japan [6, 15]. The incidence of breast cancer is 50% higher in urban compared to rural provinces.

The rate of breast cancer in immigrant women tends to become similar to the rate in native-born women from the same area indicating contribution of environmental factors [16-18].

2.2 ETIOLOGY AND TUMORIGENESIS

Long time back in 1743 Ramazzini in Italy reported that breast cancer is more common in nuns compared to other control women indicating that pregnancy and/or lactation might protect women from breast cancer.

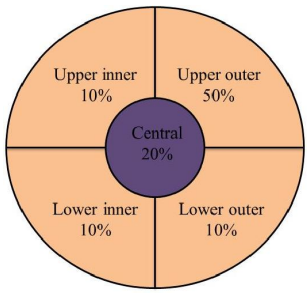

Figure 3: Rate and incidence of breast cancer in different quadrants of the breast; UO: quadrant: upper outer, UI: UI quadrant: upper inner, LO: quadrant: lower outer, LI quadrant: lower inner and central region [19]

2.3 RISK FACTORS

Scientists have discovered many risk factors to the development of breast cancer including, early menarche and late menopause, having first child late at the age of or after 35, fewer children or nulliparity and high stress levels, which can suppress the immune system. Increased consumption of animal fat, lack of exercise, high BMI, alcohol intake, cigarette

smoking [20], exogenous estrogen intake after menopause like hormone replacement therapy or oral contraceptive pills are other factors [21-24]. In some cases protective bilateral oophorectomy performed prior to the age of 35 makes the relative risk 0.36, this rate will increase with age reaching 1 at the age of 50 [25]. Another risk factor is exposure to nuclear radiation especially for younger women for example what happened in Hiroshima and Nagasaki in 1945 [26].

Genetic factors also play an important role in breast cancer genesis. Mutations in certain genes like oncogenes or tumor suppressor genes and a family history of breast or ovarian cancer, for example mutations in the tumor suppressor genes BRCA1 and BRCA2 increase the risk of early or bilateral breast cancer and 5-10% of new breast cancer cases is attributed to these mutations [15, 27, 28].

Recently it has been shown that regular antibiotic intake is another risk factor due to suppression of the immune system and increased expression of prostaglandins [29, 30]. Antidepressants may also increase the risk of breast cancer due to similarity in the chemical structure with growth regulators. Antidepressants can also suppress the immune system and increase level of intracellular estrogen [31].

On the other hand factors that decrease the incidence of breast cancer are first pregnancy before the age of 20 with a relative risk of 0.5 compared to 1 in nulliparous women [32, 33], other protective factors of breast cancer are normal BMI, regular exercise, early menopause, multiple pregnancies, breast feeding and living in Asia.

2.4 CLASSIFICATION AND STAGING

The taxonomy used for staging of breast cancer is built on the following criteria: tumor size (T), lymph node spread (N), and presence or absence of distant metastasis (M), the so called TNM classification, as shown and referenced in table 1:

Primary tumor

TX Primary tumor cannot be assessed

T0 No evidence of primary tumor (clinically occult)

Tis Carcinoma in situ: Includes both ductal and lobular carcinoma in situ and Paget's disease of the nipple without invasion and without tumor

T1 Tumor less than 2.0 cm in greatest dimension

T1mic Microinvasion present, 0.1 cm or less in greatest dimension

T1a Tumor more than 0.1 cm but not more than 0.5 cm in greatest dimension

T1b Tumor more than 0.5 cm but not more than 1.0 cm in greatest dimension

T1c Tumor more than 1.0 cm but not more than 2.0 cm in greatest dimension

T2 Tumor more than 2 cm but not more than 5 cm in greatest dimension

T3 Tumor more than 5 cm in greatest dimension

T4 Tumor of any size with direct extension to chest wall or skin, as described below

T4a Extension to chest wall

T4b Edema or ulceration of the skin of the breast or the presence of satellite nodules confined to the same breast

T4c Both T4a and T4b

T4d Inflammatory carcinoma

Regional lymph nodes (N)

NX Regional nodes cannot be assessed (or previously removed)

N0 No regional lymph node metastasis

N1 Metastasis to movable ipsilateral axillary nodes

N2 Metastasis to ipsilateral axillary nodes fixed to one another or to other structures

N3 Metastasis to ipsilateral internal mammary lymph nodes

Distant metastasis (M)

MX Distant metastasis cannot be assessed

M0 No evidence of distant metastasis

M1 Distant metastasis (includes metastasis to ipsilateral supraclavicular lymph nodes)

These previous criteria can be used in clinical staging of breast cancer as follows:

Stage	Primary tumor	Local spread	Distant metastasis	Comments
0	Tis	N0	M0	
I	T1	N0	M0	Includes T1mic
IIA	T0	N1	M0	
	T1	N1	M0	
	T2	N0	M0	
IIB	T2	N1	M0	
	T3	N0	M0	
IIIA	T0	N2	M0	
	T1	N2	M0	
	T2	N2	M0	
	T2	N2	M0	
IIIB	T4	Any N	M0	
	Any T	N3	M0	
IV	Any T	Any N	M1	

Table 1 from Gordon F Schwartz, M.D, and M.B.A: Carcinoma of the breast

2.5 MORPHOLOGICAL AND MOLECULAR SUBTYPES OF BREAST CANCER

Roughly, breast cancer is classified into either invasive or noninvasive.

Depending on the histopathological appearance, breast cancer can be sub-classified into the following subtypes, [35]:

1) Ductal carcinoma in situ (DCIS) (10-12%)
2) Ductal (75-84%)
3) Lobular (12-14%)
4) Other rare types (3-5%) including mixed ductolobular, medullary, mucinous, apocrine, metaplastic, comedocarcinoma, tubular carcinoma and Paget's disease of the nipple.

   Histopathologically breast cancer is graded into low, intermediate, and high grades. Bloom and Richardson (1957) have invented a grading system for breast cancer, revised by Elston (1991). This grading system is based on three morphological characteristics: tubular formation, extent of nuclear pleomorphism, and mitotic index [36, 37]. The overall added scores for these parameters is between 3-9, the higher the score the worse the prognosis and the poorer the survival (see table 2)

Grade	Score	5-year survival
1	3-5	90%
2	6 or 7	75%
3	8 or 9	50%

Table 2, grading score of breast cancer according to the Elston grading system and predicted 5-year survival

2.6 MOLECULAR TAXONOMY OF BREAST CANCER

According to the expression pattern of ERα, PR, and HER2/neu (also called EGFR2 encoded by the c-ERBB2 gene), breast cancer is classified into five molecular subtypes:

1. Normal like
2. Luminal A: $ERα^+$, PR^+ and $HER2^-$
3. Luminal B: $ERα^+$, PR^+ and $HER2^+$
4. HER2 or ERBB2: $ERα^-$, PR^- and $HER2^+$
5. Basal: $ERα^-$, PR^- and $HER2^-$

The basal type is called triple negative because it is characterized by absence of all markers opposite to the luminal type B where cells express all these three markers [38], DCIS cancers are usually luminal B that is $HER2^+$, while invasive carcinomas are often luminal A, usually are larger than 2 cm in size and of high grade. Basal type cancers are usually triple negative and express myoepithelial cytokeratins CK5 and CK14 while luminal type A and B cancers express the epithelial cytokeratins CK8, CK18 and CK19 [39]. Basal type breast carcinomas are enriched with breast cancer stem cell markers like CD44, which predicts poor outcome in patients with basal or ERBB2 like tumors [40-42].

Another important protein is p53, a tumor suppressor protein that acts as a guard for genome stability by enforcing cells to go into cell cycle arrest or commit suicide by apoptosis in case of DNA damage [43], 20% of breast cancers have mutations in one of the p53 alleles [44]. Bonin et al (2008) showed high mortality rate in breast cancer patients with low retinoblastoma protein (pRB), low CK8, high CDK2 and high HER2 expression [45]. Another important diagnostic and predictive marker is cathepsin D, which is found in the mammary parenchymal and stromal cells; lactate dehydrogenase 1(LDH1) is an acidic lysosomal protease expressed in tumor cells and in the lymph nodes where cancer has spread so therefore it can be viewed as a marker of metastasis in lymph nodes [46-51].

Measuring the DNA in cancer cells using FACS is another useful tool to potentially identity the malignant phenotype in breast cancer. Benign neoplasms tend to have a homogenous population of cells and the pattern of DNA called euploidy, while malignant cells tend to be heterogeneous and have an abnormal level of DNA and in addition the content and distribution between cells is unequal, a pattern called aneuploidy.

Normal tissue predominantly consists of cells in the pre-synthetic phase (G1) of the cell cycle. They have diploid karyotype (2C = 46 chromosomes). In tissue, which is not actively growing, there are only a few cells in S phase (DNA synthesis) with an increased DNA and protein content. In the post synthesis phase (G2), there will be a double set of chromosomes (4C = 92 chromosomes) [52]. Cancer cells can be diploid, but many cancers have cells of variable, near diploid DNA content and are increased in S-phase value.

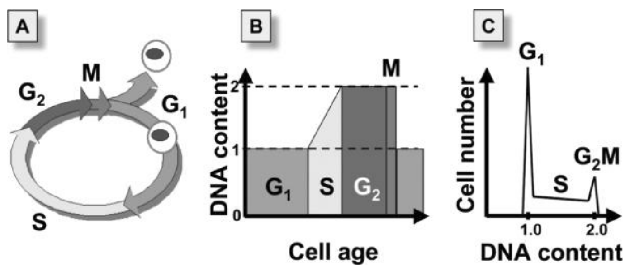

Figure 4

Schematic representation of cellular DNA content changes during cell cycle progression. (A): DNA replication is discontinuous during cell cycle occurring exclusively during S phase. (B): the postreplicative G_2-phase cell has twice the amount of cellular DNA content as compared to the G_1 cell. (C): DNA content histogram with the characteristic G_1- and G_2M-

phase peaks of DNA content DI = 1.0 and DI = 2.0, respectively and S-phase cells are distributed in between the peaks.

Adapted from Zbigniew Darzynkiewicz et al, 2010 [53]

2.7 DIAGNOSIS OF BREAST CANCER

Breast cancer is first suspected and diagnosed based on the clinical history and physical examination. Similarly benign presenting breast lesions that simulate breast cancer should be kept in mind and considered as differential diagnosis. The typical presentation is a painless lump and in addition to nipple retraction, nipple discharge, skin tethering and in the last stage skin ulceration.

Secondly using noninvasive techniques including ultrasound and mammography followed by invasive techniques like percutaneous fine needle aspiration biopsy (FNAC), core biopsy and excisional biopsy should be considered to obtain a diagnosis. The samples are subjected to histopathology, immunohistochemistry, and FISH. Other sophisticated diagnostic tools may be needed like MRI, scintimammography and positron emission tomography (PET).

Molecular diagnostics include determining gene amplification, expression and mutations of a selected number of genes and proteins in breast cancer that regulate and promote tumorigenesis and correlate with prognosis and outcome. These markers include ERα, ERβ, PR, Her2Neu, VEGF, Ki67, p53, BRCA1, BRCA2, CD24 and CD44. For example expression of ERα and PR is a sign of less aggressive disease, which associates with recurrence free survival while high expression of Her2Neu and p53 in patients with DCIS and IDC associates with local recurrence [54].

It's quite important especially for women at high risk to perform screening by breast self-examination and mammography for early detection of cancer, thereby passing the way for better prognosis and survival. Many studies have shown decreased mortality up to 23% in

women invited to the screening program, false positive cases in mammography range between 0.1-1.4% [55].

2.8 BREAST CANCER, OVARIAN HORMONES AND THEIR RECEPTORS

In mammary epithelium, estrogen and progesterone signal via three receptors; ERα, ERβ and PR. These receptors belong to the steroid receptor superfamily; they exist in the nucleus and cytoplasm. Transcriptionally active receptors translocate to the nucleus and act on nuclear targets [56].

Estrogen has a genotoxic effect on mammary epithelium due to several mechanisms including mitogenesis and aneuploidy. Estrogen acts by ERα to enhance branching and increase the number of ductal epithelia during menstrual cycle, pregnancy and lactation while ERβ will promote ducts to differentiate to form alveoli for milk secretion. Signaling via PR receptors don't increase the number of mammary epithelial cells [56, 57]. Estrogen and progesterone signal transduction via these receptors will influence mammary stem cells both in terms of cellular growth, expansion and ability to constitute all mammary gland cell types despite the lack of ER and PR expression in mother cells [58, 59].

There are two major types of epithelia in the mammary gland; luminal and myoepithelial, the latter is the basal type, which expresses mesenchymal markers CK5, CK14, p63, ASMA, vimentin, while luminal cells usually express luminal or ductal epithelial markers CK8, CK18, CK19.

During puberty, pregnancy and lactation the mammary epithelial cells grow under the influence of the steroid hormones estrogen and progesterone. ERα knockout mice will not develop a normal ductal structure [60, 61], while ERβ knockout will affect terminal differentiation of mammary epithelium but not so much the ductal growth [57]. These facts

explain why steroid hormones, mainly by estrogen over stimulation will increase the risk for breast cancer development.

More than 70% of breast cancers are ERα positive, these tumors are often of low grade, noninvasive, slowly growing and respond well to chemo, radio and hormone therapy while ERα negative tumors usually are of high grade, invasive, radio-resistant and Tamoxifen resistant with overall poor patients survival [56, 57, 62].

Studies showed that estrogen treatment of ERα positive cancers promote cancer progression while ERβ has a transcriptional suppressive effect on ERα and that is why in advanced cancer loss of the inhibitory effect of ERβ on ERα promotes cancer progression [63, 64]. In contrast, other studies showed that after ERβ knockdown reintroduction of ERβ in invasive tumor cells will increase cancer cell proliferation and progression. Both in vivo and in vitro ERβ was able to increase the proliferation and invasion of breast cancer MDA-MB-435 cells [65, 66].

ERβ is expressed in resting mammary epithelium and ERβ usually is more predominant than ERα, which is expressed only in 10-20% of mammary epithelial cells [67]. In contrast 70% of breast cancer cases express ERα while ERβ decrease is significant and is expressed much less than ERα especially in advanced cancers [68-70]. Because of that ERα but not ERβ antagonists like Tamoxifen constitutes the classic hormonal therapy in ERα positive tumors. Tamoxifen is a non-steroidal estrogen antagonist that is used as a therapeutic agent for up to 5 years as part of adjuvant therapy in patients with ERα positive breast cancer [56, 57].

In conclusion regarding ER status; ERα positive tumors have a better prognosis than the negative ones [62]. ERα positive tumors are typically low grade, associated with longer disease free survival and respond well to hormonal therapy [57]. Typically there is cross-talk between estrogen receptor and growth factor pathways as molecular targets for overcoming the endocrine resistance. Growth factors, such as epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) stimulate the ERα transcriptional activity in an estrogen-

independent manner [71]. ERα negative breast cancer is usually of high grade, aggressive, invasive, and Tamoxifen resistant and it is associated with overall poor survival [67, 72, 73].

2.9 TREATMENT OF BREAST CANCER

Despite that the incidence of breast cancer has increased over the last decades the cure rate has also increased and the key points for cure of breast cancer is early detection and better understanding of the molecular mechanisms behind breast cancer progression. The main goal of the treatment is to block growth of the primary tumor and prevent loco-regional and distant metastasis to the liver, lungs, bone and brain.

2.9.1 SURGERY

The main and standard current treatment of breast cancer is surgery in the form of lumpectomy/quadrantectomy or modified sub-partial mastectomy including axillary lymph node clearance; this is usually referred to breast conserving surgery. The old fashioned radical mastectomy is no longer used. Sentinel lymph node biopsy is a useful diagnostic procedure with or without any clinical evidence of early tumor spread to the axilla [74]; sentinel node is the first node in breast lymphatic drainage [75, 76].

After surgery patients receive chemo (adjuvant) and radiotherapy [77]. Sometimes it is necessary to shrink the tumor size and decrease blood supply to the tumor before surgery and then neo-adjuvant chemo/radiotherapy is recommended prior to surgery [78]. This usually improves overall survival (OAS) and recurrence free survival (RFS) [79].

2.9.2 CHEMOTHERAPY

Chemotherapy is used as neoadjuvant and adjuvant treatment before or/and after surgery to prevent relapse and decrease likelihood of recurrence and eradicate micro-metastasis. Usually

more than one drug is given (poly chemotherapy) to overcome single drug toxicity. The likelihood of success of chemotherapy depends on many factors such as tumor grade, clinical stage, age of patient, lymph node spread, ER status and many others.

These drugs have to be given at a high dose making them toxic to other normal cells affecting whole body physiology. They are recommended to patients first after evaluations in clinical trials. These drugs act in different ways, for example by inducing DNA damage [80] or by targeting signaling pathways like EGFR. Breast cancer cells in S phase of the cell cycle respond more to chemotherapy than other tumor cells with low S phase. In one study S-phase fractions of the primary tumour was tested in premenopausal breast cancer patients who participated in a randomized trial [81].

2.9.3 HORMONAL THERAPY

In hormonal therapy, ERα positive tumors are targeted by selective estrogen receptor modulators (SERM) like Tamoxifen and Raloxifen. They work as estrogen agonists and antagonists and they are given for up to 5 years, reducing recurrence and mortality by more than 60% in the first 10-15 years [82]. They also reduce incidence of contralateral breast cancer [82].

Aromatase inhibitors (AIs) inhibit peripheral conversion of androgens to estrogen and thereby reduce the primary source of estrogen in postmenopausal women [83].

This family of drugs also includes non-steroidal inhibitors like anastrazole and letrozole, steroidal inhibitors like exemestane, and formestane [84-86]. Some results showed that a switch to AIs after two to three years of Tamoxifen treatment might be more effective for the remaining 5 years and improve DFS [87, 88].

2.9.4 RADIOTHERAPY

Radiotherapy in breast cancer aims to decrease loco-regional spread of the disease [89]. The therapy can be external beam radiation and internal beam radiation (called also brachytherapy), external radiotherapy is more commonly used in breast cancer [90]. Radiotherapy can be neoadjuvant to shrink tumor size or decrease vascularization and can be given after surgery along with adjuvant therapy like CMF (cyclophosphamide, methotrexate, 5 fluorouracil).

2.9.5 IMMUNOTHERAPY

According to the expression pattern of ERα, ERβ and HER2 breast cancer has been re-classified using these molecular criteria into normal like, luminal A, luminal B, ERBB2 and basal.

HER2/neu is a tyrosine kinase growth factor receptor expressed in 25-30% of primary breast cancer [91]. Another upcoming therapeutic target is VEGF, which stimulates new vessels formation in breast cancer. New antibody therapeutic modalities are to target HER2/neu and VEGF using monoclonal antibodies against them (trastuzumab and bevacizumab respectively) [92, 93].

New microarray studies of gene expression patterns have recognized cancer finger-prints that can distinguish invasive gene signatures, and they are very useful predictive and prognostic tools [12-14, 94]. Finally palliative chemotherapy is given to terminal patients in stage IIIB or stage IV with disseminated disease and distant metastases. It improves patient's survival up to 12-24 months in 10-70% of terminal patients [95].

3. STEM CELLS

Mature differentiated cells in the body are derived from a single common cellular ancestor termed pluripotent stem cell; this stem cell has unique abilities of self-renewal and differentiation to give rise to multilineage differentiated progeny. All organs in the body are derived from tissue specific stem cells which have dual ability to self renew and differentiate to progeny and differentiated daughter cells (asymmetric division, figure 5A) [96]. In breast, adult mammary stem cells will divide to give rise to the three epithelial lineages; ductal, luminal and myoepithelial (figure 5B).

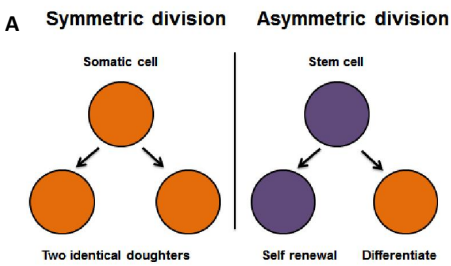

Figure 5A

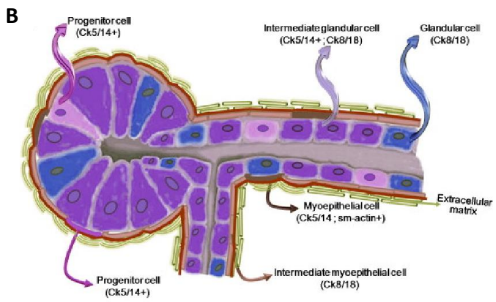

Figure 5B, modified from Bosch, A. et al, 2010. [97]

3.1 MAMMARY GLAND STEM CELLS

Stingl et al 2006 has characterized mammary tissue stem cells as Sca-1$^{-/low}$ CD45$^{-/low}$ CD31$^{-/low}$ CD24^{med}CD49f$^{+/high}$ [98]. These stem cells and bipotent progenitors will repopulate the basal cell pool which express basal markers CD5, CD14, ASMA, p63 vimentin, calponin, and myosin and also will give rise to luminal cells that express CK8, CK18, CK19, which are a type of intermediate cells, (figure 5) [97, 98]. These cells are called intermediate progenitors

or bipotent progenitors; they also express a significant amount of the GATA3 transcription factor, which is essential in mammary morphogenesis [99, 100].

3.2 CANCER STEM CELL

About fifteen years ago Dick's team provided the first evidence of the cancer stem cell concept in leukemia. Studies of different types of organs and cancers like hematopoietic cancer (leukemia), cancer of the brain, colon, prostate, lung, and ovary, melanoma, multiple myeloma and others, strongly suggest that only a small subclass or subsets of cancer cells within a tumor cell society are capable of extensive proliferation and can establish new tumors in vivo upon transplantation in immune compromised hosts. These cells are termed tumor-initiating cells (TIC) [101-112]. The special assay for detecting TICs requires that cells are injected in a low amount, even better as single cells, while the majority of cancer cells have a limited ability to divide, a population of cancer stem cells or TICs have the exclusive ability to extensively form new tumors in vivo [113]. These CSCs, which represent rare cells within the tumor, have the ability to self-renew and give rise to the phenotypically diverse tumor cell population and thereby these cells drive tumorigenesis. In colon cancer the $CD133^+$ population, accounts for about 2.5% of the tumour cells. Subcutaneous injection of $CD133^+$ colon cancer cells readily reproduced the original tumor in immunodeficient mice, whereas $CD133^-$ cells did not form tumors. Unlike $CD133^-$ cells, $CD133^+$ colon cancer cells grew exponentially for more than one year in vitro as undifferentiated tumour spheres in serum-free medium [114].

It has been shown that a small number of cancer cells express stem cell markers, including CD133 and ATP-binding cassette transporters (using exclusion by Hoechst 33342 dye), and many types of tumors and cancer cell lines contain CSCs, which self-renew, express stem cell markers, and are tumorigenic in vivo [115]. BRCA1-deficient mouse mammary tumors harbor

heterogeneous CSC populations, and $CD44^{+}/CD24^{-}$ $EpCAM^{+}$ cells represent a population that corresponds to human breast cancer stem cells [116-118].

The following question remains to be answered: Are we targeting the right cells when we treat cancer? It has been shown recently that disseminated tumor cells in blood and bone marrow are enriched in CSC phenotype, which show chemo and radioresistance [119].

2.3 STEM CELL NICHE

There is cross-talk between CSCs and the tumor microenvironment. The stem cell niche comprised of CSCs and extracellular matrix maintains CSCs in stemness state preventing their differentiation, contributing also in communication and intercellular trafficking. Evidence for the existence of CSC niches is that growth of human breast cancer cells can be obtained in vivo only in a cleared mammary fat pad of NOD/SCID mice indicating that the presence of human stroma and niche like environment is crucial and critical for human tumor growth [120, 121]. Another evidence was provided by the Weinberg group, they showed that bone marrow derived human mesenchymal cells when mixed with weakly metastatic breast cancer cells will increase their metastatic and tumorigenic potential. These mesenchymal cells release a chemokine CCL5, which acts in a paracrine fashion and stimulates CCL5 receptor signaling promoting seeding and distant metastases [122, 123].

3.4 BREAST STEM CELL AND CSC REGULATORY PATHWAYS

Self-renewal, differentiation and fate determination are influenced by many signaling pathways including sonic hedgehog, WNT, NOTCH, PTEN and p53 that regulate embryonic and adult stem cell development and differentiation. Altered regulation of these pathways might lead to development of tumors, which are resistant to conventional treatment.

3.4.1 SONIC HEDGEHOG

Sonic hedgehog (SHH) signals through its receptors PTCH1, SMO, and eventually through intracellular GLI1 and GLI2. These proteins are highly expressed in stem cells but downregulated whenever the cells differentiate [124]. Inhibition of SHH by cyclopamine or vismodegib will inhibit human glioma CSC growth [125]. SHH is important both in breast development, tumorigenesis and cancer progression [126]. It has been shown also that SHH works with the Polycomb gene silencer BMI1 to regulate stem cells both in normal breast and cancer [127]. The number and size of mammospheres will be increased upon increased expression of SHH. Furthermore SHH signaling is activated in cells with the breast CSC phenotype; $CD24^{-/ow/}CD44^{+}EpCAM^{+}$.

3.4.2 NOTCH SIGNALING

NOTCH is a cell membrane protein. Cleaved intracellular NOTCH will eventually translocate to the nucleus and act as a transcriptional regulator. NOTCH is cleaved in the cytoplasmic part by the γ-secretase enzyme. In breast, activation of NOTCH will increase both secondary spheres formation and branching morphogenesis by 10 folds, these effects will be blocked upon use of γ-secretase inhibitors [128]. NOTCH acts indirectly through mTOR, and use of γ-secretase inhibitors and mTOR inhibitor like rapamycin will suppress tumor growth [129].

2.4.3 WNT SIGNAL TRANSDUCTION

In breast cancer, activation of Wnt signal transduction through the frizzled receptor will lead to dysregulation of self-renewal and differentiation through APC and hypermethylation of Wnt gene promoter. Canonical Wnt signaling will be mediated via β-catenin, and non canonical without β-catenin. In canonical Wnt signaling binding of Wnt to frizzled receptor will free β-catenin from a complex made up of axin, glycogen synthase kinase 3β (GSK3B)

and APC, this eventually will release β-catenin in the cytosol and it will translocate to the nucleus and associate with (TCF/LEF) which will act by up-regulating c-myc, cyclin D1 and MMP7 gene transcription [130].

Wnt signaling is tightly controlled by epigenetic regulation via methylation, deacetylation, phosphorylation and ubiquitination. Methylation is mediated by DNMT1, DNMTα, DNMTβ enzymes that add methyl group on cytosine. Another enzyme responsible for removing acetyl groups is histone deacetylase (HDAC).

Wnt1 inhibitor (WIF1) is downregulated in many cancers such as prostate, lung, breast, melanoma, and bladder cancer. WIF1 is hypermethylated at the promoter region, which promotes tumorigenesis [131, 132]. Dickkopf-related protein 1 (DDK1) is another Wnt blocker that is manipulated by hypermethylation in breast and colon cancer leading to increased invasiveness and EMT [133, 134].

3.5 BREAST CANCER STEM CELL

Recently there have been substantial efforts to characterize cancer initiating or breast cancer stem cells (CSCs), which show enhanced tumorigenic property [135, 136]. The first evidence was provided by Al-Hajj et al in 2003[135] when they showed that even a small number of human breast cancer cells are able to initiate tumor formation upon transplantation into the cleared mammary fat pad of NOD-SCID mice. These cells were shown to be enriched in the $CD24^{ow/-}$, $CD44^{+}$, $EpCAM^{+}$ phenotype, they display properties innate to stem cells, they represent a minority of cancer cells in tumor, they have tumor-initiation, self-replication and self-renewal ability. The origin of CSCs is hypothesized to be either from tissue stem cells abnormally using the existing stem cell regulatory pathways to promote tissue stem cell self-renewal and therefore providing these cells with a long life span [137]. Sometimes an alternative intermediate cell type is proposed to be involved in the transformation from a stem

cell to a differentiated form. The division of the latter form results in mature cells with a partial ability of self-renewal [138].

Recently using microarray gene expression profiles and fingerprints scientists discovered what they named as invasive gene signature (IGS). Gene-expression profiles of invasive tumorigenic breast-cancer cells correlate well with the IGS of breast cancer and may be used to develop prognostic tools for assessing clinical outcome, survival and recurrence free survival in patients with breast cancer [12, 13, 94, 139].

There are different models describing cancer. Most neoplasms are thought to originate from single cells, tumor progression is initiated and propagated by acquisition of genetic modifications within the original clone resulting in subsequent selection of more aggressive clones, which is controlled by genetic and epigenetic factors [140-142]. Two alternative models have been proposed. The first is the stochastic or clonal evolution model or non-hierarchical model, which assumes the homogeneity of the tumor whereby every tumor cell is potentially tumor initiating. Progression by low-probability random events and the random mutation of cells in this model will lead to clonal selection. The second model is the CSC model or hierarchical model, which states that the tumor is functionally heterogeneous and a subset of definable tumor cells with stem cell-like properties drive tumor-initiation, progression and recurrence and promote metastasis [143], CSCs have the ability to proliferate indefinitely. However, neither model has been proven to be exclusive in nature. The likely explanation is that tumors may follow either pathway or may exhibit features of a mixed model.

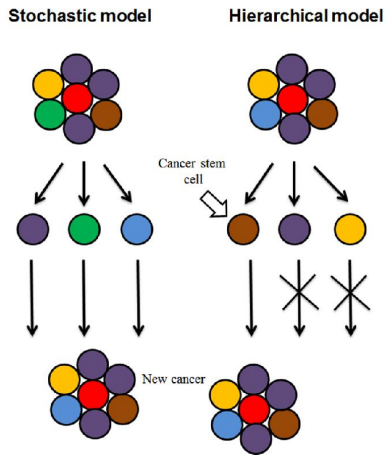

Figure 6, Cancer propagation models, modified from Monika Tataria et al, 2006. [144]

3.5.1 BREAST CSC MARKERS

Associated with Hoechst 33342 dye efflux scientists discovered a side population of cells, which have CSC properties attributed to the expression of members of the ABC transporter family [145]. Al-hajj et al 2003 showed that $CD44^{+}CD24^{-/low}$ $EpCAM^{+}$ phenotype cells are enriched for CSCs from both primary tumors and lung metastases and are tumorigenic in vivo [135]. $CD44^{+}CD24^{-/low}$ cells are claudin-low and display a gene signature of epithelial mesenchymal transition (EMT) and induction of EMT in epithelial cells led to acquisition of stem cell properties and resistance to both hormone and chemotherapy [136, 146].

Another marker of breast CSCs is CD133 or prominin-1, it's shown to be a specific marker for CSCs in $BRCA1^{+}$ breast tumors, which are triple-negative tumors [147].

Recently an important relation between CSCs and miRNAs has been found, three clusters, miR-200c-141, miR-200b-200a-429 and miR-183-96-182, were downregulated in breast CSCs. miR-200c strongly suppresses the ability of normal mammary stem cells to form

mammary ducts and tumor formation driven by human breast CSCs in vivo [148]. Another marker is ALDH1, which is an enzyme that oxidates aldehydes, increased ALDH1 activity overlaps with the $CD44^{+}CD24^{-}$ $ALDH1^{+}$ fraction [149]. $ALDH1^{+}$cells are higher in basal-like and HER2-positive tumors compared with luminal subtypes [150].

3.5.1.1 CD24

CD24 is a glycophosphatidylinositol (GPI) anchored protein, it is a mucin-like heavily glycosylated peripheral membrane protein [151]. CD24 is rarely expressed in normal tissues [152], but some normal somatic cells express CD24 for example B lymphocytes, neutrophils and neural cells in which cells it is called heat-stable antigen [153]. On the other hand CD24 is often highly expressed in undifferentiated cells and in many solid cancers like lung, brain, stomach, colorectal, prostate, breast, and ovarian carcinoma and it correlates with poor prognosis [152, 154]. Recently many studies proved the association between CD24 and tumor cell invasion, adhesion, migration, metastatic progression and spread [4, 155-161]. However, little is known about the mechanisms by which CD24 mediates these cellular effects since it is a non-transmembrane peripheral protein but is engaged with cellular signaling.

In human CD24 is composed of 31 amino acids and has molecular weight of 30-70 kDa. The ligand partner of CD24 is P-selectin. Endothelial cells and platelets use CD24/ P-selectin interaction to facilitate the passage of tumor cells to the blood stream during metastasis [162-164]. Since CD24 is located peripherally, it is incorporated in detergent-resistant membrane domains (DRMs) or the so-called lipid-rafts [165, 166]. These rafts play a central role in CD24 cellular signaling [167, 168]. Lipid rafts also mediate signaling of CD24 with a Src-kinase called c-fgr, which associates with CD24 in small cell lung cancer cells [169-172]. Recently it has been shown that CD24 associates and coexists with CD44, CD29, and CD31 in various cancers [173-175].

3.5.1.2 CD44

CD44 is a class I transmembrane glycoprotein. It binds hyaluronan (HA), and hyaluronan binds to amino acids 32-132. CD44 was discovered in 1983 as a lymphocyte homing receptor [176]. It's not simply an adhesion molecule but it's involved in multiple functions including regulation of cell growth, survival, differentiation and motility. It has many isoforms and it plays an important role in progression of many tumor types [177] and also constitutes a marker for tumor initiating cells [178].

CD44 was cloned in 1989 [179] and the protein was shown to be encoded by a single gene with variable alternative spliced exons. The CD44 has two active binding sites for other glycosaminoglycans (GAGs) [180]. The CD44v isoform is only expressed in some epithelial cells, it is expressed during lymphocyte maturation and activation and during embryonic development, and in several types of carcinoma [181]. An important interaction is between CD44v6 and vascular endothelial growth factor (VEGF) [182]. CD44 isoforms crosslink with each other and that eventually will lead to configurational changes that makes it possible for CD44 to bind to Hyaluronan [183]. The transmembrane domain of CD44 incorporates and form glycolipid-enriched microdomains (GEMs) and the GEM is an important mediator of CD44 signal transduction. The CD44 cytoplasmic tail is also important in outside in signaling since it is associated with cytoskeletal proteins like ankyrin, ezrin and others that are involved in HA-dependent cell adhesion, migration, motility and invasion by binding to actin filaments [184, 185]. CD44 is important in cell migration and is an adhesion component for the stem cell niche and in EMT serves as important factor in progression of many tumors [186], [177]. CD44 was proposed as an important CSC marker for many cancers [187, 188]. It has been shown that CD44 isoforms are strongly overexpressed in human colon dysplastic crypts and mutant adenomatous polyposis coli (APC) colon adenomas. Furthermore, the HA–CD44 complex triggers NANOG phosphorylation where it associates with Drosha, RNA helicase and p68, leading to the transcription of the oncogenic microRNA miR-21 and a reduction in the expression of the tumor suppressor programmed cell death. [189]. There is crosstalk between CD44 and CXCR4 signalling; CXCL12, the ligand of CXCR4 stimulates adhesion of progenitor cells through

CD44 [190]. In colorectal cancer with high HA staining intensity the cancer-related survival rate in Duke's stage C and D is also correlated with CD44 expression and HA mediates invasion in these cancers [191]. CD44 plays an important role in EMT in which cancer-initiating cells acquire a migratory phenotype for settlement in distant tissues and this involves CD44 and tumor necrosis factor-α (TNFα)- induced transcription. TNFα activates protein kinase C (PKC) that promotes hyaluronan synthase (HAS) transcription. CD44 complexes initiate activation of transforming growth factor-β receptor 2 (TGFβRII) and the downstream SMAD signalling complex, which essentially contributes to EMT [192], figure 7. TNFα-mediated overexpression of SLUG and the constitutively active p65 subunit of nuclear factor-κB (NF-κB) result in a dramatic shift towards the $CD44^{+}CD24^{-}$ phenotype that is associated with stem cell behavior [186]. Lastly and importantly, the invasiveness of breast cancer cells with an EMT phenotype can be inhibited by CD44-specific antibodies [193].

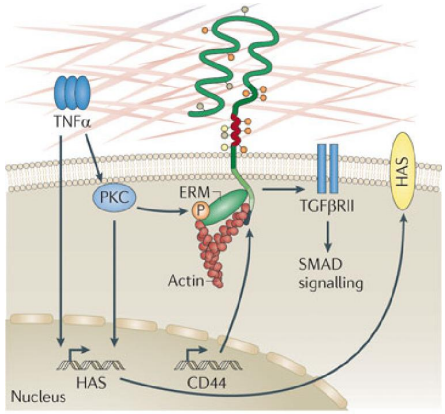

Figure 7, adapted from Margot Zöller, 2011[192].

Role of CD44 and tumor necrosis factor-α (TNF α) - induced transcription in EMT.

TNFα activates protein kinase C (PKC) that promotes hyaluronan synthase (HAS) transcription. The CD44-phosphorylated ERM (ezrin/radixin/moesin) complex initiates Activation of transforming growth factor-β receptor 2 (TGFβRII) and the downstream SMAD signalling complex, which essentially contribute to EMT.

3.5.1.3 EpCAM

EpCAM (epithelial cell adhesion molecule) is a type I transmembrane glycoprotein consisting of an extracellular domain (EpEX), a single transmembrane domain and a short 26-amino acids cytoplasmic tail or intracellular domain (EpICD) [194]. The extracellular domain comprises an epidermal growth factor-like domain, a thyroglobulin repeat domain followed by a cysteine-poor domain [195]. During epithelial mesenchymal transition (EMT) cells lose adhesion molecules and alter their polarity in order to become metastatic, EpCAM seems to

be downregulated in such a situation, followed by re-expression at the metastatic sites [196, 197].

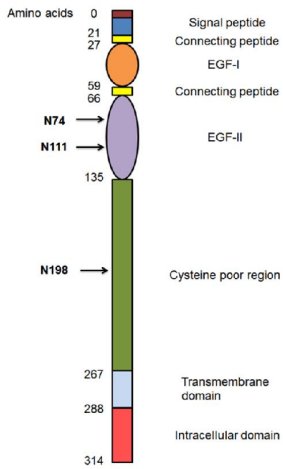

Figure 8, EpCAM domain structure and N-glycosylation sites, Gadalla et al, 2013 [198]

EpCAM is expressed by most of the epithelia of healthy individuals. In most human carcinomas, EpCAM is overexpressed [199]. EpCAM has been discovered in multiple ways and given many names according to the respective antibody used to detect the protein like epithelial glycoprotein-2 (EGP-2), epithelial specific antigen (ESA), GA733-2 and tumor-associated calcium signal transducer 1 (TACSTD1) [194]. EpCAM usually localizes in the basolateral side of epithelial cells, but in cancer cells it is present everywhere including in the apical region of the cells [200, 201].

Recently, EpCAM has been identified as an additional marker for cancer-initiating stem cells in many cancers along with CD24 and CD44 [135]. The function of EpCAM is not fully understood, EpCAM is able to abrogate E-cadherin-mediated cell–cell adhesion. It disturbs

the link between α-catenin and F-actin leading to loss of cell–cell adhesion [202]. EpCAM associates with claudin-7 leading to alteration of EpCAM-mediated homotypic cell–cell adhesion promoting cell motility, proliferation, survival, carcinogenesis and metastasis [203]. Outside in signaling of EpCAM involves shedding of its ectodomain EpEX.

EpCAM undergoes proteolytic cleavage at the cytoplasmic tail sequentially catalyzed by TACE and presenilin-2 leading to separation of EpCAM intracellular domain (EpICD) with subsequent translocation of this part into the nucleus where it regulates transcription of cyclin A, cyclin E, and c-myc promoting tumor progression. Released EpICD associates with FHL2, beta-catenin and Lef-1 to form a nuclear complex that contacts DNA at Lef-1 consensus sites, induces gene transcription and is oncogenic in immunodeficient mice, there is a cross talk between EpCAM signaling and E-cadherin and wnt pathways (figure 9) [204].

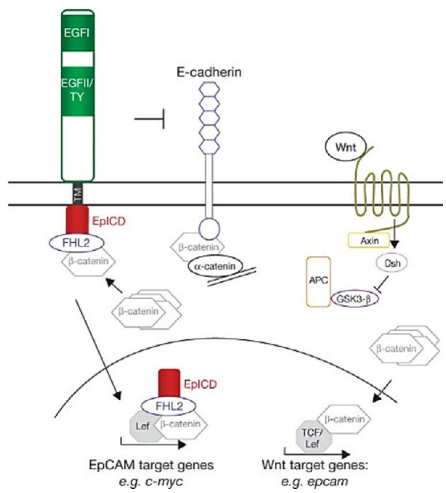

Figure 9, EpCAM nuclear signaling and possible cross-talk with E-cadherin and Wnt pathways. Adapted from Maetzel et al, *Nature Cell Biology* (2009) [204].

On the other hand studies showed that EpCAM overexpression in some carcinomas such as renal clear cell carcinoma and thyroid carcinoma is associated with improved patients survival [205-211]. It has been demonstrated that in pancreatic, breast and hepatic cancers EpCAM positive cells have 50-100 fold tumorigenic potential of the negative ones [135, 212-214]. EpCAM was also shown to be associated with poor prognosis in gall bladder, pancreas and bladder carcinomas [215, 216]. De novo expression of EpCAM resulted in the rapid up-regulation of the proto-oncogene c-Myc along with enhanced cell proliferation and metabolism.

In summary, according to some studies EpCAM is the 'good guy', being associated with improved survival in certain cancer types, whereas in other cancer types, EpCAM is the 'bad guy' being associated with decreased survival [205, 217].

EpCAM interacting and associated proteins

There are some known ligand partners and interacting proteins with EpCAM, for example the cytoplasmic intracellular domain of EpCAM contains two α-actinin binding sites, through which EpCAM interacts with α-actinin and, consequently, with the actin cytoskeleton [218]. EpCAM associates and binds directly to the tight junction protein claudin 7 [219]. EpCAM knockdown resulted in decreases in claudin-7 and claudin-1 proteins [220]. We showed also that EpCAM associates with ERAP2 in breast cancer cell lines [198]. EpCAM was shown to interact directly and form a complex with CD44v4-v7 [221], furthermore it has been shown that EpCAM is part of the tetraspanin complex and forms a primary complex with CD9 [222, 223].

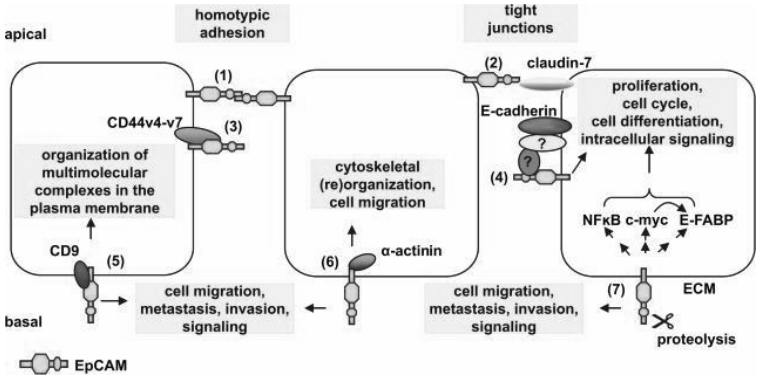

Figure 10, schematic representation of ligands identified for EpCAM and possible functions related to them. Adapted from Trzpis et al, 2007 [224]

3.5.1.4 PROM1 (CD133)

CD133 molecule (murine homologue prominin-1) was discovered in hematopoietic stem cells in 1997 [225, 226]. It is a single five pass transmembrane glycoprotein with a molecular weight of 120kDa and seven isoforms. The protein has eight N-linked glycosylation sites. Its biological function is still mysterious. Cancer initiating cells have been shown to be CD133 positive in leukemia [226], brain cancer [103], ovarian cancer [227], hepatocellular carcinoma [228], prostate [229] and pancreas cancer [230]. In colon cancer $CD133^{+}$ cells, which represent around 2.5% of tumor cells were able to form tumors in NOD-SCID mice but $CD133^{-}$ cells were not [114]. These cells usually are radioresistant [231]. The expression of CD133 in colorectal cancer has been associated with poor survival [232, 233].

CD133 is preferentially localized in highly curved plasma membrane protrusions such as microvilli and primary cilia [234, 235]. Some studies have shown a link between the expression of CD133 and the cellular resistance to apoptosis [236, 237] and CD133 positive glioma stem cells were shown to up-regulate antiapoptotic proteins such as survivin [238].

CD133 is one of the most frequently used biomarkers for CSCs especially after its initial discovery in CSCs from brain tumors [239]. However other studies have found that $CD133^-$ cells also are able to give rise to tumors in immunodeficient mice [240-245] and $CD133^-$ cells may give rise to $CD133^+$ cells [242].

Regarding tumor prognosis it has been shown that the combined tumor cell phenotype of $CD133^+/Ki67^+$ was associated with a particularly poor clinical outcome [246, 247]. In hepatocellular carcinoma increased CD133 expression corresponds to higher tumor stage, thus indicating a poor prognosis for patients [248]. Observations in brain tumor cell lines support the link between CD133 positivity and radioresistance [249-251], however, other studies with lung or breast cancer cell lines failed to show a similar correlation [252]. Liu et al described $CD133^+$ glioblastoma cells having a higher resistance to various drugs [238]. After a period of enthusiasm in CD133 research, still there is debate about its usefulness as a CSC biomarker. There are complex epigenetic and microenvironmental modulators of CD133 gene transcription that affect CD133 expression in normal tissues and solid cancers [253].

3.5.1.5 ALDEHYDE DEHYDROGENASE 1 (ALDH1)

Aldehyde dehydrogenase 1 (ALDH1) is an enzyme that belongs to a superfamily of aldehyde dehydrogenases that play a key role in the metabolism of endogenous and exogenous aldehydes. The human, ALDH superfamily comprises 19 isozymes that possess important physiological and toxicological functions. It is the key enzyme that oxidizes aldehydes to corresponding acids in an irreversible manner and this action is very crucial for alcohol metabolism. ALDH was first discovered in ox liver more than 50 years ago [254].

The ALDH1A subfamily plays a pivotal role in embryogenesis, development and maintenance of cellular homeostasis. Increased expression and activity of ALDH isozymes have been reported in various human cancers and is associated with cancer relapse [255].

Ginestier et al showed that normal and cancer human mammary epithelial cells with increased ALDH activity have stem/progenitor cell properties [256]. The measurement of aldehyde dehydrogenase isoform 1(ALDH1) activity shows promising potential as a universal marker for the identification and isolation of stem cells in many cancers [257]. In breast cancer, tumor cell ALDH1 expression significantly correlated only with basal-like and $HER2^+$ tumor types in the adjuvant series and tumor grade. On the other hand, high degree of stromal expression was significantly associated with best disease-free survival as well as overall survival [258]. The expression and distribution of breast ALDH1 and other CSC markers is largely dependent on histological type. Medullary and metaplastic carcinomas are the two types highly associated with high-grade carcinomas, basal-like and claudin-low molecular subtypes, and with the CSC phenotype $CD44^+CD24^{-/low}/ALDH1^+$ [259].

3.5.2 PURIFICATION AND ISOLATION OF CSCs IN BREAST CANCER

There are many ways to purify and isolate CSCs, combining them together will increase efficacy and purification of these cells. These methods are not specific to breast CSCs but can be generally used in any CSC isolation and includes: side population technique (SP), tumor spheres cultures, $CD44^+CD24^{-/low}$ $Lin^{-/low}$ phenotype and ALDEFLUOR® assay.

3.5.2.1 SIDE POPULATION ASSAY

The side population assay uses the fact that the ATP-binding cassette ABCG2/BCRP1 is overexpressed. In stem cells these molecules exclude vital dyes like rhodamine or Hoechst 33342, a property that is lacking in differentiated cells. This will lead to generation of side population cells in flow cytometry; these cells are around 2% of all cancer cells [260, 261].

3.5.2.2 TUMOR SPHERE CULTURES

The second method is the tumor spheres assay in which cancer cells are seeded in low attachment flasks and cultivated in serum free media with growth factors like basic fibroblast growth factor (bFGF). This will lead to formation of floating mammary spheres, which are enriched for $CD44^{+}CD24^{-/low}$cells [262].

3.5.2.3 FACS BASED CELL SURFACE MARKERS

In this third method of purifying breast stem cancer cells, single cell suspensions are sorted by FACS using anti CD24 and anti CD44 fluorescent antibodies. $CD44^{+}CD24^{-/low}$ cells constitute typically less than 5% of all the cells in the cancer and as few as 200 xenografted cells of this phenotype were able to propagate in NOD-SCID mice as serial xenogeneic transplants while 200000 and even more of non $CD44^{+}CD24^{-/low}$ cells failed to do so (figure 11) [135].

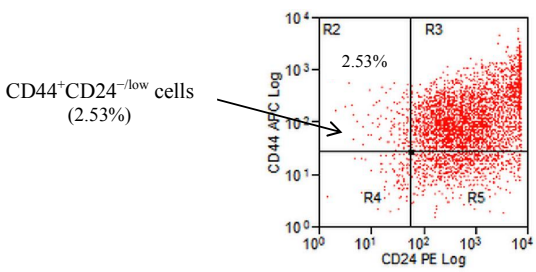

Figure 11, Percentage of $CD44^{+}CD24^{-/low}$ cells in the MCF7 breast cancer cell line (upper left quadrant) measured by flow cytometry (Gadalla et al 2011[263]).

3.5.2.4 ALDEHYDE DEHYDROGENASE METHOD

In the aldehyde dehydrogenase (ALDH1) assay, ALDH1 enzyme activity is measured. This enzyme is a detoxifying enzyme responsible for oxidation of intracellar aldehydes, which may

have a role in oxidizing retinol to retinoic acid in early stem cell differentiation [264-267]. The isolated ALDH1 positive cell fraction from human breast cancer contains cells of the breast CSC phenotype $CD44^{+}CD24^{-/low}$ and can be used as a powerful marker of poor clinical outcome [256].

3.5.3 THERAPEUTIC IMPLICATIONS OF CSCs

Most current breast cancer therapies including chemotherapy, radiotherapy, and hormonal therapy are effective against rapidly dividing cancer cells but not stem cells, which may remain partly quiescent. The most curative approach aims to target the slowly dividing stem cells, which show high degree of chemo-radioresistance [146]. That is why breast cancer stem cells have become an attractive target of therapy. Many mechanisms have been proposed to cause chemoresistance such as overexpression of the multidrug resistance transporter genes ABCG2 and ABCC1, these genes are switched off in differentiated progenitors. This can be proven by side population assays via dye exclusion test using rhodamine or Hoechst 33342. This reaction can also be inhibited by Ca^{2+} transporter inhibitors like verapamil [268, 269]; another approach is to target the $CD44^{+}CD24^{-/low}$ population.

In glioma it has been shown that $CD133^{+}$cells are enriched after irradiation due to deficiency in the DNA damage checkpoint response, a checkpoint that is normally allowing DNA repair involving the cell cycle regulatory proteins CHEK1 and CHEK2 [270, 271].

3.6 ENDOPLASMIC RETICULUM AMINOPEPTIDASES (ERAPs)

3.6.1 STRUCTURE and FUNCTION

Endoplasmic reticulum aminopeptidases (ERAP) including ERAP1 and ERAP2 are essential enzymes existing in all livings from bacteria to humans. ERAPs are zinc-metallopeptidases of the oxytocinase M1 subfamily; they regulate protein expression and maturation. Both ERAP1

and ERAP2 have been identified both in the cytosol and in the endoplasmic reticulum (ER) and they have a structural identity of 49%. Zinc-binding motifs are essential for their enzymatic activity [272]. ERAP1 and ERAP2 genes are located on chromosome 5q15 spanning 45 kilobases. ERAP enzymes exist normally in many tissues and are strongly induced after stimulation with type I and type II interferons IFNα, IFNγ [273-275] and tumor necrosis factor-alpha (TNF-α) [276].

ERAP2 is also known as leukocyte-derived arginine aminopeptidase (L-RAP), it was cloned by Tanioka et al in 2003 [275]. The ERAP2 protein contains 960 amino acids and has a calculated molecular mass of 123 kDa. ERAP2 contains a hydrophobic N-terminal region, zinc metallopeptidase (M1) motifs, and 9 potential N-glycosylation sites. Using immunofluorescence analyses ERAP2 appeared in a reticular distribution, with perinuclear enhancement staining, suggesting ER localization of the soluble protein and likely with the C terminus of the molecule hanging in the ER lumen [198].

In innate immune and inflammatory responses, class I major histocompatibility complex (MHC) relies on ERAP2 for final trimming and antigen presentation by MHC-I molecules [277]. MHC-I ERAP enzymes trim amino acid residues from the NH2 terminus of polypeptides. Antigen fraction will first be degraded by cytosolic aminopeptidases and then translocated into the ER lumen by TAP1 (Transporter associated with Antigen Processing 1) and TAP2, to be processed by ERAP1 and ERAP2 in ER and eventually associate with MHC-I molecules for presentation and further recognition by certain $CD8^+$ T cells and NK cells and triggering NK cell-mediated lysis [278].

In ERAP knockdown models using siRNA, there has been partial reduction in class I MHC proteins [278, 279]. On the other hand ERAP1 immunization has led to potent CD8 response [280, 281].

ERAP1 catalyses and cuts after neutral amino acids like valine, leucine and isoleucine while ERAP2 selectively trims polypeptides after arginine and lysine basic residues. In addition to these immunological functions, ERAP1 and ERAP2 have been implicated in the regulation of angiogenesis and blood pressure [275, 282, 283].

3.6.2 ERAP2 (LRAP)

Tumor cells are known to evade immune recognition by a variety of mechanisms such as by downregulation or loss of the antigen processing/class I MHC presentation machinery which is dictated by ERAP1 and ERAP2 via their proteolytic function [284]. Altered expression of ERAP1 and ERAP2 has been noticed in many solid tumors resulting in alteration and abnormal MHC-I expression compared to their normal counterpart [285].

3.6.3 ERAP2 EXPRESSION IN TUMORS

Highly variable levels of ERAPs expression have been observed in melanomas, leukemia-lymphomas and carcinomas of breast, colon, lung, chorion, skin, prostate, cervix, kidney and bladder compared to their normal counterparts [285-289]. The ERAP1 and ERAP2 expression patterns were independent of each other and regardless of histotype, their expression in cancer was not correlated with MHC class I expression. In a study it was shown that expression of both ERAP1 and ERAP2 was lost, acquired or retained as compared to the normal counterparts, depending on the tumor histotype [286]. Down-regulation of ERAP1 and/or ERAP2 expression was mainly detected in ovary, breast and lung carcinomas, whereas an up-regulation of these enzymes was observed in colon and thyroid carcinomas [286]. This altered expression of ERAPs can explain ERAPs role in the abnormal cell surface expression of MHC class I molecules in tumor cell lines [285]. In the most aggressive type of

neuroblastoma cells, ERAP1, ERAP2 as well as MHC class I molecules were expressed at very low levels [276].

In conclusion, low expression of either ERAP1 or ERAP2 in cancer results in low levels of functional trimming activities. Altered and/or imbalanced expression/function of ER aminopeptidases is not a unique feature of long-term cultured cells, but can be detected in vivo [286].

3.6.4 ERAP1 AND ERAP2 NON-IMMUNOLOGICAL FUNCTIONS

ERAP2 plays a role in the regulation of blood pressure through the renin-angiotensin system. ERAP2 cleaves angiotensin III to angiotensin IV converting kallidin to the vasodilator bradykinin [275, 283]. In pregnancy, variants of ERAP2 have been found to be associated with an increased risk of preeclampsia, hypertension and proteinuria [290, 291].

ERAP1 can act as a "susceptibility factor" for an infectious organism since ERAP1-deficient mice are not able to process the immunodominant decapeptide HF10 of Toxoplasma gondii and die from overwhelming infection when challenged with this pathogen [292]. Similarly, in cervical carcinoma induced by persistent infection and malignant transformation of the uterine cervical epithelium by human papillomavirus (HPV), increased cancer metastasis and decreased survival is associated with several variants of ERAP and this will lead to tumor growth and progression [293].

ERAP2 is linked to many autoimmune diseases in particular MHC class I alleles. A recent study revealed that 26% of the overall risk to develop ankylosing spondylitis (AS) is due to interaction of ERAP1 and HLA-B27 during the process of antigen trimming and presentation [294, 295]. A similar scenario was observed in studies that showed involvement of ERAP in type I diabetes [296], multiple sclerosis [296, 297], psoriasis [298, 299] and Crohn's disease [300].

3.7 ANNEXIN A2

3.7.1 STRUCTURE and FUNCTION

Annexin A2 is a pleiotropic, Ca^{2+} dependent phospholipid-binding protein. In the cell, Annexin A2 exists as a monomer and as a heterotetrameric complex with plasminogen and S100A10. Annexin A2 has several names; p36, annexin II, calpactin I, lipocortin II, chromobindin VIII, or placental anticoagulant protein IV [301]. Annexin A2 (ANXA2) contains three distinct functional regions: the N-terminal region, the C-terminal region, and the core region. The N-terminal region contains tissue plasminogen activator (tPA) and S100/A10 (also called p11) binding sites. The core region contains the Ca^{2+} and phospholipid-binding site. The C-terminal region contains the F-actin, heparin and plasminogen-binding sites. The core domain contains four repeats, and each repeat has five alpha-helices. Through tPA Annexin A2 acts as a key regulator of fibrinolysis by conversion of fibrinogen to fibrin. Extracellularly, Annexin A2 serves as a surface receptor for S100A10 and eventually regulates fibrinolysis. Annexin A2 is also a receptor for the extra cellular matrix glycoprotein protein, tenascin C (TNC) and this binding promotes breast cancer metastasis via establishment of pre-metastatic niches.

3.7.2 ANNEXIN A2 AS A CANCER BIOMARKER

ANXA2 is frequently observed in many cancer types. Overall, ANXA2 is overexpressed in acute lymphoblastic leukemia (ALL) [302], APL [303], breast cancer [304], colorectal carcinoma [305], gastric cancer [306], glioma [307, 308], hepatocellular carcinoma [309], lung cancer [310-313], ovarian cancer [314], urinary bladder cancer [315], multiple myeloma [316], oral squamous cell carcinoma [317] and pancreatic cancer [318, 319].

3.7.3 EXPRESSION OF ANNEXIN A2 IN BREAST CANCER

ANXA2 is usually undetectable in normal and hyperplastic ductal breast tissue. In contrast, ANXA2 is consistently expressed in invasive breast cancer and ductal carcinoma *in situ*. Sharma MR et al, 2006 showed that generated plasmin is capable of degrading ECM consequently facilitating cell invasion and migration of MDA-MB-231 cells. This invasion can be blocked by a monoclonal antibody to ANXA2, indicating that ANXA2-dependent localized plasmin generation by human breast cancer cells could contribute to angiogenesis and metastasis [304]. These results suggest that ANXA2 may be an attractive target for new anti-angiogenic and anti-breast cancer therapies. Also, abnormal ubiquitination of ANXA2 may promote the metastasis and infiltration of breast cancer cells by inducing high levels of ANXA2 expression [320]. Silencing of ANXA2 gene down-regulates the levels of S100A10, c-Myc, and plasmin and inhibits breast cancer cell proliferation and invasion [321].

In breast cancer siRNA mediated ANXA2 downregulation leads to increased apoptosis, decreased cell viability and migration. ANXA2 is a known substrate of Src. ANXA2 is also one of the regulators of EGFR receptor endocytosis especially in Herceptin resistant and triple negative breast cancer [322].

4. MATERIAL AND METHODS

4.1 CELL CULTURE

Four breast cancer cell lines were used in the studies, MCF-7, ZR-75-1 and MC2 are ERα$^{+/high}$ EpCAM$^{+/high}$ while MDA-MB-231 and HS-578T are ERα$^{-/low}$ EpCAM$^{-/low}$[323].

Cells were grown in D-MEM with L-glutamine and 10% fetal calf serum, supplied with sodium pyruvate and penicillin /streptomycin. The MC2 cell line is derived from MDA-MB-231 and stably transfected with ER-α [324]. MC2 cells were grown in Yellow MEM with 5% stripped bovine serum and G418. Cells were split once a week using Trypsin-EDTA.

4.2 Immunofluorescence

The cells were grown on coverslips in 6 well plates at up to 70% confluence. The cells were fixed in 99% ethanol for 15 minutes at room temperature and then washed with 1x PBS three times. Cells were blocked with 1X PBS/5% bovine serum albumin (BSA) at 4°C for one hour. Directly conjugated or primary antibody was added to the coverslips at a dilution of 1:50 to 1:100 in 1X PBS/5% (BSA) and incubated in humid chamber overnight at 4°C, followed by washing in PBS three times. After that cells were incubated with corresponding secondary antibody in 1X PBS/5% (BSA) at a dilution of 1:500 to 1:1000 and finally washed three times with 1x PBS (see Table 4). Each coverslip was inverted on a clean glass slide with a drop of Vectashield mounting medium with DAPI and subjected to microscopy in a Carl Zeiss Axioplan 2 immunofluorescence microscope. Photos were acquired using Axiovision V4 software.

4.3 Flow cytometry (FACS)

Cells were harvested at 60-80% confluence from a 10 cm tissue culture dish using 1x 0.05% trypsin-EDTA and resuspended in FACS sample buffer (3% fetal calf serum, 0.01% sodium

azide in 1x PBS). The cells were counted and tested for viability by trypan blue exclusion. The cell suspension was adjusted to 10^5 to 10^6 cells per ml, distributed into FACS tubes and centrifuged at 1000 rpm. The supernatant was discarded and the cells were incubated with the directly conjugated antibody or primary antibody (see Table 4) at a dilution of 1:50 – 1:100 for one hour followed by centrifigation in FACS buffer three times. The cells were finally suspended in 500 µl of 1x PBS. FACS data were acquired using BD-Biosciences FACS Calibur®. Analysis was performed using Cell Quest software and Flowjo™ FACS v7.

4.4 SDS-PAGE and Western blotting

Total cell extracts were prepared from the cells after washing with cold PBS three times. The cells were dissolved in 2x SDS sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% Bromophenol blue and 0.125 M Tris HCl, pH 6.8). Total protein concentration was measured. Up to 50µg total protein per lane was loaded on to 10% SDS PAGE gel and separated. The separated proteins were transferred overnight to nitrocellulose membrane using wet and semi-wet Western blot transfer. The membrane was washed with TBS-T (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20), and then blocked with 5% fat free milk as a membrane-blocking agent in PBS. Incubation with the primary antibody was overnight at 1: 2000 dilution at 4°C. The membrane was washed and subsequently incubated with the appropriate secondary antibody for one hour at room temperature (see Table 4). After washing with TBS-T, a mixture of Amersham ECL Western blotting detection reagent 1 and 2 was poured onto the membrane and incubated for one minute. The luminescence was made either manually using films or imaged electronically by Fuji Film Las-1000 and images analyzed using LAS-1000 Pro and Image Gauge software 2003.

4,5 PCR and biomarker gene expression analysis

1 µl of Oligo (dT) was added to 11 µl RNA and 1 µl dNTP, the solution was mixed well and incubated for 5 min at 65ºC and on ice for >1 min. A premix was made from the following: 4 µl 5x first strand buffer, 1 µl RNase inhibitor, 1 µl Superscript III reverse transcriptase and 1 µl 0.1 M DTT. 7 µl of the premix was added to each PCR-tube and a temperature program with 25ºC for 5 min, 50ºC for 1 hour, and 70ºC for 15 min was run. The cDNA was diluted to 100 µl and stored at -20ºC before use in the PCR reaction.

Total RNA was isolated using the RNeasy Mini Kit. Total RNA was measured spectrophotometrically by the NanoDrop® ND-1000. Purity of the isolated RNA was determined at an absorbance of 230nm, 260nm and 280nm. We aimed for A^{260}/ A^{280} ratio ≥ around 2 and A^{260}/A^{230} ratio was in the range of 1.8-2.2.

The primers for the genes of interest were taken from published articles [325-330] and the sequences were as follows:

PGR (97 bp) FP: 5'-TCAGTGGGCAGATGCT-3'

RP: 5'-GCCACATGGTAAGGCATAATGA-3'

GAPDH (151 bp) FP: 5'-ACGGATTTGGTCGTATTGGGC-3'

RP: 5'-TTGACGGTGCCATGGAATTTG-3'

EpCAM (186 bp) FP: 5'-GGACCTGACAGTAAATGGGGAAC-3'

RP: 5'-CTCTTCTTTCTGGAAATAACCAGCAC-3

CD24 (208 bp) FP: 5′-TGAAGAACATGTGAGAGGTTTGAC-3'

RP: 5'-GAAAACTGAATCTCCATTCCACAA-3'

CD44 (482 bp) FP: 5'-GACACATATTGCTTCAATGCTTCAGC-3'

RP: 5'TCGATGCCAAGATGATCAGCCATTGGAA-3'

1 µl of template (the sample DNA that contains the target sequence) was added to each tube and the thermal cycler program was set to a single starting denaturation step. The amplified products were separated by electrophoresis in a 2% agarose gel and visualized under UV-light. The molecular weight marker used was a 100 b ladder. The bands were visualized and photographed under UV-light. The PCR primers were tested for specificity.

4.6 qPCR using SYBR Green

We used the 2xSYBR Green Master Mix kit (Qiagen # 204052). Samples for the standard curve were prepared from the cDNA generated from the cell lines that were used and with serial dilution factors 1, 1:2, 1:8, 1:64 and 1:2560. A negative control for each primer set was included in every run. GAPDH was used as internal control. All reagents were kept on ice when setting up the PCR. The Master Mix was 20 µl with a final concentration of 1x. 19 µl of the mix and 1 µl of template were added in each reaction well of a 96-well plate, thermal cycler (Eppendorf) was used to amplify the target cDNA and measure the products. The results were analyzed and the amplified products were visualized by 2% agarose gel electrophoresis. A melting curve/dissociation step was included to check the quality of the PCR products and to identify any unwanted amplified products.

To evaluate the results from the real time PCR the ΔΔ Ct method was used to calculate a relative difference of the PGR, CD24, and EpCAM mRNA changes after ER-α modulation compared to the WT cells. The WT expression was given the value of 1 and the test samples were shown relative to the WT expression.

4.7 Transfection of ER-α small interfering RNAs

The silencing of ER-α was made with targeting siRNAs from Dharmacon®, using the target sequence 1: (5'-AACCTCGGGCTGTGCTCTTT-3') and target sequence 17: (5'AAACGAGAGGAAGAGCTGCCA-3') and Dharmacon® siCONTROL as a non-targeting or negative control [331]. The cell lines MC2, MCF-7 and ZR-75-1 were plated one day prior to transfection to obtain 60% confluence, in either 10 cm dishes for FACS or 6 well plates for Western blot. Mirus TransIT-TKO transfection reagent was used as a vehicle. To form a complex between the media and transfection reagent; transfection reagent was added to 1x OPTI-MEM serum free media and mixed thoroughly. The mixture was left for 20 min at room temperature to allow a complex formation between serum free OPTI-MEM and TransIT-TKO transfection reagent. After that siRNA was added to the transfection reagent –OPTI-MEM complex at 80 μM final concentration in each well, mixed gently and incubated for an additional 20 min at room temperature. After that appropriate volumes of the final complex (OPTI-MEM transfection reagent/siRNA) were added drop wise to the cells, see Table 3. After 24 hours of incubation at 37˚C the cells were re-transfected and cells were harvested after 36 h from the start of transfection for FACS and Western blotting. RNA was prepared using the RNeasy Mini Kit from Qiagen as described above.

Plate	OPTI-MEM	TransIT-TKO transfection reagent	siRNAs (80µM final concentration in well)	Complete growth media
10 cm plate for FACS	1500µl	93µl	320µl	7500µl
6 well plate for Western blotting	250µl	14µl	56µl	1250µl

Table 3 *Optimized protocol of ERα small interfering RNAs*

An experiment to find the optimal targeting siRNA concentration had been made with cells from the cell line ZR-75-1. Six different volumes of 1µM stock solution of ER-α siRNA were used ranging from a volume of 1-2.2 µl aiming for a final concentration of siRNA at 80 µM per well in a 12 well-plate.

4.8 EpCAM co-immunoprecipitation followed by gel staining

Cells were washed three times with cold PBS on ice. After centrifugation cell pellets were solubilized in 1:10 ice-cold RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Non-Idet (NP-40) and 50 mM Tris base). By using 22 G needle, a suspension was created and it was then centrifuged at 16000 rcf for 15 min. The supernatant represents the cytoplasm and membrane fraction while sediments include the nuclei.

To clear any unbound immune complexes, the supernatant was incubated for an hour with protein G agarose beads at 4°C (immunoprecipitation starter pack, GE Healthcare), beads were spun down by centrifuging for 3 min at 3000 rcf, 4°C and supernatant was incubated with the anti-human EpCAM mouse monoclonal antibody (C-10, Santa Cruz) overnight at 4°C at a dilution of 2 µg per 100–500 µg of total protein (in 1 ml of cell lysate). Immune complexes were captured with protein G agarose beads during incubation for 2 hours at 4°C; 50 µl 1x SDS sample buffer was added. EpCAM and co-

precipitated proteins were eluted by shaking and boiling the samples for 10 min at 95°C; after centrifugation supernatant represents EpCAM with possible co-precipitated proteins. This sample was run in 10% SDS PAGE and total cell extracts were run in parallel. Gel was stained overnight by mild shaking with Colloidal Blue dye (Colloidal Blue Staining Kit, Invitrogen), followed by destaining in double distilled water over 8-12 hours, and differentially stained bands were identified. When selecting differentially stained bands for analysis we considered a strict criteria, picking up those bands that were present in ZR-75-1 (EpCAM $^{+/high}$) but not in MDA-MB-231 (EpCAM $^{-/low}$), to enhance the chance of picking up EpCAM interacting proteins. Gel slices were sealed in a clean plastic bag and sent for ESI-MALDI MS-MS mass spectrometry. Following this procedure, EpCAM was identified at 40 kDa by mass spectrometry, and as expected it was present in co-precipitates from ZR-75-1 (EpCAM $^{+/high}$) but not in co-precipitates from MDA-MB-231 (EpCAM $^{-/low}$) and this finding was a suitable internal control for the procedure.

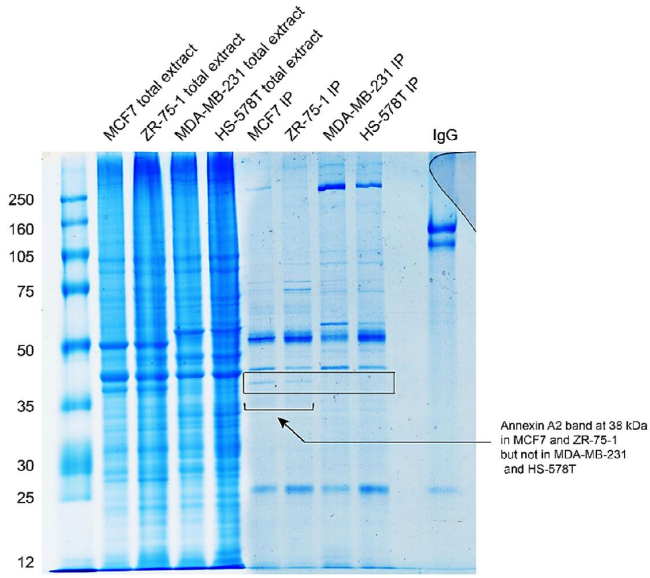

Figure 12: Scanned gel photo showing Annexin A2 bands in MCF7, ZR-75-1 but not in MDA-MB-231 and HS-578T cell lysates.

4.9 ESI-MALDI mass spectrometry, MS-MS and peptide mass fingerprints identification procedure

Gel was cut and desired bands were sliced out followed by trypsin enzymatic digestion with carbamidomethyl fixed modification. The mass and purity of the proteins were analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). Hits were correlated with known polypeptide sequences using publicly available databases; NCBI; OMIM protein, Swissprot and ExPASy. Processing of protein bands for mass spectrometry was done at the Proteomics Resource Center at the Department of Medical Biochemistry and Microbiology, Uppsala University,

Sweden.

4.10 Co-immunoprecipitation followed by Western blotting

Co-immunoprecipitated protein samples prepared as above were separated in 10% SDS PAGE and for all cell lines, total cell extracts were run in parallel. The separated proteins were transferred overnight to nitrocellulose membrane (Schleicher & Schuell) using wet and semi wet Western blot transfer as described before.

4.11 Subcellular fractionation

ZR-75-1 cells were washed three times on ice with cold PBS and scraped off, cells were then spun down for 5 minutes at 700 G, 4 °C. Cell pellets were resuspended in homogenization buffer (250 mM sucrose, 10 mM Tris HCl buffer, pH 7.4). Homogenization was performed by drawing and releasing the suspended cells many times using 22G needle and 10 cc syringe and then spun down for 15 min at 1500 G 4°C. The supernatant constitutes cytosolic proteins and membrane proteins, and the pellet contains the nuclei. The supernatant was then transferred to Sorval™, tubes were strictly balanced and centrifuged at 28000 rpm (10600 rcf) for 1 hour at 4°C. The supernatant represents cytosolic fraction while the pellet is membrane fraction.

4.12 Tunicamycin treatment of ZR-75-1 and MCF7 cells

EpCAM has three theoretical N-glycosylation sites, namely N74, N111 and N198 with three potential post glycosylation variants [332, 333]. The fully glycosylated form of EpCAM is usually at 40 kDa. The purpose of using tunicamycin is to see its effect on

EpCAM total protein apparent Mw using Western blotting. Tunicamycin (TM) is an antibiotic that specifically inhibits the asparagine-linked N-glycosylation of proteins by inhibiting the N-acetylglucosamine transferase. Tunicamycin was dissolved in 500µl Me_2SO (Dimethyl sulfoxide = DMSO) to a concentration of 10 mg/ml. In order to optimize the effect of glycosylation three different dilutions, at 1µg/µl, 5µg/µl and 10µg/µl, were used on the cells while control cells received equivalent amounts of DMSO. The viability of cells was determined by trypan blue exclusion. Cells were analyzed during 24 hours to guarantee viability and then dissolved in 2x SDS sample buffer followed by one dimensional SDS-PAGE and Western blotting.

4.13 Expression of EpCAM and ERAP2 in the presence of dog pancreatic microsomes

Canine pancreatic microsomal membranes are vesicles used to study co-translational and post-translational processing of proteins. Processing events such as signal peptide cleavage, membrane insertion, translocation and core glycosylation can be examined using appropriate cDNA/mRNA in vitro in presence of these microsomal membranes. Here we wanted to confirm EpCAM signal peptide cleavage, EpCAM glycosylation and also to see the effect on EpCAM expression in the presence of ERAP2 and possible enzyme/substrate interaction of the two proteins.

The EpCAM cDNA (GA733-2) was a kind gift from Prof. Dorothee Herlyn at the Wistar Institute of Anatomy and Biology and the ERAP2 cDNA was a kind gift from Prof. Peter van Endert at Université Paris Descartes. Both cDNA were introduced into the pGEM1 vector between an Xba1 restriction site and a Sma1 restriction site together with a

preceding Kozak sequence and verified by sequencing of the plasmid DNA at Eurofins MWG Operon (Ebersberg, Germany).

The TNT Quick coupled transcription/translation system (Promega) was used to synthesize EpCAM and ERAP2 in the presence of dog pancreas rough microsomes (RM). 150-200 ng pGEM1-EpCAM or pGEM1-ERAP2 plasmid, 5 μCi L-[^{35}S] methionine (Perkin Elmer), and 1 μl dog pancreas RM were added to 10 μl reticulocyte lysate and incubated for 90 min at 30°C. For endoglycosidase H (Endo H) treatment 6 μl of the TNT reaction was mixed with 3 μl dH_2O and 1 μl of 10X glycoprotein denaturating buffer. Following addition of 1 μl of Endo H (500,000 units/ml; NEB, MA, US) and 7 μl of dH_2O as well as 2 μl of 10X G5 reaction buffer, the sample was incubated for 1 h at 37 °C. The samples were then subjected to SDS-PAGE and protein bands were visualized using a Fuji FLA-3000 phosphoimager (Fujifilm, Tokyo, Japan).

4.14 Oncomine™ dataset accession and gene clustering analysis

We have used the Oncomine™ database premium account at www.oncomine.org to analyze a gene list that has been assembled for this purpose. The list was composed of stem cell marker and hormone receptor genes, genes related to pluripotency, epithelial and EMT related genes, EpCAM associated genes and other breast cancer related genes including cell cycle regulating genes (see paper IV). We analyzed the relation of these genes to different clinical parameters from Oncomine [1, 13, 334-341]. The analysis included the following parameters: Normal breast versus breast cancer, clinical outcome, metastasis versus primary, molecular subtypes, recurrence versus primary, pathology

grade. The statistical filter and criteria included: p-value ≤ 0.05, fold change ≥ 1.5, top 10% gene rank, mRNA samples.

For gene clustering, gene expression data for 59 breast cancer cell lines was downloaded from the Cancer Cell Line Encyclopedia portal [342]. Expression data for the selected genes was extracted by using the select feature row function on the Gene Cluster portal. The cell lines were clustered by hierarchical clustering at GenePattern [343] by using the HierarchicalClustering module [344] with standard settings. Clustering analysis of clinical breast cancer samples from the Ivshina study [1] was performed in a similar fashion. Cluster images were prepared with the HierarchicalClustering Image module.

4.15 ANTIBODIES

Antibody	Clone	source	Application	Source and Cat. #
EpCAM-FITC	B29.1	Mouse monoclonal antihuman	FC, IF	Biomeda cat. # FM010
CD44-APC	G44-26	Mouse monoclonal antihuman	FC, IF	BD Pharmingen Cat. # 559942
CD24-PE	ML5	Mouse monoclonal antihuman	FC, IF	BD Pharmingen Cat. # 555428
Negative isotype control-APC	27-35	Mouse antihuman	FC, IF	BD Pharmingen Cat. # 5557-45
Negative isotype control-APC	Mouse IgG_{2A} κ	Mouse monoclonal antihuman	FC, IF	BioLegend Cat. #400112
ER-α	MC-20	Rabbit polyclonal antihuman	FC, IF, WB	Santa Cruz Cat. # sc-542
CD44	N-18	Goat polyclonal anti-human	FC, IF, WB	Santa Cruz Cat. Cat. # sc-7051
EpCAM	C-10	Mouse monoclonal antihuman	FC, IF, WB, IP	Santa Cruz Cat. # sc-25308
EpCAM		Rabbit polyclonal antihuman	FC, IF, WB, IP	abcam Cat. # ab 71916
CD24		Mouse monoclonal antihuman	FC, IF, WB, IP	SWA11 hybridoma
Cytokeratins (CK5, 14, 8, 18)	MNF-116	Mouse monoclonal antihuman	FC, IF, WB	Dako Cat. # M0821
Vimentin		Goat polyclonal antihuman	FC, IF, WB	Sigma Cat. # V4630
Vimentin	Vim 3B4	Mouse monoclonal antihuman	FC, IF, WB	Dako Cat. # M7020
E-cadherin		Rabbit polyclonal antihuman	FC, IF, WB	Cell signaling Cat. # 4065
ERAP2	3F5	Mouse monoclonal antihuman	IF, WB	R&D systems Cat. # MAB3830

ERAP2	Q6P179	Goat polyclonal antihuman	IF, WB, IP	R&D systems Cat. # AF 3830
ERAP2		Mouse polyclonal antihuman		abcam Cat. # ab 69037
Anti-mouse-Texas Red		Horse anti-mouse secondary Ig	FC, IF	Vector Lab Cat. # TI-2000
Anti-Rabbit-Texas Red		Goat anti-rabbit secondary Ig	FC, IF	Vector Lab
Anti-rabbit- FITC		Goat anti-rabbit secondary Ig	FC, IF	Jackson ImmunoResearch Cat. # 111 095 144
ki67	MIB-1	Mouse monoclonal antihuman	FC, IF, WB	Dako # M7240
Annexin A2		Rabbit polyclonal antihuman	FC, IF, WB, IP	Santa Cruz. Cat. # sc-9061
GAPDH		Mouse monoclonal antihuman	IF, WB	Santa Cruz. Cat. # sc-32233

Table 4

5. RESULTS AND DISCUSSION

5.1 Paper I

In paper I we have studied the expression of breast cancer stem cell markers CD24, CD44 and EpCAM in selected cell lines based on their ERα status. MCF-7, ZR-75-1 and MC2 are $ERα^{+/high}$ whereas MDA-MB-231 and HS-758T are $ERα^{-/low}$. MCF-7, ZR-75-1 and MC2 have epithelial glandular-like morphology, with cells that form acinar structures similar to adult breast epithelium and more than 98% of cells express EpCAM. On the other hand MDA-MB-231 and HS-758T grow in a diffuse spread out fashion with cells that look like fibroblasts. These two cell lines are almost EpCAM negative as estimated from the surface expression of EpCAM by flow cytometry.

At the protein level we have also estimated the three markers using Western blotting. EpCAM was seen as a single band at 40 kDa in the $ERα^{+/high}$ cells of MCF-7, ZR-75-1 and MC2 while no band was detected at the same molecular weight in the $ERα^{-/low}$ MDA-MB-231 and HS-758T. Validation of the ERα status was done by immunoblotting, ERα was observed at 66 kDa in MCF-7, ZR-75-1 and MC2 cells. Estimation of total protein level of CD24 by immunoblotting revealed two patterns, while seen as two predominant bands at 45 kDa in MCF-7 and ZR-75-1 cells, the two bands moved higher in the gel at 60 kDa for MDA-MB-231 and HS-758T.

The fractions of CD24, CD44 and EpCAM positive cells were estimated by surface localized protein in live cells using flow cytometry. CD24 was predominantly positive in the $ERα^{+/high}$ cells of MCF-7 and ZR-75-1 ranging between 93-99% of positive cells, while less than 5% of the cells were positive for CD24 in the $ERα^{-/low}$ MDA-MB-231 and HS-758T clutures. EpCAM followed the same pattern as CD24 in the studied breast

cancer cell lines. An opposite pattern was observed in case of surface expression of CD44 and less than 10% of cells were positive in MCF-7 and ZR-75-1 cultures while more than 95% of the MDA-MB-231 and HS-578T cell culture were positive for CD44. The composite phenotype of $CD24^{-/low}$ $CD44^{+/high}$ $EpCAM^{+/high}$ as it was estimated by FACS ranged between 3-5% in all tested cell lines.

After ESR1 (ERα) silencing the mRNA expression of CD24, CD44, EpCAM, PGR, and ERα was estimated by PCR. There was a dramatic drop of PGR gene expression of more than 90% which can be taken as an internal positive control of the ERα down-regulation. The results correlated well with the total protein estimated expression by Western blotting. However the fraction of $CD24^{-/low}$ $CD44^{+/high}$ $EpCAM^{+/high}$ cells known as a breast cancer stem cell signature, was not significantly affected by ERα down-regulation. Vimentin increased after ERα siRNA treatment as estimated by immunoblotting, while cytokeratin 8, 9, 18 and 19 decreased. We noticed quite dramatic changes in cellular morphology of both MCF-7 and ZR-75-1 after ERα siRNA knockdown. These morphological changes included loss of epithelial ductal and lobular like glandular patterns, rather cells tended to grow in a spread out manner similar to that of fibroblasts and other mesenchymal cells and similar to the mesenchymal-like cell line MDA-MB-231.

To summarize this paper ERα seemed not to have an obvious regulatory control over CD44 and EpCAM expression while CD24, a known downstream target of ERα was shown to be affected by positive ERα regulation as it was down-regulated by ERα silencing. These findings provide an insight into the role of ERα in regulation of the three important breast cancer (stem cell) and cell surface adhesion proteins.

The aim of paper I was to elucidate whether the putative breast cancer stem cell markers CD24, CD44 and EpCAM are regulated by ERα. Also to elucidate whether the induced morphological phenotype which is consistent with EMT affected the fraction of breast cancer stem cell phenotype $CD24^{-/low}$ $CD44^{+/high}$ $EpCAM^{+/high}$. We noticed that there is an association between EpCAM positivity and $ER\alpha^{+}$ status while the cells lacking EpCAM were typically $ER\alpha^{-/low}$. The fraction of $CD24^{-/low}$ $CD44^{+/high}$ $EpCAM^{+/high}$ cells was quite low in the $ER\alpha^{+}$ cell lines, and adding $EpCAM^{+/high}$ to the signature resulted in only 3-5% cells with this composite $CD24^{-/low}$ $CD44^{+/high}$ $EpCAM^{+/high}$ phenotype in these culture. However the $ER\alpha^{-/low}$ cell lines including the $EpCAM^{-/low}$ cells of MDA-MB-231 and HS-578T had similar frequency of cells with this signature.

CD44 was detected predominantly in the more mesenchymal MDA-MB-231 and HS-578T cells. The $CD24^{-/low}$ $CD44^{+/high}$ phenotype is predominant in basal breast cancer cells while cells with $CD24^{+/high}$ $CD44^{-/low}$ phenotype tend to be more frequent in epithelial/luminal breast cancer. In summary, reduced epithelial glandular phenotype upon reduction of ERα expression was not associated with any change in the stem cell signature. Also recent literature has shown that epithelial–mesenchymal transition of breast carcinoma is association with loss of E-Cadherin and up-regulation of the mesenchymal intermediate filament protein vimentin. We could confirm a similar change in these proteins upon ERα down-regulation supporting that ERα down-regulation resulted in an EMT-like phenotype.

5.2 Paper II

In paper II we showed for the first time an association between the adhesion and stem cell marker EpCAM and ERAP2 after EpCAM co-immunoprecipitation in the $EpCAM^{+/high}$ ZR-75-1 cell line and subsequent ESI-MALDI, MS-MS mass spectrometry and peptide mass fingerprinting. The $EpCAM^{-/low}$ MDA-MB-231 cell line was included in the study as a negative control. Using Western blot analysis of total cell lysates, EpCAM was detected as a 40 kDa protein in MCF-7, ZR-75-1, and MC2, but not in MDA-MB-231 and HS-578T. ERAP2 was detected at 110 kDa in all breast cancer cell lines used in this study. An additional band of ERAP2 with the estimated molecular weight of 65kDa was observed in MCF-7 and ZR-75-1. The EpCAM ERAP2 interaction was confirmed by co-immunoprecipitation (IP) and reverse CO-IP followed by Western blotting. We next tested if the two proteins colocalized in the cells. A strong membranous and cytoplasmic immunofluorescence staining for EpCAM was seen in MCF-7 and ZR-75-1. In contrast, immunostaining of MDA-MB-231 and HS-758T showed almost no EpCAM protein, while ERAP2 was present almost equally in all tested breast cancer cell lines. ERAP2 staining was observed in the cytoplasm and as (sub) plasma membrane staining in MCF-7 and ZR-75-1, while mainly as cytoplasmic staining in MDA-MB-231 and HS-578T. ERAP2 membrane staining could be continuous forming lines separating two adjacent cells or in the form of punctate staining and as reticulated pattern scattered in the cytoplasm leaving an empty peripheral halo in the cytoplasm. This pattern confirmed potential co-localization of ERAP2 and EpCAM in the cytoplasm and the cell membrane.

We used *in vitro* transcription/translation assay supplemented with dog pancreas RMs in order to confirm signal peptide cleavage, glycosylation sites and proteolytic cleavage segments of EpCAM potentially due to ERAP2. (See figure 10 for other known EpCAM associated proteins).

EpCAM and ERAP2 were successfully expressed. EpCAM was expressed as a single band at 40 kDa, but when expressed in the dog microsome EpCAM was migrated in the SDS-PAGE as four products, probably due to signal peptide cleavage by signal peptidase (SPase) and N-linked glycosylation (N74, N111 and N198) by the oligosaccharyl transferase (OST) enzyme. ERAP2 was detected at 100 kDa in vitro. When expressed in the microsomes that is in the presence of ER vesicles an additional weak product of ERAP2 appeared at 110-120 kDa that may be due to several potential N-linked glycosylation sites (N85, N119, N219, N294, N405, N431, N650, N714 and N879) located in the C-terminal luminal part of the protein. Glycosylation sites were determined and glycosylation confirmed using endoglycosidase H treatment of the translated product. The size of the cleaved and unglycosylated product of EpCAM was set at 35 kDa. By using the NetNGlyc 1.0 Serve program, glycosylation sites of human EpCAM protein (NP_002345) were analyzed, we found three possible sites for N-linked glycosylation of EpCAM. Later MCF-7, ZR-75-1 and MDA.MB-231 cells were treated with the glycosylation inhibitor tunicamycin. We used tunicamycin in order to confirm different EpCAM glycosylated variants and EpCAM proteolytic cleavage segments. After EpCAM Western blotting an EpCAM band appeared at 40 kDa in MCF-7 and ZR-75-1 but not in MDA-MB-231. There was another extra band at 35 kDa in both MCF-7 and ZR-75-1. Tunicamycin treatment led to almost disappreance of the upper band leaving

only the EpCAM band at 35 kDa. Tunicamycin treatment also produced a double band around 30kDa in MCF-7 and less in ZR-75-1. Tunicamycin did not affect the fraction of cells expressing breast cancer adhesion proteins CD24, CD44 and EpCAM on the cell surface as estimated by FACS.

In summary, we have concluded in paper II that the association and co-localization of ERAP2 and EpCAM at the surface of breast cancer cells is a unique and novel finding. This might provide new ideas on EpCAM processing and brings up the question if it is related to antigen presentation which is a critical step dys-regulated in cancer. The presence of membranous EpCAM in carcinoma cells may help localize the associated ERAP2 molecule near the surface of such cells. Future work might be needed to address EpCAM polypeptides is trimmed by ERAP1 and ERAP2.

5.3 RESULTS, PAPER III

After EpCAM co-immunoprecipitation assay followed by mass spectrometry, Annexin A2 was also identified as EpCAM co-immunoprecipitated protein with a score of 163/66 in the $EpCAM^{+}$ ZR-75-1 cell line. MDA-MB-231 cells in which EpCAM is almost absent was included in IP as a control $EpCAM^{-/low}$ cell line. Using Annexin A2 antibody Annexin A2 was detected by Western blotting after EpCAM co-immunoprecipitation, and the converse co-immunoprecipitation experiment using Annexin A2 antibodies followed by Western blot analysis identified EpCAM in the Annexin A2 precipitates. Using Western blotting, Annexin A2 was observed at 38 kDa in all breast cancer cell lines under study, while EpCAM was seen only in $EpCAM^{+}$ ZR-75-1 and MCF7 cells.

Annexin A2 showed cytoplasmic, membranous as well as nucleolar staining patterns in the $ER\alpha^+$ cell lines MCF7, ZR-75-1 and a subset of MC2 cells (the later is an ERα transfected MDA-MB-231), while membranous localization was not detected in MDA-MB-231 and HS578T cells. Both proteins co-localized as a line or dots, mainly at the plasma membrane. Co-localization was enhanced in peripheral lamellae and membrane ruffles. The co-localization was also enhanced in places where cells were closely attached to each other and became less in single cells or sparse cells. In MC2 expressing less EpCAM, Annexin A2 tends to be confined to the cytoplasm and less at the cell membrane.

The binding partners of EpCAM are most likely not known yet. In this paper we aimed to search for new EpCAM associated proteins that could clarify the mechanisms by which EpCAM acts in the cell. Annexin A2 is an essential component for export and import vesicles and plays a major role in cellular proteins trafficking. Since EpCAM must be excreted through the endoplasmic reticulum and outside, there is a possibility that Annexin A2 act as a vehicle for EpCAM. The initial discovery of Annexin A2 and its binding partner S100A10 and its role in coagulation and vasculogenesis has been marked. Annexin A2 facilitates the conversion of fibrinogen to fibrin via a direct interaction with tissue plasminogen activator (tPA) which is deeply involved in an anticoagulation process [345, 346].

Annexin A2 was also shown to bind tenascin C which is localized in the interstitial space as an extracellular matrix protein [318, 347]. Annexin A2 was also observed to be up regulated in invasive and metastatic cells and also in EMT transformed cells.

High Annexin A2 expression was seen in metastatic breast cancer and colon cancer cells compared with the non-metastatic cells [304]. Annexin A2 binds S100A10/p11 at the N-terminus close to the tyrosine phosphorylation site affected by pp60src and the serine phosphorylation site affected by PKC.

Over-expression of Annexin A2 is observed in many human cancers including brain, lung, pancreas and breast cancers, choriocarcinoma, leukemia, osteosarcoma and pheochromocytoma [304, 316, 348-351].

5.4 PAPER IV

As a confirmation and continuation of paper I, we have chosen to select 124 genes associated with breast cancer stem cells and EMT phenotypes and analyzed them by gene clustering and also question them in the Oncomine microarray database of array data combined with clinical data. We also want to know if ER alpha down-regulation, which is known to be linked to an EMT-like state in breast is related to a breast CSC phenotype. We observed that cell lines with low ER alpha cluster into two groups (beta and gamma) while ERα^{+} cell lines form a separate cluster (alpha), the two ERα low clusters show distinct and overlapping gene expression patterns correlating mainly to EMT and breast cancer stem cells. We named these clusters cluster beta and cluster gamma, respectively. MDA-MB-231 and HS57BT cells belonged to cluster beta with a predominant EMT phenotype. Genes which are highly expressed in both cluster beta and cluster gamma include CD44, ETS1, VIM, FSCN1 and LAMC1. The epithelial cluster includes ERα positive breast cancer cell lines which express more epithelial genes like DSP, CDH1, CLDN3, CLDN4, CLDN7, EPCAM and KRT19, while the following genes are more expressed in the ERα negative cell lines: genes involved in EMT and TGFβ signaling

(ACTA2, COL1A1, COL1A2, CDH2, MMP2, FGF2, ZEB1, ZEB2, FN1, ITGA5, TGFBR2, SNAI2, MMP14, SERPINH1, TGFB1, LAMA2, LAMA4, ILK, NRG1, SNAI1, TNC and TWIST1). Cluster gamma cell lines were ER alpha negative and found to express genes involved in stem cell regulation including CDH3, ITGB4, LAMA5, ITGB6, CLDN1, KLF5, FOXQ1, TGFA, KRT5, EGFR, LAMA3, PECAM1, KLF6, FOXC1, MMP7, TGFB2, SOX9, STAT3, PROM1, and MYC.

ER alpha silencing by siRNA has up-regulated CDH1, NANOG and down-regulated ZEB1 and MYC.

On the other hand data mining using Oncomine database showed EpCAM, CDH2 and ERα are frequently elevated in cancer compared to normal breast. Genes associated with shorter survival are CD24, MYC, CDH2, EpCAM and low expression of PGR. Same finding was observed in microarray analysis using clinical samples in Ivshina dataset.

In summary our clustering analysis of the cell lines has confirms the existence of two categories of ESR1 negative breast cancers one with a more EMT-like phenotype and another with a more CSC-like phenotype.

6. ACKNOWLEDGMENTS

I would like to start by saying a big thanks to **Monica!** For being a good supervisor and a humble person. I also want to thank my friend and co-supervisor **Christer** for the intellectual discussion, fika and dinner together with my best Chinese friend **Peng**!

Thanks to Professor **Jonas Bergh's laboratory** at the department of Oncology-Pathology, Karolinska Institutet for providing breast cancer cell lines. Thanks to Professor **V. Craig Jordan**, Fox Chase Cancer Center's Division of Medical Science, USA for providing MC2 cell line. I would like to say thanks to Dr**. Juan Castro** for his assistance in flow cytometry and also for his sense of humor and jokes. Thanks to Professor **Anders Zetterberg** for sharing your great knowledge and enthusiasm.

I want to convey my appreciation to all my colleagues in Monica's lab without exception: Mikael, Anna, Ulrica, Sanna, Xiaobing, Inga, Karl, Tong, Elisa, Josef, James, Jian, Frank, Rong, Kaveh, Shiva and Teresita for the nice company and useful discussions. Thanks to my co-supervisor Daniel for all the useful discussions and helpful advises. I want also to thank my co-supervisor Johanna for help and patience I would like also to send my appreciation and thanks to Anna Alexandraki who did the PCR work for the first paper. I want here also to convey my thanks to IngMarie Nilsson and her researchers Karin Öjemalm and Patricia Lara Vasquez for working with us in the second paper and I would like also to thank Professor Åke Engström and his lab at BMC, Uppsala University for helping us in processing protein bands analysis by mass spectrometry. I want also to thank the co-authors **Cecilia Williams**

and **Philip Jonson** from University of Houston, USA, for intellectual input and contribution to paper IV.

Thanks **Tamador (Toota),** my little sister for everything, sharing conversations, who helped me not miss the Arabic culture spirit.

I want here to thank Ann-Britt for her professional administrative help during my PhD. I want also to thank the administration team at the department including Anne Jensen and Erika Rindsjö.

I thank people at the Libyan Embassy in Stockholm for their cooperation and hospitality; namely the Ambassador **Dr. Abdulmajeed**, the cultural attaché and the student organizer **Faraj Al-Mutaser**. I would like to acknowledge **Khaled**; the son of the Ambassador for nice company and inspiration.

Now it's the turn of my family and friends in Tobruk, Libya; Thanks to my brothers **Mohammad** and **Abdulhameed**, and my sisters **Tooha, Jaiwaa, Najah and Aziza,** for moral support and generous feelings which without I wouldn't make it!!

Now I want to convey my respect and love to my family members who died before hoping they can hear me when I am defending my thesis; my Father **Khairallah**, my mother **Ghalia**, my sisters **Aysha** and **Magboulah**.

Thanks to my brothers in law **Saleh, Hashim, Hamad and Hafed.**

And also all my nephews and nieces especially **Eiman, Ahmad, Montacer, Aysha, Malik, Anas, Hajooria and Erhaim, Maram!** Especial thanks to my schoolmates and friends in Libya. **Emad, Jumaah, Dr. Hisham**, **Dr. Atif, Salem Abdulraheem**, **Fayed, Mohammad Naseeb and Ali Algubba**. Great acknowledgment to my colleagues in **Tobruk: Dr. Najlaa, Dr. Amina, Dr. Fadia!**

Big hug and appreciation to my brother and best friend in Libya **Dr. Alaa Khames**; the great pathologist of my home city **Tobruk**.

7. References

1. Ivshina, A.V., et al., *Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer.* Cancer Res, 2006. **66**(21): p. 10292-301.
2. Lynch, M.D., M. Cariati, and A.D. Purushotham, *Breast cancer, stem cells and prospects for therapy.* Breast Cancer Res, 2006. **8**(3): p. 211.
3. Vogelstein, B. and K.W. Kinzler, *The multistep nature of cancer.* Trends Genet, 1993. **9**(4): p. 138-41.
4. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
5. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
6. Parkin, D.M., F.I. Bray, and S.S. Devesa, *Cancer burden in the year 2000. The global picture.* Eur J Cancer, 2001. **37 Suppl 8**: p. S4-66.
7. Schwartsmann, G., et al., *Anticancer drug discovery and development throughout the world.* J Clin Oncol, 2002. **20**(18 Suppl): p. 47S-59S.
8. Botha, J.L., et al., *Breast cancer incidence and mortality trends in 16 European countries.* Eur J Cancer, 2003. **39**(12): p. 1718-29.
9. Zahl, P.H., B.H. Strand, and J. Maehlen, *Incidence of breast cancer in Norway and Sweden during introduction of nationwide screening: prospective cohort study.* BMJ, 2004. **328**(7445): p. 921-4.
10. Coleman, M.P., et al., *EUROCARE-3 summary: cancer survival in Europe at the end of the 20th century.* Ann Oncol, 2003. **14 Suppl 5**: p. v128-49.
11. Sant, M., et al., *EUROCARE-3: survival of cancer patients diagnosed 1990-94--results and commentary.* Ann Oncol, 2003. **14 Suppl 5**: p. v61-118.
12. van 't Veer, L.J., et al., *Gene expression profiling predicts clinical outcome of breast cancer.* Nature, 2002. **415**(6871): p. 530-6.
13. Sorlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10869-74.
14. van de Vijver, M.J., et al., *A gene-expression signature as a predictor of survival in breast cancer.* N Engl J Med, 2002. **347**(25): p. 1999-2009.
15. Cianchetti, E., et al., *[Breast cancer. Epidemiology and other factors related to incidence in various populations].* Minerva Med, 1985. **76**(12): p. 555-61.
16. Yi, M., et al., *Comparative analysis of clinicopathologic features, treatment, and survival of Asian women with a breast cancer diagnosis residing in the United States.* Cancer, 2012. **118**(17): p. 4117-25.
17. Locke, F.B. and H. King, *Cancer mortality risk among Japanese in the United States.* J Natl Cancer Inst, 1980. **65**(5): p. 1149-56.
18. King, H. and F.B. Locke, *Cancer mortality among Chinese in the United States.* J Natl Cancer Inst, 1980. **65**(5): p. 1141-8.
19. Darbre, P.D., *Recorded quadrant incidence of female breast cancer in Great Britain suggests a disproportionate increase in the upper outer quadrant of the breast.* Anticancer Res, 2005. **25**(3c): p. 2543-50.
20. Pascual, M.R., et al., *Clinical factors related to the presence of estrogen receptors in breast cancer: a prognostic stratification analysis.* Neoplasma, 1982. **29**(4): p. 453-61.
21. Coyle, Y.M., *The effect of environment on breast cancer risk.* Breast Cancer Res Treat, 2004. **84**(3): p. 273-88.
22. Cho, E., et al., *Premenopausal fat intake and risk of breast cancer.* J Natl Cancer Inst, 2003. **95**(14): p. 1079-85.

23. Schatzkin, A., et al., *Alcohol consumption and breast cancer in the epidemiologic follow-up study of the first National Health and Nutrition Examination Survey.* N Engl J Med, 1987. **316**(19): p. 1169-73.
24. Le Marchand, L., et al., *Body size at different periods of life and breast cancer risk.* Am J Epidemiol, 1988. **128**(1): p. 137-52.
25. Trichopoulos, D., B. MacMahon, and P. Cole, *Menopause and breast cancer risk.* J Natl Cancer Inst, 1972. **48**(3): p. 605-13.
26. Tokunaga, M., et al., *Incidence of female breast cancer among atomic bomb survivors, 1950-1985.* Radiat Res, 1994. **138**(2): p. 209-23.
27. Proia, T.A., et al., *Genetic predisposition directs breast cancer phenotype by dictating progenitor cell fate.* Cell Stem Cell, 2011. **8**(2): p. 149-63.
28. Wolff, T.A. and J.E. Wilson, *Genetic risk assessment and BRCA mutation testing for breast and ovarian cancer susceptibility.* Am Fam Physician, 2006. **74**(10): p. 1759-60.
29. Couper, M.P., et al., *Use of the Internet and ratings of information sources for medical decisions: results from the DECISIONS survey.* Med Decis Making, 2010. **30**(5 Suppl): p. 106S-114S.
30. Wei, E.K., K.Y. Wolin, and G.A. Colditz, *Time course of risk factors in cancer etiology and progression.* J Clin Oncol, 2010. **28**(26): p. 4052-7.
31. Moysich, K.B., et al., *Use of common medications and breast cancer risk.* Cancer Epidemiol Biomarkers Prev, 2008. **17**(7): p. 1564-95.
32. Lambe, M., et al., *Maternal risk of breast cancer following multiple births: a nationwide study in Sweden.* Cancer Causes Control, 1996. **7**(5): p. 533-8.
33. Lambe, M., et al., *Parity, age at first and last birth, and risk of breast cancer: a population-based study in Sweden.* Breast Cancer Res Treat, 1996. **38**(3): p. 305-11.
34. Singletary, S.E. and J.L. Connolly, *Breast cancer staging: working with the sixth edition of the AJCC Cancer Staging Manual.* CA Cancer J Clin, 2006. **56**(1): p. 37-47; quiz 50-1.
35. Toikkanen, S., L. Pylkkanen, and H. Joensuu, *Invasive lobular carcinoma of the breast has better short- and long-term survival than invasive ductal carcinoma.* Br J Cancer, 1997. **76**(9): p. 1234-40.
36. Bloom, H.J. and W.W. Richardson, *Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years.* Br J Cancer, 1957. **11**(3): p. 359-77.
37. Elston, C.W. and I.O. Ellis, *Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up.* Histopathology, 1991. **19**(5): p. 403-10.
38. Tamimi, R.M., et al., *Comparison of molecular phenotypes of ductal carcinoma in situ and invasive breast cancer.* Breast Cancer Res, 2008. **10**(4): p. R67.
39. Morrison, B.J., et al., *Breast cancer stem cells: implications for therapy of breast cancer.* Breast Cancer Res, 2008. **10**(4): p. 210.
40. Shipitsin, M., et al., *Molecular definition of breast tumor heterogeneity.* Cancer Cell, 2007. **11**(3): p. 259-73.
41. Fulford, L.G., et al., *Basal-like grade III invasive ductal carcinoma of the breast: patterns of metastasis and long-term survival.* Breast Cancer Res, 2007. **9**(1): p. R4.
42. Polyak, K., *Breast cancer stem cells: a case of mistaken identity?* Stem Cell Rev, 2007. **3**(2): p. 107-9.
43. Mills, A.A., *p53: link to the past, bridge to the future.* Genes Dev, 2005. **19**(18): p. 2091-9.

44. Jerry, D.J., K.A. Dunphy, and M.J. Hagen, *Estrogens, regulation of p53 and breast cancer risk: a balancing act.* Cell Mol Life Sci, 2010. **67**(7): p. 1017-23.
45. Bonin, S., et al., *Molecular characterisation of breast cancer patients at high and low recurrence risk.* Virchows Arch, 2008. **452**(3): p. 241-50.
46. Foekens, J.A., et al., *Cathepsin-D in primary breast cancer: prognostic evaluation involving 2810 patients.* Br J Cancer, 1999. **79**(2): p. 300-7.
47. Tandon, A.K., et al., *Cathepsin D and prognosis in breast cancer.* N Engl J Med, 1990. **322**(5): p. 297-302.
48. Westley, B.R. and F.E. May, *Prognostic value of cathepsin D in breast cancer.* Br J Cancer, 1999. **79**(2): p. 189-90.
49. Losch, A., et al., *Prognostic value of cathepsin D expression and association with histomorphological subtypes in breast cancer.* Br J Cancer, 1998. **78**(2): p. 205-9.
50. Brouillet, J.P., et al., *Immunoradiometric assay of pro-cathepsin D in breast cancer cytosol: relative prognostic value versus total cathepsin D.* Eur J Cancer, 1993. **29A**(9): p. 1248-51.
51. Isola, J., et al., *Cathepsin D expression detected by immunohistochemistry has independent prognostic value in axillary node-negative breast cancer.* J Clin Oncol, 1993. **11**(1): p. 36-43.
52. Zimmermann, A. and F. Truss, *[Comparative cytologic and flow-through cytophotometric studies in prostate cells (author's transl)].* Urologe A, 1978. **17**(6): p. 391-4.
53. Darzynkiewicz, Z., H.D. Halicka, and H. Zhao, *Analysis of cellular DNA content by flow and laser scanning cytometry.* Adv Exp Med Biol, 2010. **676**: p. 137-47.
54. Esteva, F.J. and G.N. Hortobagyi, *Prognostic molecular markers in early breast cancer.* Breast Cancer Res, 2004. **6**(3): p. 109-18.
55. Boyle, P., *Current situation of screening for cancer.* Ann Oncol, 2002. **13 Suppl 4**: p. 189-98.
56. Hennighausen, L. and G.W. Robinson, *Information networks in the mammary gland.* Nat Rev Mol Cell Biol, 2005. **6**(9): p. 715-25.
57. Forster, C., et al., *Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15578-83.
58. Asselin-Labat, M.L., et al., *Control of mammary stem cell function by steroid hormone signalling.* Nature, 2010. **465**(7299): p. 798-802.
59. Joshi, P.A., et al., *Progesterone induces adult mammary stem cell expansion.* Nature, 2010. **465**(7299): p. 803-7.
60. Carroll, J.S., et al., *Genome-wide analysis of estrogen receptor binding sites.* Nat Genet, 2006. **38**(11): p. 1289-97.
61. Feng, Y., et al., *Estrogen receptor-alpha expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice.* Proc Natl Acad Sci U S A, 2007. **104**(37): p. 14718-23.
62. Korach, K.S., *Insights from the study of animals lacking functional estrogen receptor.* Science, 1994. **266**(5190): p. 1524-7.
63. Hartman, J., et al., *Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts.* Cancer Res, 2006. **66**(23): p. 11207-13.
64. Nilsson, S., et al., *Mechanisms of estrogen action.* Physiol Rev, 2001. **81**(4): p. 1535-65.
65. Hou, Y.F., et al., *ERbeta exerts multiple stimulative effects on human breast carcinoma cells.* Oncogene, 2004. **23**(34): p. 5799-806.
66. Tonetti, D.A., et al., *Stable transfection of an estrogen receptor beta cDNA isoform into MDA-MB-231 breast cancer cells.* J Steroid Biochem Mol Biol, 2003. **87**(1): p. 47-55.

67. Platet, N., et al., *Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion.* Crit Rev Oncol Hematol, 2004. **51**(1): p. 55-67.
68. Esslimani-Sahla, M., et al., *Increased estrogen receptor betacx expression during mammary carcinogenesis.* Clin Cancer Res, 2005. **11**(9): p. 3170-4.
69. Palmieri, C., et al., *Estrogen receptor beta in breast cancer.* Endocr Relat Cancer, 2002. **9**(1): p. 1-13.
70. Park, B.W., et al., *Expression of estrogen receptor-beta in normal mammary and tumor tissues: is it protective in breast carcinogenesis?* Breast Cancer Res Treat, 2003. **80**(1): p. 79-85.
71. Schiff, R., et al., *Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance.* Clin Cancer Res, 2004. **10**(1 Pt 2): p. 331S-6S.
72. Bardou, V.J., et al., *Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases.* J Clin Oncol, 2003. **21**(10): p. 1973-9.
73. Knight, W.A., et al., *Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer.* Cancer Res, 1977. **37**(12): p. 4669-71.
74. Veronesi, U., et al., *Sentinel lymph node biopsy as an indicator for axillary dissection in early breast cancer.* Eur J Cancer, 2001. **37**(4): p. 454-8.
75. Krag, D., et al., *The sentinel node in breast cancer--a multicenter validation study.* N Engl J Med, 1998. **339**(14): p. 941-6.
76. Veronesi, U., et al., *A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer.* N Engl J Med, 2003. **349**(6): p. 546-53.
77. Sharabi, S.E., et al., *The need for breast cancer screening in women undergoing elective breast surgery: an assessment of risk and risk factors for breast cancer in young women.* Aesthet Surg J, 2010. **30**(6): p. 821-31.
78. Wallgren, A., et al., *Timing of radiotherapy and chemotherapy following breast-conserving surgery for patients with node-positive breast cancer. International Breast Cancer Study Group.* Int J Radiat Oncol Biol Phys, 1996. **35**(4): p. 649-59.
79. Overgaard, M., et al., *Postoperative radiotherapy in high-risk postmenopausal breast-cancer patients given adjuvant tamoxifen: Danish Breast Cancer Cooperative Group DBCG 82c randomised trial.* Lancet, 1999. **353**(9165): p. 1641-8.
80. Chuthapisith, S., et al., *Breast cancer chemoresistance: emerging importance of cancer stem cells.* Surg Oncol, 2010. **19**(1): p. 27-32.
81. Stal, O., et al., *S-phase fraction and survival benefit from adjuvant chemotherapy or radiotherapy of breast cancer.* Br J Cancer, 1994. **70**(6): p. 1258-62.
82. *Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials.* Lancet, 2005. **365**(9472): p. 1687-717.
83. Bilynskyj, B.T., *The breast cancer treatment as a marker of progress in oncology.* Exp Oncol, 2010. **32**(3): p. 190-4.
84. Mouridsen, H.T. and N.J. Robert, *The role of aromatase inhibitors as adjuvant therapy for early breast cancer in postmenopausal women.* Eur J Cancer, 2005. **41**(12): p. 1678-89.
85. Mouridsen, H.T. and A.S. Bhatnagar, *Letrozole in the treatment of breast cancer.* Expert Opin Pharmacother, 2005. **6**(8): p. 1389-99.
86. Mouridsen, H., et al., *Phase III study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: analysis of survival and update of*

efficacy from the International Letrozole Breast Cancer Group. J Clin Oncol, 2003. **21**(11): p. 2101-9.
87. Coombes, R.C., et al., *A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer.* N Engl J Med, 2004. **350**(11): p. 1081-92.
88. Jakesz, R., et al., *Switching of postmenopausal women with endocrine-responsive early breast cancer to anastrozole after 2 years' adjuvant tamoxifen: combined results of ABCSG trial 8 and ARNO 95 trial.* Lancet, 2005. **366**(9484): p. 455-62.
89. Veronesi, U., et al., *Radiotherapy after breast-conserving surgery in small breast carcinoma: long-term results of a randomized trial.* Ann Oncol, 2001. **12**(7): p. 997-1003.
90. Matsunaga, S., et al., *Gamma Knife surgery for metastatic brain tumors from primary breast cancer: treatment indication based on number of tumors and breast cancer phenotype.* J Neurosurg, 2010. **113 Suppl**: p. 65-72.
91. Slamon, D.J., et al., *Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer.* Science, 1989. **244**(4905): p. 707-12.
92. Vogel, C.L., et al., *Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer.* J Clin Oncol, 2002. **20**(3): p. 719-26.
93. Slamon, D.J., et al., *Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2.* N Engl J Med, 2001. **344**(11): p. 783-92.
94. van 't Veer, L.J., et al., *Expression profiling predicts outcome in breast cancer.* Breast Cancer Res, 2003. **5**(1): p. 57-8.
95. Bergh, J., et al., *A systematic overview of chemotherapy effects in breast cancer.* Acta Oncol, 2001. **40**(2-3): p. 253-81.
96. Lobo, N.A., et al., *The biology of cancer stem cells.* Annu Rev Cell Dev Biol, 2007. **23**: p. 675-99.
97. Bosch, A., et al., *Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research.* Cancer Treat Rev, 2010. **36**(3): p. 206-15.
98. Stingl, J., et al., *Purification and unique properties of mammary epithelial stem cells.* Nature, 2006. **439**(7079): p. 993-7.
99. Asselin-Labat, M.L., et al., *Gata-3 negatively regulates the tumor-initiating capacity of mammary luminal progenitor cells and targets the putative tumor suppressor caspase-14.* Mol Cell Biol, 2011. **31**(22): p. 4609-22.
100. Asselin-Labat, M.L., et al., *Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation.* Nat Cell Biol, 2007. **9**(2): p. 201-9.
101. Lapidot, T., et al., *A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.* Nature, 1994. **367**(6464): p. 645-8.
102. Reya, T., et al., *Stem cells, cancer, and cancer stem cells.* Nature, 2001. **414**(6859): p. 105-11.
103. Singh, S.K., et al., *Identification of human brain tumour initiating cells.* Nature, 2004. **432**(7015): p. 396-401.
104. O'Brien, C.A., et al., *A human colon cancer cell capable of initiating tumour growth in immunodeficient mice.* Nature, 2007. **445**(7123): p. 106-10.
105. Qin, J., et al., *The PSA(-/lo) prostate cancer cell population harbors self-renewing long-term tumor-propagating cells that resist castration.* Cell Stem Cell, 2012. **10**(5): p. 556-69.

106. Zhang, S., et al., *Identification and characterization of ovarian cancer-initiating cells from primary human tumors.* Cancer Res, 2008. **68**(11): p. 4311-20.
107. Schatton, T., et al., *Identification of cells initiating human melanomas.* Nature, 2008. **451**(7176): p. 345-9.
108. Boiko, A.D., et al., *Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271.* Nature, 2010. **466**(7302): p. 133-7.
109. Civenni, G., et al., *Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth.* Cancer Res, 2011. **71**(8): p. 3098-109.
110. Matsui, W., et al., *Characterization of clonogenic multiple myeloma cells.* Blood, 2004. **103**(6): p. 2332-6.
111. Matsui, W., et al., *Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance.* Cancer Res, 2008. **68**(1): p. 190-7.
112. Eramo, A., et al., *Identification and expansion of the tumorigenic lung cancer stem cell population.* Cell Death Differ, 2008. **15**(3): p. 504-14.
113. Al-Hajj, M. and M.F. Clarke, *Self-renewal and solid tumor stem cells.* Oncogene, 2004. **23**(43): p. 7274-82.
114. Ricci-Vitiani, L., et al., *Identification and expansion of human colon-cancer-initiating cells.* Nature, 2007. **445**(7123): p. 111-5.
115. Kondo, T., *Stem cell-like cancer cells in cancer cell lines.* Cancer Biomark, 2007. **3**(4-5): p. 245-50.
116. Molyneux, G., et al., *BRCA1 basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells.* Cell Stem Cell, 2010. **7**(3): p. 403-17.
117. Miyoshi, Y., K. Murase, and K. Oh, *Basal-like subtype and BRCA1 dysfunction in breast cancers.* Int J Clin Oncol, 2008. **13**(5): p. 395-400.
118. Wright, M.H., et al., *Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics.* Breast Cancer Res, 2008. **10**(1): p. R10.
119. Balic, M., et al., *Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype.* Clin Cancer Res, 2006. **12**(19): p. 5615-21.
120. Yang, W., et al., *Breast cancer metastasis in a human bone NOD/SCID mouse model.* Cancer Biol Ther, 2007. **6**(8): p. 1289-94.
121. Kuperwasser, C., et al., *A mouse model of human breast cancer metastasis to human bone.* Cancer Res, 2005. **65**(14): p. 6130-8.
122. Karnoub, A.E., et al., *Mesenchymal stem cells within tumour stroma promote breast cancer metastasis.* Nature, 2007. **449**(7162): p. 557-63.
123. Ince, T.A., et al., *Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes.* Cancer Cell, 2007. **12**(2): p. 160-70.
124. Rudin, C.M., *Vismodegib.* Clin Cancer Res, 2012. **18**(12): p. 3218-22.
125. Clement, V., et al., *HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity.* Curr Biol, 2007. **17**(2): p. 165-72.
126. Hatsell, S. and A.R. Frost, *Hedgehog signaling in mammary gland development and breast cancer.* J Mammary Gland Biol Neoplasia, 2007. **12**(2-3): p. 163-73.
127. Liu, S., et al., *Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells.* Cancer Res, 2006. **66**(12): p. 6063-71.
128. Dontu, G., et al., *Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells.* Breast Cancer Res, 2004. **6**(6): p. R605-15.

129. Chan, S.M., et al., *Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia.* Blood, 2007. **110**(1): p. 278-86.
130. Kim, M.K., et al., *An integrated genome screen identifies the Wnt signaling pathway as a major target of WT1.* Proc Natl Acad Sci U S A, 2009. **106**(27): p. 11154-9.
131. Ai, L., et al., *Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer.* Carcinogenesis, 2006. **27**(7): p. 1341-8.
132. Wissmann, C., et al., *WIF1, a component of the Wnt pathway, is down-regulated in prostate, breast, lung, and bladder cancer.* J Pathol, 2003. **201**(2): p. 204-12.
133. Suzuki, H., et al., *Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer.* Br J Cancer, 2008. **98**(6): p. 1147-56.
134. Aguilera, O., et al., *Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer.* Oncogene, 2006. **25**(29): p. 4116-21.
135. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-8.
136. Battula, V.L., et al., *Ganglioside GD2 identifies breast cancer stem cells and promotes tumorigenesis.* J Clin Invest, 2012. **122**(6): p. 2066-78.
137. Kleffel, S. and T. Schatton, *Tumor dormancy and cancer stem cells: two sides of the same coin?* Adv Exp Med Biol, 2013. **734**: p. 145-79.
138. Li, F., et al., *Beyond tumorigenesis: cancer stem cells in metastasis.* Cell Res, 2007. **17**(1): p. 3-14.
139. Chang, H.Y., et al., *Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds.* PLoS Biol, 2004. **2**(2): p. E7.
140. Nowell, P.C., *The clonal evolution of tumor cell populations.* Science, 1976. **194**(4260): p. 23-8.
141. Stingl, J. and C. Caldas, *Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis.* Nat Rev Cancer, 2007. **7**(10): p. 791-9.
142. Bjerkvig, R., et al., *Opinion: the origin of the cancer stem cell: current controversies and new insights.* Nat Rev Cancer, 2005. **5**(11): p. 899-904.
143. Pardal, R., M.F. Clarke, and S.J. Morrison, *Applying the principles of stem-cell biology to cancer.* Nat Rev Cancer, 2003. **3**(12): p. 895-902.
144. Tataria, M., S.V. Perryman, and K.G. Sylvester, *Stem cells: tissue regeneration and cancer.* Semin Pediatr Surg, 2006. **15**(4): p. 284-92.
145. Christgen, M., et al., *Detection of putative cancer stem cells of the side population phenotype in human tumor cell cultures.* Methods Mol Biol, 2012. **878**: p. 201-15.
146. Creighton, C.J., et al., *Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 13820-5.
147. Liu, T.J., et al., *CD133(+) cells with cancer stem cell characteristics associates with vasculogenic mimicry in triple-negative breast cancer.* Oncogene, 2012.
148. Shimono, Y., et al., *Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells.* Cell, 2009. **138**(3): p. 592-603.
149. Alison, M.R., et al., *Finding cancer stem cells: are aldehyde dehydrogenases fit for purpose?* J Pathol, 2010. **222**(4): p. 335-44.
150. Morimoto, K., et al., *Stem cell marker aldehyde dehydrogenase 1-positive breast cancers are characterized by negative estrogen receptor, positive human epidermal growth factor receptor type 2, and high Ki67 expression.* Cancer Sci, 2009. **100**(6): p. 1062-8.

151. Kay, R., P.M. Rosten, and R.K. Humphries, *CD24, a signal transducer modulating B cell activation responses, is a very short peptide with a glycosyl phosphatidylinositol membrane anchor.* J Immunol, 1991. **147**(4): p. 1412-6.
152. Kristiansen, G., M. Sammar, and P. Altevogt, *Tumour biological aspects of CD24, a mucin-like adhesion molecule.* J Mol Histol, 2004. **35**(3): p. 255-62.
153. Springer, T., et al., *Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens.* Eur J Immunol, 1978. **8**(8): p. 539-51.
154. Lee, J.H., et al., *CD24 overexpression in cancer development and progression: a meta-analysis.* Oncol Rep, 2009. **22**(5): p. 1149-56.
155. Mierke, C.T., N. Bretz, and P. Altevogt, *Contractile forces contribute to increased glycosylphosphatidylinositol-anchored receptor CD24-facilitated cancer cell invasion.* J Biol Chem, 2011. **286**(40): p. 34858-71.
156. Baumann, P., et al., *CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis.* Cancer Res, 2005. **65**(23): p. 10783-93.
157. Aigner, S., et al., *CD24 mediates rolling of breast carcinoma cells on P-selectin.* FASEB J, 1998. **12**(12): p. 1241-51.
158. Hahne, M., et al., *The heat-stable antigen can alter very late antigen 4-mediated adhesion.* J Exp Med, 1994. **179**(4): p. 1391-5.
159. Smith, S.C., et al., *The metastasis-associated gene CD24 is regulated by Ral GTPase and is a mediator of cell proliferation and survival in human cancer.* Cancer Res, 2006. **66**(4): p. 1917-22.
160. Overdevest, J.B., et al., *CD24 offers a therapeutic target for control of bladder cancer metastasis based on a requirement for lung colonization.* Cancer Res, 2011. **71**(11): p. 3802-11.
161. Fukushima, T., et al., *Silencing of insulin-like growth factor-binding protein-2 in human glioblastoma cells reduces both invasiveness and expression of progression-associated gene CD24.* J Biol Chem, 2007. **282**(25): p. 18634-44.
162. Sammar, M., et al., *Heat-stable antigen (CD24) as ligand for mouse P-selectin.* Int Immunol, 1994. **6**(7): p. 1027-36.
163. Aigner, S., et al., *CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells.* Blood, 1997. **89**(9): p. 3385-95.
164. Friederichs, J., et al., *The CD24/P-selectin binding pathway initiates lung arrest of human A125 adenocarcinoma cells.* Cancer Res, 2000. **60**(23): p. 6714-22.
165. Schabath, H., et al., *CD24 affects CXCR4 function in pre-B lymphocytes and breast carcinoma cells.* J Cell Sci, 2006. **119**(Pt 2): p. 314-25.
166. Simons, K. and M.J. Gerl, *Revitalizing membrane rafts: new tools and insights.* Nat Rev Mol Cell Biol, 2010. **11**(10): p. 688-99.
167. Runz, S., et al., *CD24 induces localization of beta1 integrin to lipid raft domains.* Biochem Biophys Res Commun, 2008. **365**(1): p. 35-41.
168. Varma, R. and S. Mayor, *GPI-anchored proteins are organized in submicron domains at the cell surface.* Nature, 1998. **394**(6695): p. 798-801.
169. Zarn, J.A., et al., *Association of CD24 with the kinase c-fgr in a small cell lung cancer cell line and with the kinase lyn in an erythroleukemia cell line.* Biochem Biophys Res Commun, 1996. **225**(2): p. 384-91.
170. Sammar, M., et al., *Mouse CD24 as a signaling molecule for integrin-mediated cell binding: functional and physical association with src-kinases.* Biochem Biophys Res Commun, 1997. **234**(2): p. 330-4.

171. Stefanova, I., et al., *GPI-anchored cell-surface molecules complexed to protein tyrosine kinases.* Science, 1991. **254**(5034): p. 1016-9.
172. Baumann, P., et al., *CD24 interacts with and promotes the activity of c-src within lipid rafts in breast cancer cells, thereby increasing integrin-dependent adhesion.* Cell Mol Life Sci, 2012. **69**(3): p. 435-48.
173. Shi, Y., et al., *CD24: a novel cancer biomarker in laryngeal squamous cell carcinoma.* ORL J Otorhinolaryngol Relat Spec, 2012. **74**(2): p. 78-85.
174. Lee, H.J., et al., *CD24, a novel cancer biomarker, predicting disease-free survival of non-small cell lung carcinomas: a retrospective study of prognostic factor analysis from the viewpoint of forthcoming (seventh) new TNM classification.* J Thorac Oncol, 2010. **5**(5): p. 649-57.
175. Visvader, J.E. and G.J. Lindeman, *Cancer stem cells in solid tumours: accumulating evidence and unresolved questions.* Nat Rev Cancer, 2008. **8**(10): p. 755-68.
176. Gallatin, W.M., I.L. Weissman, and E.C. Butcher, *A cell-surface molecule involved in organ-specific homing of lymphocytes.* Nature, 1983. **304**(5921): p. 30-4.
177. Naor, D., et al., *Involvement of CD44, a molecule with a thousand faces, in cancer dissemination.* Semin Cancer Biol, 2008. **18**(4): p. 260-7.
178. Ratajczak, M.Z., *Cancer stem cells--normal stem cells "Jedi" that went over to the "dark side".* Folia Histochem Cytobiol, 2005. **43**(4): p. 175-81.
179. Stamenkovic, I., et al., *A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family.* Cell, 1989. **56**(6): p. 1057-62.
180. Toyama-Sorimachi, N. and M. Miyasaka, *A novel ligand for CD44 is sulfated proteoglycan.* Int Immunol, 1994. **6**(4): p. 655-60.
181. Ruiz, P., C. Schwarzler, and U. Gunthert, *CD44 isoforms during differentiation and development.* Bioessays, 1995. **17**(1): p. 17-24.
182. Tremmel, M., et al., *A CD44v6 peptide reveals a role of CD44 in VEGFR-2 signaling and angiogenesis.* Blood, 2009. **114**(25): p. 5236-44.
183. Lesley, J., R. Hyman, and P.W. Kincade, *CD44 and its interaction with extracellular matrix.* Adv Immunol, 1993. **54**: p. 271-335.
184. Lokeshwar, V.B., N. Fregien, and L.Y. Bourguignon, *Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function.* J Cell Biol, 1994. **126**(4): p. 1099-109.
185. Takai, Y., et al., *Structural basis of the cytoplasmic tail of adhesion molecule CD43 and its binding to ERM proteins.* J Mol Biol, 2008. **381**(3): p. 634-44.
186. Bhat-Nakshatri, P., et al., *SLUG/SNAI2 and tumor necrosis factor generate breast cells with CD44+/CD24- phenotype.* BMC Cancer, 2010. **10**: p. 411.
187. Allan, A.L., et al., *Tumor dormancy and cancer stem cells: implications for the biology and treatment of breast cancer metastasis.* Breast Dis, 2006. **26**: p. 87-98.
188. Lopez, J.I., et al., *CD44 attenuates metastatic invasion during breast cancer progression.* Cancer Res, 2005. **65**(15): p. 6755-63.
189. Bourguignon, L.Y., et al., *Hyaluronan-CD44 interaction with protein kinase C(epsilon) promotes oncogenic signaling by the stem cell marker Nanog and the Production of microRNA-21, leading to down-regulation of the tumor suppressor protein PDCD4, anti-apoptosis, and chemotherapy resistance in breast tumor cells.* J Biol Chem, 2009. **284**(39): p. 26533-46.
190. Avigdor, A., et al., *CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow.* Blood, 2004. **103**(8): p. 2981-9.

191. Kim, H.R., et al., *Hyaluronan facilitates invasion of colon carcinoma cells in vitro via interaction with CD44.* Cancer Res, 2004. **64**(13): p. 4569-76.
192. Zoller, M., *CD44: can a cancer-initiating cell profit from an abundantly expressed molecule?* Nat Rev Cancer, 2011. **11**(4): p. 254-67.
193. Uchino, M., et al., *Nuclear beta-catenin and CD44 upregulation characterize invasive cell populations in non-aggressive MCF-7 breast cancer cells.* BMC Cancer, 2010. **10**: p. 414.
194. Baeuerle, P.A. and O. Gires, *EpCAM (CD326) finding its role in cancer.* Br J Cancer, 2007. **96**(3): p. 417-23.
195. Balzar, M., et al., *The biology of the 17-1A antigen (Ep-CAM).* J Mol Med (Berl), 1999. **77**(10): p. 699-712.
196. Frederick, B.A., et al., *Epithelial to mesenchymal transition predicts gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non-small cell lung carcinoma.* Mol Cancer Ther, 2007. **6**(6): p. 1683-91.
197. Santisteban, M., et al., *Immune-induced epithelial to mesenchymal transition in vivo generates breast cancer stem cells.* Cancer Res, 2009. **69**(7): p. 2887-95.
198. Gadalla, S.E., et al., *EpCAM associates with endoplasmic reticulum aminopeptidase 2 (ERAP2) in breast cancer cells.* Biochem Biophys Res Commun, 2013. **439**(2): p. 203-8.
199. Went, P.T., et al., *Frequent EpCam protein expression in human carcinomas.* Hum Pathol, 2004. **35**(1): p. 122-8.
200. Gosens, M.J., et al., *Loss of membranous Ep-CAM in budding colorectal carcinoma cells.* Mod Pathol, 2007. **20**(2): p. 221-32.
201. Yanamoto, S., et al., *Clinicopathologic significance of EpCAM expression in squamous cell carcinoma of the tongue and its possibility as a potential target for tongue cancer gene therapy.* Oral Oncol, 2007. **43**(9): p. 869-77.
202. Winter, M.J., et al., *Expression of Ep-CAM shifts the state of cadherin-mediated adhesions from strong to weak.* Exp Cell Res, 2003. **285**(1): p. 50-8.
203. Nubel, T., et al., *Claudin-7 regulates EpCAM-mediated functions in tumor progression.* Mol Cancer Res, 2009. **7**(3): p. 285-99.
204. Maetzel, D., et al., *Nuclear signalling by tumour-associated antigen EpCAM.* Nat Cell Biol, 2009. **11**(2): p. 162-71.
205. Ensinger, C., et al., *EpCAM overexpression in thyroid carcinomas: a histopathological study of 121 cases.* J Immunother, 2006. **29**(5): p. 569-73.
206. Hwang, E.Y., et al., *Decreased expression of Ep-CAM protein is significantly associated with the progression and prognosis of oral squamous cell carcinomas in Taiwan.* J Oral Pathol Med, 2009. **38**(1): p. 87-93.
207. Kimura, H., et al., *Prognostic significance of EpCAM expression in human esophageal cancer.* Int J Oncol, 2007. **30**(1): p. 171-9.
208. Seligson, D.B., et al., *Epithelial cell adhesion molecule (KSA) expression: pathobiology and its role as an independent predictor of survival in renal cell carcinoma.* Clin Cancer Res, 2004. **10**(8): p. 2659-69.
209. Songun, I., et al., *Loss of Ep-CAM (CO17-1A) expression predicts survival in patients with gastric cancer.* Br J Cancer, 2005. **92**(9): p. 1767-72.
210. Klatte, T., et al., *Cytogenetic and molecular tumor profiling for type 1 and type 2 papillary renal cell carcinoma.* Clin Cancer Res, 2009. **15**(4): p. 1162-9.
211. Ralhan, R., et al., *EpCAM nuclear localization identifies aggressive thyroid cancer and is a marker for poor prognosis.* BMC Cancer, 2010. **10**: p. 331.
212. Li, C., C.J. Lee, and D.M. Simeone, *Identification of human pancreatic cancer stem cells.* Methods Mol Biol, 2009. **568**: p. 161-73.

213. Li, C., et al., *Identification of pancreatic cancer stem cells.* Cancer Res, 2007. **67**(3): p. 1030-7.
214. Yamashita, T., et al., *EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features.* Gastroenterology, 2009. **136**(3): p. 1012-24.
215. Fong, D., et al., *Ep-CAM expression in pancreatic and ampullary carcinomas: frequency and prognostic relevance.* J Clin Pathol, 2008. **61**(1): p. 31-5.
216. Scheunemann, P., et al., *Occult tumor cells in lymph nodes as a predictor for tumor relapse in pancreatic adenocarcinoma.* Langenbecks Arch Surg, 2008. **393**(3): p. 359-65.
217. van der Gun, B.T., et al., *EpCAM in carcinogenesis: the good, the bad or the ugly.* Carcinogenesis, 2010. **31**(11): p. 1913-21.
218. Guillemot, J.C., et al., *Ep-CAM transfection in thymic epithelial cell lines triggers the formation of dynamic actin-rich protrusions involved in the organization of epithelial cell layers.* Histochem Cell Biol, 2001. **116**(4): p. 371-8.
219. Ladwein, M., et al., *The cell-cell adhesion molecule EpCAM interacts directly with the tight junction protein claudin-7.* Exp Cell Res, 2005. **309**(2): p. 345-57.
220. Wu, C.J., et al., *Epithelial cell adhesion molecule (EpCAM) regulates claudin dynamics and tight junctions.* J Biol Chem, 2013. **288**(17): p. 12253-68.
221. Schmidt, D.S., et al., *CD44 variant isoforms associate with tetraspanins and EpCAM.* Exp Cell Res, 2004. **297**(2): p. 329-47.
222. Claas, C., et al., *The tetraspanin D6.1A and its molecular partners on rat carcinoma cells.* Biochem J, 2005. **389**(Pt 1): p. 99-110.
223. Le Naour, F., et al., *Profiling of the tetraspanin web of human colon cancer cells.* Mol Cell Proteomics, 2006. **5**(5): p. 845-57.
224. Trzpis, M., et al., *Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule.* Am J Pathol, 2007. **171**(2): p. 386-95.
225. Miraglia, S., et al., *A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning.* Blood, 1997. **90**(12): p. 5013-21.
226. Yin, A.H., et al., *AC133, a novel marker for human hematopoietic stem and progenitor cells.* Blood, 1997. **90**(12): p. 5002-12.
227. Ferrandina, G., et al., *Expression of CD133-1 and CD133-2 in ovarian cancer.* Int J Gynecol Cancer, 2008. **18**(3): p. 506-14.
228. Suetsugu, A., et al., *Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells.* Biochem Biophys Res Commun, 2006. **351**(4): p. 820-4.
229. Collins, A.T., et al., *Prospective identification of tumorigenic prostate cancer stem cells.* Cancer Res, 2005. **65**(23): p. 10946-51.
230. Hermann, P.C., et al., *Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer.* Cell Stem Cell, 2007. **1**(3): p. 313-23.
231. Ong, C.W., et al., *CD133 expression predicts for non-response to chemotherapy in colorectal cancer.* Mod Pathol, 2010. **23**(3): p. 450-7.
232. Choi, D., et al., *Cancer stem cell markers CD133 and CD24 correlate with invasiveness and differentiation in colorectal adenocarcinoma.* World J Gastroenterol, 2009. **15**(18): p. 2258-64.
233. Lugli, A., et al., *Prognostic impact of the expression of putative cancer stem cell markers CD133, CD166, CD44s, EpCAM, and ALDH1 in colorectal cancer.* Br J Cancer, 2010. **103**(3): p. 382-90.
234. Weigmann, A., et al., *Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells.* Proc Natl Acad Sci U S A, 1997. **94**(23): p. 12425-30.

235. Dubreuil, V., et al., *Midbody and primary cilium of neural progenitors release extracellular membrane particles enriched in the stem cell marker prominin-1*. J Cell Biol, 2007. **176**(4): p. 483-95.
236. Chiou, S.H., et al., *Identification of CD133-positive radioresistant cells in atypical teratoid/rhabdoid tumor.* PLoS One, 2008. **3**(5): p. e2090.
237. Zobalova, R., et al., *CD133-positive cells are resistant to TRAIL due to up-regulation of FLIP.* Biochem Biophys Res Commun, 2008. **373**(4): p. 567-71.
238. Liu, G., et al., *Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma.* Mol Cancer, 2006. **5**: p. 67.
239. Singh, S.K., et al., *Identification of a cancer stem cell in human brain tumors.* Cancer Res, 2003. **63**(18): p. 5821-8.
240. Beier, D., et al., *CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles.* Cancer Res, 2007. **67**(9): p. 4010-5.
241. Joo, K.M., et al., *Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas.* Lab Invest, 2008. **88**(8): p. 808-15.
242. Wang, J., et al., *CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells.* Int J Cancer, 2008. **122**(4): p. 761-8.
243. Ogden, A.T., et al., *Identification of A2B5+CD133- tumor-initiating cells in adult human gliomas.* Neurosurgery, 2008. **62**(2): p. 505-14; discussion 514-5.
244. Chu, P., et al., *Characterization of a subpopulation of colon cancer cells with stem cell-like properties.* Int J Cancer, 2009. **124**(6): p. 1312-21.
245. Dalerba, P., et al., *Phenotypic characterization of human colorectal cancer stem cells.* Proc Natl Acad Sci U S A, 2007. **104**(24): p. 10158-63.
246. Pallini, R., et al., *Cancer stem cell analysis and clinical outcome in patients with glioblastoma multiforme.* Clin Cancer Res, 2008. **14**(24): p. 8205-12.
247. Tong, Q.S., et al., *Expression and clinical significance of stem cell marker CD133 in human neuroblastoma.* World J Pediatr, 2008. **4**(1): p. 58-62.
248. Song, W., et al., *Expression and clinical significance of the stem cell marker CD133 in hepatocellular carcinoma.* Int J Clin Pract, 2008. **62**(8): p. 1212-8.
249. Blazek, E.R., J.L. Foutch, and G. Maki, *Daoy medulloblastoma cells that express CD133 are radioresistant relative to CD133- cells, and the CD133+ sector is enlarged by hypoxia.* Int J Radiat Oncol Biol Phys, 2007. **67**(1): p. 1-5.
250. Lin, J., et al., *gamma-secretase inhibitor-I enhances radiosensitivity of glioblastoma cell lines by depleting CD133+ tumor cells.* Arch Med Res, 2010. **41**(7): p. 519-29.
251. Chen, K.H., et al., *Celecoxib enhances radiosensitivity in medulloblastoma-derived CD133-positive cells.* Childs Nerv Syst, 2010. **26**(11): p. 1605-12.
252. Mihatsch, J., et al., *Selection of radioresistant tumor cells and presence of ALDH1 activity in vitro.* Radiother Oncol, 2011. **99**(3): p. 300-6.
253. Grosse-Gehling, P., et al., *CD133 as a biomarker for putative cancer stem cells in solid tumours: limitations, problems and challenges.* J Pathol, 2013. **229**(3): p. 355-78.
254. Yoshida, A., et al., *Human aldehyde dehydrogenase gene family.* Eur J Biochem, 1998. **251**(3): p. 549-57.
255. Koppaka, V., et al., *Aldehyde dehydrogenase inhibitors: a comprehensive review of the pharmacology, mechanism of action, substrate specificity, and clinical application.* Pharmacol Rev, 2012. **64**(3): p. 520-39.
256. Ginestier, C., et al., *ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome.* Cell Stem Cell, 2007. **1**(5): p. 555-67.

257. Douville, J., R. Beaulieu, and D. Balicki, *ALDH1 as a functional marker of cancer stem and progenitor cells.* Stem Cells Dev, 2009. **18**(1): p. 17-25.
258. Resetkova, E., et al., *Prognostic impact of ALDH1 in breast cancer: a story of stem cells and tumor microenvironment.* Breast Cancer Res Treat, 2010. **123**(1): p. 97-108.
259. Beca, F.F., et al., *Cancer stem cells markers CD44, CD24 and ALDH1 in breast cancer special histological types.* J Clin Pathol, 2013. **66**(3): p. 187-91.
260. Petriz, J., *Flow cytometry of the side population (SP).* Curr Protoc Cytom, 2013. **Chapter 9**: p. Unit9 23.
261. Petriz, J., *Flow cytometry of the side population (SP).* Curr Protoc Cytom, 2007. **Chapter 9**: p. Unit9 23.
262. Ponti, D., et al., *Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties.* Cancer Res, 2005. **65**(13): p. 5506-11.
263. Gadalla, S.E., et al., *Uncoupling of the ERalpha regulated morphological phenotype from the cancer stem cell phenotype in human breast cancer cell lines.* Biochem Biophys Res Commun, 2011. **405**(4): p. 581-7.
264. Duester, G., *Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid.* Eur J Biochem, 2000. **267**(14): p. 4315-24.
265. Brocker, C., et al., *Aldehyde dehydrogenase (ALDH) superfamily in plants: gene nomenclature and comparative genomics.* Planta, 2013. **237**(1): p. 189-210.
266. Sophos, N.A., et al., *Aldehyde dehydrogenase gene superfamily: the 2000 update.* Chem Biol Interact, 2001. **130-132**(1-3): p. 323-37.
267. Ziegler, T.L. and V. Vasiliou, *Aldehyde dehydrogenase gene superfamily. The 1998 update.* Adv Exp Med Biol, 1999. **463**: p. 255-63.
268. Britton, K.M., et al., *Breast cancer, side population cells and ABCG2 expression.* Cancer Lett, 2012. **323**(1): p. 97-105.
269. Kim, J.B., et al., *Berberine diminishes the side population and ABCG2 transporter expression in MCF-7 breast cancer cells.* Planta Med, 2008. **74**(14): p. 1693-700.
270. Bao, S., et al., *Glioma stem cells promote radioresistance by preferential activation of the DNA damage response.* Nature, 2006. **444**(7120): p. 756-60.
271. Hambardzumyan, D., M. Squatrito, and E.C. Holland, *Radiation resistance and stem-like cells in brain tumors.* Cancer Cell, 2006. **10**(6): p. 454-6.
272. Hattori, A., et al., *Molecular cloning of adipocyte-derived leucine aminopeptidase highly related to placental leucine aminopeptidase/oxytocinase.* J Biochem, 1999. **125**(5): p. 931-8.
273. Saric, T., et al., *An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides.* Nat Immunol, 2002. **3**(12): p. 1169-76.
274. Shin, E.C., et al., *Proteasome activator and antigen-processing aminopeptidases are regulated by virus-induced type I interferon in the hepatitis C virus-infected liver.* J Interferon Cytokine Res, 2007. **27**(12): p. 985-90.
275. Tanioka, T., et al., *Human leukocyte-derived arginine aminopeptidase. The third member of the oxytocinase subfamily of aminopeptidases.* J Biol Chem, 2003. **278**(34): p. 32275-83.
276. Forloni, M., et al., *NF-kappaB, and not MYCN, regulates MHC class I and endoplasmic reticulum aminopeptidases in human neuroblastoma cells.* Cancer Res, 2010. **70**(3): p. 916-24.
277. Cui, X., et al., *Shedding of the type II IL-1 decoy receptor requires a multifunctional aminopeptidase, aminopeptidase regulator of TNF receptor type 1 shedding.* J Immunol, 2003. **171**(12): p. 6814-9.

278. Neefjes, J., et al., *Towards a systems understanding of MHC class I and MHC class II antigen presentation.* Nat Rev Immunol, 2011. **11**(12): p. 823-36.
279. York, I.A., et al., *The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues.* Nat Immunol, 2002. **3**(12): p. 1177-84.
280. Hammer, G.E., et al., *In the absence of aminopeptidase ERAAP, MHC class I molecules present many unstable and highly immunogenic peptides.* Nat Immunol, 2007. **8**(1): p. 101-8.
281. Hammer, G.E., et al., *The aminopeptidase ERAAP shapes the peptide repertoire displayed by major histocompatibility complex class I molecules.* Nat Immunol, 2006. **7**(1): p. 103-12.
282. Sato, Y., *Role of aminopeptidase in angiogenesis.* Biol Pharm Bull, 2004. **27**(6): p. 772-6.
283. Hattori, A., et al., *Characterization of recombinant human adipocyte-derived leucine aminopeptidase expressed in Chinese hamster ovary cells.* J Biochem, 2000. **128**(5): p. 755-62.
284. Marincola, F.M., et al., *Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance.* Adv Immunol, 2000. **74**: p. 181-273.
285. Fruci, D., et al., *Expression of endoplasmic reticulum aminopeptidases in EBV-B cell lines from healthy donors and in leukemia/lymphoma, carcinoma, and melanoma cell lines.* J Immunol, 2006. **176**(8): p. 4869-79.
286. Fruci, D., et al., *Altered expression of endoplasmic reticulum aminopeptidases ERAP1 and ERAP2 in transformed non-lymphoid human tissues.* J Cell Physiol, 2008. **216**(3): p. 742-9.
287. Kamphausen, E., et al., *Distinct molecular mechanisms leading to deficient expression of ER-resident aminopeptidases in melanoma.* Cancer Immunol Immunother, 2010. **59**(8): p. 1273-84.
288. Kazeto, H., et al., *Expression of adipocyte-derived leucine aminopeptidase in endometrial cancer. Association with tumor grade and CA-125.* Tumour Biol, 2003. **24**(4): p. 203-8.
289. Watanabe, Y., et al., *Adipocyte-derived leucine aminopeptidase suppresses angiogenesis in human endometrial carcinoma via renin-angiotensin system.* Clin Cancer Res, 2003. **9**(17): p. 6497-503.
290. Hill, L.D., et al., *Fetal ERAP2 variation is associated with preeclampsia in African Americans in a case-control study.* BMC Med Genet, 2011. **12**: p. 64.
291. Johnson, M.P., et al., *The ERAP2 gene is associated with preeclampsia in Australian and Norwegian populations.* Hum Genet, 2009. **126**(5): p. 655-66.
292. Blanchard, N., et al., *Immunodominant, protective response to the parasite Toxoplasma gondii requires antigen processing in the endoplasmic reticulum.* Nat Immunol, 2008. **9**(8): p. 937-44.
293. Mehta, A.M., et al., *Single nucleotide polymorphisms in antigen processing machinery component ERAP1 significantly associate with clinical outcome in cervical carcinoma.* Genes Chromosomes Cancer, 2009. **48**(5): p. 410-8.
294. Fierabracci, A., et al., *The putative role of endoplasmic reticulum aminopeptidases in autoimmunity: insights from genomic-wide association studies.* Autoimmun Rev, 2012. **12**(2): p. 281-8.
295. Evans, D.M., et al., *Interaction between ERAP1 and HLA-B27 in ankylosing spondylitis implicates peptide handling in the mechanism for HLA-B27 in disease susceptibility.* Nat Genet, 2011. **43**(8): p. 761-7.

296. Fung, E.Y., et al., *Analysis of 17 autoimmune disease-associated variants in type 1 diabetes identifies 6q23/TNFAIP3 as a susceptibility locus.* Genes Immun, 2009. **10**(2): p. 188-91.
297. Guerini, F.R., et al., *A functional variant in ERAP1 predisposes to multiple sclerosis.* PLoS One, 2012. **7**(1): p. e29931.
298. Strange, A., et al., *A genome-wide association study identifies new psoriasis susceptibility loci and an interaction between HLA-C and ERAP1.* Nat Genet, 2010. **42**(11): p. 985-90.
299. Sun, L.D., et al., *Association analyses identify six new psoriasis susceptibility loci in the Chinese population.* Nat Genet, 2010. **42**(11): p. 1005-9.
300. Franke, A., et al., *Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci.* Nat Genet, 2010. **42**(12): p. 1118-25.
301. Hajjar, K.A. and S. Krishnan, *Annexin II: a mediator of the plasmin/plasminogen activator system.* Trends Cardiovasc Med, 1999. **9**(5): p. 128-38.
302. Spijkers-Hagelstein, J.A., et al., *Src kinase-induced phosphorylation of annexin A2 mediates glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia.* Leukemia, 2013. **27**(5): p. 1063-71.
303. Menell, J.S., et al., *Annexin II and bleeding in acute promyelocytic leukemia.* N Engl J Med, 1999. **340**(13): p. 994-1004.
304. Sharma, M.R., et al., *Angiogenesis-associated protein annexin II in breast cancer: selective expression in invasive breast cancer and contribution to tumor invasion and progression.* Exp Mol Pathol, 2006. **81**(2): p. 146-56.
305. Emoto, K., et al., *Annexin II overexpression correlates with stromal tenascin-C overexpression: a prognostic marker in colorectal carcinoma.* Cancer, 2001. **92**(6): p. 1419-26.
306. Emoto, K., et al., *Annexin II overexpression is correlated with poor prognosis in human gastric carcinoma.* Anticancer Res, 2001. **21**(2B): p. 1339-45.
307. Roseman, B.J., et al., *Annexin II marks astrocytic brain tumors of high histologic grade.* Oncol Res, 1994. **6**(12): p. 561-7.
308. Zhai, H., et al., *Annexin A2 promotes glioma cell invasion and tumor progression.* J Neurosci, 2011. **31**(40): p. 14346-60.
309. Mohammad, H.S., et al., *Annexin A2 expression and phosphorylation are up-regulated in hepatocellular carcinoma.* Int J Oncol, 2008. **33**(6): p. 1157-63.
310. Sharma, M.R., et al., *Antibody-directed targeting of angiostatin's receptor annexin II inhibits Lewis Lung Carcinoma tumor growth via blocking of plasminogen activation: possible biochemical mechanism of angiostatin's action.* Exp Mol Pathol, 2006. **81**(2): p. 136-45.
311. Luo, C.H., et al., *Prognostic significance of annexin II expression in non-small cell lung cancer.* Clin Transl Oncol, 2013. **15**(11): p. 938-46.
312. Jia, J.W., et al., *Clinical significance of annexin II expression in human non-small cell lung cancer.* Tumour Biol, 2013. **34**(3): p. 1767-71.
313. Wang, C.Y., et al., *Annexin A2 silencing induces G2 arrest of non-small cell lung cancer cells through p53-dependent and -independent mechanisms.* J Biol Chem, 2012. **287**(39): p. 32512-24.
314. Lokman, N.A., et al., *Annexin A2 is regulated by ovarian cancer-peritoneal cell interactions and promotes metastasis.* Oncotarget, 2013. **4**(8): p. 1199-211.

315. Lu, C.M., et al., *A panel of tumor markers, calreticulin, annexin A2, and annexin A3 in upper tract urothelial carcinoma identified by proteomic and immunological analysis.* BMC Cancer, 2014. **14**: p. 363.
316. Bao, H., et al., *Overexpression of Annexin II affects the proliferation, apoptosis, invasion and production of proangiogenic factors in multiple myeloma.* Int J Hematol, 2009. **90**(2): p. 177-85.
317. Rodrigo, J.P., et al., *Clinical significance of annexin A2 downregulation in oral squamous cell carcinoma.* Head Neck, 2011. **33**(12): p. 1708-14.
318. Esposito, I., et al., *Tenascin C and annexin II expression in the process of pancreatic carcinogenesis.* J Pathol, 2006. **208**(5): p. 673-85.
319. Vishwanatha, J.K., et al., *Enhanced expression of annexin II in human pancreatic carcinoma cells and primary pancreatic cancers.* Carcinogenesis, 1993. **14**(12): p. 2575-9.
320. Deng, S., et al., *Overexpression of annexin A2 is associated with abnormal ubiquitination in breast cancer.* Genomics Proteomics Bioinformatics, 2012. **10**(3): p. 153-7.
321. Zhang, J., et al., *Silencing of the annexin II gene down-regulates the levels of S100A10, c-Myc, and plasmin and inhibits breast cancer cell proliferation and invasion.* Saudi Med J, 2010. **31**(4): p. 374-81.
322. Shetty, P.K., et al., *Reciprocal regulation of annexin A2 and EGFR with Her-2 in Her-2 negative and herceptin-resistant breast cancer.* PLoS One, 2012. **7**(9): p. e44299.
323. Neve, R.M., et al., *A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes.* Cancer Cell, 2006. **10**(6): p. 515-27.
324. Pearce, S.T., H. Liu, and V.C. Jordan, *Modulation of estrogen receptor alpha function and stability by tamoxifen and a critical amino acid (Asp-538) in helix 12.* J Biol Chem, 2003. **278**(9): p. 7630-8.
325. Bolodeoku, J., et al., *Demonstration of CD44 gene expression in cells from fine needle aspirates of breast lesions by the polymerase chain reaction.* Clin Mol Pathol, 1995. **48**(6): p. M307-M309.
326. Ermak, G., et al., *Novel CD44 messenger RNA isoforms in human thyroid and breast tissues feature unusual sequence rearrangements.* Clin Cancer Res, 1996. **2**(8): p. 1251-4.
327. Kaipparettu, B.A., et al., *Estrogen-mediated downregulation of CD24 in breast cancer cells.* Int J Cancer, 2008. **123**(1): p. 66-72.
328. Raynor, M., et al., *Optimisation of the RT-PCR detection of immunomagnetically enriched carcinoma cells.* BMC Cancer, 2002. **2**: p. 14.
329. Schostak, M., et al., *Quantitative real-time RT-PCR of CD24 mRNA in the detection of prostate cancer.* BMC Urol, 2006. **6**: p. 7.
330. Sorbello, V., et al., *Quantitative real-time RT-PCR analysis of eight novel estrogen-regulated genes in breast cancer.* Int J Biol Markers, 2003. **18**(2): p. 123-9.
331. Leu, Y.W., et al., *Loss of estrogen receptor signaling triggers epigenetic silencing of downstream targets in breast cancer.* Cancer Res, 2004. **64**(22): p. 8184-92.
332. Pauli, C., et al., *Tumor-specific glycosylation of the carcinoma-associated epithelial cell adhesion molecule EpCAM in head and neck carcinomas.* Cancer Lett, 2003. **193**(1): p. 25-32.
333. Munz, M., et al., *Glycosylation is crucial for stability of tumour and cancer stem cell antigen EpCAM.* Front Biosci, 2008. **13**: p. 5195-201.
334. Ma, X.J., et al., *Gene expression profiling of the tumor microenvironment during breast cancer progression.* Breast Cancer Res, 2009. **11**(1): p. R7.

335. Turashvili, G., et al., *Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis.* BMC Cancer, 2007. **7**: p. 55.
336. Sorlie, T., et al., *Repeated observation of breast tumor subtypes in independent gene expression data sets.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8418-23.
337. Curtis, C., et al., *The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups.* Nature, 2012. **486**(7403): p. 346-52.
338. Richardson, A.L., et al., *X chromosomal abnormalities in basal-like human breast cancer.* Cancer Cell, 2006. **9**(2): p. 121-32.
339. Zhao, H., et al., *Different gene expression patterns in invasive lobular and ductal carcinomas of the breast.* Mol Biol Cell, 2004. **15**(6): p. 2523-36.
340. Finak, G., et al., *Stromal gene expression predicts clinical outcome in breast cancer.* Nat Med, 2008. **14**(5): p. 518-27.
341. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
342. Barretina, J., et al., *The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity.* Nature, 2012. **483**(7391): p. 603-7.
343. Reich, M., et al., *GenePattern 2.0.* Nat Genet, 2006. **38**(5): p. 500-1.
344. Eisen, M.B., et al., *Cluster analysis and display of genome-wide expression patterns.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14863-8.
345. Hajjar, K.A. and S.S. Acharya, *Annexin II and regulation of cell surface fibrinolysis.* Ann N Y Acad Sci, 2000. **902**: p. 265-71.
346. Hajjar, K.A. and J.S. Menell, *Annexin II: a novel mediator of cell surface plasmin generation.* Ann N Y Acad Sci, 1997. **811**: p. 337-49.
347. Chung, C.Y. and H.P. Erickson, *Cell surface annexin II is a high affinity receptor for the alternatively spliced segment of tenascin-C.* J Cell Biol, 1994. **126**(2): p. 539-48.
348. Lokman, N.A., et al., *The role of annexin A2 in tumorigenesis and cancer progression.* Cancer Microenviron, 2011. **4**(2): p. 199-208.
349. Hancox, R.A., et al., *Tumour-associated tenascin-C isoforms promote breast cancer cell invasion and growth by matrix metalloproteinase-dependent and independent mechanisms.* Breast Cancer Res, 2009. **11**(2): p. R24.
350. Diaz, V.M., et al., *Specific interaction of tissue-type plasminogen activator (t-PA) with annexin II on the membrane of pancreatic cancer cells activates plasminogen and promotes invasion in vitro.* Gut, 2004. **53**(7): p. 993-1000.
351. Ohno, Y., et al., *Annexin II represents metastatic potential in clear-cell renal cell carcinoma.* Br J Cancer, 2009. **101**(2): p. 287-94.