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Björner, Sofie

2013

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Lund University, Faculty of Medicine Doctoral Dissertation Series 2013:6
ISBN 978-91-87189-74-6
ISSN 1652-8220

Printed in Sweden by E-husets Tryckeri, LTH, Lund University
Lund 2012

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Skåne University Hospital Malmö, Lund University, Sweden

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Academic Dissertation
By due permission of the Faculty of Medicine, Lund University, Sweden, to be defended at the main lecture hall, Dept of Pathology, Skåne University Hospital, Malmö, on Friday 25th of January, 2013, at 9.00 am for the degree of Doctor of Philosophy, Faculty of Medicine

Faculty Opponent
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University of Leeds, UK

Organization	Document name
LUND UNIVERSITY Department of Laboratory Medicine Center for Molecular Pathology Skåne University Hospital, Malmö	DOCTORAL DISSERTATION
	Date of issue 2013-01-25
	Sponsoring organization
Author(s) Sofie Björner	

Title and subtitle

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Abstract

Early diagnosis and reliable prognosis and treatment prediction of breast cancer will ultimately lead to a decreased mortality rate. This can be achieved by identification of prognostic and treatment predictive biomarkers, and by understanding the mechanisms behind early changes in the breast.
The cell cycle is a closely controlled process, involving multiple components with regulation on several levels. Loss of adequate cell proliferation control and cell cycle regulation is one of the main characteristic of cancer. In this thesis we have found that low level of the cell cycle regulatory protein p27 was associated with impaired response to tamoxifen in premenopausal breast cancer patients, but not with prognosis. We have also observed that the expression of the microRNA (miRNA) miR-92a could provide independent prognostic information in breast cancer patients, and loss of miR-92a was associated with more severe breast cancer traits.
The earliest histologically identifiable breast lesion with an increased risk for developing breast cancer is called columnar cell hyperplasia (CCH). We have identified miRNA expression changes in CCH compared to normal mammary gland tissue in both epithelial cells and in the surrounding stroma. In addition we have linked epithelial expression of miR-27a, miR-92a and let-7c to negative cell proliferation regulation, and stromal miR-132 expression to alteration of genes associated with extra cellular matrix and actin-motility pathways in fibroblasts, and metabolic genes and pathways in co-cultured CCH epithelial cells.
Finally, cyclin D1 associated miR-483-5p affected cell migration in opposite directions depending on estrogen receptor status, highlighting the importance of subdivision of breast cancers in order to correctly understand the biology and subsequently for correct treatment of breast cancer subgroups.
In summary, these studies identified two potential biomarkers; one for predicting tamoxifen sensitivity, and one with prognostic value. We also revealed changes in miRNA expression in early premalignant breast lesions involved in cell proliferation, and opposing roles in cell migration for a cell cycle-related miRNA in breast cancer subgroup models.

Key words: Breast cancer, premalignant, biomarker, cell cycle, p27, miRNA, let-7c, miR-92a, stroma

Classification system and/or index termes (if any):

Supplementary bibliographical information:		Language
ISSN and key title: 1652-8220		ISBN 978-91-87189-74-6
Recipient's notes	Number of pages	Price
	Security classification	

Distribution by (name and address)
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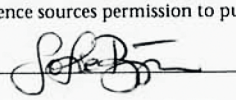
Signature  Date 2012-12-06

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This thesis is based on the following papers, referred to in the text by their roman numerals.

Paper I

p27Kip is a predictive factor for tamoxifen treatment response but not a prognostic marker in premenopausal breast cancer patients.

Stendahl M, **Nilsson S**, Wigerup C, Jirström K, Jönsson PE, Stål O, Landberg G.

International Journal of Cancer. 127(12):2851-8 (2010)

Paper II

Downregulation of miR-92a is associated with aggressive breast cancer features and increased tumour macrophage infiltration.

Nilsson S, Möller C, Jirström K, Lee A, Busch S, Lamb R, Landberg G.

PLoS One. 7(4):e36051 (2012)

Paper III

Epithelial and stromal microRNA signatures of columnar cell hyperplasia linking let-7c to precancerous breast cancer cell proliferation.

Björner S, Fitzpatrick P, Li Y, Allred C, Howell A, Ringberg A, Olsson H, Miller C, Landberg G.

Submitted for publication (2012)

Paper IV

Cyclin D1 associated miR-483-5p has distinct roles in cell migration in subgroups of breast cancer cell lines.

Nilsson S, Möller C, Diffner E, Gregersson P, Fitzpatrick P, Landberg G.

Manuscript (2012)

Abbreviations

ADH	Atypical ductal hyperplasia
AGO	Argonaute
AI	Aromatase inhibitor
AIB1	Amplified in breast cancer 1
ALA	Atypical lobule type A
AREG	Amphiregulin
BRCA	Breast cancer susceptibility gene
CAF	Cancer associated fibroblast
CCH	Columnar cell hyperplasia
CDK	Cyclin dependent kinase
ceRNA	Competing endogenous RNA
CKI	Cyclin dependent kinase inhibitor
CSC	Cancer stem cell
DCIS	Ductal carcinoma *in situ*
DGCR8	DiGeorge syndrome critical region gene 8
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
FEA	Flat epithelial atypia
FFPE	Formaling fixed paraffin embedded
FISH	Fluorescent *in situ* hybridization
HELU	Hyperplastic enlarged lobular unit
HER2	Human epidermal receptor 2
IBC	Invasive breast cancer
ISH	*In situ* hybridization
LCIS	Lobular carcinoma *in situ*
LNA	Locked nucleic acid
lncRNA	Long non-coding RNA
MAPK	Mitogen activated protein kinase
miRISC	MiRNA-induced silencing complex
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MRE	MiRNA response element
mRNA	Messenger RNA
NHG	Nottingham histological grade
piRNA	Piwi RNA
PR	Progesterone receptor
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
qRT-PCR	Quantitative real-time poly chain reaction
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
SERD	Selective oestrogen receptor downregulator
SERM	Selective oestrogen receptor modulator
siRNA	Small interfering RNA
α-SMA	α-Smooth muscle actin
TAM	Tumour associated macrophage
TDLU	Terminal duct lobular unit
TIMP	Tissue inhibitor of metalloproteinase
TLDA	Taqman low density array
TMA	Tissue microarray
UTR	Untranslated region

Introduction to tumour biology

Cancer

Cancer was thought to be one incurable disease. Today we know that cancer is several different diseases. Years of research have provided us with effectual treatment strategies, and these therapies are constantly improving thanks to the immense amount of current research.

In common for all cancer types is the transformation of normal cells to malignant cancer cells which is characterized by uncontrolled cell division. The underlying mechanism for this complex multistep transformation process includes certain traits and has been described as the "hallmarks of cancer" by Hanahan and Weinberg. In the first version in 2000, the authors described six hallmarks;[1] 1) sustaining proliferative signalling by providing self-supplied growth factors, 2) evading growth suppressors and thus be unresponsive to growth-inhibitory signalling, 3) activating invasion and metastasis to enter the circulation in order to invade surrounding tissues, 4) enabling replicative immortality meaning a limitless replication potential, 5) inducing angiogenesis which provides the tumour with new blood vessels, and 6) resisting cell death by not responding to apoptotic signalling. Since then, a lot of research in the cancer biology field has provided more knowledge and additional hallmarks have been suggested in an updated version of the report by Hanahan and Weinberg;[2] 7) deregulating cellular energy metabolism, 8) avoiding immune destruction, 9) genome instability and mutation, and 10) tumour-promoting inflammation. Cancer cells are also masters of manipulating their surrounding neighbours. For example, normal cells in the tumour stroma (e.g. fibroblasts) get signals from the cancer cells and start to produce and provide the cancer cells with growth factors.[3,4] This close interaction between the epithelial and stromal compartments emphasizes the complexity that underlies cancer diseases.

Genetic alterations, tumour suppressors and oncogenes

The genome instability is a basic hallmark of cancer and is manifested by different genomic alterations. Deletions lead to inactivation or "loss of function" of tumours suppressors, whereas amplifications of chromosomal segments are associated with

activation or "gain-of-function" of oncogenes. Tumour suppressors are genes that inhibit cellular growth. Two of the first described tumour suppressors were p53 and Retinoblastoma protein (Rb). p53 is a key regulator in multiple cancer associated processes including apoptosis and cell cycle control. Its essential roles are tightly regulated on transcriptional, post-transcriptional as well as post-translational levels.[5,6] The Rb protein is a component of the cell cycle machinery where it holds the cell in a non-proliferative state in G_0/G_1 phase until it gets phosphorylated leading to cell cycle entry.[7] For tumour suppressor genes, both alleles must be lost in order to loose the functions.[8] Conversely, cancer-promoting oncogenes are often amplified, overexpressed or expressed in a mutant form leading to proliferative advantages.[9] For example, members of the Ras protein family act as transducers in the MAPK pathway which is a proliferative signalling network. Ras is frequently mutated to a constantly active state, resulting in an activation of downstream signalling pathways and cellular growth.[10]

The pathogenesis of cancer

Risk factors

Even though we know a lot about the progression of tumours through accumulation of beneficial genetic changes, it is not entirely delineated why and how a tumour is initially formed. Apart from age, which often is highly associated with cancer incidence, most risk factors are associated with lifestyle. For some factors there is an obvious link to a certain cancer form, e.g. smoking and lung cancer,[11] and ultraviolet (UV) light exposure and melanoma.[12] Other known lifestyle factors are diet, physical activity, body weight, and alcohol consumption.[13] There are also environmental factors linked to cancer, e.g. exposure to asbestos and radon.[14] Some viruses have the ability to cause cancer as well, one example being the human papilloma virus that can cause cervical cancer.[15] Family history of cancer can also be considered a risk factor. It is estimated that 5-10% of certain cancer forms, mainly breast, colorectal, prostate and melanoma, are hereditary.[16]

The cause of cancer

The origin of cancer is a debated topic without a clear answer. The theories depend naturally on recent discoveries in the cancer biology field. Today there are two main theories; the cancer stem cell hypothesis and the clonal evolution model.

The cancer stem cell (CSC) hypothesis is based on the heterogeneity of cancer cells and proposes that there is a hierarchical organization with a subpopulation of cells with stem-like properties that is responsible for the sustained tumour growth. CSCs have been observed in acute myeloid leukaemia as well as from several solid tumours

including breast. Nevertheless it does not appear to be applicable to all cancer types.[17] CSCs have the ability to self-renew and to differentiate like normal stem cells, however it has been suggested that CSCs originate from committed progenitor cells that already have undergone some differentiation.[18] Evidence supporting the existence of CSCs comes mainly from serial transplantation studies in mice where the injected human CSC population was able to regenerate the primary tumour and to self-renew on serial transplantation and *in vitro* studies in non-adherent conditions.[19,20] The theory that CSCs originate from dedifferentiated cells that had undergone epithelial to mesenchymal transition (EMT) is partly based on the resemblance of their gene expression profile to mesenchymal stem cells.[21] The CSC hypothesis postulates an explanation for the continuous growth of tumours, but the CSC is not necessarily the first cell that gets transformed into a malignant cancer cell.

The clonal evolution model was postulated 35 years ago and is in contrast to the CSC hypothesis not based on a hierarchical cell organization.[22] In fact, the self-renewal capacity of CSCs is considered an acquired property that is an advantage for the transformed cancer cell. The hypothesis proposes that a tumour is formed by the selection and subsequent clonal expansion of a random single cell with acquired growth advantages via accumulation of genetic alterations.[23] Sub-populations are generated when single clones acquire advantage properties of their own, creating a heterogeneous cell population, possibly due to signalling from the tumour microenvironment.[24]

Taken that both theories are well documented and validated, it is possible that a combination of the two is contributing to the heterogeneous tumour formation and progression.[25] Nevertheless, neither of these two hypotheses can explain the first stride in this multistep process. It was suggested that certain people can be more prone to developing cancer depending on an altered homeostasis, leading to an overall imbalance in the body that tilts the otherwise tightly controlled system. This would result in the possibility of single cells to start the transformation towards a cancer cell.[26]

Normal breast development and breast cancer

The normal breast

The breast starts to develop during fetal development but the major developmental stages occur after birth i.e. during puberty, pregnancy, lactation and involution. Until puberty, the breast is identical between male and female and grows in the same tempo as the rest of the body.[27] During puberty, the hormonal status changes dramatically mainly due to the influence of oestrogen. The cells in the mammary gland start to proliferate and form terminal duct lobular units (TDLUs) that begin to expand and branch to finally form the mature epithelial ductal tree (Figure 1). The TDLUs are hollow structures with a central lumen, consisting of two epithelial cell layers surrounded by a basement membrane separating the TDLU from the surrounding stroma. During pregnancy and lactation, the ductal tree expands further, filling the stroma with lobuloalveolar structures in which the luminal epithelial cells produce milk and the contractile basal myoepithelial cells constrict in hormonal response to aid milk release.[28] It has also been suggested that mammary stem cells reside within the basal myoepithelial cell layer along the ducts.[29] After lactation follows a stage termed involution during which the lobuloalveolar structures undergo massive apoptotic cell death and the ductal tree is restored to the pre-pregnancy status.[28]

Breast cancer

Breast cancer is the most common cancer form among women comprising approximately 16% of all female cancers.[30] In Sweden it is estimated that 1 out of 10 women has a risk of developing breast cancer before the age of 75, with ~7000 new cases every year. Early detection of breast cancer is the key to prevent progression of the disease. This is mainly accomplished by routine mammographic screening of women. In Sweden, women between 40/50-69 years of age are invited to be screened with an average attendance of 75%.[31,32] The incidence of breast cancer cases has

increased during the last years, which is most likely due to the increased age of the population and possibly because of better screening methods, however mortality has decreased. It is reasonable to assume that the observed decreased mortality rate is a result of better and earlier detection of breast cancer and improved treatment, including individualized therapy. The 5-year breast cancer survival is 88% and the 10-year survival is close to 80%.[33] Most breast cancers (90-95%) are sporadic and the remaining 5-10% of the cases are thought to be caused by mutations in high risk genes such as *BRCA1* and *BRCA2*.[34]

Apart from the previously mentioned cancer contributing factors in general, there are a few breast cancer specific risk factors. The exposure to hormones is the underlying mechanism to several risk factors; the increased risk associated with longer reproductive cycling especially with an early onset of menarche,[35] the increased risk with no childbirth and with a late first childbirth,[36] and the increased risk associated with hormone replacement therapy to alleviate menopausal symptoms.[37] Studies investigating the impact of oral contraceptives as a risk factor for breast cancer conclude that there is a slightly increased risk for current users or women who used them in the past ten years.[38]

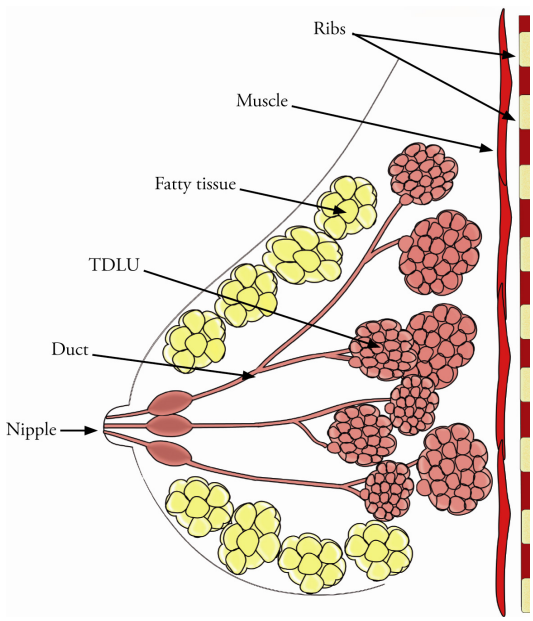

Figure 1. Illustration of the breast.
The breast consists of ~25 lobes, each of which is drained in a collecting duct terminating in the nipple. On the other end of each duct is a TDLU located, consisting of a small segment of duct and a lobule made up of aggregated milk-producing acini. These basic functional units of the breast are surrounded by stroma and fatty tissue.

Breast cancer development

There are several similarities between normal breast development and the development of breast cancer. For the mammary gland to fully develop, the cells have to both proliferate and to expand into the surrounding stroma. However, even though the basement membrane is thinner at the invasion border, there is always that boundary keeping the epithelial cells separated from the stromal compartment, a feature lost in invasive breast cancer (IBC). The stromal cells including fibroblasts, macrophages and adipocytes to name a few, all contribute to the ductal branching morphogenesis by production of growth and promigratory factors and extracellular matrix proteases. These processes are also present in breast cancer development, although with deviant signalling in cells and between compartments which leads to uncontrolled cell proliferation. In IBC the cancer cells escape from the basement membrane boundary and eventually invade the surrounding tissues.[28,39]

Figure 2 illustrates the histological model of breast cancer evolution. The progression of breast cancer from normal breast is suggested to start in certain premalignant lesions in the breast. There are several types of these lesions and only a few are thought to be able to propagate to IBC. The best described and most studied are atypical hyperplasia (AH) and carcinoma *in situ* (CIS). Both can occur in the ducts as well as in the lobules. They are characterized by increased proliferation without metastatic properties.[40] These premalignant lesions are less common in non-cancerous breasts,[41,42] and women with AH and CIS have an approximately 5 and 10 fold increased risk, respectively, of developing IBC.[43-45] For ductal AH (ADH) and the premalignant lesion in the lobule (LDH), the elevated risks are bilateral meaning that they provide risk information for both breasts, suggesting that they are more likely markers for IBC than precursors. However, they are often observed in both breasts and in greater numbers in cancerous breast, proposing that they can be both precursors and markers to IBC. Ductal CIS (DCIS) is usually ipsilateral and considered a determined IBC precursor.[40]

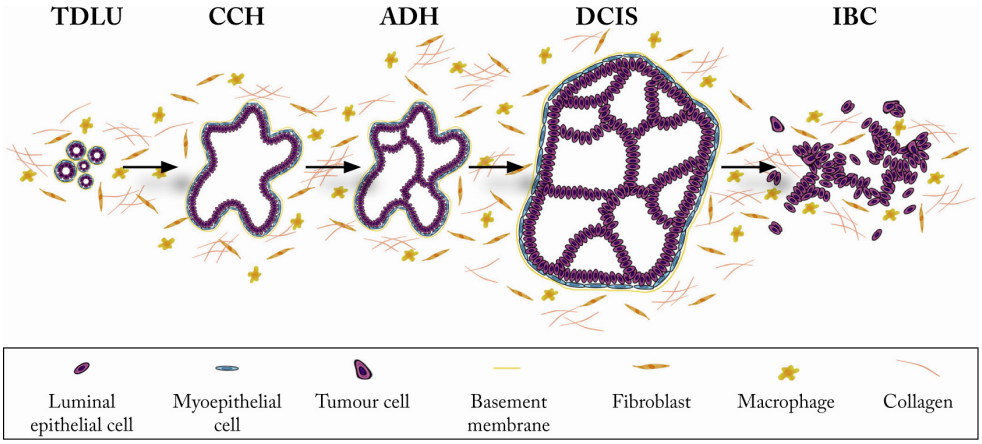

Figure 2. Schematic presentation of the natural history of breast cancer.
The epithelial cells in the TDLU start to proliferate, resulting in the enlarged structures called CCH that have the potential to further develop into ADH and DCIS. Upon disruption of the basement membrane, the luminal epithelial cells can invade the stroma and with released growth factors and other migratory stimulating agents from stromal cells, the cancer cells can continue to the circulation and form metastasis at distant sites in the body.

Columnar cell hyperplasia - CCH

In addition to ADH and DCIS, it has been suggested that there is an intermediate lesion that may be involved in the transition from normal TDLU to ADH, DCIS and beyond. This was proposed in the early 1970s by Wellings *et al* who called the structures atypical lobules type A (ALA).[42] These common structures in the breast, referred to as columnar cell hyperplasia (CCH), have been called several alternative names including hyperplastic enlarged lobular unit (HELU) and flat epithelial atypia (FEA).[46] CCHs are very common and do not necessarily develop into ADH suggesting that there may be underlying biological abnormalities that causes some CCH to progress. However, they are more common in breasts that later progress to breast cancer.[47] Lee and co-workers observed that in accordance with ADH and DCIS, cells in CCHs have a higher proliferative capacity, lower apoptotic rate, and an increased expression of the oestrogen receptor α (ERα) (Figure 3).[48] The same research group further demonstrated that CCHs have a distinct gene expression profile compared to TDLUs.[49] The development from TDLU to CCH appears to be caused by reactivation of breast developmental pathways and an inhibition of pathways involved in terminal differentiation, involving ERα- and epidermal growth factor- (ERBB) regulated genes. The 10-fold upregulation in CCH of amphiregulin (*AREG*), which is important for normal embryonic breast development and an epidermal growth factor receptor (EGFR) ligand, could possibly be due to the

elevated expression of ERα and increased oestrogen signalling.[50,51] *AREG* was also upregulated in ADHs that evolved from the CCHs and has been shown to enhance tumourogenesis of breast cancer cells in mice.[52] Another EGFR ligand, *EGF* which is involved in the differentiation of the adult breast, was greatly downregulated (14-fold) in CCH compared to TDLUs. These results indicate that there appears to be a switch of EGFR ligands in CCH leading to a shut-down of an adult breast terminal differentiation program and a reactivation of embryonic breast developmental pathways.[49]

Due to the common presence of CCH in normal breasts that do not develop breast cancer, it is possible that the observed alterations and differences from normal TDLUs, both histological and on a gene expression level, are rather preparing the cell and microenvironment for a malignant transformation if the opportunity arises. Perhaps the tissue must have certain properties in order to nourish the cancer signal leading to a genetic alteration.

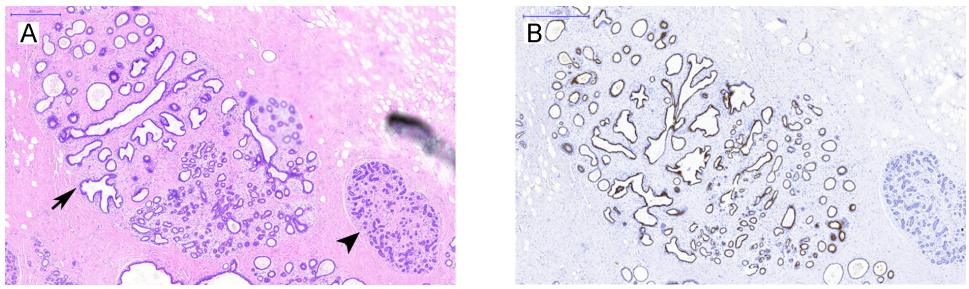

Figure 3. Identification of TDLU and CCH
(A) CCHs (arrow) are enlarged TDLUs (arrow head) that are tightly packed with columnar shaped epithelial cells. These premalignant lesions are characterized by increased expression of ERα (*B*), a higher proliferation rate and fewer apoptotic cells. CCH is the first histological identifiable lesion with premaligant potential to develop invasive breast cancer.

Oncogenes and tumour suppressor genes

A cancer cell accumulates genetic alterations over time. Some of these genetic modifications are common in several cancer types. For example as mentioned above, the deletion or inactivation of tumour suppressor genes like *p53* and *Rb*, and the overexpression, amplification or aberrant activation of oncogenes like *Ras* and several growth factor receptors (e.g. EGFR) is observed in many cancers. More specific genetic alterations for breast cancer are mutations in *BRCA1* and *BRCA2*. These mutations are also observed in ovarian cancer. *BRCA1* and *BRCA2* are involved in the repairing of double stranded DNA breaks by homologous recombination.[53] These mutations are directly linked to hereditary breast cancer and are present in 3-8% of all

breast cancers.[34] Studies in different patient cohorts have estimated the increased risk of developing breast cancer by the age of 70 to 45-60% among mutation carriers.[54]

Breast cancer subgroups

Breast cancer is a very heterogeneous disease and can be subdivided based on several parameters. However, tumours classified to one subgroup may not always be divided into the same group when using another classification.

Histological classification

Breast cancer can roughly be categorized into two groups based on histological differences: carcinoma *in situ* (CIS) and invasive breast cancer. These two are separated based on the presence of the myoepithelial cell layer.[55] CIS is a non-invasive premalignant breast lesion with a 10-fold increased risk of developing IBC and is further subdivided into ductal CIS (DCIS) or lobular CIS (LCIS). DCIS is the most common form and is subclassified into additional structures with relevance for patient outcome based on architectural features.[56]

The two most prominent types of invasive breast cancer are ductal and lobular carcinoma, accounting for approximately 75% and 15% respectively of all invasive breast cancer cases. The remaining 10% consist of the rare histological cancer types; mucinous, tubular, comedo, inflammatory, medullary, and papillary carcinoma. The different subtypes differ in histopathological features e.g. hormonal status and histological grade, as well as in aggressiveness of the tumours.[57]

Classification based on protein expression

Breast cancers can also be divided into groups depending on their expression of certain cellular markers, often referred to as biomarkers. These markers are usually proteins but can also be other detectable molecules, such as oligonucleotides. The emerging evidence that certain microRNAs (miRNAs) are extensively involved in breast cancer progression has led to the search for good miRNA candidates for classifying tumours. Nevertheless, the most common and routinely used biomarkers are ERα, progesterone receptor (PR) and human epidermal receptor 2 (HER2).[58] These biomarkers are treatment predictive indicators for targeted therapies. ER+/PR+ tumours are predicted to respond to endocrine therapies,[59] and HER2+ tumours can be targeted with the monoclonal antibody trastuzumab.[60]

In addition to predict treatment response, these biomarkers are also used as prognostic markers. Approximately 70% of all breast cancers are ER+.[57] Both ductal and lobular carcinomas are usually ER+/PR+ whereas the rare and aggressive inflammatory breast cancer subtype is more often ER-/PR-, indicating that the

presence of hormone receptors is associated with good prognosis.[57] ER is expressed in the luminal epithelial cells. Roughly 10-15% of the luminal cells in a normal breast express ER, however these are not the proliferating cells. This is altered in breast cancer where approximately 70% of the proliferating cells express ER.[61] HER2 is a growth receptor that is overexpressed in 20-30% of breast cancer cases.[60] Tumours expressing HER2, as measured both with immunohistochemistry (IHC) for protein and with fluorescence *in situ* hybridization (FISH) for copy numbers of the gene used to be associated with poor outcome. However, the prognosis has improved due to the targeted therapy with trastuzumab.[62] Patients with tumours that are negative for all three receptors, triple-negative (TN) tumours have the worst clinical outcome and treatment response.[63]

Classification based on gene expression

With the entrance of high-throughput screening and gene expression arrays came the possibility to classify tumours based on their molecular profile. In 2000, Perou and co-workers presented four molecular subtypes of breast cancer; ER+/luminal-like, basal-like, HER2 and normal breast.[64] The following year, Sørlie *et al* from the same research group, demonstrated that one of the subtypes (ER+/luminal-like) could be further divided into luminal A and luminal B, and that the molecular subtypes were associated with different patient outcomes.[65]

Among the two luminal subtypes, luminal A has the best prognosis although luminal B is still better than the basal, claudin-low and HER2 subtypes.[65] Luminal A is the most common subtype of all representing 50-60% of all breast cancers whereas luminal B only comprises 10-20%. These subtypes are hormone receptor positive but differ in aggressiveness where luminal B tumours have a higher histological grade and proliferative index.[64,65]

The basal subtype, representing 10-20% of all breast carcinomas, has the worst patient outcome, and display aggressive cancer features like high histological grade and large tumour size.[65] These tumours are negative for ER, PR and HER2 and thus shares phenotypical characteristics with TN tumours. However these profiles are not completely overlapping.[66]

The HER2 subtype comprises 15-20% of breast cancer cases and although it can be treated with targeted therapy (trastuzumab), patients with theses tumours have a poor prognosis and the tumours are often highly proliferative and of high grade.[65] The HER2 subtype can also be further divided into prognostic relevant subgroups.[67]

Since the first report, numerous researchers have contributed to the molecular classification of breast cancers. An additional subtype, claudin-low tumours, was added in 2007 and is characterized by low expression of cell adhesion molecules.[68] Genes involved in epithelial-mesenchymal-transition are overexpressed in this

subtype, features that are associated with a cancer stem cell phenotype.[69] Between 12-14% of all breast cancers are classified as claudin-low and patients with these tumours have a poor prognosis.[70]

Prognostics

The prognosis for a breast cancer patient is based on a number of clinocopathological parameters. The histological entity of the tumours, as described above, provides prognostic information. However, the most common parameters are histological grade, tumour stage and immunohistological biomarkers. The immunohistological biomarkers are described above and include ER, PR and HER2.

The tumour grade

The Nottingham Histological Grade (NHG) classification is routinely used and provides a measurement of the tumour based on three different morphological features. These are; the percentage of tubule formation, the degree of nuclear pleomorphism, and mitotic count. The tumour is scored from 1-3 for each feature, and the total score of all three gives a description of the differentiation status of the tumour. Well-differentiated tumours get a combined score of I (3-5 points), moderately differentiated score II (6-7 points), and poorly differentiated score III (8-9 points).[71]

The tumour stage

The tumour stage provides additional clinical information about the prognosis for a certain patient. The TNM classification is also a combined score from 0-IV including the parameters tumour size (T), lymph node status (N) and distant metastasis (M). Stage 0 is *in situ* cancer (DCIS), at stage I the tumour is <20 mm and has no nodal involvement, at stage II the tumour is 20-50 mm with or without positive lymph nodes, stage III tumours are >50 mm and have spread to proximal lymph nodes, and at stage IV the tumour has spread and formed distant metastases.[72]

Breast cancer therapy

Surgery, radiotherapy and chemotherapy

Prior to surgery, patients with advanced breast cancer receive neoadjuvant treatment in order to decrease the size of the tumour and to give an indication if the tumour responds to the selected treatment. Neoadjuvant treatment can be chemotherapy or endocrine treatment and has also been shown to improve survival.[73] The standard treatment for breast cancer patients is surgery, either by breast-conserving surgery

(lumpectomy), or in some cases by removing the whole breast (mastectomy), followed by radiotherapy.[74] To minimize the risk of recurrence by potential remaining cancer cells after surgery, the patients receive adjuvant systemic therapy in terms of chemotherapy and/or some form of endocrine treatment depending on type of tumour. Hormone receptor negative tumours are treated with chemotherapy. There are different types of chemotherapy and they are often administered in combination (polychemotherapy) to give an enhanced effect. The most common polychemotherapies are anthracycline-based combinations like FAC (fluorouracil, doxorubicin, cyclophosphamide) or FEC (fluorouracil, epirubicin, cyclophosphamide), as well as the well-tested CMF (cyclophosphamide, methotrexate, fluorouracil).[75] Taxanes, like paclitaxel and docetaxel, are another type of potent chemotherapeutic agents that can be used alone or in combination.[76]

Inhibitors and monoclonal targeted therapies

Breast cancer patients with tumours overexpressing HER2 are treated with the monoclonal antibody trastuzumab (Herceptin) and/or the HER2 inhibitor Lapatinib. Despite the expression of the target HER2, some patients develop resistance to the treatment, possibly due to crosstalk between receptors and amplified HER2 signalling.[77,78]

Endocrine therapy

Most breast cancers are ER+ and depend on oestrogen for sustained growth. These tumours can be treated with endocrine therapy since this type of treatment inhibits the action of oestrogen. This can be achieved in several manners, either by inhibiting the synthesis of oestrogen (aromatase inhibitors) or by blocking the receptor (SERMs and SERDs). Which one to use is based on the menopausal status of the patient. In general, premenopausal women are treated with SERMs and SERDs, and postmenopausal women receive aromatase inhibitors.[79]

The most widely used selective ER modulator (SERM) in ER+ breast cancer during the last three decades is tamoxifen. Tamoxifen competes with oestrogen for the binding of ER. This results in recruitment of co-repressors, instead of co-activators which would be the result of oestrogen-binding. The binding of tamoxifen to ER induces a conformation change in ER which still leads to dimerization and DNA-binding, but no activation of ER target genes.[80] PR is an indirect marker for functional ER signalling and has been reported to be an even better predictor for tamoxifen response than ER.[81] Tamoxifen acts as an antagonist in breast, however agonistic effects are observed in bone, uterus and the cardiovascular system, and use of tamoxifen is associated with an elevated risk of uterine cancer.[82-84] Raloxifene is another SERM that is used by postmenopausal patients in the prevention of breast cancer. Similar to tamoxifen, raloxifene has antagonistic effect in the breast and is

used to prevent and treat osteoporosis, but in contrast to tamoxifen there is no evidence pointing toward a stimulatory effect in the uterus.[85]

Fulvestrant is a selective ER downregulator (SERD) i.e. a pure oestrogen antagonist. It competitively binds to the ER with high affinity and downregulates the receptor by a conformational change, leading to complete inhibition of oestrogen signalling via the ER. Fulvestrant is used in patients who have developed resistance to tamoxifen.[86]

Another way of inhibiting the oestrogen-dependent growth is by hindering the production of oestrogens. This is accomplished by the use of aromatase inhibitors (AIs). These agents inhibit the enzyme aromatase, which is responsible for the synthesis of oestrogens.[87] The currently used non-steroidal AIs letrozole and anastrozole, and the steroidal AI exemestane are used in postmenopausal women and have been shown to significantly increase disease-free survival in these patients compared to tamoxifen.[88]

Endocrine resistance

In theory, all endocrine treatment strategies should have effects in ER+ tumours. Unfortunately that is not the case in approximately 50% of all ER+ breast cancers treated with tamoxifen.[89] Endocrine resistance can be either intrinsic (*de novo*) or acquired, and it appears to be several mechanisms working together in order to sustain the resistance. Deregulated ER expression and activity loss by mutations, gene regulation or modification at transcriptional and/or translational level, as well as down- and overexpression of ER co-repressors and co-activators respectively, and overexpression of transcription factors are mechanisms reported to be involved in this process. In addition, crosstalk between ER and growth factor receptors e.g. HER2 is also a mode of action for endocrine resistance.[90]

The cell cycle

The cell cycle is a strictly controlled machinery involving multiple components and processes. It is regulated by numerous intra- and extra cellular stimuli. Normal function of cell division is required for maintained homeostasis in the body. In cancer cells, this perfect balance is disrupted and cells start to divide in an uncontrolled manner, which is the number one characteristic of cancer.

The cell cycle is divided into four phases. In the first gap phase, G_1, the cell is preparing for DNA replication which occurs in the following S phase, proceeded by the second gap phase, G_2, in which the cell prepares for mitosis in M phase. Before entering S phase, cells in G_1 can enter a non-proliferative state in G_0 phase. Most normal cells are resting in G_0.[91]

The key components of the cell cycle

The cyclin dependent kinases and the cyclins

The progression through the cell cycle is governed by cyclin dependent kinases (CDKs) and their association partners consisting of different cyclins (Figure 4). Different CDKs and cyclins act at different phases during the cell cycle, and this is regulated by the cycling synthesis and destruction of the cyclins. The D cyclins are the only cyclins that are induced by mitogens and integrins.[92] They bind to CDK4 and CDK6 and are required for the entry in G_1[93] by phosphorylation of Rb which is further hyperphosphorylated by the cyclin E-CDK2 complex which is also present in G_1 phase.[94-96] The hyperphosphorylation of Rb releases bound molecules important for cell cycle progression. The cyclin E-CDK2 complex is thus important for the transition to S phase.[97] In S phase, CDK2 forms a new complex with cyclin A[98] and in late G_2 phase and early M phase cyclin A binds to CDK1 to prepare for mitosis which occurs in M phase where cyclin B is in complex with CDK1.[99] Genetic alterations are found in both CDKs and cyclins in different cancers, stressing the importance of keeping this process intact.[100,101]

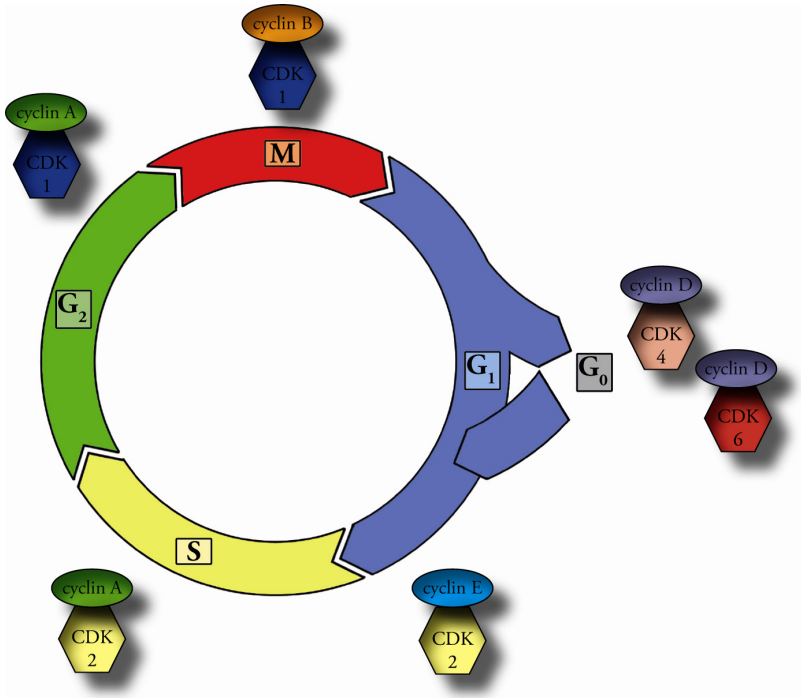

Figure 4. The phases and components of the cell cycle.
Upon proliferative stimuli, the levels of D-cyclins increase and they form complexes with CDK4/6, leading to phosphorylation of Rb, which is further phosphorylated by cyclin E-CDK2, resulting in transition from G_1 to S phase. CDK2 binds with cyclin A in S phase, and cyclin A later forms a complex with CDK1 in G_2/M phase. CDK1 is in complex with cyclin B during mitosis in M phase.

Cyclin D1 and cancer

Cyclin D1 is frequently overexpressed in many cancer types.[102] In breast cancer, approximately 50% of all cases have an increased expression of cyclin D1 and the chromosomal region for the cyclin D1 gene (*CCND1)* is amplified in ~15% of all breast cancers.[103,104] Amplification of cyclin D1 has been correlated with poor outcome.[105,106] However, there is no clear consensus in the reports regarding the prognostic value of overexpression of cyclin D1 in breast cancer. Some studies have observed that cyclin D1 is a negative predictor,[107,108] whereas others have observed the opposite.[109,110] Several studies have reported a link between cyclin D1 and ER.[109,111] In a study comparing the AI anastrozole with tamoxifen it was shown that low expression of cyclin D1 and *CCND1* amplification correlated with poor outcome.[106] Amplification of *CCND1* has also been linked to agonistic effects of tamoxifen.[112]

Based on these observations, it has been suggested that cyclin D1 may have different effects in different subgroups of breast cancers.[113] *CCND1* amplified tumours are distinct from cyclin D1 overexpressed tumours and separating the two groups could be a better way to analyze the prognostic value. It was further suggested that the link between low cyclin D1 expression, ER and poor outcome,[109] and low cyclin D1 and weaker response to endocrine treatment[106] could be due to a role for cyclin D1 as a marker for a functional ER signalling pathway. Low levels of cyclin D1 would rather be an indicator of a functional ER signalling pathway than a cell cycle component. In this case, low cyclin D1 would indicate that the cells were not dependent on ER signalling and therefore did not respond to endocrine treatment.[113]

The cell cycle inhibitors

In order to keep the cell in a controlled state, the CDKs and cyclins are tightly regulated through inhibition, degradation and subcellular localization. The CDK inhibitors (CKI) bind CDKs alone or in combination with cyclins and thereby regulate the CDK activity. There are two distinct families for inhibitors; the INK4 family and the Cip/Kip. The INK4 inhibitors (p15, p16, p18 and p19) specifically inhibit the G_1 CDKs 4 and 6,[114] whereas the Cip/Kip family (p21, p27, p57) can inhibit both the cyclin D-CDK4/6 complex as well as cyclin B-CDK1 and cyclin E-CDK2.[115] These inhibitors are in turn regulated by both internal and external signals. For example, p21 is transcriptionally regulated by the tumour suppressor p53 and induced upon DNA damage.[116]

p27 and cancer

The role of p27 is mainly considered to be tumour suppressive due to its inhibition of cyclin E-CDK2, but it can also promote cell cycle progression by acting as an assembly factor for the cyclin D-CDK4/6 complex.[117] Mutations in the p27 gene is not very common and the observed decreased levels of p27 in cancers are often due to increased degradation of the protein or mislocalization of p27 to the cytoplasm.[118] Most clinical studies have investigated the nuclear expression of p27 and observed that loss of p27 is associated with poorer prognosis in breast cancer.[119,120] Taken that the majority of breast cancer patients receive some form of adjuvant treatment, it is difficult to conclude that the observed expression of p27 is determining the actual prognosis for the patient or if it in fact predicts the response of the selected adjuvant treatment. Either way, loss of p27 is correlated with worse clinical outcome for the patient. In a study of premenopausal early breast cancer patients receiving adjuvant treatment, high level of p27 was again a predictor of better outcome.[121] They also concluded that patients with high expression of p27 responded better to a combination of the two endocrine therapies tamoxifen and goserelin, and the

tamoxifen+goserelin combination was more effective than tamoxifen+chemotherapy (CMF).

MicroRNAs

It has been twenty years since the discovery that small RNAs could act as gene regulators in addition to known protein encoded transcription factors. This has revolutionized the conception of gene regulation and the researchers behind the finding of the cellular process called RNA interference, Andrew Z Fire and Craig C Mello, were awarded with the Nobel Prize in 2006. The term "junk-DNA" has been extensively revised and with high-throughput transcriptomics it has become apparent that essentially the whole genome, consisting of functionally coding and non-coding genes, is transcribed. Although the involved components and the whole process, both in normal and during malignant circumstances, are being thoroughly investigated, we are still in the beginning of trying to understand the underlying mechanisms of these cell and context specific regulators.

Non-coding RNAs

The first microRNAs

The first microRNA (miRNA) was first discovered in the nematode *Caenorhabditis elegans* in 1993 by Rosalind Lee and Rhonda Feinbaum in Victor Ambros's lab. They showed that *lin-4,* the known negative regulator of the developmental gene *lin-14*, was not a protein-coding gene. It encoded two small RNA transcripts with sequences complementary to a repeated sequence in the 3' untranslated region (UTR) of the *lin-14* mRNA.[122] Gary Ruvkun and colleagues had simultaneously observed that *lin-4* could posttranscriptionally downregulate lin-14 protein through binding to conserved sequence elements in the 3'UTR of *lin-14*.[123] It would then take several years until the next miRNA, *let-7*, was discovered in 2000, again in *C.elegans.*[124] The term miRNA was introduced in 2001 and was defined as a usually highly conserved endogenously expressed 21-24 nt long non-coding single stranded RNA.[125-127] Today there are more than 2000 mature human miRNAs listed in the latest release (19.0) of the main miRNA database, miRBase.[128] Each miRNA is estimated to influence hundreds of downstream targets and it is thought that approximately one third or more of all genes are under the regulation of miRNAs.[129]

MiRNAs can both be found in clusters of several miRNAs and as single miRNA genes.[130] They are mainly located in introns of protein-coding genes (40%) and in introns of non-coding genes (40%). Approximately 10% of the miRNAs are found in exons of non-coding RNAs and the remaining miRNAs can be located in either introns or exons as a result of alternative splicing patterns.[131]

Other non-coding RNAs

In a screen for structural, i.e. functional, non-coding RNAs in the human genome it was predicted that there are about 30 000 structured RNA elements and roughly 1000 of these are conserved across all vertebrates.[132] Several other non-coding RNAs have been described apart from miRNAs e.g. small interfering RNAs (siRNAs), piwi RNAs (piRNAs) and the long non-coding RNAs (lncRNAs) to mention a few. They are separated from each other by their length of nucleotides and their biogenesis. MiRNAs, siRNAs and piRNAs are all small non-coding RNAs with similar mode of action. They can be divided into two groups depending on whether they are processed by the RNA III ribonuclease Dicer (miRNAs and siRNAs) or not (piRNAs). MiRNAs are further distinct from siRNAs by being single stranded in contrast to double stranded, and by being additionally processed by the nuclear RNA III ribonuclease (RNase) Drosha.[131] MiRNAs and siRNAs also have different types of transcripts from which the small RNA is derived. In the case of miRNAs, the transcripts are able to form distinctive hairpin structures.[130] These are different from the longer hairpin structures, from which siRNAs are derived, and which generate a larger variety of small RNAs.[133]

Biogenesis

A schematic picture of the biogenesis pathway for miRNAs is presented in figure 5. MiRNAs are primarily transcribed by RNA polymerase II into stem-loop structured primary transcripts (pri-miRNAs) containing cap structures and being polyadenylated,[134,135] although transcription by RNA polymerase III occurs in some cases.[136] The pri-miRNA is processed and cleaved near the stem by the Microprocessor complex, which primarily consists of nuclear RNase III Drosha[137] and the DiGeorge syndrome critical region gene 8 (DGCR8)[138-141] generating a ~70 nt hairpin precursor miRNA (pre-miRNA) with ~2 nt 3' overhang. This step is however bypassed for the group of miRNAs located in introns of genes, referred to as "mirtrons" which produce hairpin precursors in conjunction with splicing.[142-144] All

pre-miRNAs are actively exported to the cytoplasm by the dsRNA recognizing complex consisting of nuclear receptor Exportin-5 and Ran-GTP co-factor.[145,146]

In the cytoplasm, the pre-miRNA is further processed by cleavage and removal of the loop of the hairpin. This is completed by a second RNase III, Dicer[147-150] and its co-factor TRBP[151,152] leading to the release of a ~22 nt long double stranded RNA molecule. It is normally the strand with the thermodynamically unstable 5' terminal from the miRNA-miRNA* duplex that will be the guide strand. The other strand is called the star sequence (miRNA*) or passenger strand and is often degraded.[153,154] Yet the passenger strand is also functional having mRNA targets and there are examples of the miRNA* being the more abundant strand.[155-157] The guide strand is less tightly paired at the terminal resulting in a preference for RNA helicase proteins to unwind the duplex and load that strand onto the miRNA-induced silencing complex (miRISC).[130,153,154] The miRISC is a large multi-protein complex. The key functional component is one of the members of the Argonaute protein family (AGO1-4/eIF2C1-4)[158,159] which is able to bind the RNA.[160-162] Another essential protein of the miRISC is GW182 which is a marker for processing bodies (P-bodies) and is required for the silencing of the target mRNA.[163,164]

Regulation

Target recognition

Once the miRNA is incorporated into the miRISC, the complex is directed to its targets where it regulates the mRNAs. The mechanism that the target mRNA was recognized by pairing (sometimes imperfectly) to residues 2-7 or 2-8 at the 5' of the miRNA[123] has later been supported by other studies pointing out the importance of these specific residues to be highly conserved,[165] as well as showing that the specificity of the miRNA:mRNA interaction is largely influenced by the basepairing of the first eight nucleotides at the 5' region of the miRNA.[166] This region is referred to as the "seed" region and is used for prediction of potential targets. There are several bioinformatic target prediction programs available including miRanda,[167] miRDB,[168,169] PicTar,[170] miRecords [171] and DIANA-microT.[172,173] Usage of this kind of tools has resulted in the estimation that >60% of the human protein-coding genes are under the regulation of miRNAs.[174] However, it is of great importance to emphasize that sequences outside of the "seed" are also crucial for gene regulation, and target recognition and regulation are extremely dependent on surrounding circumstances and must be investigated in its proper tissue and cellular context.

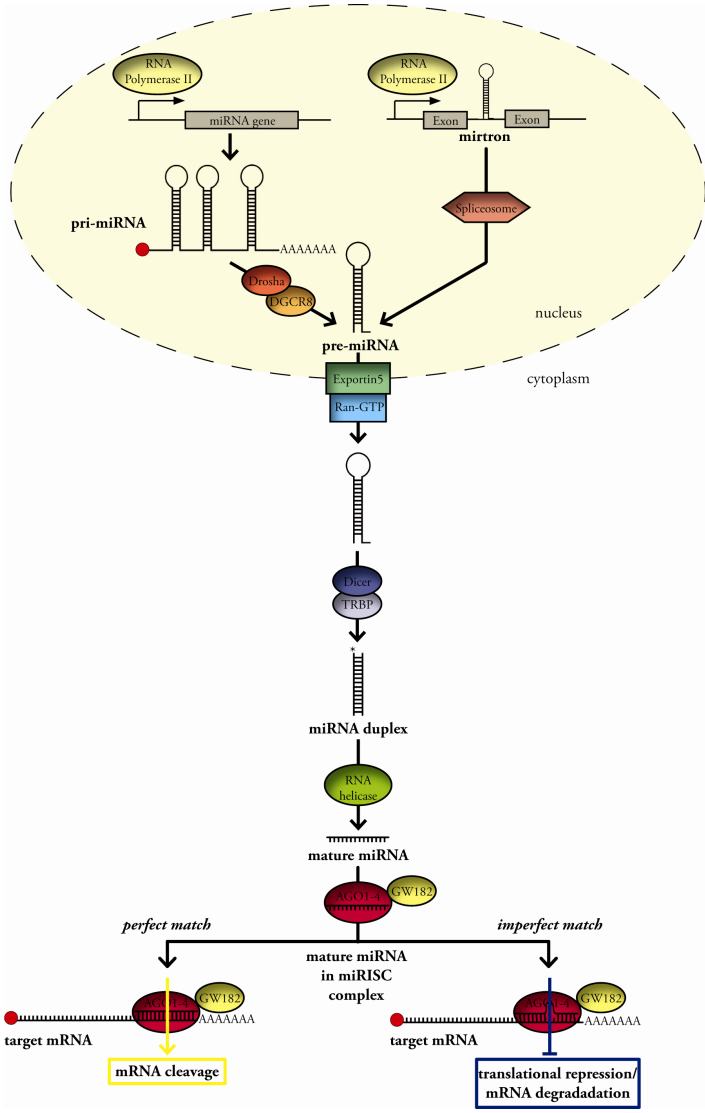

Figure 5. The miRNA biogenesis.
For most miRNAs, the miRNA gene is transcribed into a stem-loop structure (pri-miRNA) and processed by Drosha to a ~70 nt long hairpin precursor called pre-miRNA. Mirtrons are miRNA genes located in introns and bypass Drosha by being processed to pre-miRNA during splicing. The pre-miRNA is exported to the cytoplasm by Exportin5 and Ran-GTP, further cleaved by Dicer and TRBP to a miRNA-miRNA* duplex. The strands are unwinded and the guide strand is incorporated into the miRISC complex and directed to its target mRNAs, leading to either mRNA cleavage or translational repression depending on perfect or imperfect match between miRNA and mRNA.

Regulation of target mRNAs

MiRNA binding sites in the mRNA transcript

Initial studies proposed that miRNAs could only regulate target mRNAs in a negative manner by binding to the 3'UTR of the target mRNA.[122,123] Now it has been demonstrated that miRNAs can also associate with binding sites within the amino acid coding sequence (CDS) of mRNA transcripts in order to repress translation.[175] By doing so, the network of miRNAs can fine tune the regulation of mRNA translation. Tay *et al* observed that miRNAs can span over two exons, suggesting that the miRNA can selectively target splice variants.[176] In addition, miRNAs have been found to bind to 5'UTR of mRNA transcripts[177,178] and to gene promoters.[179]

Repression

The most common and well-studied forms of mRNA target regulation is translational cleavage and repression. Cleavage of the mRNA occurs when the miRNA sequence is completely complementary to a part of the mRNA sequence. This is the case of siRNAs and miRNAs in plants.[180,181] The actual cleavage is performed by the Argonaute protein family member AGO2 which is the only Argonaute protein with "slicer" activity.[182-184] It is nevertheless unusual with complete sequence matches between miRNA and target mRNA in animals.

The mechanism behind translational repression is not completely clarified. A suggested mode of action is inhibition of the initiation of translation by binding of AGO2 to the 5'cap of the mRNA[185-187] or by interactions with ribosomal subunits resulting in release from the ribosome.[188-190] It has also been shown that miRNAs can promote deadenylation of mRNAs leading to degradation.[191,192] RNA-binding factors like Importin 8 has been observed to be able to modulate the repression of target mRNAs by increasing the repressive effects of miRNAs by means of facilitating the miRNA:mRNA recognition.[193] This protein is also present in the cytoplasmic P-bodies or GW-bodies, which are structures where untranslated mRNAs and mRNA-processing enzymes are assembled for degradation.[163,194] These P-bodies can act as storage for miRNA-repressed mRNAs. It has been shown that under certain circumstances the repression can be withdrawn resulting in the release of mRNAs.[195]

Activation

Even though the most common mode of action for miRNAs is translational repression, emerging studies are also suggesting a role for miRNAs in positive regulation. MiRNAs that bind to promoter regions have been reported to activate translation resulting in increased levels of the specific protein product.[179,196] MiRNAs coupled to the regulation of the cell cycle can have both repressive and activation functions. Vasudevan *et al* reported that miRNAs can only activate translation in

quiescent cells, and miRNAs act as repressors in cycling cells.[197] This observation, that miRNAs can have both activating and repressing effects depending on cell cycle state, puts forward the complexity of mRNA regulation by miRNAs.

Regulation of miRNAs

Transcriptional, epigenetic and bidirectional regulation

MiRNAs have different expression patterns in distinct developmental stages both during normal and malignant conditions. Exactly how this regulation of miRNAs occurs is not completely delineated and appears to take place at many different levels. The transcription of a miRNA gene is influenced by several factors, one being the genomic localization of the gene. Some miRNAs in introns of protein-coding genes are co-expressed with their pre-mRNA host.[198] Others have their own promoters as similar and complex as those for protein-coding genes.[199] A few regulatory factors that bind directly to the miRNA promoter have been identified. The oncogenic transcription factor c-Myc was demonstrated to bind directly to the locus where the miR-17-92 miRNA cluster is located and could upregulate the expression of all included miRNAs.[200] C-Myc also had the opposite function and repressed several tumour suppressive miRNAs by direct binding to their promoters.[201] In addition, it was shown by several research groups that the well-known tumour suppressor p53 could bind to the promoter region for the miR-34 family (miR-34a-c) and upregulate the miRNAs.[202-206]

Another way of regulating miRNA transcription is through epigenetic modifications. It has been suggested that ~5-10% of all miRNAs are under epigenetic regulation.[207-209] The miRNA let-7a-3 gene was observed to be heavily methylated in normal human tissues and thus silenced, however in some lung adenocarcinoma the methylation was absent leading to upregulation of the miRNA and enhanced tumour features.[210] There are several reports about cancer-associated miRNAs often being epigenetically regulated, observations that can be used in therapeutic strategies.[211]

To add to the complexity, miRNAs can be transcribed in two directions (sense and antisense) meaning that both DNA strands can be transcribed.[157,212,213] This eventually gives rise to two distinct mature miRNAs with different seed sequences. The hypothesis that this type of bidirectional regulation exists in order to secure the tightly controlled regulation of miRNA pairs having similar targets or target pathways is supported by studies in yeast.[214]

Translational miRNA regulation

The processing of pri-miRNAs to mature sequences is an additional layer of regulation. Several positive and negative regulatory factors have been identified.

Among the positive regulators are members of the SMAD protein family. They have been shown to enhance the production of certain mature miRNAs from pri-miRNA.[215] Similar enhancement of miRNA processing has been observed in the cytoplasm where the pre-miRNA is processed to mature miRNA.[216] This may also indicate that the nuclear export of pre-miRNA is implicated in the selection of what miRNAs that will be completely processed. Indeed, Exportin-5 and Dicer were both influenced by steroids in mouse uterus suggesting that levels of miRNA biogenesis components affect the processing of mature miRNAs.[217] One of the identified negative post-transcriptional regulators is Lin-28. Lin-28 inhibits pri-miRNA processing of all let-7 family members, potentially by blocking the association between Drosha and the let-7 hairpin structure, which leads to implications in stem cell differentiation.[218-221]

Another mechanism to inhibit miRNA biogenesis is by A-to-I RNA editing, meaning that an adenosine nucleoside is converted to inosine. This usually occurs in introns of non-coding genes and in 3'UTR regions.[222,223] For miRNA processing, it has been reported that this results in the inhibition of the Drosha cleavage.[224] It has also been observed that A-I editing in the seed sequence of the miRNA leads to a different set of target mRNAs.[225] This process has been observed in approximately 6% of human miRNAs

On top of these regulatory events, is the hypothesis of competing endogenous RNAs (ceRNAs).[226] These non-coding RNA transcripts contain miRNA response elements (MREs) that act like miRNA sponges and thereby reduce the levels of the miRNAs corresponding to the MREs found in the mRNAs. It has been shown that the non-coding pseudogene to the tumour suppressor *PTEN*, *PTENP1*, also contains many of the MREs found in *PTEN*. *PTENP1* functions as a positive regulator for *PTEN* by sequester miRNAs that otherwise would all bind to *PTEN* leading to repression. It was also observed that the locus from where the pseudogene is transcribed was lost in several cancers, suggesting that *PTENP1* could be considered a tumour suppressor gene.[227]

MiRNAs and cancer

The emerging evidence during the last two decades that miRNAs are potent regulators in practically all cellular processes and play powerful roles in the maintenance of normal functions, has not surprisingly resulted in observations that deregulated miRNAs are greatly implicated in most, if not all, human diseases, including cancer. The first report linking miRNAs and cancer came in 2002 from

Carlo Croce's group.[228] They were initially looking for a gene at chromosome 13q14, a region that is deleted in more than half of B cell chronic lymphocytic leukemias (B-CLL). To their surprise they did not find a protein coding gene, but instead they discovered the two miRNAs miR-15 and miR-16. The expression of these miRNAs was then found to be lost in approximately 68% of all B-CLLs, and the first link to cancer was established. Since then, the number of papers being published on miRNAs and cancer has increased from 8 reports in 2002 to over 2000 in 2012.

Oncomirs and tumour suppressor miRNAs

Many research groups have generated miRNA signatures for cancers in general[229] and for specific cancers e.g. breast,[230,231] leukemia,[232] liver,[233] colorectal[234] and lung.[235] These studies have led to the conclusion that miRNAs play different roles in different tissues, even under cancerous conditions. This is probably due to different target mRNA contents in the different tissues. Moreover, studies in the same tissue demonstrating a contradictory miRNA expression profile can for example possibly be explained by the fact that these studies have been carried out on a mixture of cellular compartments (epithelial and stromal), at different stages of the disease or without proper matching of samples.

Oncomirs

In the analysis of solid cancers by Volinia *et al*, it was shown that the miRNAs miR-21 and miR-191 were upregulated across different tissues.[229] MiR-21 has previously been reported to be highly upregulated in glioblastoma and to have an antiapoptotic function.[236] MiR-191 is upregulated in hepatocellular carcinoma caused by hypomethylation of the genomic locus of miR-191 and this was associated with poor prognosis.[237] Other suggested oncomirs are the seven members of the miRNA cluster miR-17-92a which is overexpressed in B-cell lymphoma and lung cancer.[238,239] Yet individual members of the cluster have been reported to have distinct roles in different cell types and tissues. In breast cancer, miR-17-5p is often downregulated and has been shown to target the oncogene AIB1 (amplified in breast cancer 1).[240]

A general downregulation of miRNA expression

MiRNAs have been mapped in the genome to be located in fragile sites, that are often deleted or amplified, and to cancer-associated genomic regions.[241] There are also several studies demonstrating that miRNAs are generally downregulated in cancer samples compared to normal tissue, suggesting a protecting role for miRNAs.[242,243] The hypothesis that most miRNAs are tumour suppressive has also been suggested based on studies of the miRNA processing proteins Dicer and Drosha. Loss of one or both of these proteins was inversely correlated with outcome in several types of

cancers, including breast and ovarian cancer.[244,245] On the other hand, there are studies demonstrating the opposite for other cancer forms such as colorectal and prostate cancer.[246,247] Even though several studies have reported a general decreased expression of miRNAs in cancer tissues, a large-scale analysis of solid cancers in multiple tissues revealed a solid cancer miRNA signature with a large fraction of overexpressed miRNAs with targets being significantly enriched for tumour suppressors and oncogenes.[229] These contradictory results can possibly be explained by differences in microarray platforms, normalization and preparation of tissues. As the technology improves, these differences are likely to diminish, the technical errors can be abolished and the generated data can be confidently comparable. It is, however, very likely that miRNA expressions vary in different cancers, during different stages and in different subsets of cells, indicating again the importance of taking each specific tissue and cell type into account when studying individual miRNAs.

MiRNAs and breast cancer

MiRNA signatures in breast cancer

The reported results from miRNA expression arrays of breast cancer tumours have generated signatures with both similarities and differences. In 2005, Iorio *et al* reported a study where they used biotin-labelled samples hybridized to a miRNA microarray chip to compare the expression of 245 human and mouse miRNAs in 76 primary tumours and a total of 34 pooled samples of normal breast tissue divided into 10 samples.[230] They obtained a miRNA signature of 15 miRNAs that could distinguish normal breast from breast cancer. They also found miRNA signatures that could subdivide tumours based on presence of ER and PR, tumour stage, positive lymph nodes, vascularisation, proliferation index, and p53 status.

Two years later in 2007, Blenkiron and colleagues demonstrated that miRNA expression profiles could be used for dividing primary invasive breast carcinoma tumours into subgroups based on the molecular subtypes luminal A, luminal B, basal-like, HER2 and normal-like.[231] They used a bead-based flow cytometric miRNA expression platform to analyse 309 miRNAs in fresh frozen tissue from 93 tumours, 5 normal breasts and 21 breast cancer cell lines, and observed that approximately one third of the miRNAs were expressed. They too noticed major differences in miRNA expression between ER+ and ER- tumours and could confirm the decreased expression of miR-30a-5p in the ER- tumours observed by Iorio and co-workers.[230] In

contrast with other studies, they did not observe a perfect separation between normal and cancer samples, however that could be due to small number of normal samples.

Given that the routine and standard processing of clinical material for histological analysis is fixation in formalin followed by paraffin embedding (FFPE), Hui *et al* investigated in 2009 the expression of 365 miRNAs in 36 invasive breast carcinoma and 6 normal breast tissue FFPE samples.[248] They used the quantitative real-time PCR (qRT-PCR) based microfluid card TaqMan Low Density Array (TLDA), validated the array results with single-well qRT-PCR and concluded that this platform was both technically and biologically robust for analysis of FFPE samples. They observed that the normal tissue and the breast cancer samples were separated in an unsupervised hierarchical clustering analysis based on the expression of all miRNAs. In concordance with the previous studies in breast cancer, they observed downregulation of miR-125b and upregulation of miR-21, miR-155, miR-191, miR-196, miR-210 and miR-213.

MiRNAs as biomarkers and therapeutics in breast cancer

MiRNAs as biomarkers in tissue and body fluids

The observation that miRNAs are preserved in FFPE and appear to be differentially expressed in normal and breast cancer samples suggests that there is potential for miRNAs to be used as routine biomarkers in the clinical setting. Several studies has investigated single or a subset of miRNAs and used the methods qRT-PCR and *in situ* hybridization (ISH) for this purpose. There are a number of specifically validated miRNAs harbouring prognostic value in breast cancer. For example, the expression of miR-7b and miR-205 was evaluated using ISH in a large cohort of invasive breast cancers.[249] The expressions of both miRNAs were shown to be inversely correlated with several key clinicopathological parameters and were predictive for survival. The expression of miR-21 was investigated using qRT-PCR and confirmed previous studies that miR-21 is upregulated in breast cancer.[250] High expression of miR-21 was positively linked to larger tumours, higher stage, grade and proliferation index, ER-/HER2+ tumours, provided prognostic information and was significantly associated with poorer survival.

Recent discovery that miRNAs can be measured in body fluids, such as plasma and serum is promising for a non-invasive method of measuring tumour biomarkers.[251] A pilot study was performed with blood serum from patients with primary breast cancer, metastatic disease and healthy women using qRT-PCR.[252] They concluded that the elevated expression of the tumour-associated miRNAs miR-155, miR-10b and miR-34a measured in plasma could discriminate between patients with primary

and metastatic tumours. In addition, high level of miR-155 was also a significant marker for separation between healthy individuals and primary breast cancer patients.

MiRNAs as treatment predictive markers and role as therapeutic agent

Emerging studies propose that miRNAs have potential as treatment predictive markers. Overexpression of miR-221 and miR-222 in breast cancer cell lines induced tamoxifen resistance via downregulation of p27[253] and by regulation of ER.[254] MiR-221 and miR-222 were also able to induce resistance to the ER antagonist fulvestrant.[255] Recent studies reported that high expression of miR-30c[256] and miR-26a[257] were both independent treatment predictive markers for tamoxifen response in primary breast cancer tumours, and similar results were observed for miR-342.[258] Resistance to the aromatase inhibitor letrozole was associated with upregulation of miR-128a by targeting the TGFβ signalling pathway,[259] and high levels of miR-34a was shown to be linked to docetaxel resistance in human breast cancer cells.[260]

The potential for miRNAs to have therapeutic implications is evident. There are a few studies in mice of targeted breast cancer treatment with miRNAs delivered either as an expression vector or in exosomes.[261,262] An example that miRNAs can be used as treatment has been evaluated in a phase II clinical trial for hepatitis C using an LNA (locked nucleic acid)-modified oligonucleotide to target miR-122.[263,264]

The tumour microenvironment and breast cancer

The body of evidence of a role for an essential interplay between the epithelial and stromal compartment during both normal development and cancer progression has increased tremendously. There is no doubt that there is a crosstalk involving the epithelial cells and the components of the surrounding microenvironment. The theory about "seed and soil" was proposed over a century ago and suggests that a seed can only grow if the soil is right, meaning that a cancer cell is dependent on signals from the microenvironment.[265]

Components of the tumour microenvironment

The stroma consists of several cell types and the extracellular matrix (ECM) that communicate with the epithelial cell compartment via paracrine signalling mediated by cytokines and different growth factors. This interaction can in turn modify multiple cellular processes including differentiation, survival, proliferation, polarity, and the capacity of mammary epithelial cells to invade surrounding tissues.[266] The major components are ECM molecules (i.e. collagens, laminins and fibronectin), fibroblasts, endothelial cells, adipocytes and immune cells. The proportions of the different components vary during different stages of development.[267]

The structural ECM is a large part of the stroma. The ECM plays an essential role during both developmental processes and through direct and indirect regulation of cellular behaviour. It does so by dynamically changes of production, degradation and remodelling of its components. Disturbances in this tightly regulated process is associated with malignancies including cancer.[268] Studies have shown that women with denser breasts have an increased risk of developing breast cancer.[269] Collagen I, which is one of the major structural proteins in the ECM, has been reported to promote invasion,[270] whilst increased expression of collagen V has been demonstrated to reorganize the collagen fibres resulting in an increased ECM deposition.[271-273] The dynamics depend largely on the expression and/or activities of ECM enzymes

including matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). These enzymes are produced by different cells both in the stroma (fibroblasts and immune cells) and by epithelial cells, and are important during branching morphogenesis.[267]

The fibroblasts are responsible for the production of ECM components, and they are the most abundant cell type in the stroma. They are normally in a dormant state and are only activated during wound healing and fibrosis and are then called myofibroblasts.[274] Fibroblasts present in the tumour stroma, the cancer-associated fibroblasts (CAFs), resemble activated fibroblasts mainly by their expression of α-smooth muscle actin (α-SMA).[275] CAFs have the ability to modulate the tumour stroma, which is associated with higher-grade tumours and poor outcome.[276]

It has also been demonstrated that CAFs have a pro-inflammatory gene signature that promotes the infiltration of macrophages.[277] These tumour-associated macrophages (TAMs) correlate with poor prognosis in breast cancer.[278] TAMs can increase cancer cell proliferation and survival as well as promote cell migration, invasiveness, angiogenesis, and ECM reorganization.[279]

Abnormalities during breast cancer progression

In order to investigate the molecular alterations underlying the changes in the tumour microenvironment during breast cancer development, several research groups have characterized both the different compartments (epithelial and stromal), and also the different cell types within the two compartments. Ma and colleagues microdissected the epithelial cells and the stroma of normal, pre-invasive and invasive breast cancer tissue and analysed the gene expression. They observed extensive changes in both the epithelium and stroma in the malignant tissue, and particularly an increase of ECM genes, matrix metalloproteases and cell cycle genes in the stroma of both the pre-invasive samples as well as the invasive breast cancer tissue. This suggests that the microenvironment is involved in the tumourogenesis prior to invasion of surrounding tissues. The gene expression profile from the stroma could also define histological grade, whilst high-grade tumours had an increased expression of immune response genes.[280]

Allinen *et al* investigated gene expression and genetic changes of the major cell types (luminal epithelial, myoepithelial, and endothelial cells, infiltrating leukocytes, fibroblasts, and myofibroblasts) in both epithelial and stromal compartments of normal breast, *in situ* carcinoma and invasive breast cancer. Interestingly they found that the gene expression differed in all cell types during cancer progression, but

genetic alterations were only detected in the cancer epithelial cells. They further observed in myoepithelial cells and in myofibroblasts an overexpression of certain chemokines that bind to receptors on the luminal epithelial cells resulting in increased proliferation.[281]

The present investigation

Aims

The overall aim of this thesis was to study cell cycle associated regulators in invasive breast cancer and premalignant breast lesions, with regards to potential biomarkers for cancer progression and tamoxifen treatment prediction as well as possible involvement of identified epithelial miRNAs in the surrounding micro environment.

Specific aims

- Delineate the prognostic and tamoxifen treatment predictive role for the CDK inhibitor p27 in premenopausal breast cancer patients.
- Investigate the correlation between the expression of miR-92a and breast cancer features and tumour environmental components using miRNA *in situ* hybridization and objective evaluation by automated digital analysis.
- Identify miRNAs with altered expression in the earliest histologically identifiable lesion in the breast with premalignant potential, and investigate their role in cell proliferation and interactions with the surrounding stroma.
- Study cyclin D1-associated miRNAs and their role in cell migration, proliferation and mammosphere formation, and delineate the effects in different breast cancer subgroups.

Results and Discussion

The importance of early detection of breast cancer and robust clinical biomarkers are known to increase patient survival and treatment efficiency among women suffering from breast cancer. For a heterogeneous disease such as breast cancer, it is essential to gain understanding about the underlying biology and the molecular differences observed in the distinct subgroups and clinical stages of the progression. This thesis describes potential biomarkers associated with regulation of cell proliferation and their role in premalignant breast lesions as well as invasive breast cancer.

MiRNA microarray platforms

We used quantitative real time PCR (qRT-PCR) miRNA microarrays to evaluate the global expression of miRNAs in paper III and IV. This method is based on the TaqMan technology and uses a stem-loop structure specific for binding mature miRNAs. The arrays are called TaqMan Low Density Arrays (TLDAs). These arrays have previously been validated and reported to generate robust results from FFPE samples, both biologically and technically.[248]

There are different types of miRNA microarray platforms available. The classical microarray platform developed for gene profiling has been adapted to miRNAs by introducing LNA-modified probes and thereby enhancing the binding affinity resulting in more reliable readouts.[282] Briefly, miRNA labelled with a fluorescent dye is hybridized to its complementary detection probe on a glass array and emits fluorescence in the presence of a laser which is detected by a scanner. This type of array can contain several thousand probes and thereby analyze the expression of all known human miRNAs simultaneously. However, they often require specific scanners.

The bead-based technology is based on coloured beads covered with capture probes for one specific miRNA that are hybridized to labelled miRNAs.[243] They are further stained and sorted by a flow cytometer with two lasers detecting both the identity of the miRNA (the bead colour), and the quantity (the staining intensity). The bead technology is more flexible than the glass array platform due to the ability to change bead composition. It is also less expensive and faster, but it does not cover the same amount of miRNAs.

Several studies have compared the different platforms and concluded that most platforms have good intra-platform repeatability, meaning that each platform is stable and can repeat the same result. There is however a problem when comparing data from different platforms.[283,284] This is probably due to problems in normalization of the data. Reliable controls should be consistently stable and highly abundant across samples, as well as being of the same size as miRNAs.[285] The intra- and inter-platform

repeatability as well as normalization was examined in a study including LNA microarrays, bead arrays and TLDAs. In line with previous studies, the platforms showed better intra- than inter-platform reproducibility, however the endogenous controls in the TLDAs performed better than the other platforms across the investigated samples, suggesting a robust normalization of the data.[286]

p27 is a predictive factor for tamoxifen treatment response (*paper I*)

p27 has dual contradictory roles in cell cycle regulation by acting both as a CDK2-inhibitor and as an assembly factor for cyclin D-CDK4/6 complexes. Previous studies have suggested p27 as a prognostic marker in breast cancer and low levels of p27 have been associated with worse outcome in several cancers. However, in order to truly identify a prognostic marker it is important to perform the study in a cohort of untreated patients. Due to obvious reasons these patient cohorts are not very common. This study was performed in a cohort of 500 premenopausal patients with stage II invasive breast cancer randomized to either two years of adjuvant tamoxifen or no adjuvant treatment, irrespective of hormone receptor status. This makes this cohort one of its kind in the search for prognostic and treatment predictive markers. The patient samples were arranged in a tissue microarray (TMA).

p27 is associated with tumour grade, tumour size and the expression of cyclin D1 and ER

The expression of p27 was evaluated using immunohistochemical staining. Protein expression was scored as 0 (0-10%), 1 (11-50%), 2 (51-75%) and 3 (76-100%) p27-positive tumour cell nuclei. The results revealed a significant inverse correlation between p27 and tumour grade and tumour size. It was also more common with high levels of p27 in older patients and in patients with lymph node metastases. Ductal and lobular tumours were also associated with high expression of p27 whereas low p27 was more often observed in medullary tumours. The analysis further revealed that there was a significant positive correlation between p27 expression and previously analysed expression of cyclin D1 and ERα and an inverse association was observed between p27 and proliferation marker Ki-67 and many mitoses. However this inverse correlation was only present in the ER- tumours.

These results suggest that loss of p27 is associated with a more aggressive phenotype which is in line with its role as a tumour suppressor. They also indicate that p27 is required for cyclin D1 dependent cell proliferation in ER+ but not ER- tumours, suggesting that the ER+/p27 low tumours rely on a different proliferative pathway not depending on oestrogen, possibly involving cyclin E. This supports a role for p27 as an assembly factor for functional cyclin D1 promoting growth in ER+ tumours. It also highlights the heterogeneity within the subgroup ER+ breast cancer tumours.

p27 is not a prognostic factor in premenopausal women

In order to correctly investigate the prognostic value of a certain biomarker, it is ideal to study an untreated group of patients. It is however difficult to find these collections of patient material since most patients receive some kind of adjuvant treatment after surgery. The cohort for this study is therefore unique when searching for prognostic markers in premenopausal patients. Analysis of the prognostic value of p27 was thus performed in the group of patients that had been randomized to no adjuvant treatment. In contrast to other studies that suggested p27 to be of prognostic value, we did not observe this property in this patient material. There could be several reasons for this. The most likely reason is that the studies have been performed in different patient cohorts consisting of a heterogeneous collection of tumour samples. Additionally, in this study we only included stage II premenopausal women while in other studies patients of all stages and ages have usually been included. Furthermore, when the patients were stratified based on ER status, it became clear that ER- patients with low expression of p27 had a significantly better survival compared to patients with high expression of p27. This highlights the essential need of dividing breast cancers into subgroups in order to fully understand the biology behind the different subdivisions of breast cancer and to subsequently be able to foresee the prognosis for patients.

Loss of p27 is a negative predictor of tamoxifen response

We next investigated whether p27 could predict if a patient would respond to the endocrine treatment tamoxifen or not. Tamoxifen competes with oestrogen to bind to the ER and is therefore theoretically only active in the ER+ tumours. In addition, PR has been shown to be an even better predictor of tamoxifen response than ER.[81] The treatment predictive analysis was consequently performed in the patient group with ER+ or ER+/PR+ tumours. There was no beneficial effect of tamoxifen, as measured by recurrence free survival, in patients with tumours having low expression of p27 whereas the opposite was observed in the subgroup of ER+ tumours expressing high levels of p27. The effect of tamoxifen was even more noticeable when tumours expressing both ER and PR were analysed. The treatment response to tamoxifen relative to the expression of p27 was also significant in a multivariate interaction analysis adjusted for age, tumour grade, nodal status and tumour size. The underlying mechanism for why low p27 expressing tumours do not respond to tamoxifen remains to be investigated, however one can speculate that p27 is required for a functional ER signalling pathway by cyclin D1 due to its assembly factor effects. In fact, it was only in this subgroup of tumours (high p27, ER+) that cyclin D1 was associated with increased proliferation as measured by Ki67. Only in cells with a functional ER pathway is it possible for tamoxifen to have an effect. Moreover, p27 is also likely to be required for its second function, as a CDK-inhibitor arresting the cell cycle.

Taken together, this study suggests that loss of p27 can be used as a treatment predictive marker for weak tamoxifen response in ER+/PR+ premenopausal breast cancer patients, but not as a prognostic marker.

MiR-92a is inversely correlated with breast cancer characteristics (*paper II*)

MiRNAs are small endogenously expressed non-coding RNAs that regulate their target mRNAs on a posttranscriptional level. Each miRNA has many different targets and has been shown to be able to influence whole pathways.[129] This has led to the suggestion that miRNAs can be valuable as both biomarkers for prognostic purposes and even as therapeutic targets. There is a large amount of reports about deregulated miRNAs in the majority of cancers. However it is becoming more and more evident that miRNAs have tissue specific effects and therefore should be evaluated in their proper context. In this study we investigated the expression of the miRNA miR-92a in formalin fixed breast cancer samples arranged in a TMA. We developed a miRNA *in situ* hybridization protocol and evaluated the expression by digital image analysis to ensure that the results were objective.

The miRNA in situ *hybridization method*

In situ hybridization is a useful method for investigation of specific nucleotide sequences in tissue samples and when specimens are arranged in TMAs it is possible to analyse a large amount of samples in a short period of time. The method has however been criticised for the challenging and time consuming optimization procedure in order to establish a high specificity of the probe. In our study we used double-DIG labelled probes enhanced with LNA (locked nucleic acid) molecules leading to a more stable interaction between the probe and the target miRNA. We also included an extra fixation step in order to retain the miRNAs that would otherwise diffuse out of the tissue during the preparations. The miR-92a probe was validated in a cell line where the miRNA was downregulated. To further ensure that it was indeed miR-92a expression we measured and not an artefact from the method, a scramble probe was hybridized to a second set of the TMA as a negative control. Any signal from the negative control was then subtracted from the miR-92a signal. The TMA slides were subsequently scanned and objectively analysed by the Digital Image Hub software. The settings for the image analysis were adjusted manually before analysis, and were identical for all samples.

MiR-92a adds independent prognostic information and loss of miR-92a is associated with high grade tumour, poor clinical outcome and increased cell migration

The expression of miR-92a was analysed in a cohort of 144 breast cancer samples. From the analysis of associations between miR-92a and clinico-pathological parameters we observed that tumours of high histological grade had a significant lower level of miR-92a. Patients with high levels of miR-92a also had a better outcome compared to patients with lower levels of miR-92a. We also observed in a multivariate Cox proportional hazard regression analysis with adjustments for known prognostic factors that patients with high levels of miR-92a had a lower risk of recurrence demonstrating that miR-92a provides independent prognostic information in breast cancer.

To study the effects of miR-92a on cellular functions we modulated the level of miR-92a in the breast cancer cell line MDA-231 and monitored proliferation and cell migration. We observed a highly significant increase in cell migration after downregulation of miR-92a proposing a potential mechanism for the observed poor clinical outcome associated with loss of miR-92a.

The role of miR-92a in the tumour stroma

The involvement of the stroma surrounding tumours has been shown to greatly influence the initiation and progression of breast cancer.[287] One of the properties in malignant stroma is the presence of activated fibroblasts, characterized by increased levels of α-smooth muscle actin (α-SMA), and of activated macrophages which are positive for CD68. We did not find a link between activated fibroblasts and miR-92a, nonetheless there was a clear inverse correlation between high macrophage content in the tumour and expression of miR-92a. This observation proposes that the expression of miR-92a in the epithelial cells is involved in the interaction of the immune cells in the surrounding tumour stroma.

Epithelial and stromal miRNA signatures and effects in premalignant breast lesions (*paper III*)

Columnar cell hyperplasia (CCH) is a commonly occurring feature in the female breast. However, it has also been linked to different stages of breast cancer, making it the first histologically identifiable lesion with premalignant potential.[288-290] The lesions are characterized by the enlargement of normal terminal duct lobular units (TDLUs) with tightly packed columnar-shaped epithelial cells lining the lumen. These alterations often display an increased expression of ERα and have an elevated proliferation rate.[48] The studies performed in paper III are focusing on the underlying

mechanisms for this first potential premalignant transformation of the normal breast with focus on miRNAs as regulators.

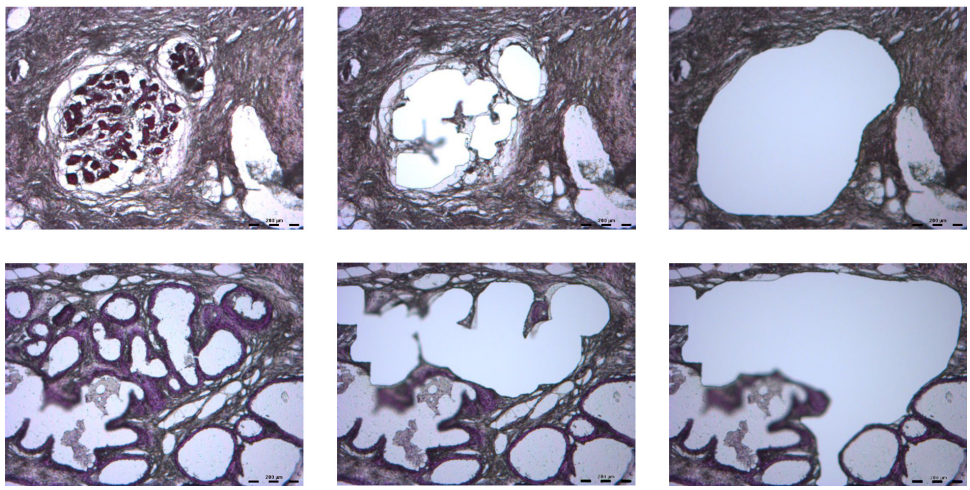

Figure 6. Laser capture microdissection of TDLU and CCH
TDLUs (upper panel) and CCH (lower panel) were stained with hematoxylin (left panel) and epithelial cells (middle panel) and the surrounding stroma (right panel) were cut out using laser capture microdissection. The material was collected for further miRNA microarray analysis.

MiRNA expression changes occur already in premalignant CCH and miRNAs are mainly downregulated in both epithelial and stromal compartments of CCH compared to TDLU

It has become evident that the interplay between the epithelial cells and the surrounding stroma is of great importance for the normal function of the breast. Disturbances of this balance can result in changes in cell behaviour at early stages and can eventually lead to breast cancer.[291,292] In order to distinguish between the epithelial cells and the stroma, we used laser capture microdissection to separate the two compartments (Figure 6). In the study we used patient samples from women who had undergone prophylactic mastectomy. TDLUs and CCH with sufficient material from both epithelial and stromal compartments from the same patient were collected. The global miRNA expression was analyzed using TaqMan Low Density Array (TLDA) and the expressions of 663 miRNAs for each sample were obtained. The results revealed a general downregulation of miRNA expression in CCH compared to TDLU in both the epithelial and stromal compartment. This overall decrease in expression has previously only been investigated and observed in cancers and it has been proposed that this pattern reflects the general inhibitory function of miRNAs and contributes to a less differentiated cell which is characteristic for cancer cells.[243]

MiR-27a, miR-92a and let-7c have anti-proliferative effects in epithelial CCH cells

Among the downregulated miRNAs in the epithelial compartment of CCH were miR-27a, miR-92a and let-7c. Since increased cell proliferation was a characteristic of CCH we studied if the selected miRNAs had any involvement in this cellular function. In order to do functional studies we used human mammary cells from a clinical sample of CCH that had been cultured during several passages. In an attempt to mimic the *in vivo* situation of TDLU to CCH progression, we used two passages of cells, referred to as low and high passage CCH cells. The low passage CCH cells were less proliferative than the high passage cells and expressed higher levels of the selected miRNAs, features that also were observed in the clinical samples. We modulated the miRNA levels with mimics and inhibitors and monitored the effects on cell proliferation. Downregulation of miR-27a increased proliferation in both passages and let-7c downregulation significantly increased the number of low passage CCH cells. We observed the opposite effect on proliferation in both passages after upregulation of all three miRNAs with the exception of let-7c that again only affected the low passage CCH cells. One of these miRNAs, miR-27a, has previously been implicated in regulation of proliferation however it was evidenced to promote cell proliferation.[293] Similar observations have been reported for the cluster in which miR-92a is located.[238] Nevertheless we have observed a decreased expression of miR-92a as an individual miRNA in breast cancer, as described in paper II. Comparable results were reported in hepatocellular carcinoma.[294] We have observed that miR-27a and miR-92a have anti-proliferative roles in mammary epithelial CCH cells, suggesting that one miRNA can have opposite effects depending on cell type and tissue. The third studied miRNA, let-7c, is part of the let-7 miRNA family in which two members have been reported to have anti-proliferative effects.[295] We have now also demonstrated that let-7c has anti-proliferative effects in the earliest mammary epithelial cells with premalignant potential.

Let-7c can potentially target the oncogenic transcription factor Myb

In order to investigate potential targets, we compared a published gene expression list from the epithelial cell compartment of CCH with predicted target sites for the selected miRNAs *in silico* using the online DIANA LAB web server. We found that the transcription factor Myb was a potential target for let-7c. In the patient material we could confirm the elevated levels of Myb in CCH as reported previously.[49] We then manipulated the levels of let-7c and observed that the Myb mRNA levels were indeed negatively regulated by let-7c. With the aim of investigating a direct binding of let-7c to Myb mRNA, we constructed a luciferase reporter vector with the potential let-7c binding site in the 3'UTR of Myb. The vector was introduced into let-7c-high expressing CCH cells (low passage) and let-7c-low expressing CCH cells (high passage). The results revealed a lower luciferase activity in the let-7c-high expressing CCH cells, suggesting that let-7c binds to the predicted site and suppresses

the activity. Thus the observed decreased levels of let-7c can potentially explain the elevated Myb expression and the increased proliferation observed in epithelial cells in CCH.

Stromal miR-132 affects metabolic and actin-associated pathways in mammary fibroblasts and co-cultured epithelial CCH cells

One of the few highly upregulated miRNAs in the stromal compartment of CCH was miR-132. This miRNA has previously been reported to be essential for the mouse mammary development and the specific expression in the stromal fibroblasts played a significant role in the interplay between the epithelial cells and the stroma.[296] Our observation in combination with the reported role in mouse mammary development made us interested to study if this interplay also was important in human and what potential downstream effects of miR-132 could be, both in the stromal fibroblasts and in the epithelial CCH cells. We observed that overexpression of miR-132 in human immortalized fibroblasts affected several cellular pathways and individual genes involved in extracellular matrix production, metabolism and cytoskeleton. Similar pathways were affected in epithelial CCH cells when co-cultured with miR-132 overexpressed fibroblasts, however not specific individual genes. This clearly demonstrates that alteration of miR-132 levels in fibroblasts have a major effect on adjacent epithelial cells, but the exact effect can only be determined in an *in vivo* setting and needs to be further studied.

MiRNA expression levels are primarily decreasing in cancer progression

In addition to the patients used in the analyses described above, we evaluated the global epithelial and stromal miRNA expression in TDLU, CCH and IBC from one additional patient displaying all three structures. From this case study and the CCH microarray analysis we could conclude that the miRNA expression in CCH and IBC is in general lower compared the healthy tissue. This fits with the overall elevated levels of gene expression from the microdissected CCHs compared to TDLUs, performed by Lee and colleagues.[49] Similar observations of a general downregulation of miRNAs have previously been reported in both tumours and cancer cell lines.[242,243] This suggests that the inhibitory role of miRNAs are applying a general suppression on cells and if this is turned off the cells' tightly monitored regulation is unable to keep the cells in a controlled state and they can start to transform. It has also been shown that miRNAs are frequently located in chromosomal regions that often displays abnormalities and are considered cancer-associated genomic regions.[241] Taken together; we have observed that the general downregulation of miRNAs is present already in the premalignant lesion CCH and continues as cancer progress. This is in line with other studies and suggests that miRNAs appear to play a general role as gatekeepers to a more malignant state of the cells. A downregulation of miRNAs may

increase a cells susceptibility to malignant signals, which ultimately can lead to genetic alterations.

MiR-483-5p is associated with cyclin D1 and has opposite effects on migration depending on ER expression status in breast cancer cell lines (*paper IV*)

Cyclin D1 plays an important role in the cell cycle regulatory network. Its effects on the G_1-S phase transition is well-established, however cyclin D1 also possesses additional cellular functions. Cyclin D1 is involved in cell motility and depletion of the protein leads to increased cell migration in the ER+ breast cancer cell line MDA-231.[297] Further investigation in MDA-231 and three other breast cancer cell lines, the ER+ cell lines MCF-7 and T47D and the ER- cell line MDA-468, revealed a cell migration pattern in line with previous report and demonstrated that this was depending on the ER status of the cell lines.[298] In addition, Lamb *et al* also showed that cyclin D1 played different roles in ER+ and ER- cell lines regarding mammosphere forming capacity. In our study we have investigated whether these observed effects on cell migration and mammosphere formation are mediated by downstream miRNAs and if they can have breast cancer subgroup specific functions. These observations can be of importance for the understanding of the different effects of cyclin D1 and downstream mediators observed in breast cancer subgroups.

Cyclin D1 associated miRNA expression

Cyclin D1 protein was downregulated in the four studied breast cancer cell lines and RNA was extracted. The same type of miRNA microarrays used in paper III (TLDAs) were used for miRNA microarray analyses by measuring the expression of 643 miRNAs. About two thirds of the miRNAs were not expressed. Of the remaining miRNAs, 52% were downregulated and less than 1% was upregulated in all cell lines, 42% were altered independent of ER status and the remaining twelve miRNAs were expressed in opposite directions depending on ER status. From these 12 miRNAs we selected miR-483-5p in order to study whether cellular functions observed after cyclin D1 depletion could be carried out by miR-483-5p.

MiR-483-5p affects certain cellular functions depending on ER status

The cell lines were transiently transfected with either control mimic or miR-483-5p mimic and the effects on cell migration, mammosphere formation and proliferation were monitored. We observed trends towards a decrease in cell migration in the ER+ cell lines and an increase in the ER- cell lines with a significant difference in the MDA-231 cell line. For the cell migration experiments we used the Boyden chamber assay which is a method based on chemotaxis, and measures the migration capacity of

cells moving toward a gradient of serum. This method is widely used in cell migration studies. Cells with high migration capacity are suitable for this method. Despite lower migration ability for some cell lines, this method can still give an indication whether or not the cells acquire a more migratory phenotype during certain conditions or not. We also observed a significant decrease of mammospheres in the ER+ cell line MCF-7, and no effect in the remaining cell lines. The mammosphere assay is a measurement of the cells ability to form mammospheres which is a trait for cancer initiating cells or potential cancer stem cells. Another method for studying cancer stem cells in breast cancer is by sorting the cells based on the cell surface markers $CD24^{-}/CD44^{+}$.[299] MiR-483-5p did not have a clear effect on proliferation in either cell line. These data suggests that some of the observed effects by cyclin D1 may be, if not executed, at least enhanced by the action of miR-483-5p. It is possible that the combined effect of several miRNAs from our established list of 12 miRNAs would result in a more pronounced effect on these cellular properties.

Cyclin D1 and miR-483-5p

The downregulation of cyclin D1 resulted in an increase of miR-483-5p expression in the ER+ cell lines and a decrease in the ER- cell lines. MiRNAs are occasionally regulated by their own targets and even if our results indicated opposite expression patterns of miR-483-5p as a consequence of cyclin D1 depletion, we were interested to see if miR-483-5p could influence the expression of cyclin D1. We observed that upregulation of miR-483-5p increased cyclin D1 levels in the ER+ cell line T47D while a decrease was observed in the ER- cell line MDA-231. It is not likely that cyclin D1 is a direct target for miR-483-5p based on the observed opposite effects on expression, and indeed the *in silico* analysis for a potential target site came out negative. However, miR-483-5p had an impact on the expression level of cyclin D1, suggesting that there might be intermediates that are affected by miR-483-5p and in turn affect cyclin D1. What these intermediates are can only be determined by investigating what mRNAs are physically bound to miR-483-5p. However, cyclin D1 is involved in a number of pathways including the Wnt and the MAPK pathway,[300,301] suggesting that the candidate intermediates can be components in these signalling networks.

This data gives an example of how complex and heterogeneous breast cancer tumours are, and truly emphasizes the magnitude of dividing breast cancers into subgroups based on well defined, yet to be established, biomarkers for a better and more precise evaluation of both prognosis and treatment prediction for breast cancer patients.

Conclusions

We have identified cell cycle associated prognostic and treatment predictive biomarkers in breast cancer, detected proliferation regulating miRNAs in the earliest premalignant lesion of the breast, and investigated the role of a cell cycle associated miRNA in models of breast cancer subgroups.

- Low levels of the cell cycle regulator p27 is a tamoxifen treatment predictive factor but not a prognostic marker in premenopausal women with stage II invasive breast cancer.
- Decreased expression of miR-92a is associated with more severe breast cancer characteristics including cancer-related stromal features, and can add independent prognostic information in breast cancer patients.
- MiRNA expression is generally decreased in both epithelial cells and surrounding stroma of the premalignant breast lesion CCH. Epithelial expression of let-7c can inhibit cell proliferation, potentially via regulation of Myb. Stromal expression of miR-132 is increased in CCH stroma and affects ECM genes in fibroblasts and metabolic pathways in co-cultured CCH epithelial cells.
- Cyclin D1-associated miR-483-5p has contrasting roles in cell migration, affects mammosphere formation, but not proliferation, in breast cancer cell lines depending on expression of ER.

Populärvetenskaplig sammanfattning

Cancer är ett laddat ord, och för de flesta av oss är cancern inte längre bort än hos en nära släkting eller vän. Trots den gemensamma benämningen, är cancer är inte bara en sjukdom, utan ca 200 olika som alla har gemensamt att cellerna börjar dela sig okontrollerat. Cancer är väldigt vanligt och uppskattningsvis kommer var tredje person att drabbas av cancer under sin livstid. Bland män är prostatacancer vanligast och bland kvinnor är bröstcancer den vanligaste formen.

I Sverige är ungefär 30 % av alla cancerfall bland kvinnor bröstcancer, och cirka en av tio kvinnor riskerar att få diagnosen innan 75 års ålder. Antalet nya fall har ökat för vart år, och detta kan troligen kopplas till att fler blir upptäckta tack vare mammografiundersökningar. Samtidigt har dödligheten stadigt sjunkit under de senaste åren, vilket vi förmodligen har nya och effektivare behandlingsmetoder att tacka för, samt att brösttumörerna upptäcks på ett tidigt stadium.

Förmågan för en normal cell att kunna dela sig är ytterst viktig, bl.a. vid sårläkning då nya celler måste bildas för att återställa huden. Denna delningsprocess kallas för cellcykel då cellen går igenom en cyklisk process där den återvänder till samma utgångspunkt som där den började, fast i två kopior. Cellcykeln är under sträng reglering och involverar många komponenter, både innanför och utanför cellerna. I normala fall när en delning ska ske, signalerar cellerna sinsemellan under väldigt kontrollerade former. En cancercell har däremot inte denna kontroll och kan dela sig utan att den får signaler. De cellulära proteinerna som driver cellcykeln kallas cykliner och CDK-proteiner. Dessa bildar komplex som får cellen att gå igenom cykeln och slutligen dela sig. I bröstcancerceller finns det ofta för mycket av den första cyklinen i cykeln, cyklin D1. För en kontrollerad celldelning krävs det att dessa komplex regleras och försvinner när de inte behövs längre. För det ändamålet finns proteiner som kallas för CDK-inhibitorer, varav en heter p27. I cancerceller, som kännetecknas av att de har genetiska förändringar, sker ofta förändringarna i just proteiner och molekyler som är involverade i celldelning.

MikroRNA-gener är en relativt nyfunnen klass av gener som verkar genom att reglera uttrycket av specifika proteiner. Enkelt uttryckt kan man säga att om det är proteinerna som utför de olika arbetsuppgifterna i cellen, är det mikroRNA-generna som ser till så att det finns rätt mängd proteiner på rätt arbetsplats. Uppskattningsvis är cirka en tredjedel av alla 25 000 protein-kodande gener reglerade av olika mikroRNA och det har visat sig att de är med i många olika processer i cellerna. Ett

mikroRNA kan reglera flera proteiner och ett protein kan vara reglerat av flera mikroRNA, allt för att cellen ska vara så kontrollerad som möjligt för ett korrekt uppförande. Då mikroRNA-gener i stort sett kontrollerar alla viktiga processer i cellerna, är det inte förvånande att dessa gener är förändrade i cancerceller. Vi har studerat mikroRNA-uttryck i förstadier till bröstcancer och funnit att redan där finns det skillnader. Vi har också undersökt ett specifikt mikroRNA i bröstcancertumörer och sett att minskade nivåer av det mikroRNAt är förknippat med sämre prognos. Vidare har vi sett att ett mikroRNA associerat med cellcykelproteinet cyklin D1 har olika roller i olika typer av bröstcancer vilket är en viktig observation för förståelse för uppförandet av olika bröstcancertyper.

Majoriteten av brösttumörer opereras bort genom att ta bort en del av bröstet eller i vissa fall hela bröstet. För att behöva ta bort så lite som möjligt kan patienter med avancerad bröstcancer få behandling innan operation, s.k. neoadjuvant behandling, som förhoppningsvis gör att tumören krymper. Denna förbehandling, t.ex. kemoterapi, kan också ge en indikation på huruvida tumörcellerna svarar på den typen av terapi och om den är effektiv för vidare behandling. Efter operation behandlas patienten med strålning för att eliminera cancerceller som eventuellt blivit kvar. Patienten får också en efterbehandling, kallad adjuvant behandling, som kan bestå av kemoterapi eller anti-hormonell behandling. Den anti-hormonella behandlingen ges till patienter vars tumörer utrycker östrogenreceptorn. Det gör cirka 70 % av alla brösttumörer, och patienter med denna typ av tumör har oftast en bättre prognos. Östrogenreceptorn tillsammans med östrogen stimulerar cellen att dela sig. Genom att hämma antingen östrogenreceptorn eller produktionen av östrogen stoppas celldelningen. Det vanligaste preparatet för behandling av denna typ av brösttumörer heter tamoxifen och verkar genom att interagera med östrogenreceptorn, och därmed hindra östrogen från att få cellen att dela sig.

Precis som cancer är ett samlingsnamn på flera sjukdomar, är också bröstcancer uppdelad i ett flertal mindre subgrupper med olika karaktär. Dessa tumörgrupper kan skilja sig åt med avseende på hur prognosen för patienterna ser ut och huruvida en patient svarar på en viss behandling eller inte. För att kunna klassificera och hitta dessa grupper behöver man mätbara biomarkörer, dvs. specifika komponenter i tumören som är annorlunda från normal frisk vävnad. Dessa molekylära komponenter i cellen är vanligtvis proteiner, men kan även vara RNA och DNA sekvenser. Östrogenreceptorn är ett bra exempel på en biomarkör som både kan ge information om patientens framtida sjukdomsbild, samt om tumören kommer att svara på anti-hormonell behandling, t.ex. tamoxifen. Trots detta finns det en del patienter med östrogenreceptor-positiva tumörer där tamoxifen inte har någon effect. För att kunna urskilja dem och direkt ge dem en annan effektiv behandling, är det viktigt att hitta ytterligare behandlingsprediktiva biomarkörer. I denna avhandling har vi identifierat att låga nivåer av det cellcykelreglerande proteinet p27, vars uttryck ofta

är minskat i bröstcancertumörer, är associerat med sämre tamoxifensvar i östrogenreceptor-positiva tumörer, dvs. p27 är en behandlingsprediktiv biomarkör för tamoxifen. Däremot kunde vi inte se att p27 kunde förutse patientens sjukdomsförlopp.

Bröstet består av flera olika celltyper och kan grovt delas in i två avdelningar; den epiteliala och den stromala. I den epiteliala avdelningen finns det två celltyper, luminala epitelceller och basala myoepitelceller, och det är dessa två som utgör själva strukturen av det funktionella bröstet, dvs. står för produktionen av mjölk. Den förgrenade strukturen i bröstet består av mjölkgångar. I ena änden mynnar dessa ut i bröstvårtan, och i andra änden finns en struktur som till formen är lik en klase med vindruvor. Denna vindruveklase är bröstets minsta funktionella del och kallas terminal duct lobular unit (TDLU) och består av en mjölkproducerande körtel och tillhörande mjölkgångar. Runt omkring finns den stromala avdelningen som upprätthåller formen samt är väldigt viktig för hur cellerna i den epiteliala avdelningen beter sig. Bröstcancer börjar oftast i TDLU och i mjölkgångarna. Man tror att ett vanligt händelseförlopp för utvecklingen av bröstcancer går från förändringar i epitelcellerna i TDLU vidare till en ökad celldelning som till slut leder till att de tidigare normala epitelcellerna blir cancerceller som tar sig ut ur bröstet och sprider sig till andra organ och bildar dottertumörer, så kallade metastaser. Det är när cancern sprider sig som den utvecklas till en väldigt allvarlig sjukdom. Det är därför viktigt att hitta brösttumören innan den hinner komma till det stadiet. Det är också viktigt att hitta tecken i förstadier till bröstcancer som tyder på att cellerna har en elakartad potential. Det kan man göra genom att titta på vilka gener som är förändrade. Den första strukturella förändringen i bröstet med ökad risk att utvecklas till bröstcancer kallas för columnarcellshyperplasi (CCH). Vi har identifierat förändringar i ett flertal mikroRNA-gener i CCH som kan ligga till grund för denna tidiga premaligna förändring i bröstet. Vi har även kopplat ihop några av dessa, bl.a. let-7c, med ökad celldelningsförmåga. Detta kan vara viktigt för att upptäcka tidiga förändringar hos kvinnor som löper risk att utveckla bröstcancer.

Sammanfattningsvis har studierna i denna avhandling identifierat cellcykelrelaterade biomarkörer för behandlingssvar av tamoxifen och prognostisk information, upptäckt celldelningsreglerande mikroRNA-gener i den tidigaste förändringen av bröstet med koppling till bröstcancer, samt visat hur ett cellcykelassocierat mikroRNA har olika effekter i olika subgrupper av bröstcancer, och därmed påvisat vikten av att behandla bröstcancer utefter subgruppstillhörighet.

Acknowledgements

I would like to express my gratitude to everyone who supported my work and contributed to this thesis, and I would especially like to thank:

Göran Landberg; my supervisor, for giving me the opportunity to learn about breast cancer in Sweden, Houston and Manchester. It has been interesting, exciting, fun and challenging at the same time, a perfect combination!

Håkan Axelson; my co-supervisor, for taking good care of "the orphans" and teaching me about cloning and kidney cancer.

Christina; for your positive attitude and for always helping out in the lab. Your expertise in ISH has been invaluable. ***Elise***; for your fast and very impressive production of IHC slides. ***Siv***; for very nice conversations. ***Elisabeth***; for sorting out all ordering issues. ***Kristin***; for with excellent knowledge helping me out with all the administrative tasks.

My wonderful office mates; ***Sophie***; my fantastic travelling partner and friend, for sharing practically all steps during the last six years, both inside and outside the lab. ***Anna-Karin***; for introducing me to running, and for being such a great friend and co-rider in this roller-coaster. ***Louise***; for experiencing the first 10km run together, and for being a brilliant listener. ***Greta***; for always being so positive and full of energy, and for surprising us every now and then with bread and cookies.

Katja; for being a magnificent room mate in Manchester, and for nice tapas dinners in Madrid. ***Eva***; for your optimism, and for also being an extraordinary apartment friend in Manchester. ***Nick***; for providing me with numerous episodes of TV series, and for all nice dinners in Manchester. You are all truly good friends.

Carro, Pontus, Tina; for introducing me to the lab and sharing your knowledge. ***Anna, Åsa, Gry,***; for making my first years in the lab very nice. ***Matilda and Marica***; for good times in the lab as well as in Orlando and for always making me laugh.

Emma, Kris, David, Jennifer, Elinn, Birgitte; for teaching me about renal cell carcinoma and Notch signalling by welcoming me to your interesting journal clubs.

All the remaining colleagues at CMP, both past and present; thank you all for creating an amazing working environment and for all nice discussions during breakfasts and coffee breaks.

My colleagues in Manchester, ***Susann, Lynsey, Becky, Hannah G, Hannah H, Christina*** and ***The Breast Biology Focus Group***; for making me feel at home in Manchester, and thank you all for the introduction to English sport activities and for the visits to the pub for good traditional food.

My colleagues in Göteborg, ***Paul, Pernilla, Nina;*** who made a Manchester conference extra amusing, and thanks for taking good care of me in Göteborg.

All my paper ***co-authors***, for nice collaborations.

Mamma och Pappa; för att ni alltid har stöttat mig och ställt upp på allt från sportaktiviteter och resor, till studier och barnpassning. Ni är exemplariska förebilder och utan tvekan de bästa föräldrarna man kan ha.

Sara; min fantastiska syster. Du är ovärderlig för mig, både som syster och som kompis. Det är en lyx att ha dig, ***Carl*** och ***Siri*** så nära.

Erik och Ida; min underbara lilla familj. Ni är det finaste som finns.

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