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IDENTIFICATION OF NOVEL REGULATORS FOR TUMOR PROGRESSION IN BREAST CANCER

by

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To my beloved family and dear friends

“Wonder is the beginning of wisdom.”
-Socrates

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Identification of novel regulators for tumor progression in breast cancer
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ABSTRACT

Breast cancer is the most frequent solid tumor among women and the leading cause of cancer related death in women worldwide. The prognosis of breast cancer patients is tightly correlated with the degree of spread beyond the primary tumor. In this thesis, the aim was to identify novel regulators of tumor progression in breast cancer as well as to get insights into the molecular mechanisms of breast cancer progression and metastasis. First, the role of phospholipid remodeling genes and enzymes important for breast cancer progression was studied in breast cancer samples as well as in cultured breast cancer cells. Tumor samples displayed increased *de novo* synthesized fatty acids especially in aggressive breast cancer. Furthermore, RNAi mediated cell based assays implicated several target genes critical for breast cancer cell proliferation and survival. Second, the role of arachidonic acid pathway members 15-hydroxyprostaglandin dehydrogenase (HPGD) and phospholipase A2 group VII (PLA2G7) in tumorigenesis associated processes was explored in metastatic breast cancer cells. Both targets were found to contribute to epithelial-mesenchymal transition related processes. Third, a high-throughput RNAi lysate microarray screen was utilized to identify novel vimentin expression regulating genes. Methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) was found to promote cellular features connected with metastatic disease, thus implicating MTHFD2 as a potential drug target to block breast cancer cell migration and invasion. Taken together, this study identified several putative targets for breast cancer therapy. In addition, these results provide novel information about the mechanisms and factors underlying breast cancer progression.

Keywords: Breast cancer, EMT, tumor progression

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Uusien rintasyövän etenemistä säätelevien geenien identifiointi
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TIIVISTELMÄ

Rintasyöpä on naisten yleisin syöpä, joka aiheuttaa naisilla eniten syöpään liittyviä kuolemia maailmanlaajuisesti. Rintasyövässä potilaan ennuste riippuu syövän leviämisasteesta sekä mahdollisten etäpesäkkeiden määrästä. Tässä väitöskirjatutkimuksessa pyrittiin tunnistamaan uusia rintasyövän etenemistä säätteleviä geenejä. Ensimmäisessä osatyössä tutkittiin fosfolipidejä muokkaavia geenejä ja entsyymejä lipidomisten ja immunohistokemiallisten analyysien sekä RNA interferenssin (RNAi) avulla rintasyöpäkudosnäytteissä ja rintasyöpäsoluviljelmissä. Rasvahappojen *de novo* –synteesin havaittiin olevan huomattavasti vilkkaampaa syöpäkudoksissa ja siihen osallistuvien geenien ilmentyvän voimakkaammin syöpä- kuin normaalikudoksissa. Lisäksi näiden geenien havaittiin olevan tärkeitä syöpäsolujen kasvulle ja selviytymiselle. Toisessa osatyössä tutkittiin arakinoidihappo-aineenvaihduntaan osallistuvien geenien HPGD:n ja PLA2G7:n toimintaa rintasyövän etenemiseen ja etäpesäkkeiden muodostumiseen liittyvissä prosesseissa. Molempien geenien havaittiin osallistuvan epiteeli-mesenkymaalimuuntumista (EMT) sääteleviin prosesseihin. Kolmannessa osatyössä identifioitiin uusia vimentiinin ilmentymistä säätteleviä geenejä lysaattimikrosiru –tehoseulontamenetelmää käyttäen. Tulokset osoittivat MTHFD2 –geenin edistävän etäpesäkkeiden muodostukseen liitettyjä prosesseja ja täten olevan mahdollinen kohdegeeni rintasyöpäsolujen liikkumiseen ja invaasiokykyyn vaikuttavien lääkehoitojen kehittämiselle. Tässä väitöskirjatutkimuksessa tunnistettiin useita potentiaalisia rintasyövän etenemiseen vaikuttavia kohdegeeneja sekä saatiin uutta tärkeää tietoa rintasyövän leviämiseen liittyvistä tekijöistä.

Avainsanat: rintasyöpä, etäpesäke, EMT

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ABBREVIATIONS

AA	Arachidonic acid
ACACA	acetyl-CoA carboxylase alpha
ACTB	actin, beta
AR	androgen receptor
BMP	bone morphogenetic protein
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
CAF	cancer associated fibroblast
CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)
CD44	CD44 antigen (homing function and Indian blood group system)
CDC42	cell division cycle 42
CDH1	cadherin 1, type 1, E-cadherin
CDH2	cadherin 2, type 2, N-cadherin
cDNA	complementary deoxyribonucleic acid
CTC	circulating tumor cell
DNA	deoxyribonucleic acid
DTC	disseminated tumor cell
ECM	extracellular matrix
EGFR	epidermal growth factor
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
EPHB4	ephrin type-B receptor 4
ER	estrogen receptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
FASN	fatty acid synthase
FGF	fibroblast growth factor
FOXC2	forkhead box C2
FZD7	frizzled family receptor 7
HER2	human epidermal growth factor receptor *2*
HPGD	15-hydroxyprostaglandin dehydrogenase
HT	high throughput
HTS	high throughput screen
IHC	immunohistochemistry
KIF11	kinesin family member 11
MAPK	mitogen-activated protein kinase

MET	mesenchymal-epithelial transition
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTHFD1L	methylenetetrahydrofolate dehydrogenase 1-like
MTHFD2	methylenetetrahydrofolate dehydrogenase 2
NCAD	N-cadherin
p53	tumor protein *p53*
PC	phosphatidylcholine
PGE_2	prostaglandin E2
PgR	progesterone receptor
PI3K	phosphatidylinositol 3-kinase
PLA2G7	phospholipase A2, group VII
PLK1	polo-like kinase 1
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real-time polymerase chain reaction
RAC1	ras-related C3 botulinum toxin substrate 1
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
SHMT2	serine hydroxymethyltransferase 2
shRNA	small hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
Slug	zinc finger protein SNAI2
SNAIL	zinc finger protein SNAI1
SREBP1	sterol regulatory element binding transcription factor 1
TGF-β	transforming growth factor, beta
THRSP	thyroid hormone responsive
uPAR	urokinase plasminogen activator receptor
VIM	vimentin
VTN	vitronectin
WIPF2	WAS/WASL interacting protein family, member 2
ZEB	zinc finger E-box binding homeobox

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred in the text by Roman numerals I-III. The original communications have been reproduced with the permission of the copyright holders. Unpublished data is also included.

I Hilvo M, Denkert C, **Lehtinen L**, Müller B, Brockmöller S, Seppänen-Laakso T, Budczies J, Bucher E, Yetukuri L, Castillo S, Berg E, Nygren H, Griffin J, Fiehn O, Loibl S, Richter-Ehrenstein C, Radke C, Hyötyläinen T, Sysi-Aho M, Kallioniemi O, Iljin K and Orešič M. Novel theranostic opportunities offered by characterization of altered membrane lipid metabolism in breast cancer progression. *Cancer Res*. (2011) 71:3236-3245.

II **Lehtinen L**, Vainio P, Wikman H, Reemts J, Hilvo M, Issa R, Pollari S, Brandt B, Orešič M, Pantel K, Kallioniemi O and Iljin K. 15-Hydroxy-prostaglandin dehydrogenase associates with poor prognosis in breast cancer, induces epithelial-mesenchymal transition and promotes cell migration in cultured breast cancer cells**.** *J Pathol.* (2012) 226:674-86.

III **Lehtinen L**, Ketola K, Mäkelä R, Mpindi JP, Viitala M, Kallioniemi O and Iljin K. High-throughput RNAi screening for novel modulators of vimentin expression identifies MTHFD2 as a potent regulator of breast cancer cell migration and invasion. *Submitted.*

1 INTRODUCTION

Tumor growth and cancer progression are enabled by ten key processes considered as hallmarks of cancer (Hanahan and Weinberg, 2011). These include genomic instability and mutation, tumor promoting inflammation, replicative immortality, resistance to cell death and growth suppressors, deregulation of cellular energetics, sustained proliferative signaling, avoidance of immune destruction, and activation of angiogenesis, invasion and metastasis. Thus management of cancer is difficult, as successful treatment should target several of these features efficiently enough to stop cancer progression, and to avoid development of distant metastases. Solid tumors derived from epithelial tissues (i.e. carcinomas), such as breast tumors, represent the majority of cancers. Epithelial cells form the skin and line the body cavities protecting other cells from the environment, thus exposing themselves to pathogens, toxins and radiation as well as mechanical stress, and hence are more prone to mutations.

Breast cancer is the most frequent solid tumor among women and the leading cause of cancer related death worldwide (Jemal *et al*., 2011). Several factors contribute to the initiation and progression of breast carcinomas. To be able to proliferate at an accelerated rate, cancer cells need elevated levels of nutrients (Dang, 2012). This is achieved by inducing several metabolic pathways such as glycolysis and *de novo* synthesis of fatty acids. Most breast cancer related deaths are caused by tumor metastases and the prognosis of breast cancer patients is tightly correlated with the degree of spread beyond the primary tumor (Woodward *et al*., 2003). Metastases arise as a result of a series of steps such as tumor cell detachment and dissemination from the primary site, invasion of disseminated tumor cells into blood vessels, survival in the blood stream, exit from the circulation, and adaptation into new microenvironment.

This thesis work aimed to identify novel regulators of breast cancer progression as well as putative therapeutic targets for breast cancer treatment. Therefore, the *de novo* fatty acid synthesis and the involved genes were studied in breast cancer cells. In addition, the role of arachidonic acid pathway in breast cancer was explored, as previous studies have proposed tumorigenesis promoting properties for arachidonic acid and its metabolites

(Natarajan and Nadler, 1998; Wang and Dubois, 2006; Sahin *et al*., 2009; Martinez-Orozco *et al*., 2010). Furthermore, this study aimed to elucidate the mechanisms leading to tumor metastasis and the function of the genes contributing to epithelial-mesenchymal as well as mesenchymal-epithelial transition processes.

2 REVIEW OF THE LITERATURE

2.1 Breast cancer

Approximately 14 % (1 in 7) of women will develop breast cancer during their lifetime (Jemal *et al*., 2011). The most common risk factor for developing breast cancer is age, as over 70 % of breast cancers occur in women over 50 years old. Other well-known factors contributing to increased risk of breast cancer are early age at menarche, nulliparity or first birth after the age of 35, late menopause, a first degree relative with breast cancer, exposure to estrogen (e.g. post-menopausal hormone replacement therapy), and exposure to radiation (Kumar *et al.*, 2005). Increasing evidence implicates smoking, alcohol intake, physical inactivity, as well as adult and post-menopausal obesity in developing breast cancer (Kushi *et al*., 2012). In addition, dietary fatty acids have been associated with increased risk of breast cancer (Rose, 1997; Escrich *et al*., 2011).

Breast cancers can be divided in two types according to their origin; sporadic cancers which are largely related to estrogen exposure, and hereditary cancers which associate to family history of first degree relative with breast cancer or germ-line mutations. The incidence of breast cancer has been increasing throughout the world during the last decades, probably due to elevated life expectance of women as well as decreased birth rate (Jemal *et al*., 2011). Similar development has been recently observed also in developing countries, possibly associating with improved living standards and availability of birth control options for women (Jemal *et al*., 2011). Despite the inclining number of yearly breast cancer cases, the number of breast cancer related deaths has not followed this trend, indicating earlier and better diagnosis as well as the existence of improved treatment options and availability of therapeutic agents (Jemal *et al*., 2011).

2.1.1 Breast cancer subtypes

The breast consists of the mammary gland and stroma which includes blood vessels, lymphatic vessels, connective tissue as well as fat tissue. The mammary gland consists of lobules which produce milk when terminally differentiated during pregnancy and milk carrying ducts. Both are formed by a bi-layered epithelium of luminal and myoepithelial (basal) cells. These cells are

hypothesized to originate from same progenitors but subsequently after lineage differentiation give rise to distinct types of breast tumors (Vargo-Gogola and Rosen, 2007). According to current understanding the initiation of breast carcinogenesis occurs in the terminal ductal lobular unit (Wellings, 1980). Breast tumors are nonetheless still classified according to the histological appearance and termed as ductal or lobular.

Breast cancers can be divided into nine different types according to their histological appearance and localization (Table 1, Kumar *et al.*, 2005). As a pre-malignant lesion progresses towards malignancy, it develops into a local unspread carcinoma (carcinoma in situ, CIS). Approximately 20% of tumors observed in mammography are classified as ductal carcinoma in situ (DCIS) (Virnig *et al*., 2010) which is frequently detected as mammographic calcifications and densities. Although DCIS has not penetrated the basement membrane, it is estimated that most cases progress and become invasive (Page *et al*., 1995). Lobular in situ carcinomas (LCIS) are more rarely visible in mammography and are commonly identified by biopsies (Simpson *et al*., 2003). Majority of LCIS are diagnosed in premenopausal women and frequently bilateral i.e. appearing in both breasts.

In invasive, also termed as infiltrating carcinomas, the cancer cells have penetrated the basement membrane and progressed from a locally confined tumor into a larger spreading mass of tumor cells. According to current classification invasive carcinomas are divided further to seven subtypes; ductal or non-specific type (NST), lobular type, tubular carcinomas, mucinous carcinomas, medullary carcinomas, papillary carcinomas and metaplastic carcinomas (Table 1, Kumar *et al.*, 2005). The most common types of breast cancers are solid invasive ductal and lobular carcinomas. In invasive ductal carcinoma the tumor has started from the mammary ducts and invaded the surrounding stroma. These types of tumors account for approximately 80% of all invasive breast cancers and do not show distinctive properties but usually display a wide selection of features. 10% of invasive breast cancers are harder to detect lobular cancers. These tumors often show loss of genes in chromosome 16 responsible for cell adhesion (Berx and Van Roy, 2001). In addition, lobular invasive carcinomas harbor a different pattern of metastasis compared to other breast tumor types (Kumar *et al.*, 2005).

Table 1: Different breast cancer types and their incidence.

Type of breast cancer	%
In Situ Carcinoma	**15-30**
Ductal carcinoma in situ (DCIS)	*80*
Lobular carcinoma in situ (LCIS)	*20*
Invasive Carcinoma	**70-85**
Non-specific type ("ductal")	*79*
Lobular carcinoma	*10*
Tubular/cribriform carcinoma	*6*
Mucinous carcinoma	*2*
Medullary carcinoma	*2*
Papillary carcinoma	*1*
Metaplastic carcinoma	*<1*

Data adapted from Kumar *et al.*, 2005.

Hereditary breast cancers account for 5-10 % of all breast cancers (Lacroix and Leclercq, 2005). The most common cause of hereditary breast cancer is an inherited mutation in the DNA repair gene(s) BRCA1 and/or BRCA2. Both of these genes act as tumor suppressors and their loss-of-function promotes malignancy in breast and ovarian cancers. BRCA1 forms a complex with BRCA2 and another DNA repair gene RAD51 (Venkitaraman, 2002). BRCA2 can also bind to DNA directly. BRCA1 and BRCA2 mutations increase the risk of breast cancer by 10- to 20-fold compared to the general population and the patients often suffer earlier cancer onset (Chen and Parmigiani, 2007; Allain, 2008). Compared to sporadic cancers BRCA1 associated carcinomas are more frequently poorly differentiated high grade tumors (Gage *et al*., 2012). BRCA2 associated cancers do not show a distinctive phenotype although displaying often higher grade tumors (Allain, 2008). Less than 10% of hereditary breast cancers are due to mutations in other genes such as CHEK2, P53, PTEN, LKB1, STK11, BRIP1, PALB2, and ATM (de Jong *et al*., 2002; Gage *et al*., 2012). The genetic testing of patients for these mutations is currently not as well established than in the case of BRCAs and further research is needed for more detailed profiling of patients with hereditary breast cancer.

2.1.2 Different gene expression patterns of breast tumors

The treatment and prognosis of breast cancer is highly dependent on the hormonal status and genetic background of the tumor. Breast cancers are

defined according to the expression of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). Studies utilizing gene-expression profiling have further defined the subtypes according to their gene-expression patterns. These are commonly described as luminal A, luminal B, HER2/neu/ERBB2 positive, basal and normal breast like (Perou *et al*., 2000; Sorlie *et al*., 2001; Sorlie *et al*., 2003) and they are largely in clinical use. Luminal A and luminal B are commonly ER positive, and possess expression patterns typical for luminal type cells. Patients with luminal tumors generally have better prognosis (Sorlie *et al*., 2001). The HER2/ERBB2 positive cancers are characterized by expression of genes in the HER2 amplicon. These tumors are mostly ER- and show more aggressive growth-properties (Kumar *et al.*, 2005). Basal-type tumors are derived from basal cells and largely correspond to triple-negative cancers (ER-, PgR- and HER2-) (Nielsen *et al*., 2004; Carey *et al*., 2007). In addition, these cancers are often associated with BRCA1 mutations (Sorlie *et al*., 2003). Basal and HER2 positive tumors are considered as the most aggressive breast carcinoma types. Supporting this, ample evidence shows association of the two subtypes with decreased patient survival and high susceptibility to disease relapse (Sorlie *et al*., 2003). Recent studies indicate even more subtypes with distinct gene expression signatures. Evidence from an integrated analysis of gene copy number and gene expression data indicates as many as ten subgroups with distinct clinical outcomes and therapeutic response (Curtis *et al*., 2012). This may allow selection of targeted medication for individual patients with certain types of tumors.

2.2 Current treatments for breast cancer

Breast cancer is primarily treated by surgical removal of the primary tumor and in the case of spread disease, also removal of the axillary lymph nodes. Surgical management of the primary tumor can be obtained either by breast-conserving surgery in combination with radiation therapy, complete breast removal i.e. mastectomy combined with breast reconstruction, or mastectomy alone. Ample evidence shows equal patient survival between these options (van Dongen *et al*., 2000; Veronesi *et al*., 2002). Further treatment options are determined according to the characterization of the primary tumor. The course of action is largely based on clinical prognostic parameters such as ER, PgR and HER2 status as well as tumor stage and tumor grade.

For early stage tumors in situ (DCIS, LCIS) and lower grade small tumors, breast-conserving surgery and radiation therapy alone can be a sufficient treatment. However, larger tumors of higher grade and possible lymph node positivity require in addition adjuvant chemotherapy to prevent cancer spreading. Patients under the age of 35 are in high risk of disease relapse and are thus always given adjuvant chemotherapy after surgery (Aebi *et al*., 2000). Importantly, the genetic expression profile of the primary tumor has to be taken into account when selecting correct adjuvant chemotherapy (Table 2).

The hormonal status of the tumor i.e. whether the tumor is hormone receptor positive is a determining factor for the selection of treatment. Hormone receptor positive tumors and more importantly estrogen receptor positive tumors (ER+) are responsive to hormonal treatments, which can have a major impact on tumor growth and patient survival (Bundred, 2001; Elston and Ellis, 2002). In the case of hormone responsive tumors, the most important aspect is to suppress estrogen hormone function. Thus the treatment of ER positive breast cancer includes the use of selective estrogen receptor modulators and selective estrogen receptor down-regulators (Howell *et al*., 2004). These include tamoxifen, toremifene and fulvestrant. Tamoxifen can be used for up to five years and has been shown to reduce the relapse risk of ER+ cancer by 50% and decrease the incidence of breast cancer related death by 30% (Howell *et al*., 2004). Aromatase inhibitors anastrozole, letrozole and exemestane are used for treatment of hormone-receptor positive breast cancer only in post-menopausal women (Ito *et al*., 1993). In younger pre-menopausal women with active ovaries and estrogen production these are not effective enough.

Table 2. Current therapeutic agents for treatment of breast cancer.

Indication	Therapeutic agent	Mechanism
Hormone receptor positive *Suppression of estrogen function and ER-signaling*	Tamoxifen	anti-estrogen
	Toremifene	anti-estrogen
	Fulvestrant	anti-estrogen
	Megestrole acetate	progestine
	Gosereline	suppression of ovaries
	Anastrozole	aromatase inhibitor
	Letrozole	aromatase inhibitor
	Exemestane	aromatase inhibitor
Hormone receptor negative *Chemotherapy*	Doxorubicin	interference with DNA replication
	Epirubisine	interference with DNA replication
	Doxetaxel	inhibition of DNA replication
	Paclitaxel	inhibition of DNA replication
	Cyclophosphamide	interference with DNA replication
	Methotrexate	interference with DNA replication
	Fluorouracil	inhibition of thymidylate synthase
	Vinorelbine	inhibition of mitosis
	Capecitabine	inhibition of DNA synthesis
HER2 positive *Suppression of HER2-receptor*	Trastuzumab	HER2 antibody
	Lapatinib	inhibition of HER2-receptor

Another option for blocking estrogen production in pre-menopausal women is ovarian ablation with gosereline, radiation therapy or surgical removal (International Breast Cancer Study Group (IBCSG) *et al*., 2003). Patients with hormone receptor negative tumors are treated with chemotherapeutics such as taxanes and antracyclines since the use of hormonal suppression would not give any additional advantages (Metzger-Filho *et al*., 2012). The treatment

aims to suppress the proliferation of possible cancer cells left in the body after surgery and radiation. Commonly used treatment for HER2 positive breast cancer is the HER2 receptor blocking antibody trastuzumab (Piccart-Gebhart *et al*., 2005). Another HER2 inhibitor, lapatinib has been successfully used in clinical trials in combination with trastuzumab (Baselga *et al*., 2012). In cases of very large tumors or the desire for breast-conserving surgery, neo-adjuvant chemotherapy i.e. therapy prior to surgery can be administered to achieve tumor size reduction. These commonly comprise of different cytostatic drugs. In the case of ER+ or HER2+ tumors also estrogen suppressing agents or trastuzumab is included, respectively.

The treatment of metastatic breast cancer is principally the same as in the case of non-spread cancer. Hormonal suppression and cytostatic agents are commonly used in combination with palliative therapy e.g. surgery or radiation therapy and management of pain (Finnish Medical Society Duodecim, 2007). Since the disease is mostly incurable at this stage, the treatment aims to diminishing the size of the distant metastases as well as blocking further spreading. In addition, obtaining better life quality for the remaining time and longer asymptomatic periods are intended. In bone metastatic breast cancer surgery and radiation therapy can relief bone pain and induce bone healing (Koswig and Budach, 1999). Bone resorption and related skeletal events can be suppressed with bisphosphonates and denosumab, thus improving and increasing the survival time of breast cancer patients with bone metastases (Coleman, 2008; Stopeck *et al*., 2010).

Although breast cancer as well as the different factors contributing to its progression have been extensively studied for decades and the treatment options are well established, morbidity and mortality rates remain high. The fact that breast cancer is not a single disease but a collection of diseases with different clinical and pathological features forms the major challenge for current treatments options (Hanahan and Weinberg, 2000). Early detection of breast cancer is crucial for successful treatment as the tumor size and stage correlate with patient survival (Kumar *et al.* 2005). Although in most cases the primary tumor can be efficiently removed by surgery, distant metastases remain the main cause for breast cancer mortality. Metastasized cells are hard to detect and can lay dormant in the body for years, even decades. In addition, cancer cells which possess metastatic capabilities are often resistant to conventional systemic therapies such as radiation and chemotherapy. The metastatic cascade controlling breast cancer progression and spreading is

complicated and versatile, still requiring more investigation and knowledge of these processes in order to find ways for more efficient cancer therapies.

2.3 Molecular mechanisms of breast cancer progression

Tumor progression is driven by accumulated genetic and epigenetic alterations in developing tumor cell genomes. Although a single mutation is not sufficient to initiate malignant growth, majority of cancers rise from a single abnormal cell. Carcinogenesis is thus a continuum of events all contributing to the acquisition of the aforementioned hallmarks of cancer.

Changes in gene expression are the first signs as well as drivers of cellular transformation. Aberrant expression of just a few genes can be sufficient for tumorigenic transformation (Clark *et al*., 2000; Kang *et al*., 2003), which indicates the fragile nature of cellular homeostasis. In order to maintain their normal state and behavior, cells strive to retain homeostasis of intracellular signaling and are able to establish negative-feedback loops (Amit *et al*., 2007; Cabrita and Christofori, 2008; Mosesson *et al*., 2008; Wertz and Dixit, 2010) which normally downregulate the overly expressed signals. However, in cancer these mechanisms are malfunctioning which can lead to uncontrolled proliferative signaling. Overexpression of several oncogenes such as RAS, MYC and RAF has been associated with increased proliferation and growth of cancer cells (Hanahan and Weinberg, 2011). Recent studies however, suggest that the excessively elevated expression of these genes would in turn provoke induction of apoptosis or cell senescence as a cellular defense mechanism (Lowe *et al*., 2004; Evan and d'Adda di Fagagna, 2009; Collado and Serrano, 2010). Cancer cells are able to evade these countermeasures by adaptation to these signals and by disabling their own apoptosis-senescence inducing signaling (Hanahan and Weinberg, 2011).

2.3.1 Pathways contributing to breast tumorigenesis

Phosphoinositide-3-kinase (PI3K) is an important downstream mediator of several tyrosine kinase signaling pathways regulating cell proliferation and contributing to suppression of apoptotic signals. Loss-of-function of PI3K pathway inhibiting enzyme, phosphoinositide phosphatase PTEN, results in upregulated PI3K which in turn promotes tumorigenesis (Yuan and Cantley, 2008; Jiang and Liu, 2009). In breast cancer, the PI3K pathway contributes to alterations of ER-signaling and acquisition of resistance to hormonal therapies. It is the most commonly altered pathway in breast cancer, accompanied by

mutation and/or amplification of the genes encoding the PI3K catalytic and regulatory subunits PIK3CA and PIK3CB as well as PIK3R1; the PI3K effectors AKT1 and AKT2; receptor tyrosine kinases such as HER2 (ERBB2); the fibroblast growth factor receptor 1 (FGFR1) as well as the oncogene K-Ras (Miller *et al*., 2011). In addition to PI3K, several other oncogenic kinase pathways modulate ER expression and function in breast cancer cells. These include MAPK, the proto-oncogene c-Jun as well as the small GTPase Ras (Campbell *et al*., 2001; Massarweh and Schiff, 2007; Miller *et al*., 2011).

In addition to inducing pathways promoting tumorigenesis, cancer cells must evade the influence of tumor suppressor genes. These are often genes involved in DNA repair, such as the well-known players in breast cancer, BRCA1 and BRCA2. Mutations in these genes result in impaired ability to repair breaks in the DNA double-strand (Lacroix and Leclercq, 2005). In addition, BRCA1 has been shown to repress ER, c-MYC and Stat5a transcription activities, suppressing their proliferation promoting effects (Wang *et al*., 1998; Fan *et al*., 1999; Vidarsson *et al*., 2002). Thus, downregulation of BRCA1 leads to uncontrolled proliferation of the mutated cells. In addition to means for mending damaged DNA, cells possess intrinsic mechanisms for controlling the cell cycle. These mechanisms enable the cells to decide whether the cell is qualified for proliferation, or are the damages too severe for obtaining healthy progeny. As an example, a defect in the expression of a critical cell-cycle gatekeeper RB protein induces persistent cell proliferation, and aberrant expression of another important cell-cycle regulatory protein p53 enables the proliferation of cells with severely damaged DNA (Hanahan and Weinberg, 2011). By disabling the cell cycle control mechanisms a mutated cell with damaged DNA is able to divide and these defects accumulate, eventually leading to malignancy.

2.3.2 Changes in cell metabolism

The increased rate of cancer cell proliferation requires elevated levels of nutrients and enhanced protein synthesis. The accumulation of highly proliferating cancer cells into tumor mass results in shortage of oxygen and nutrients, which the cells attempt to compensate by inducing angiogenesis for the formation of new blood vessels (Zeng *et al*., 2011). However, due to uncontrolled growth rate and distorted signaling, this tumor neovasculature is often poorly formed and inefficient which leads to consistent lack of nutrients and persistent hypoxic stress in the tumor. Hypoxic conditions have been shown to induce tumorigenesis further supporting the carcinogenic process

(Hockel *et al*., 1999; Hockel and Vaupel, 2001) and promote resistance to cancer therapy (Harris, 2002). These conditions create a self-feeding cycle of malignancy. Under hypoxic conditions cellular glucose is converted to lactate (i.e. glycolysis). This is enhanced in cancer tissues and persists despite the presence of oxygen (Dang, 2012). Several studies show that sustained aerobic glycolysis, called the Warburg effect, leads to activation of oncogenes and loss of tumor suppressor expression (Vander Heiden *et al*., 2009; Levine and Puzio-Kuter, 2010; Cairns *et al*., 2011; Koppenol *et al*., 2011).

In addition to lack of oxygen, tumor cells suffer from malnutrition and defects in the normal cellular metabolism. Several metabolic pathways and their metabolites such as glycine, serine, threonine and sarcosine have been implicated in cellular transformation (Locasale *et al*., 2011; Possemato *et al*., 2011; Zhang *et al*., 2012) and cancer metastasis (Sreekumar *et al*., 2009). The chemical reactions involved in cellular metabolism lead to mitochondria mediated release of reactive oxygen species (ROS), which are chemically extremely reactive. ROS are involved in tumorigenesis as well as regulation of several pathways in normal and malignant cells (Weinberg and Chandel, 2009). In breast, estrogen undergoes oxidative metabolism and generates ROS, which has been shown to induce oxidant-induced damage to DNA as well as lipid peroxidation (Roy *et al*., 2007; Okoh *et al*., 2011). Thus the exposure to estrogen and its metabolic byproducts promotes the malignant processes in breast cancer cells. This further highlights the importance of estrogen inactivation in breast cancer treatment.

2.3.2.1 Fatty acid metabolism

Cellular membranes consist of fatty acids which cells obtain either from dietary lipids or by *de novo* synthesis (Menendez and Lupu, 2007). These fatty acids are crucial components for cell division and proliferation. For the synthesis of structural lipids normal tissues favor the use of dietary (exogenous) lipids over *de novo* (endogenous) synthesis, which is usually suppressed in normal cells (Mashima *et al*., 2009). Several studies show that breast cancer cells display increased *de novo* fatty acid synthesis and that many of the involved lipid metabolism genes are connected with malignant processes (Milgraum *et al*., 1997; Swinnen *et al*., 2000; Kuhajda, 2006). The most important enzymes taking part in *de novo* synthesis of fatty acids are fatty-acid synthase (FASN) and acetyl-CoA carboxylase (ACACA). ACACA is involved in carboxylation of acetyl-CoA to malonyl-CoA which in turn is subsequently converted to long-chain fatty acids by FASN (Mashima *et al*., 2009). Supporting the importance of

these genes in breast carcinogenesis, the expression of FASN is regulated by growth factors and their receptors, including epidermal growth factor receptor (EGFR) and HER2, as well as by steroid hormones and their receptors, such as estrogen receptor (ER), androgen receptor (AR) and progesterone receptor (PgR) (Mashima *et al*., 2009). The involvement of HER2, ER and PgR in regulation of FASN and thus in the regulation of *de novo* fatty acid synthesis in breast, highlights the therapeutic possibilities of targeting this process. Other important players in *de novo* synthesis of fatty acids during breast cancer progression are sterol regulatory element binding transcription factor 1 (SREBP1) and thyroid hormone-inducible protein (SPOT14, or THRSP). In addition to growth factors and hormones, FASN is regulated by SREPB1 via PI3K-Akt pathway (Campa *et al*., 2009). Furthermore, the transcription factor p53 has been implicated in FASN regulation (D'Erchia *et al*., 2006).

2.3.2.2 Arachidonic acid metabolism in breast cancer

Arachidonic acid (AA) is one of the major fatty acids in the breast. It is known to stimulate breast cancer cell growth and contribute to tumor metastasis. AA mediates several cellular processes, such as cell survival, angiogenesis, chemotaxis, mitogenesis, migration and apoptosis (Navarro-Tito *et al*., 2008; Wang and Dubois, 2010; Martinez-Orozco *et al*., 2010). It is released from plasma membranes by phospholipase A2 (PLA2) and subsequently converted to various eicosanoids and signaling molecules by cyclooxygenases (COX), lipoxygenase (LOX) and cytochrome P-450 (CYP) (Figure 1). Arachidonic acid is converted via these pathways to several types of biologically active eicosanoids; including prostaglandins, leukotrienes, hydroxyeicosatetraenoic acid, epoxyeicosatrienoic acid and hydroperoxy-eicosatetraenoic acids (Cathcart *et al*., 2011). Prostaglandins, including Prostaglandin E_2 (PGE2), are produced by COX enzymes and involved in several important biological processes such as inflammation, sensitization of nociceptive nerves (pain signals), modulation of renal blood flow and protection of gastric mucosa. Overexpression of COX-2 has been shown in 40% of breast cancers (Howe, 2007) and COX inhibiting agents such as non-steroidal anti-inflammatory drugs (NSAIDs) have been indicated as potential therapeutics in breast cancer treatment (Harris, 2009). PGE2 is thought to be the principal factor responsible for the tumorigenic effects of prostaglandins (Chen and Smyth, 2011). 15-hydroxyprostaglandin dehydrogenase (HPGD) is the key PGE2 degrading enzyme converting it to inactive metabolites (Tai *et al*., 2002). HPGD has been mostly reported to be downregulated in cancer and indicated as a tumor suppressor (Backlund *et al*., 2005; Ding *et al*., 2005; Wolf *et al*., 2006; Huang *et*

al., 2008; Liu *et al*., 2010). However, overexpression of HPGD has been observed in invasive apocrine subtype of breast cancer (Tseng-Rogenski *et al*., 2010), as well as in advanced prostate cancer (Vainio *et al*., 2011a), and indicated as a potential drug target.

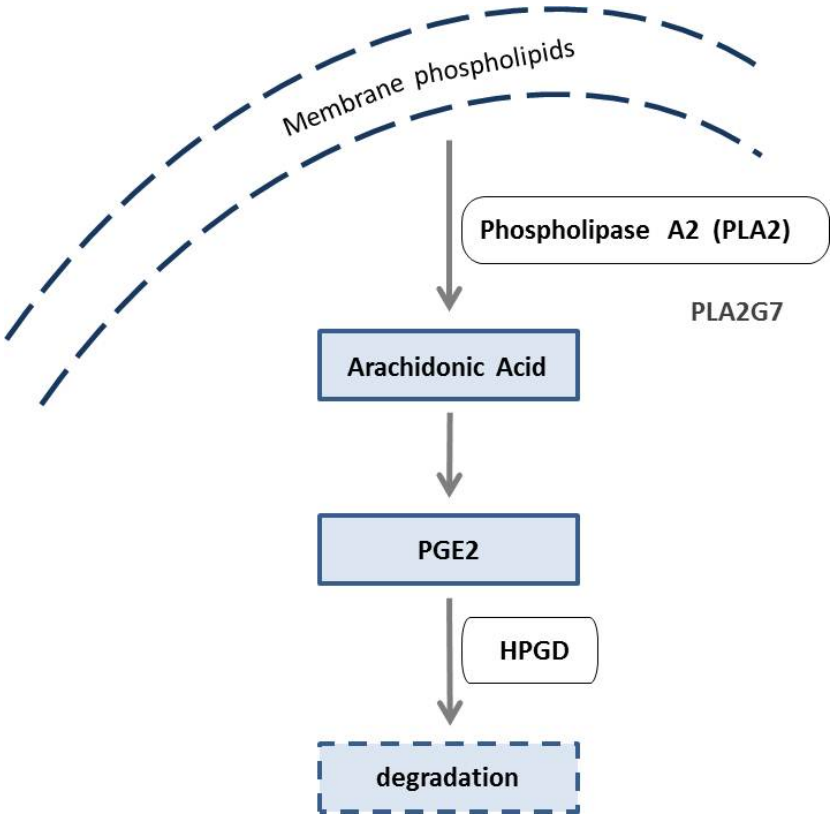

Figure 1: A simplified presentation of the function of arachidonic acid (AA) pathway.

PLA2G7 (platelet-activating factor acetylhydrolase) belongs to the family of phospholipases, lipid degrading enzymes. It functions in the arachidonic acid pathway by mobilizing arachidonic acid from the cell membranes (Figure 1). Currently PLA2G7 is used as a biomarker for cardiovascular diseases (Packard *et al*., 2000; Oei *et al*., 2005; May *et al*., 2006). However, a previous study reports that PLA2G7 promotes prostate cancer cell migration and invasion and indicates PLA2G7 as a potential drug target for prostate cancer (Vainio *et al*., 2011b). In addition, silencing of PLA2G7 has been shown to reduce xenograft

growth of colon cells expressing activated Ras and mutant p53 (McMurray *et al.* 2008).

2.3.3 Establishing tumor supporting microenvironment

The extracellular matrix is an important regulator of cell behavior and is involved in major developmental processes (Wiseman *et al*., 2003; Stickens *et al*., 2004; Rebustini *et al*., 2009; Lu *et al*., 2011). It comprises of an interstitial matrix situated between the cells as well as the basement membrane on which the epithelial cells lay. The interstitial matrix contributes to the tissue strength and flexibility as well as protects the ECM from mechanical forces. It consists of fibrillar collagens, different proteoglycans such as heparin sulphate, and glycoproteins such as fibronectin and tenascin C (Egeblad *et al*., 2010; Lu *et al*., 2012). The basement membrane is a thin sheet formed by fibers underlying epithelial and endothelial cells, separating the epithelium or endothelium from the stroma. It comprises of laminins, fibronectin, type IV collagen as well as collagen connecting linker proteins, and is thus more compact and less porous structure than the interstitial matrix (Lu *et al*., 2012).

The ECM provides an anchorage site for the cells and it can both enable and disable cell invasion by functioning either as a barrier or as a tract for migration (Spano *et al*., 2012). It is therefore an important component in regulating tumor progression and cancer cell metastasis. Cells are connected to the ECM via the focal adhesion complex which undergoes conformational changes upon exposure to mechanical force (Sawada *et al*., 2006; del Rio *et al*., 2009; Wang *et al*., 2011). In addition to its mechanical role in supporting tumor progression, the ECM binds several tumorigenesis promoting growth factors and regulates their signaling and concentration in the tumor environment. These include the wingless family glycoproteins (WNTs), bone morphogenic proteins (BMPs and TGFβ family members) and fibroblast growth factors (FGFs) (Norton *et al*., 2005; Hynes, 2009). In addition to stimulating the ECM, cancer cells recruit surrounding stromal cells and exploit them in the formation of tumor growth supporting microenvironment (Joyce and Pollard, 2009). These include fibroblasts, endothelial cells, bone marrow derived neutrophils, mast cells, myeloid cells, macrophages, as well as mesenchymal stem cells. Together with the ECM these components form a versatile, cellular growth and tumor progression supporting environment.

2.3.4 Formation of distant metastases

The prognosis of breast cancer patients is tightly correlated with the degree of spread beyond the primary tumor (Woodward *et al*., 2003). Although the primary tumor can be efficiently removed by surgery, 90% of cancer related deaths are caused by disease relapse in the form of metastases in secondary organs (Hanahan and Weinberg, 2000). Breast cancer frequently metastasizes to lungs, liver, brain, skin, and bone. It has been estimated that almost 70% of breast cancer patients with hematogenous spread harbor bone metastases (Coleman, 2006). Despite the high numbers of patients bone metastatic breast cancer still remains incurable.

2.3.4.1 Epithelial-mesenchymal transition

A prerequisite to tumor metastasis, the embryonic program called epithelial to mesenchymal transition (EMT), is activated during embryonic development enabling cells to migrate to the desired site for tissue formation (Thiery, 2002; Peinado *et al*., 2004). In adults EMT-like processes occur during wound healing i.e. when cells detach and migrate to the site of damage to form new tissue (Martin, 1997). In addition, EMT is activated in carcinogenesis enabling the dissemination and invasion of cancer cells and allowing the tumor cells to extravasate from the blood stream to the sites of metastases-to-be. During EMT, epithelial cells lose their epithelial characteristics such as cell-to-cell contacts as well as cell polarity, and downregulate epithelial-associated genes (Table 3). In addition, EMT leads to upregulation of mesenchymal-associated genes as well as initiates major cytoskeletal changes in the cells in order to enable acquisition of fibroblast-like migratory phenotype (Hanahan and Weinberg, 2011) . A number of transcription factors such as Twist, Snail/Slug, as well as reactivation of several developmental signaling pathways inducing transcriptional changes, such as transforming growth factor-β and Wnt/β-catenin signaling have been shown to trigger EMT in breast cancer cells (Micalizzi *et al*., 2010).

As one of its hallmarks, EMT induces a cadherin switch, downregulation of E-cadherin and upregulation of N-cadherin. Several EMT associated transcription factors, such as Twist (Yang *et al*., 2004), Snail (Cano *et al*., 2000) as well as ZEB1 and ZEB2 (Schmalhofer *et al*., 2009) are known to repress E-cadherin expression. Although E-cadherin deficiency is not alone sufficient for the cells to lose their epithelial phenotype, loss of E-cadherin correlates with poor prognosis in most carcinoma types (Thiery, 2002; Peinado *et al*., 2004).

Acquired expression of the intermediate filament protein vimentin is considered as another hallmark of EMT. Along with microtubules and actin microfilaments it is an important structural component of the cell cytoskeleton. Vimentin is expressed also in migratory epithelial cells during embryonic development, wound healing and tumor progression (Guarino, 1995; Gilles *et al*., 1999). Recently, vimentin has been suggested to be a biomarker for hematogenous metastasis in melanoma (Li *et al*., 2010).

Table 3: Known EMT-markers and their function.

Marker	**Transcription**	**Implication**	**Reference**
Snail	upregulated	transcriptional repression of CDH1, induction of EMT	(Barrallo-Gimeno and Nieto, 2005)
Slug	upregulated	transcriptional repression of CDH1	(Savagner *et al*., 1997)
Twist	upregulated	induction of EMT, promotes metastasis, represses CDH1	(Castanon and Baylies, 2002)
ZEB1/EF1	upregulated	transcriptional repression of CDH1	(Chua *et al*., 2007)
ZEB2/SIP1	upregulated	transcriptional repression of CDH1, activates VIM expression	(Chua *et al*., 2007)
E47	upregulated	transcriptional repression of CDH1	(Moreno-Bueno *et al*., 2006)
Foxc2	upregulated	regulates EMT, upregulates mesenchymal gene transcription	(Carlsson and Mahlapuu, 2002)
E-cadherin	downregulated	loss of cell-cell adhesion	(Birchmeier and Behrens, 1994)
Desmoplakin	downregulated	loss of cell-cell adhesion	(Boyer *et al*., 1989)
N-cadherin	upregulated	promotes cell-matrix adhesions, facilitates cell invasion	(Islam *et al*., 1996)
Vimentin	upregulated	promotes cell migration	(Kokkinos *et al*., 2007)
MMPs	upregulated	degradation of ECM to aid migration	(Heino, 1996)

2.3.4.2 Dissemination from the primary tumor and intravasation to the blood stream

In order to form metastases, cells must disseminate from the primary tumor and invade through the basement membrane to the vasculature. Disseminated tumor cells (DTCs) can enter the blood vessels (intravasation) either directly or first circulate via the lymphatic vessels to the regional lymph nodes. In breast cancer both dissemination routes are important (Pantel and Brakenhoff, 2004). Breast cancer cells commonly spread to the axillary lymph nodes, which is thought to be one the first clinical signs of metastatic disease. Surgical removal of the sentinel lymph node is done simultaneously with the primary tumor in order to diagnose the level of cancer spreading (Lyman *et al*., 2005).

Ample evidence from recent studies demonstrate that cancer cells gain the metastatic capacity early during primary tumor development and transfer this capability to most of their progeny (Bernards and Weinberg, 2002). DTCs can be detected in regional lymph nodes, peripheral blood, and in the bone marrow of cancer patients at early stages of tumor progression (Pantel and Brakenhoff, 2004). In light of the data from experimental studies suggesting that millions of disseminated cells from the primary tumor are shed daily to the bloodstream and lymphatic vessels (Butler and Gullino, 1975; Hunter *et al*., 2008) targeting these cells is critical. Most of the available chemotherapeutic agents are aimed to arrest the cell cycle in cancer cells (cytostatic effect). Therefore, only a relatively small number of patients with DTCs benefit from current chemotherapy, since most the disseminated tumor cells are not in mitosis at the time of administration, and thus escape the cytostatic effect (Pantel *et al*., 1993; Braun *et al*., 2000; Muller *et al*., 2005).

To facilitate cell penetration through tissue boundaries, such as the basement membrane, and further facilitate cell invasion, the ECM is proteolytically degraded by activation of urokinase plasminogen activator receptor (uPAR) and plasminogen cleavage, leading to activation of matrix metalloproteinases (MMPs) (Egeblad and Werb, 2002; Riisbro *et al*., 2002; Dano *et al*., 2005). Stromal cells such as fibroblasts and myofibroblasts can assist tumor cell invasion by acting as pathfinders through ECM (Joyce and Pollard, 2009). Upon dissemination and getting access to the blood stream, the DTCs are referred as circulating tumor cells (CTCs). Having disseminated from the original primary tumor, these cells still express their original genotype. CTCs express the markers of the primary tumor; in epithelial tumors such as breast cancer CTCs

can be distinguished from other cell types in the blood stream by expression of cytokeratins, epithelial cell adhesion molecule EpCAM and absence of leucocyte marker CD45 (Mego *et al*., 2010). Whether the majority of these cells die in the circulation, or survive and extravasate, is somewhat under debate (Wong *et al*., 2001; Chambers *et al*., 2002; Podsypanina *et al*., 2008). However, owing to the physical forces of circulation as well as the presence of immune cells, the bloodstream is a highly unfavourable environment for cancer cells. Thus majority of the CTCs are bound to face anoikis, a programmed cell death induced in anchorage-dependent cells detached from the ECM (Zhan *et al*., 2004).

2.3.4.3 Extravasation from the blood stream and establishing the metastasis

Following dissemination and intravasation, the cells must survive in the blood stream, extravasate from the vasculature, and establish a new tumor in a foreign microenvironment. Extravasation is enabled by tumor cell binding to the blood coagulation factors which allows the cells to arrest, adhere to the capillary walls and subsequently intravasate to the tissue for establishment of metastasis (Palumbo *et al*., 2000; Mego *et al*., 2010).

The reversal process to EMT, mesenchymal to epithelial transition (MET), yields to loss of migratory potential and expression of several mesenchymal markers. MET normally occurs during developmental processes such as somitogenesis, kidney development, cardiogenesis, hepatogenesis and celomic cavity formation (Nakajima *et al*., 2000; Rubio *et al*., 2008; Li *et al*., 2011). In carcinogenesis MET has been proposed to be involved in redifferentiation of tumor cells into epithelial phenotype resembling the primary tumor following extravasation (Christiansen and Rajasekaran, 2006). Thus this process may explain why tumor cells at metastatic site often re-express epithelial markers and features of the primary tumor. Both EMT and MET are important in tumor metastasis by enabling tumor cell intravasation and extravasation (Figure 2).

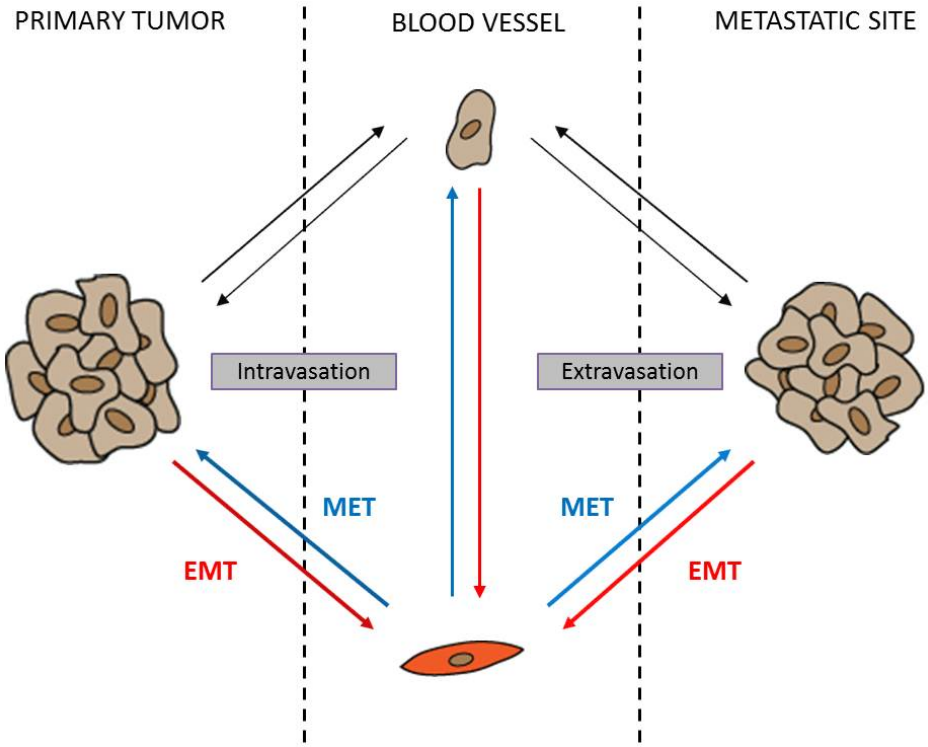

Figure 2. A schematic presentation of the metastatic cascade. Both EMT and MET are important processes in cancer cell intravasation and extravasation. These processes are reversible and dynamic, and involved in different steps of tumor progression. (Adapted and modulated from Mego *et al.* 2010).

Although MET has not been as comprehensively investigated as EMT, experimental data are available about its induction and regulation. The hepatocyte growth factor receptor c-met has been implicated in MET induction (Scotlandi *et al*., 1996; Naka *et al*., 1997; Ouyang, 1998). A study of carcinoid organization in colorectal cancer suggests the trans-membrane Wnt-receptor FZD7 necessary for MET (Vincan *et al*., 2007). In addition, inhibition of DNA methylation with 5-azacytidine induced MET in several malignant cell lines (Darmon *et al*., 1984). As EMT contributes to acquisition of therapeutic resistance, cells undergoing MET could be more sensitive to conventional cancer therapies. This highlights the importance of therapeutic targeting of also MET in addition to EMT.

2.3.4.4 Tumor dormancy and regulation of metastatic progression

As another malignant feature, breast cancer progression can be systemically regulated by the primary tumor. Number of studies suggest that co-existing tumors in a single patient are able to interact and modulate tumor progression (Mullen *et al*., 1985; Kim *et al*., 2009; McAllister and Weinberg, 2010). After dissemination tumor cells can lay dormant for several years often never establishing a metastasis. Adaptation to new microenvironment requires time and the lack of proper growth signals as well as impaired cell-cell signaling can contribute to tumor cell dormancy (Aguirre Ghiso *et al*., 1999). However, in a mouse isograft study multiple tumors were observed to enhance the growth of latent tumors (Mullen *et al*., 1985). In addition, some tumors have been shown to secrete pro-angiogenic factors which mobilize hematopoietic and endothelial precursors from the bone marrow to the circulation in order to support the tumor neoangiogenesis (Rafii, 2000; Moore *et al*., 2001; Heissig *et al*., 2002; Orimo *et al*., 2005). This supports the progression of micrometastases risen from disseminated and extravasated cancer cells (Castano *et al*., 2011), demonstrating that tumors can self-establish a tumorigenesis supporting environment and promote their own development as well as the growth of the distant metastases. Thus more comprehensive understanding of the mechanisms of metastatic progression is crucial for successful treatment of breast cancer and most importantly, the prevention of tumor metastasis.

3 AIMS OF THE STUDY

The general motivation for this study was to get more insight into the molecular mechanisms of breast cancer progression and metastasis as well as to identify novel regulators of tumor progression in breast cancer. The specific aims for the three subprojects were:

1. To elucidate the role of *de novo* fatty acid synthesis in breast cancer progression as well as to study the genes involved in metabolomic changes in breast tumors and cultured breast cancer cells.

2. To study the role of selected arachidonic acid pathway members in breast cancer.

3. To identify novel regulators of tumor progression in breast cancer cells by utilizing high-throughput lysate microarray (LMA) screens.

4 MATERIALS AND METHODS

Detailed description of the materials and methods is available in the original publications (I-III).

In silico gene expression analysis

Gene sapiens database (www.genesapiens.org, Kilpinen *et al*. 2008) was utilized in the *in silico* gene expression analyzes. The database consists of Affymetrix microarray data collected from publicly available sources such as ArrayExpress and Gene Expression Omnibus. All data have been normalized using a custom algorithm and re-annotated.

Cell lines

Cell line	Tissue of origin	Used in
BT-549	Breast ductal carcinoma	II, III
KPL-1	Breast ductal carcinoma	II
MCF-10A	Breast epithelium	I,II
MCF-7	Breast adenocarcinoma	II
MDA-MB-231	Breast adenocarcinoma	II, III
MDA-MB-231(SA)	Breast adenocarcinoma, spontaneously derived from MDA-MB-231 cells during long in vitro culture	II, III
MDA-MB-468	Breast adenocarcinoma	I, II unpublished
SK7-B-NC	Breast adenocarcinoma, isolated from bone metastases in the tibia of mice 17 weeks after injection of MDA-MB-231 cells into the left cardiac ventricle	II
ZR-75-1	Breast ductal carcinoma	I,II

High-throughput screening (HTS)

RNAi mediated screening methods (Figure 3 and Figure 4) allow the possibility of studying loss-of-function effects of thousands of genes at the same assay setting and under the same conditions.

Plate based RNAi screen

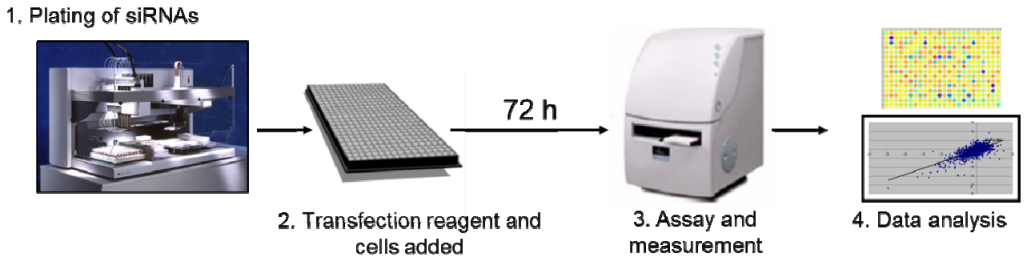

Figure 3: Plate based RNAi screen. The siRNAs are plated on 384-well plates, followed by addition of transfection reagent and cells. The end-point assays are performed after 72 h incubation, followed by data normalization and analysis.

Protein lysate microarray screening

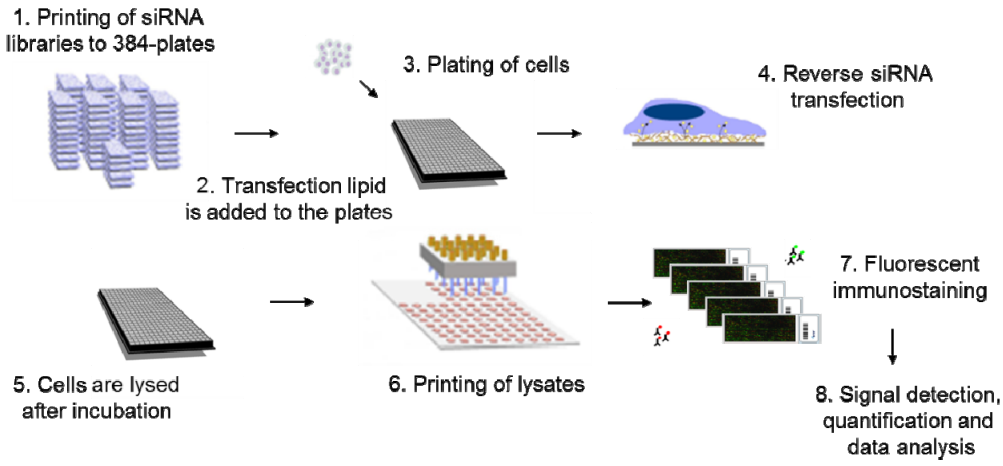

Figure 4: Protein lysate microarray screening. The siRNA transfected cells are incubated in 384–well format and then lysed by adding SDS lysis buffer. Protein lysates are printed onto slides and antibodies for specific proteins are used as endpoint markers to screen the samples.

siRNAs

All siRNAs were purchased from Qiagen.

Target gene	ID	Used in
ACACA	SI00013643	I
ELOVL1	SI04159015	I
EPHB4	SI00063784	III
	SI00288589	III
FASN	SI00059759	I
HPGD	SI00017171	II
	SI00017178	II
INSIG1	SI02650074	I
MTHFD2	SI00090510	III
	SI02664928	III
SCAP	SI00098378	I
SCD	SI03019177	I
THRSP	SI00744443	I
VIM	SI02655198	III
WIPF2	SI00150066	III
	SI03035116	III
AllStars negative control	1027281	I, II, III
Plk1	SI02223844	I, II, III
KIF11	SI02653770	I, II, III

In addition, custom made siRNA oligonucleotide libraries containing 2024 siRNAs were used in a cell based screen (III).

shRNA constructs

All shRNA constructs were purchased from Sigma-Aldrich.

Target gene	ID	Used in
HPGD	NM_000 860.x-292s1c1	II
PLA2G7	NM_005084.2-1046s1c1	unpublished
Non-targeting negative control	SHC002V	II, unpublished

Antibodies

Antigen	Supplier	Antibody ID	Used in
β-actin	Sigma-Aldrich	A5441	II, III
CD44	BD Pharmingen	555478	III
EPHB4	R&D Systems	AF3038	III
HPGD	Sigma-Aldrich	HPA005679	II
MTHFD2	Santa Cruz Biotechnology	sc-100750	III
PLA2G7	Cayman Chemical	160603	unpublished
RAC1	Cytoskeleton	ARC03	II
SNAI1	Cell Signaling Technology	SN9H2	II
VIM	Sigma-Aldrich	V6630	II, III

qRT-PCR primers and probes

Gene	Forward	Reverse	Probe	Used in
ACACA	aacgtgaagacggataagcag	cttcttcggagaatctgacca	#68	I
ACTB	ccaaccgcgagaagatga	ccagaggcgtacagggatag	#64	I, II, III
AHR	tctggaggaatctggtctgg	cagagtctggacaaggaattga	#54	II
CD24	ccaactaatgccaccacca	gtgagaccacgaagagactgg	#28	II
CD44	gacaccatggacaagttttgg	cggcaggttatattcaaatcg	#13	II
CDC42	tgcagtatcaaaaagtccaagagta	gcagtgttccaagttctaaagagg	#22	II
CDH1	cccgggacaacgtttattac	gctggctcaagtcaaagtcc	#35	II
ELOVL1	acttctctctggccctgct	tcacctcttggtacaagttcaca	#58	I
EPHB4	gccagacccaactggatg	accaggaccaggaccacac	#32	III

FASN	caggcacacacgatggac	cggagtgaatctgggttgat	#11	I
FOXC2	ggggacctgaaccacctc	aacatctcccgcacgttg	#3	II
GPAM	ggaaagtttatccagtatggcatt	tgatatcttcctggtcatcgtg	#59	I
HPGD	cacgtgaacggcaaagtg	cttcaagattccaatccacca	#13	II
INSIG1	tcttttcctccgcctggt	gaatatagaggaaatctggggatgt	#56	I
MTHFD2	tactccatggggtgtgtgg	tgggcattccaacgtttt	#5	III
NCAD	gcacagtggccacctaca	tgaaaggtttttatctctatcagacct	#29	II
RAC1	ctgatgcaggccatcaagt	caggaaatgcattggttgtg	#77	II
SCAP	ggaaccacgtgctgagagac	ggtcggtcacttgcagaca	#25	I
SCD	cctacctgcaagttctacacctg	gacgatgagctcctgctgtt	#37	I
SNAI1	gctgcaggactctaatccaga	atctccggaggtgggatg	#11	II
THRSP1	tcatgcacctcaccgaga	gtcttctatcatgtgaagggatctt	#79	I
VIM	aaagtgtggctgccaagaac	agcctcagagaggtcagcaa	#16	II, III
WIPF2	agcaaaacaaaccgagctg	gccaggcttcacctgaga	#53	III
VTN	accttacccttttgccagctc	ctttctccggtgggaacc	#23	II
PLA2G7	tggctctaccttagaaccctga	ttttgctctttgccgtacct	#63	unpublished

Reagents and chemicals

Reagent	**Supplier**	**Used in**
Alexa conjugated Phalloidin	Molecular Probes, Invitrogen	II, III
Alexa Fluor antibodies	Molecular Probes, Invitrogen	II, III
ApoONE	Promega	I
Arachidonic acid	Sigma Aldrich	II
Calcein AM live cell dye	Invitrogen	II, III
CellTitre Glow	Promega	I, II, III
Cignal Smad Reporter Assay System	SABiosciences	II
Dual-Glow Luciferase Assay System	Promega	II
ECL IgG HRP-linked antibodies	Amersham Life Sciences	II, III
ECL reagent	Amersham Biosciences	II, III
Fibronectin	CalbioChem	II
Growth Factor reduced Matrigel	BD Biosciences	III
High Capacity cDNA Reverse Transcription kit	Applied Biosystems	I, II, III
Lipofectamine	Invitrogen	II
Paraformaldehyde	Sigma-Aldrich	II, III
Prostaglandin E_2	Sigma-Aldrich	II

Recombinant human TGF-β	R&D Systems	II, III
Rneasy Mini Kit	Qiagen	I, II, III
SiLentFect	Bio-Rad Laboratories	I, II, III
Soybean Trypsin inhibitor	Sigma-Aldrich	II
Triton X-100	Sigma-Aldrich	II, III
Vectashield	Vector Laboratories	II, III

Methods

Method	**Used in**
Apoptosis assay	I
Cell adhesion assay	II
Cell culture	I, II, III
Cell invasion assay	II
Cell viability assay	I, II, III
Eicosanoid analysis	II
Flow cytometric analysis	III
Gene expression analysis	II
HTS	I, III
IHC	I, II
Immunofluorescence staining	II, III
In silico data mining	I, II, III
Lipidomic analysis	I
Luciferase reporter assay	II
qRT-PCR	I, II, III
RNA interference	I, II, III
Single cell motility assay	II
Statistical analysis	I, II, III
Western blot analysis	II, III
Wound healing assay	II, III

5 RESULTS

5.1 Characterization of lipidomic changes in breast cancer progression (I)

5.1.1 Lipidomic analyses of breast cancer tissue

A comprehensive lipidomic analysis of 257 breast cancer and 10 normal breast tissue samples revealed significant changes in the lipidomic profiles between the two sample sets (I: Table 1). The most prominent alterations observed in tumors were related to the ER status and tumor grade. Overall, the phospholipid concentrations were higher in grade 3 tumors compared to grades 1 and 2 (I: Figure 1B). Especially lipids with *de novo* synthesized fatty acid chains were elevated in high grade ER- tumors. The results showed increase of the membrane phospholipid incorporated *de novo* synthesized fatty acids in tumors, and also suggested that this process is further increased during cancer progression towards more aggressive, ER- and grade 3 tumors. In addition, the association of the lipid profiles and patient survival was studied in this patient group. The results showed that the membrane lipid composition of the tumor related to patient survival (I: Figures 1C and 1D).

5.1.2 Expression of selected lipid metabolism–related proteins associates with altered lipid profiles

The association of the the observed lipidomic changes and expression of lipid-related genes was assessed by carrying out *in silico* bioinformatics analysis in the GeneSapiens database (Kilpinen *et al*., 2008). The mRNA expression profiles of multiple genes were studied in a data set consisting of 9,783 tissue samples from 43 healthy and 68 malignant tissue types. Based on the expression and function of the genes (I: Figure 2 and Supplementary Fig S1), eight genes (ACACA, ELOVL1, INSIG1, SCAP, SCD, SREBP1 and THRSP) were selected for further studies. Especially ACACA, SCD, SREPB1 and THRSP were highly expressed in clinical breast cancer samples.

In order to connect the observed changes in lipidomic profiles with gene expression, immunohistochemical staining of the same tissue samples used in lipidome analyses was carried out (I: Figure 3A). The results indicated that while ER and HER2 status did not associate with altered protein expression,

differences were found for the tumor grade (I: Supplementary Table S8). An unsupervised hierarchical clustering analysis revealed that tumors with high expression of ACACA and FASN together with low expression of SREBP1 had significantly higher levels of phospholipids than tumors in the other cluster (I: Figure 3B).

5.1.3 Plate-based RNAi screen to identify important metabolomic enzymes

To elucidate the role of selected genes in regulating the observed phospholipid remodeling as well as to identify enzymes important in breast cancer progression, a plate based RNAi screen was carried out in ZR-75-1 and MDA MB-468 breast carcinoma cells. The siRNA library consisted of altogether 44 siRNAs targeting 11 genes which were selected based on previously made bioinformatics analyzes. Induction of apoptosis (caspase-3 and 7 activities) as well as changes in cell viability were used as end-point measurements. Scrambled siRNA and lipid (transfection without siRNA) were used as negative controls and siRNAs against KIF11 (kinesin family member 11) and PLK1 (polo-like kinase 1) as positive controls. To validate the silencing of the target genes, qRT-PCR analysis was carried out in ZR-75-1 cells (I: Figure 4A). The results confirmed at least 50% target gene silencing after 72 hours for all except ACACA and FASN. Silencing of SCD, ACACA, INSIG1, ELOVL1, THRSP and FASN had a strong impact on cell viability in ZR-751 cells (I: Figure 4B-C, Supplementary Table S9). In addition, FASN knockdown resulted in induction of apoptosis. In MDA-MB-468 cells, silencing of ACACA, FASN, INSIG1, and THRSP reduced cell viability, whereas apoptosis was induced in response to FASN, INSIG1, SCAP, SCD, and THRSP silencing. The results indicate that these genes are critical for breast cancer survival.

In order to determine whether the observed changes were specific for malignant cells, the RNAi screen was also carried out in a non-malignant but immortalized MCF-10A breast cell line. The results indicated that while these genes seemed to affect cell survival also in non-malignant cells, the effects were stronger in cancer cells (I: Figure 4E-F). Following the RNAi studies, 7 siRNAs (and 2 positive controls) were selected for lipidomics analysis in ZR-75-1 cells based on the cell viability measurements (I: Figure 4B). A significant decrease of PC(14:0/16:0) and PC(32:1) was observed in response to silencing of nearly all studied lipid synthesis modulating genes. These lipids also showed the strongest associations with ER status, grade and survival of the patients. Taken together, this study indicates phospholipids as a potential tool for breast cancer diagnostics. In addition, the results suggest that modulation of

phospholipid metabolism may provide novel therapeutic opportunities for breast cancer treatment.

5.2 Arachidonic acid pathway in breast cancer (II)

5.2.1 HPGD is highly expressed in advanced breast cancer and associates with poor prognosis

The expression of HPGD in clinical breast cancer samples was first studied in GeneSapiens database (Kilpinen *et al*., 2008), in a dataset consisting of samples from normal breast (n = 13) and breast cancer (n = 957). Elevated mRNA expression was seen in a subset of cancer samples although the median expression was higher in normal breast tissues than in breast cancer samples (II: Figure 1A). Similar pattern was observed by immunohistochemical staining of HPGD in a tumor microarray (TMA) comprising 556 samples from 411 primary breast cancer patients, including 89 cases with matched primary and lymph node metastatic samples. High expression of HPGD was detected in 21% of samples from primary tumors and in 16% of lymph node metastases.

The clinical relevance of high HPGD expression in breast tumors was assessed with Kaplan-Meier survival analyzes in both independent datasets. The results indicated that high HPGD expression had a significant association with poor outcome (II: Figure 1B and Figure 2B). Furthermore, a multivariate analysis showed that this was regardless of the other tumor characteristics (II: Table 2), implicating HPGD as an independent marker for poor outcome.

Triple-negative breast tumors are aggressive and often resistant to therapy. Since the *in silico* and TMA analyzes of HPGD expression in clinical samples showed association with poor outcome, we analyzed whether high HPGD expression associated with clinical parameters typical for aggressive type of tumors. Co-expression analyzes of HPGD, estrogen receptor (ER, encoded by *ESR1*), progesterone receptor (PgR, encoded by *PGR*) and HER2/neu (encoded by *ERBB2*) mRNAs in the *in silico* dataset indicated that HPGD was mainly expressed in samples with low expression of ESR1, PGR, and ERBB2 (II: Supplementary Figure S2). Additionally, in the TMA samples from lymph node metastases, high HPGD expression associated significantly with negative hormone receptor status whereas such association was not seen in primary tumors.

5.2.2 HPGD regulates breast cancer cell migration

In accordance with the data obtained from clinical samples, HPGD was highly expressed especially in aggressive breast cancer cells (II: Figure 3A). A qRT-PCR analysis of HPGD mRNA in a panel of nine breast cancer cell lines (six ER negative cell lines: MDA-MB-231, MDA-MB-231(SA), SK7-B-NC, MCF10A, BT-549, MDA-MB-468 and three ER positive cell lines: MCF-7, ZR-75-1, KPL-1), showed substantially elevated HPGD expression in MDA-MB-231 cells and its more metastatic variants MDA-MB-231(SA) and SK7-B-NC, indicating that high HPGD expression is associated with increased metastatic potential.

In order to study the biological function, HPGD was silenced transiently with siRNA and stably with shRNA in MDA-MB-231(SA) cells (II: Figure 3B). The knockdown did not induce visible morphological changes or affect cell viability. Genome-wide gene expression profiling and validation experiments indicated changes in pathways controlling cell migration. Interestingly, one of the most prominently altered pathways was aryl hydrocarbon signaling which has been increasingly studied in terms of cancer progression (II: Table 2 and Figure 3C). In addition, HPGD knockdown decreased the expression of small RhoGTPases Rac1 and Cdc42 (II: Figure 3D).

Results from functional studies further supported the role of HPGD in regulating cell migration. Single cell motility assay showed that transient silencing of HPGD induced a less motile phenotype in MDA-MB-231(SA) cells (II: Figure 4A). In addition, HPGD knockdown resulted in significantly impaired wound healing in several cell lines (II: Figure 4B, C and Supplementary information). Reduced migratory ability and decreased expression of small RhoGTPases suggested possible alterations in actin and intermediate filament network formation. The effect of stable HPGD knockdown on cell cytoskeleton in migrating breast cancer cells was studied by staining scratch wounded shHPGD transducted MDA-MB-231(SA) cells (MDA(SA)/HPGD- cells) for F-actin and vimentin. These cells displayed impaired formation of F-actin and vimentin networks especially on the migrating wound edge (II: Figure 5A). These results further support that HPGD is required for the cytoskeletal alterations important for cell migration.

5.2.3 HPGD functions in the EMT process

The results from clinical validation and functional experiments in HPGD impaired metastatic breast cancer cell models suggested a potential link

between HPGD and EMT. Importantly, a study by Tong *et al*. indicated induction of HPGD by TGF-β in lung cancer (Tong *et al.*, 2006). Results from a reporter assay showed that SMAD signaling was significantly more activated in TGF-β stimulated MDA-MB-231(SA) cells than in HPGD deficient cells (II: Figure 5B). In addition, stimulation with TGF-β induced cell migration more efficiently in HPGD impaired MDA-MB-231(SA) and SK7-B-NC cells than in the control cells (II:Figure 5C). Furthermore, TGF-β stimulation enhanced HPGD expression in MDA(SA)/HPGD- cells (II: Supplementary Figure 8). These results suggest that HPGD expression is regulated by TGF-β also in breast cancer cells.

HPGD functions in the arachidonic acid pathway by degrading PGE_2, a downstream product of AA metabolism. Lipidomic profiling of cellular eicosanoids showed consistent decrease in cellular arachidonic acid levels in response to HPGD silencing in MDA-MB-231(SA) cells (II: Figure 6A). Furthermore, stimulation with AA and PGE2 rescued the migratory defect seen in HPGD impaired cells (II: Figure 6B).

Possible alterations induced by silencing of HPGD in the expression of other epithelial and mesenchymal markers as well as transcription factors shown to control EMT/MET was also studied. As the previous results suggested, HPGD was shown to induce MET in the metastatic MDA-MB-231(SA) cell line (II: Figures 6C and D). HPGD deficient cells displayed increased expression of the epithelial marker E-cadherin as well as reduced expression of the mesenchymal markers vimentin and N-cadherin, an event considered as a turning-point in EMT-MET process. In addition, expression of the EMT-inducing transcription factors FOXC2 and Snail was significantly reduced. Previous studies suggest that FOXC2 controls the events in the mesenchymal portion of EMT and that its expression is triggered during EMT by transcription factors including Snail (Mani *et al*., 2007). Snail is a potent inducer of EMT and a determinant for cancer cell invasiveness (Peinado *et al*., 2004; Olmeda *et al*., 2008). These results indicate that HPGD contributes to the maintenance of tumorigenic and metastatic properties of the mesenchymal phenotype.

5.2.4 PLA2G7 in breast cancer (unpublished results)

To elucidate the role of PLA2G7 in breast cancer, PLA2G7 mRNA expression was studied in GeneSapiens database (Kilpinen *et al*., 2008). The results indicated upregulation of PLA2G7 expression in breast cancer and cancer in general compared to normal tissues (Figure 5A). Kaplan-Meier survival analysis associated high PLA2G7 expression to poor prognosis (Figure 5B).

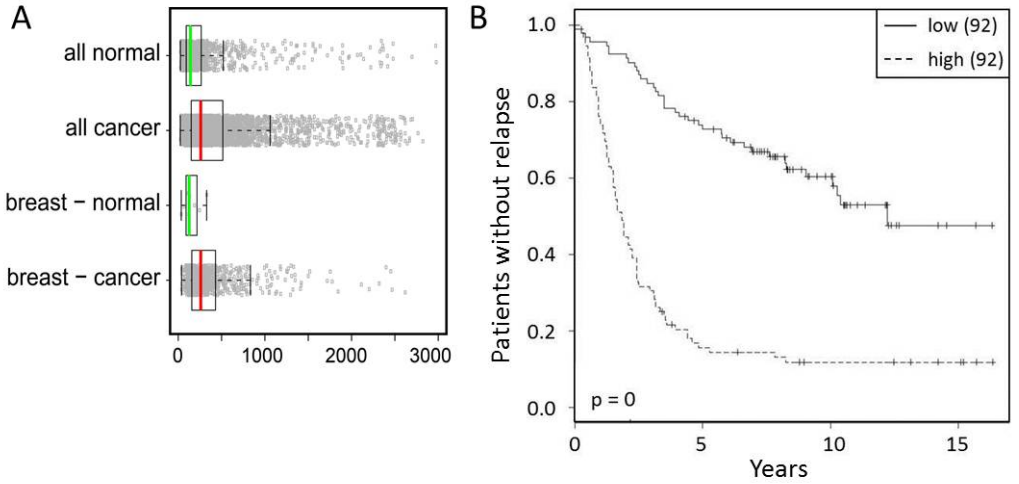

Figure 5. PLA2G7 mRNA expression in clinical breast cancer samples.
A: PLA2G7 is highly expressed in breast cancer and cancer in general compared to normal tissues. B: High expression of PLA2G7 significantly (p = 0) associates with shorter time to relapse.

Further *in silico* analysis indicated high PLA2G7 mRNA expression especially in advanced, hormone receptor negative (ER-, PgR-) basal type breast cancers (data not shown). These analyses demonstrated the clinical relevance of this gene and indicated it as a potential drug target also for breast cancer treatment.

5.2.4.1 PLA2G7 expression relates to breast cancer cell migration and invasion

Genome-wide gene expression profiling of MDA-MB-468 breast cancer cells with stable knockdown of PLA2G7, followed by functional analysis with Ingenuity Pathway analysis –tool, indicated association with tumor cell invasion, cellular movement and embryonic development (data not shown). Supporting this data, our previous results showed that PLA2G7 promotes prostate cancer cell migration and invasion (Vainio *et al*., 2011b) and indicated PLA2G7 as a potential drug target for prostate cancer. Results from the transcriptome analysis indicated reduced vimentin expression in PLA2G7 deficient MDA-MB-468 cells. Immunofluorescence staining of these cells confirmed a significant reduction of vimentin protein expression and network formation (Figure 6).

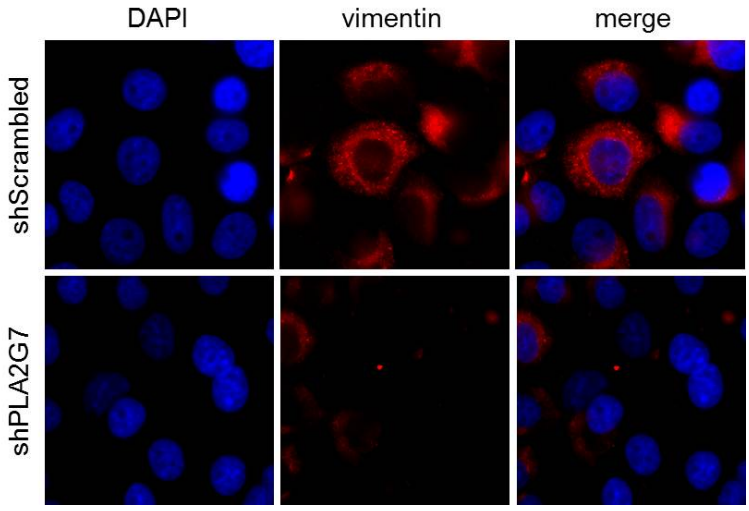

Figure 6. Immunofluorescence staining of PLA2G7 deficient MDA-MB-468 breast cancer cells. Stable silencing of PLA2G7 with shRNA results in decreased vimentin protein expression.

Furthermore, preliminary results from a wound healing experiment indicated reduced cell migration in response to transient silencing of PLA2G7 in MDA-MB-468 cells (data not shown). This indicates a role for PLA2G7 in regulating cell migration and a possible contribution to EMT process.

5.3 RNAi screen for novel regulators of vimentin expression (III)

5.3.1 RNAi lysate microarray screen in metastatic breast cancer cells

In order to identify novel vimentin expression regulating genes, we conducted RNAi lysate microarray screen in metastatic basal-like breast cancer cells. The siRNA library included 2024 siRNAs targeting 596 genes either highly expressed in breast or prostate cancer samples, previously associated with metastasis or over-expressed in bone metastatic vs. parental MDA-MB-231 breast cancer cells. The effect of target gene silencing on vimentin expression was assessed using lysate microarray technology which has been previously described (Leivonen *et al*., 2009). The control siRNAs were validated both at mRNA and protein level (III: Figure 1A). Total protein amount of each spot was measured in order to exclude cell proliferation decreasing siRNAs (III: Figure 1B). Hit selection was based on the Z-score values (≤ -2, measuring standard deviations from the mean) and the amount of total protein in the spot (≥ 0.75). The siRNAs qualified for the selection criteria in both or either of the screens were

regarded as hits (III: Table 1). As a result, *WIPF2, MTHFD2* and *EPHB4* were identified as potential vimentin expression modulating targets (III: Figures 1 and 2A, B).

5.3.2 Validation of putative vimentin expression modulators

The RNAi lysate microarray screen identified *WIPF2, MTHFD2* and *EPHB4* as potential vimentin expression modulating targets. The first target WIPF2 (WAS/WASL interacting protein family, member 2) belongs to the family of Wiskott-Aldrich syndrome proteins (WASP), which participate in WASP-mediated organization of the actin cytoskeleton (Aspenstrom, 2004). There are currently no previous studies linking WIPF2 and breast cancer. The second target MTHFD2 (methylenetetrahydrofolate dehydrogenase 2) is a mitochondrial enzyme with methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activities. High MTHFD2 expression is associated with increased risk of bladder cancer (Andrew *et al*., 2009). The third target EPHB4 (ephrin type-B receptor 4) belongs to a family of ephrin binding receptors. EPHB4 has been shown to be overexpressed in breast cancer, promoting cell migration and invasion (Wu *et al*., 2004; Kumar *et al*., 2006; Wu *et al*., 2007).

Four siRNAs per gene targeting *WIPF2, MTHFD2* or *EPHB4* were introduced to MDA-MB-231(SA) cells and vimentin protein expression was analyzed 120 hours after transfection (III: Fig 2A). For each target, two siRNAs reducing vimentin protein expression by at least 25% were selected for further validation. Target gene and vimentin silencing was validated with qRT-PCR (III: Fig 2B). In addition, EPHB4 and MTHFD2 target gene silencing was validated on protein level (III: Supplementary Figure S1).

Vimentin expression was studied *in silico* in clinical breast cancer samples (Kilpinen *et al*., 2008, dataset described in III). In concordance to the numerous studies describing vimentin in breast cancer, the analyses showed vimentin overexpression in breast cancer samples and associated high vimentin expression to poor survival (Figure 7).

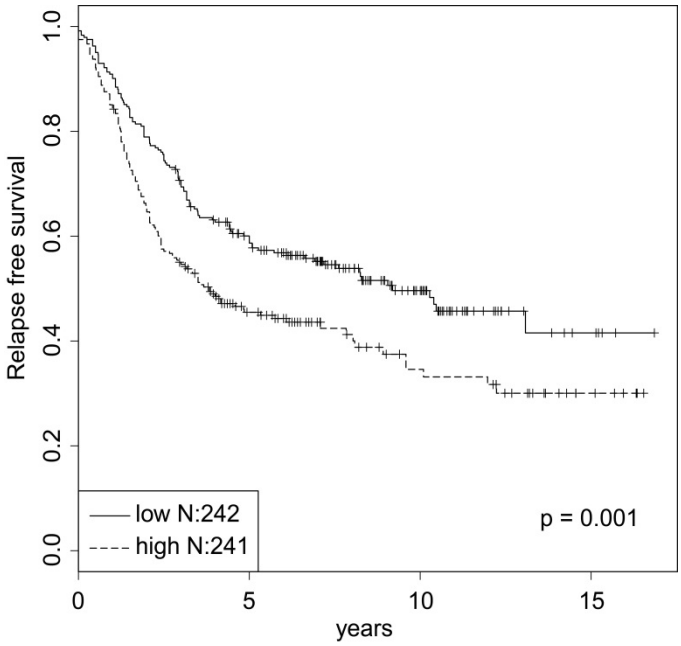

Figure 7. Analysis of vimentin mRNA expression in clinical breast cancer samples. Kaplan-Meier analysis of relapse free survival shows significant (p=0,001) association of high VIM expression and decreased probability of survival.

The clinical relevance of the hit genes was evaluated *in silico* in the same dataset (III: Figure 2C). Kaplan-Meier analyses indicated significant association of high EPHB4 or MTHFD2 mRNA expression and shorter relapse-free survival in breast cancer, whereas high WIPF2 expression was related to better outcome. Since MTHFD2 was not previously known to function in breast cancer and was linked to poor outcome, it was of special interest and therefore chosen for functional studies *in vitro*.

5.3.3 MTHFD2 expression in clinical samples

The expression profile of MTHFD2 was further studied by analyzing the overall MTHFD2 mRNA expression across several non-malignant and malignant human tissues (III: Supplementary Figure S2). Analysis of normal tissues indicated MTHFD2 expression especially in blood myeloid cells as well as in hematopoietic and mesenchymal stem cells. In cancer samples, lymphoma and neuroblastoma displayed the highest MTHFD2 mRNA expression. Overall, MTHFD2 was overexpressed in malignant tissues (III: Figure 3A). Importantly, a strong overexpression of MTHFD was seen in breast cancer samples compared to normal breast tissue. The results are supported by previous studies reporting MTHFD2 expression in tumor cells, but not in normal cells in adult mice (Di Pietro *et al*., 2004).

In addition, the possible association of high MTHFD2 expression with clinically relevant features was studied in breast cancer samples and found to be significantly correlated with hormone receptor negativity (ER-, PgR-), p53 mutation, lymph node positivity, basal subtype, increased cell proliferation (high Ki67) and HER2 positivity. Analysis of MTHFD2 mRNA expression in a cDNA panel of 96 clinical samples from several human cancers (n=8) confirmed higher MTHFD2 expression in breast, colon, kidney, liver, lung and ovarian cancers than in the corresponding normal tissues (III: Figure 4). Interestingly, samples derived from tumor metastases displayed the highest expression levels, further indicating MTHFD2 as a promoter of tumor progression.

5.3.4 MTHFD2 knockdown inhibits breast cancer cell migration and invasion and results in impaired vimentin network

To further elucidate the function of MTHFD2 in breast cancer, a gene co-expression analysis was conducted in clinical breast cancer samples. High MTHFD2 correlated with the expression of genes involved in regulation of cell cycle, cellular movement as well as cellular assembly and organization processes. Silencing vimentin in breast cancer cells has been shown to impair cell migration and invasion (Vuoriluoto *et al*., 2011). Therefore, the effect of MTHFD2 knockdown was studied in metastatic breast cancer cells. While transient silencing of VIM or MTHFD2 in MDA-MB-231, MDA-MB-231(SA) or BT-549 cells did not affect the cell morphology, the cells displayed impaired migratory phenotype in wound healing assays (III: Figure 5 and Supplementary Figure 3B). Analysis of cell viability showed that this was not due to decreased cell proliferation (Supplementary Figure 3C), but rather a defect in cell migration. Interestingly, in BT-549 cells MTFD2 knockdown had stronger effect than VIM knockdown, indicating that MTHFD2 may not regulate cell migration merely by decreasing vimentin expression. In addition, cell invasion to Matrigel matrix was significantly decreased in MTHFD2 deficient MDA-MB-231(SA) cells (III: Figure 6A).

Since vimentin has been shown to regulate formation of lamellopodia and an organized vimentin network is required for cell motility (Helfand *et al*., 2011), MTHFD2 and vimentin deficient MDA-MB-231(SA) cells were stained in order to detect deformation of vimentin network. The cells displayed clear reduction in vimentin protein expression as well as impaired network formation (III: Figure 6B). These results indicate that MTHFD2 promotes cellular properties important for metastasis in breast cancer.

5.3.5 TGF-β induces MTHFD2 expression in breast cancer cells

To get more insights into the regulation of MTHFD2, we studied whether stimulation of metastatic breast cancer cells with TGF-β affects MTHFD2 expression (III: Figure 7A). An induction of expression was observed in TGF-β stimulated MDA-MB-231 and MDA-MB-231(SA) cells, indicating that in addition to vimentin, TGF-β may also regulate MTHFD2 expression in breast cancer cells.

5.3.6 MTHFD2 silencing reduces cancer stem cell properties in breast cancer cells

The transmembrane glycoprotein CD44 is a biomarker of cancer stem cells (CSCs); the chemotherapy resistant metastatically active cells capable of forming other cancer cell populations (Dalerba and Clarke, 2007; Li *et al*., 2007; Klingbeil *et al*., 2009; Floor *et al*., 2011). In order to determine whether MTHFD2 associated with cancer stem cell marker CD44, fluorescence-activated cell-sorting was utilized for analysis of the $CD44^{low}$ and $CD44^{high}$ cell populations in response to MTHFD2 or vimentin knockdown in MDA-MB-231(SA) cells (III: Figure 7B). These results showed that both MTHFD2 and vimentin silencing reduced the amount of $CD44^{high}$ MDA-MB-231(SA) cells supporting the association of MTHFD2 and CSC properties.

6 DISCUSSION

6.1 Characterization of lipidomic changes in breast cancer progression (I)

Human cells have two sources of fatty acids, diet and *de novo* synthesis. In normal adult tissues cells rely on dietary fatty acids whereas in cancer the fatty acids are synthesized *de novo*. Increased de novo fatty acid synthesis is considered as a hallmark of cancer, also in breast cancer (Chajes *et al*., 1995). This study utilized a novel approach on studying *de novo* fatty acid metabolism in breast cancer. Combined lipidomic and protein expression analysis as well as RNAi mediated studies were used to obtain a comprehensive view of the importance of this feature in breast cancer progression.

Lipidomic profiling was done from clinical tissue samples of malignant and normal breast tissues to find which metabolic enzymes were most strongly altered in progression towards malignancy. The results displayed elevation of the membrane phospholipid incorporated *de novo* synthesized fatty acids in malignant tissues compared to normal tissues. Furthermore, the results indicated further increase of this process during cancer progression towards more aggressive, ER- and grade 3 tumors. In addition, the observed lipid profile changes were found to associate with worse patient survival.

In silico database was utilized to associate the detected lipidomic changes to the expression of lipid-related genes as well as to evaluate the overall expression of these genes in breast cancer. On the basis of this data, eight genes were selected for functional studies in cultured breast cancer cells. Interestingly, especially ACACA, SCD, SREPB1 and THRSP were highly expressed in clinical breast cancer samples. The results from the lipidomics analysis were then compared to protein expression patterns of the selected targets in the same tissue samples with immunohistochemical analysis. The results showed differential expression according to the tumor grade, while ER and HER2 status did not associate with altered protein expression. This is somewhat contradictory results since previous studies have indicated a major effect of HER2 signaling on the lipid metabolism (Kumar-Sinha *et al*., 2003).

The role of these eight targets was further studied with RNAi mediated analyses of proliferation and induction of apoptosis. The results indicated ACACA, FASN, INSIG1 and THRSP critical for breast cancer survival. Validation of target gene silencing showed less than 50% efficacy for siRNAs targeting ACACA and FASN. This indicates that these genes had particularly strong effects since already a partial silencing led to strong decrease in cells viability and increase in apoptosis, respectively. The RNAi screen was carried out also in a nonmalignant but immortalized MCF-10A to see whether the observed changes were specific for malignant cells. Interestingly, these genes appeared to affect cell survival also in non-malignant cells, the effects were more prominent in cancer cells indicating a cancer specific function. This is supported by the *in silico* analyses since the target genes were overexpressed in cancer compared to normal tissues. To bring all the observed data together, lipidomic profiling was done for samples from breast cancer cells with target gene knockdown. Importantly, the same lipids most altered in clinical breast cancer samples were also the most changed in the cultured cells.

Taken together, this study suggests that increased *de novo* fatty acid synthesis and phospholipid remodeling pathways are required for breast cancer cell growth and are important for breast tumorigenesis. The overall amounts of saturated fatty acids regulating the cell membrane fluidity have been shown to be elevated in the most aggressive tumors (Ollila *et al*., 2007). Furthermore altered membrane fluidity has been suggested to affect the sensitivity of tumor cells to chemotherapy and contribute to therapy resistance (Rysman *et al*., 2010). Our study indicates phospholipids as a potential tool for breast cancer diagnostics as it differs greatly between normal and malignant tissues. Furthermore, these results suggest that modulation of phospholipid metabolism may provide novel therapeutic opportunities for breast cancer treatment. Interestingly, after the publication of our study, lipidomic profiling of breast tumors has been investigated further (Kang *et al*., 2011). The study reports that tumors can be classified to different subtypes according to their protein and lipid profiles. Also, the analysis method is suggested as a diagnostic aid for breast cancer treatment.

Future studies could address the subject by more detailed functional characterization of the identified targets. Studying for example the changes in migration and invasion phenotype as well as possible knock-down induced morphological changes in different breast cancer cells would be of great interest. Gene expression profiling of the target deficient breast cancer cells

and non-malignant cells in combination with detailed lipidomic profiling would enhance the results obtained from this study. These additional analyses would further elucidate the mechanisms by which these target genes function in the *de novo* fatty acid synthesis pathway and promote breast cancer progression.

6.2 Arachidonic acid pathway in breast cancer (II)

The role of arachidonic acid (AA) pathway has been studied in many cancers (Wang and Dubois, 2010). In breast cancer, arachidonic acid has been shown to promote EMT (Martinez-Orozco *et al*., 2010), and cell migration (Navarro-Tito *et al*., 2008). Based on a previous study in prostate cancer conducted in our research group by Vainio *et al.* (Vainio *et al*., 2011a), two members of arachidonic acid pathway, HPGD and PLA2G7, were studied also in breast cancer.

The results suggested that HPGD functions as an oncogene, rather than as a tumor suppressor in a subset of breast cancers. HPGD was found to be overexpressed in clinical breast cancer samples from advanced disease and significantly associate with breast cancer survival. Furthermore, HPGD impairment significantly reduced breast cancer cell migration and led to reduced expression of small RhoGTPases, suggesting possible alterations in actin and intermediate filament network formation. Indeed, immunofluorescence analysis of wounded HPGD deficient cells displayed poorly formed actin and vimentin networks on the wound edge. These HPGD impairment induced defects in the migratory properties of metastatic breast cancer cells indicated a possible connection of HPGD and induction of MET.

Supporting our study, recent studies have elucidated the role of arachidonic acid in breast cancer progression and implicated it in processes supporting tumor invasion i.e. epithelial-mesenchymal transition (EMT). AA has been shown to increase vimentin expression and decrease E-cadherin in cell junctions, thus promoting cell migration and epithelial-mesenchymal transition (Martinez-Orozco *et al*., 2010). In addition, TGF-beta induced COX-2 expression has been shown to induce EMT via prostaglandin E2 (PGE2) production (Neil *et al*., 2008). Analysis of cellular arachidonic acid content in metastatic breast cancer cells in response to HPGD knockdown indicated that silencing HPGD impairs arachidonic acid metabolism and leads to reduced AA levels in the cells. When the HPGD deficient cells were stimulated with AA or PGE2, the defect in migration was partially rescued indicating that HPGD regulates cell

motility via AA and PGE2. Furthermore, analysis of the mRNA expression of several key EMT-related markers and transcription factors in HPGD-impaired cells revealed a switch towards a more epithelial and less tumorigenic phenotype.

Another important member of the arachidonic acid pathway, PLA2G7, has been previously reported to have an oncogenic role in prostate cancer and contribute to the malignant state of murine and human colon cells (McMurray *et al.* 2008; Vainio *et al*., 2011b). In this study, PLA2G7 was found to be overexpressed in breast cancer and associated with poor survival. A transcriptome analysis indicated that PLA2G7 functions in processes connected to cell migration and invasion as well as embryonic development. Further functional studies are needed to reveal the role of PLA2G7 in breast cancer progression. However, results from this study indicate PLA2G7 as an interesting candidate for evaluation as a biomarker also for breast cancer. Taken together, this study indicates a significant role for AA in breast cancer progression. Our findings connecting two key enzymes in this pathway, HPGD and PLA2G7, to the regulation of cell migration and invasion as well as EMT, highlight the importance of further studies on the subject. In addition, this study indicates therapeutic possibilities in regards of preventing metastatic spreading of breast cancer by targeting AA pathway.

Future studies should concentrate on elucidating the function of this pathway in breast cancer by studying the pathway cascade with wider selection of targets. In addition to COX subpathway, the studies could address also the two other important arachidonic acid metabolizing subpathways LOX and CYP. This way a more comprehensive view could be obtained concerning the events underlying tumorigenesis promoting properties of arachidonic acid and its metabolites in breast cancer.

6.3 RNAi screen for novel regulators of vimentin expression (III)

6.3.1 RNAi screening method

The development of high-throughput screening methods has enabled efficient use of large-scale RNAi mediated functional cell based analyses in basic research. The possibility of studying the loss-of-function effects of thousands of genes with the same assay setting and under the same conditions has opened new prospects for cancer research. Initially RNAi mediated gene silencing was described in *C. elegans* and *D. melanogaster* in late nineties (Fire

et al., 1998; Kennerdell and Carthew, 1998). Soon after the same technique was demonstrated in human cells (Elbashir *et al*., 2001), which subsequently led to the development of siRNA molecule synthesis methods for creation of commercially available reagent libraries for the use of researchers.

The basic principle of plate based functional high-throughput RNAi screens is fairly simple, thus facilitating reproducibility. Cells are cultured in multiwell format plates and the siRNAs are introduced to the cells by lipid transfection reagents. After an appropriate incubation period typically ranging from 48 to 72 hours, the cells are assayed for the phenotype or end point of interest. These can include for example cell morphology, cell viability or induction of apoptosis. In addition, it is possible to include different reporter assays to the siRNA mediated gene silencing for detailed analysis of specific pathways. For example, in order to analyze the effect of gene silencing to the expression of specific proteins, the transfected cells can be subsequently lysed and lysates printed to nitrocellulose slides. The slides are then stained with specific antibodies targeting the protein of interest and the protein expression quantified using fluorescent secondary antibodies. This enables the detection of expression for several different targets at the same time from the same cells and allows the use of numerous replicates for reliability of the results. RNAi mediated screening has been successfully used in numerous functional studies addressing different biological questions such as cell adhesion (Winograd-Katz *et al*., 2009), cell cycle regulation (Kittler *et al*., 2007; Fuchs *et al*., 2010), responses to DNA damage (Paulsen *et al*., 2009), anchorage-independent cancer cell growth (Irie *et al*., 2010), and drug resistance (Iorns *et al*., 2008; Turner *et al*., 2008).

The major challenges of RNAi screening relate to the specificity of the reagents. The siRNA molecules are known to induce secondary off-target effects (Jackson *et al*., 2003). Therefore it is important to screen several siRNAs targeting the same gene. To confirm the silencing of certain end-point target or induction of desired phenotype, validated positive and negative control siRNAs must be included in the siRNA library. In addition, the screens should be done in replicates, preferably with separate transfections. The correlation of these replicates indicates the reliability of the resulting data. Verification of the results by smaller scale secondary validation under the same conditions as the primary screen is also important for the reliability of the assay. The lysate-microarray screening method (LMA) used in this study is highly dependable on the specificity of the antibodies utilized for the end point analysis. Thus the

antibodies should be validated prior to the screen to prevent possible false hits. In addition to the reagents, the cell lines have to be selected carefully in order to facilitate the functionality of the screen. Usage of inappropriate cells for a certain end point analysis may result in unusable data and waste of valuable reagents.

In this study, the correlation between two replicate RNAi screens was very good which indicates reliability and reproducibility of the observed effects. The vimentin antibody was validated and shown to be specific. In addition, all the siRNAs targeting vimentin were among the strongest to reduce vimentin protein expression in MDA-MB-231(SA) cells. Two of the identified targets, EPHB4 and WIPF2 were by some means connected previously to cell migration or cytoskeleton, indicating the relevance of our approach. Deeper functional studies indicated similar role for also the third target, MTHFD2.

6.3.2 MTHFD2 in breast cancer

As a result of this study, MTHFD2 was found to be overexpressed in samples from malignant tumors compared to normal tissues in several tissue types, importantly especially in breast cancer. Similar results have been reported also in two other studies (Selcuklu *et al*., 2012). Relatively little is known about the function of MTHFD2 in breast carcinogenesis. Interestingly, a recent study implicates mitochondrial glycine synthesis pathway in promoting breast cancer cell proliferation (Figure 8), and associates elevated expression of the pathway enzymes SHMT2, MTHFD1L as well as MTHFD2 with worse prognosis (Jain *et al*., 2012).

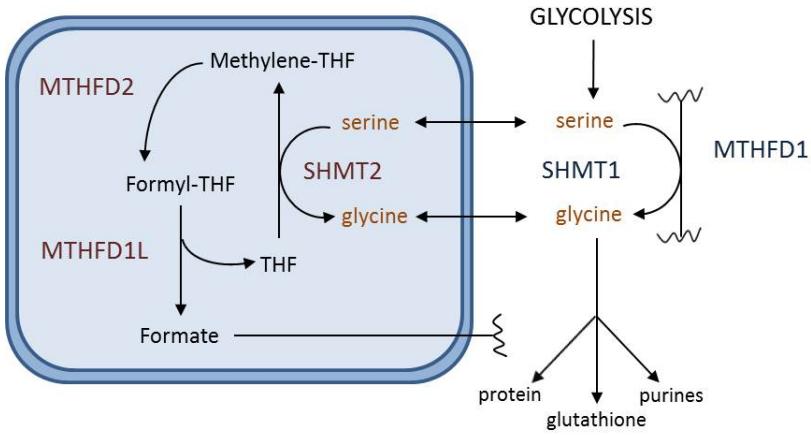

Figure 8. A schematic presentation of the mitochondrial glycine synthesis pathway. (Adapted and modulated from Jain *et al.* 2012).

MTHFD2 acts in one-carbon metabolism in the folate pathway (Christensen and Mackenzie, 2008; Tibbetts and Appling, 2010). Interestingly, this pathway can be therapeutically targeted in cancer with methotrexate (Bertino, 2009), which results in impaired DNA synthesis and repair.

We observed no modulation in MTHFD2 expression in response to vimentin silencing. Since MTHFD2 silencing had such a strong effect on cell migration and invasion, in part even more prominent than direct silencing of vimentin, the observed vimentin impairment could also be a consequence of the chanced migratory phenotype. The detailed mechanisms by which MTHFD2 induces the decrease in vimentin expression remain to be clarified. A recent study suggests that MTHFD2 is a direct target of miR-9, a micro RNA which has tumor-suppressor properties (Selcuklu *et al*., 2012). This study also concurs with our results on cell migration and invasion.

Despite the known role of vimentin in EMT, we could not observe indications of MET induction in response to MTHFD2 impairment in breast cancer cells, although reduction of vimentin expression and adaptation of less motile and invasive phenotype are key features of MET undergone cells. However, the EMT inducer TGF-β was found to regulate MTHFD2 expression. TGF-β has been implicated in regulation of vimentin and promotion of cell migration (Wu *et al*., 2007; Gal *et al*., 2008). This led us to study the association of MTHFD2 and cancer stem cell properties instead of EMT, since the tumor propagating cancer stem cells (CSCs) and cells that have undergone EMT, have been shown to have similar characteristics (Floor *et al*., 2011). We found that the expression of the cancer stem cell marker CD44 was decreased in response to MTHFD2 impairment. Interestingly, CD44 has also been implicated to contribute in cancer cell migration and invasion (Pusch *et al*., 2010; To *et al*., 2010; Yilmaz and Christofori, 2010). This suggests that the MTHFD2 knockdown induced reduction of tumorigenic properties in breast cancer cells is possibly not due to reversion of EMT and induction of MET, but due to the decreased stem cell characteristics.

For future studies on EMT and for identification of novel EMT regulators, this siRNA library should be screened in additional cells lines. This study indicates the relevance of the library in question for studying breast cancer progression related events. In addition, including both mesenchymal type and epithelial type cells would increase the validity of the obtained data by showing that the observed modulation of expression is not cell line dependent. Furthermore,

staining of other known EMT markers, such as E-cadherin or the transcription factor Snail, would add the amount of information and give more insight to the processes involved in breast cancer metastasis.

7 SUMMARY AND CONCLUSIONS

The major aim initially set for this thesis work was to identify novel regulators of breast cancer progression and find clinically relevant putative therapeutic targets for treatment of aggressive breast cancer. This was addressed in three separate studies utilizing data obtained from clinical breast cancer samples as well as several breast cancer cell models. These studies resulted in following findings:

1. The results showed increased *de novo* synthesized fatty acids in tumors and suggested that this process is further increased during cancer progression towards more aggressive tumors. In addition, several lipidomic enzymes were found to be upregulated in breast cancer and associated with poor outcome. Taken together, this study describes phospholipids as a potential tool for breast cancer diagnostics and suggests that modulation of phospholipid metabolism may provide novel therapeutic opportunities for breast cancer treatment.

2. The results support the role of arachidonic acid (AA) pathway members HPGD and PLA2G7 in EMT-related processes. AA pathway member HPGD was found to contribute to breast cancer progression by promoting cell migration and inducing EMT. High expression of HPGD correlated significantly with worse prognosis and associated with clinical features typical for poor outcome. Our study suggests that HPGD targeted therapy could be beneficial for patients suffering from advanced and metastatic breast cancer. In addition, preliminary studies of the role of PLA2G7 in breast cancer indicated overexpression of PLA2G7, and associated this with significantly higher probability to disease relapse.

3. RNAi lysate microarray screen in metastatic basal breast cancer cells identified *WIPF2, MTHFD2* and *EPHB4* as potential vimentin expression modulating targets. Further validation of MTHFD2 indicated overexpression in breast cancer as well as association with poor prognosis and revealed putative association with metastatic disease. Functional studies showed that MTHFD2 promotes cellular features

connected with metastatic disease, thus implicating MTHFD2 as a potential drug target to block breast cancer cell migration and invasion.

In conclusion, this study identified novel genes contributing to breast cancer progression via different processes. These complex and versatile events controlling breast cancer progression still require more investigation. The different clinical and pathological features of breast cancer form the major challenge for current treatments options. Understanding the underlying mechanisms of metastatic progression and development of better models for breast cancer are essential for obtaining more effective and targeted treatment options.

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