The impact of the progranulin-sortilin axis on breast cancer stem cell activity and patient outcome

Karoline Berger

Department of Laboratory Medicine
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration “Lung metastasis in mice” by Karoline Berger.

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Karoline.berger@gu.se

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To my family

“If we knew what it was we were doing,
it would not be called research, would it?”

-Albert Einstein

Abstract

Breast cancer is the most common cancer in women worldwide. Still today, despite current breast cancer therapies, many patients experience treatment resistance and relapse, which are believed to be due to failure in targeting treatment-resistant cancer stem cells. Cytokines and growth factors secreted by various cell types present in the tumor microenvironment have the potential to affect this challenging cell subpopulation. This thesis focuses on a complex cellular communication system based on hypoxia-induced secretion, where we identified the growth factor progranulin as one of the key mediators driving cancer stem cell propagation. In this thesis, we demonstrate that progranulin mediates cancer stem cell propagation in various breast cancer cell lines. By chemically degrading and modulating sortilin expression, or using a small sortilin binding molecule, AF38469, we could reduce the progranulin-induced cancer stem cell propagating effect *in vitro*, suggesting that the progranulin-induced cancer progression is dependent on sortilin. Importantly, using breast cancer xenograft models, we were able to confirm the progranulin-mediated cancer stem cell propagating effect *in vivo.* Strikingly, progranulin induced a significant increase in lung metastasis, which could be reduced by oral administration of AF38469. Moreover, when investigating the mechanisms behind sortilin-driven progranulin-induced cancer stem cell activation, we found that progranulin induced secretion of the inflammatory cytokine interleukin-6 and could demonstrate a crosstalk between progranulin and interleukin-6 protein expression. Similar to progranulin, interleukin-6 affected breast cancer stem cell expansion via sortilin, altogether suggesting that sortilin is a highly relevant biological target in breast cancer. Furthermore, in a tissue microarray of breast cancer patients, high co-expression of progranulin and sortilin defined a novel and highly malignant subgroup of breast cancer, suggesting that these proteins can be used as prognostic biomarkers. Combined, results presented in this thesis propose that targeting the progranulin-sortilin communication axis represents a potential novel breast cancer therapeutic approach, inhibiting tumor progression driven by secretion and microenvironmental influences. Accordingly, we are currently in the proses of developing sortilin-targeting drugs for the treatment of breast cancers with high expression of progranulin and sortilin.

Keywords: Breast cancer, biomarker, cancer stem cells, microenvironment, progranulin, sortilin, interleukin-6, targeted therapy, prognostic

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Sammanfattning på svenska

Bröstcancer är den vanligaste cancerformen bland kvinnor i Sverige, med över 8000 nydiagnostiserade fall varje år. Trots den ökade överlevnaden de senaste åren får många patienter återfall, och fortfarande avlider ungefär 1300 kvinnor i Sverige varje år. Bröstcancer är en mycket heterogen och komplex sjukdom, och kännetecknas av stora variationer i tumörerna mellan olika patienter. Tumörerna påverkas i stor grad av omgivningen kring tumören, den så-kallade mikromiljön, bestående av proteinfibernätverk, immunceller, cytokiner och andra celler som kan påverka hur tumören svarar på olika behandlingar. Här finns också en liten population av celler som kallas cancerstamceller som är mer aggressiva och behandlingsresistenta än andra cancerceller och kan i högre grad ge upphov till spridning och återfall. Det är därför viktigt att rikta behandlingen mot dessa celler. Syftet med detta arbete är att bättre förstå hur cancerceller påverkas av omgivningen, och vi har valt att fokusera på ett antal proteiner som just påverkar mängden cancerstamceller i bröstcancer. I en screen av utsöndrade proteiner från cancerceller där vi undersökte hur hypoxi (lågt syretryck) påverkar olika tumöregenskaper, identifierade vi progranulin som ett viktigt protein som både utsöndrades av cancerceller under hypoxi, men som också utsöndras i höga nivåer i hormonreceptornegativa bröstcancerceller. Vi har vidare studerat hur progranulin påverkar receptorn sortilin och vad detta får för effekter på cancercellerna. Våra resultat visar att progranulin, via sortilin, ökar mängden cancerstamceller i bröstcancer. Dessutom har vi detaljstuderat hur progranulin och sortilin uttrycks på proteinnivå i bröstcancer och funnit att ungefär 20% av alla premenopausala bröstcancerfall uttrycker höga nivåer av både progranulin och sortilin, vilket också visade sig vara starkt kopplat till dålig prognos för patienterna. Dessa data tyder på att signalering via sortilin kan driva elakartade egenskaper. Genom att blockera eller bryta ner sortilin i cancerceller kan vi minska progranulins effekt på cancerstamcellerna. Vi har även sett att progranulin-behandling ger en ökad mängd lungmetastaser i möss, något som också kan hämmas vid användning av en liten molekyl som binder till sortilin. Vi har ytterligare definierat hur olika klyvda peptid-delar av progranulin påverkar cancerstamceller via sortilin, såväl som undersökt samspelet mellan progranulin, sortilin och den inflammatoriska cytokinen IL-6, som även den visade sig kunna bidra till att påverka cancerstamcellsegenskaper i bröstcancer via receptorn sortilin. Sammanfattningsvis har vi genom detta arbete identifierat ett nytt sätt att angripa elakartade egenskaper i cancer. Blockering av sortilin kan vara ett effektivt sätt att inhibera tumörprogression som drivs av sekretion i tumörens mikromiljö.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. Rhost S, Hughes É, Harrison H, Rafnsdóttir S, Jacobsson H, Gregersson P, Magnusson M, Fitzpatrick P, Andersson D, **Berger K**, Ståhlberg A and Landberg G. **Sortilin inhibition limits secretion-induced progranulin-dependent breast cancer progression and cancer stem cell expansion**.
Breast Cancer Res. 2018 Nov 20;20(1):137.

II. **Berger K,** Rhost S, Rafnsdóttir S, Hughes É, Magnusson Y, Ekholm M, Stål O, Rydén L and Landberg G. **Tumor co-expression of progranulin and sortilin as a prognostic biomarker in breast cancer**.
BMC Cancer. 2021 Feb 22;21(1):185.

III. **Berger K***, Persson E*, Gregersson P, Jonasson E, Ståhlberg A, Landberg G and Rhost S. **Interleukin-6 induces stem cell propagation through liaison with the sortilin-progranulin axis in breast cancer**.
*Authors contributed equally. (*Manuscript*)

IV. **Berger K***, Rhost S*, Hughes É, Gregersson P and Landberg G. **Granulin peptide domains induce breast cancer stem cell propagation vi sortilin**.
*Authors contributed equally. (Manuscript)

V. **Berger K**, Pauwels E, Parkinson G, Landberg G, Le T, Demillo V.G, Lumangtad L.A, Jones D.E, Islam M.A, Olsen R, Kapri T, Intasiri A, Vermeire K, Rhost S and Bell T.W. **Reduction of Progranulin-Induced Breast Cancer Stem Cell Propagation by Sortilin-Targeting Cyclotriazadisulfonamide (CADA) Compounds.**
(*Manuscript under revision*)

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Abbreviations

5-FU: fluorouracil
BCSS: breast cancer-specific survival
BRCA: breast cancer
CADA: cyclotriazadisulfonamide
CD4: cluster of differentiation 4
cDNA: complementary DNA
ctDNA: circulating tumor DNA
CLL: chronic lymphocytic leukemia
CPH: cox proportional hazards
CSC: cancer stem cells
CXCL: chemokine (C-X-C motif) ligand
CXCR: chemokine (C-X-C motif) receptor
ECM: extracellular matrix
ELISA: enzyme-linked immunosorbent assay
EMT: epithelial mesenchymal transition
EphA2: Ephrin type-A receptor 2
ERBB2: receptor tyrosine-protein kinase 2
ERK1/2: extracellular signal-regulated kinase 1 and 2
ERα: estrogen receptor alpha
ERß: estrogen receptor beta
FAK: focal adhesion kinase
FASL: Fas ligand
FPA: fluorescence polarization binding assay
gp130: glycoprotein 130
GRN: progranulin gene
HER2: human epidermal receptor 2
HIF: hypoxia inducible factor
HIV: human immunodeficiency virus
HR: hazard ratio
HRE: hypoxic response element
HRP: horseradish peroxidase
IHC: immunohistochemistry

IL: interleukin
IL-6R: interleukin-6 receptor
IL-8RA/B: interleukin-8 receptor A or B (Also called CXCR1 and 2)
IVIS: In Vivo Imaging Software
JAK: Janus tyrosine family kinase
LDL: low-density lipoprotein
MAPK: mitogen-activated protein kinase
MPEP: 1-[2-(2-tert-butyl-5-methylphenoxy)-ethyl]-3-methylpiperidine
mRNA: messenger RNA
MYC: cellular myelomatosis
NGS: next generation sequencing
NOD/SCID: non-obese diabetic/severe combined immunodeficiency
PDX: patient derived xenografts
PEA: proximity extension assay
PI3-K: phosphoinositide 3-kinase
PR: progesterone receptor
qPCR: quantitative polymerase chain reaction
RB: retinoblastoma protein
SERD: selective estrogen receptor degrader
SERM: selective estrogen receptor modulator
sIL-6R: soluble interleukin-6 receptor
siRNA: small interfering RNA
SLPI: secretory leukocyte protease inhibitor
SORT1: sortilin gene
sSortilin: soluble sortilin
STAT: the signaling transducer and activator of transcription
TIL: tumor infiltrating lymphocyte
TMA: tissue microarray
TNF: tumor necrosis factor
TNFR1/2: tumor necrosis factor receptors 1 and 2
TNM: tumor node metastasis
TP53: tumor protein p53
Vps10p: vacuolar protein sorting 10 protein

Introduction

Breast cancer

Breast cancer is the most common cancer among the female population and the leading cause of death to cancer in women worldwide [1]. In Sweden, 30% of all cancers in women are breast cancer and the numbers are increasing every year [2]. In 2019, almost 8300 women were diagnosed with breast cancer in Sweden [3]. Nevertheless, earlier detection and improved treatment strategies have led to a relatively good prognosis, with 5-year and 10-year survival rates of 92% and 86.1% (Sweden, 2016) [2].

There are a number of risk factors associated with breast cancer [4, 5]. Some are linked to hormones, like early menstruation, late menopause, having your first child late in life or having no children at all. Other risk factors are associated with age, gender, genetic predisposition, family history of breast cancer, radiation exposure, obesity, diet, exercise-level and alcohol consumption [5].

Normal breast development

From newborn until menopause, the female breast undergoes cycles of development and differentiation [6]. Through distinct developmental stages, the mammary glands develop from a few stem cells and give rise to phenotypically and functionally different cell types in the breast (Figure 1) [7]. In an infant, the breast tissue consists only of a tiny duct composed of epithelial cells, which is similar in both genders until puberty. During puberty, hormones like estrogen allows the breast duct to grow rapidly and divide into primary and secondary ducts. The lobules develop and grow into tree-like structures. Throughout pregnancy, the breasts fully develop. Estrogen promotes further proliferation and differentiation of the ductal trees, and progesterone induces additional growth of the lobules. Additional increase in growth hormones and prolactin promotes the complete development of the mammary glands.

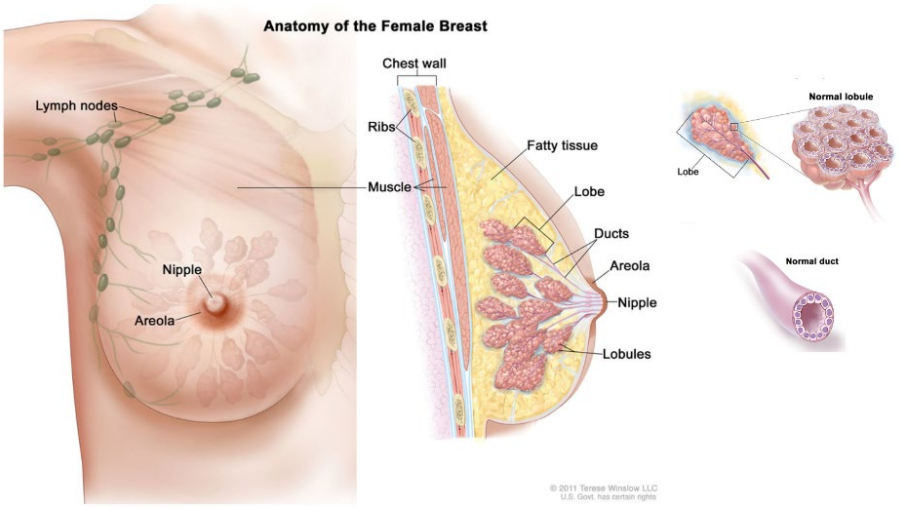

Figure 1. Normal breast tissue. The normal breast consists of milk-producing glands called lobules, which are connected by small tubes, called ducts. Lobules and ducts are surrounded by the breast stroma, consisting of fat cells, immune cells and connective tissue, as well as blood and lymph vessels. Image modified from https://www.teresewinslow.com.

Breast cancer development and progression

Cancer occur when a cell transforms to a malignant state through a series of processes characterized as the "Hallmarks of Cancer", described by Hanahan and Weinberg in 2000 [8]. The six original hallmarks, shown in Figure 2, include: (1) cells becoming self-sufficient in growth signals, leading to sustainable proliferation, (2) loss of sensitivity to growth-inhibition signals, (3) apoptotic resistance, (4) unlimited replication, (5) angiogenesis, providing the tumor with new blood vessels, and (6) activating invasion and metastasis, where the tumor becomes able to invade surrounding tissues. After new evidence linking inflammation and cancer, it became evident that additional hallmarks should be added [9]. They include: (7) avoiding immune surveillance and destruction, as well as (8) reprogramming of cellular metabolism, resulting from the cancer cells providing the surrounding microenvironment with various growth signals driving tumor progression [10]. Additionally, (9) genomic instability and mutations, along with (10) tumor-promoting inflammation were added. These are so-called enabling characteristics, leading to the acquirement of all the listed hallmarks.

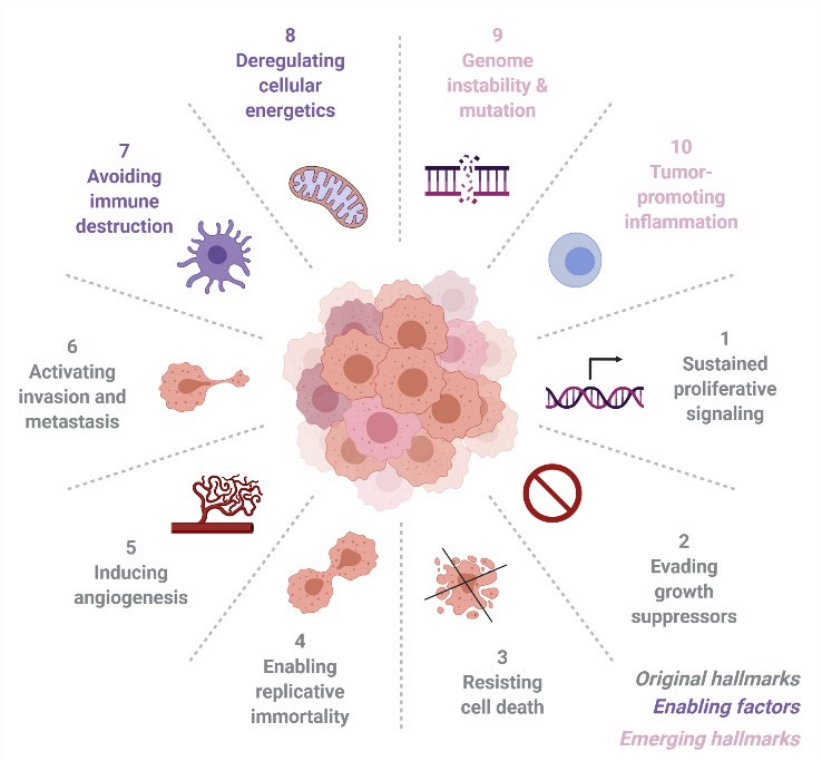

Figure 2. The Hallmarks of Cancer. The origin of cancer are described through processes including the six original hallmarks, as well as two assisting factors and two emerging hallmarks. Image created with BioRender.com.

In breast cancer, the tumor progresses from a normal cell to hyperplasia, where the cells appear normal, but divide and proliferate uncontrollably (Figure 3) [11-13]. Tumor progression can occur through a gradual accumulation of genetic and epigenetic changes, from deletions that alter tumor suppressor genes, such as tumor protein p53 (TP53) or retinoblastoma protein (RB), to amplifications or activation of oncogenes, such as receptor tyrosine-protein kinase 2 (ERBB2) or MYC (cellular myelomatosis). Most of the mutations are sporadic, but some can be inherited, such as the germline mutations breast cancer 1 (BRCA1) and BRCA2. Through further alterations, the cells proliferative capacity increases and gradually become more abnormal in shape and orientation (atypical hyperplasia). In carcinoma *in situ* (ductal or lobular), the cells appear abnormal and grow uncontrollably, but have not yet broken through the tumor boundary. In time, more genetic alterations and mutations occur. Some might inactivate DNA repair genes leading to more genetic

instability, giving rise to invasive lobular/ductal carcinoma. In invasive carcinoma, some tumor cells have gained a more motile phenotype by remodeling of the extracellular matrix (ECM). The cells are then able to break through the membrane boundary and started invading nearby tissues [12, 13]. Eventually, cells can enter the blood or lymphatic circulation where they can form colonies (metastasize) at distant sites.

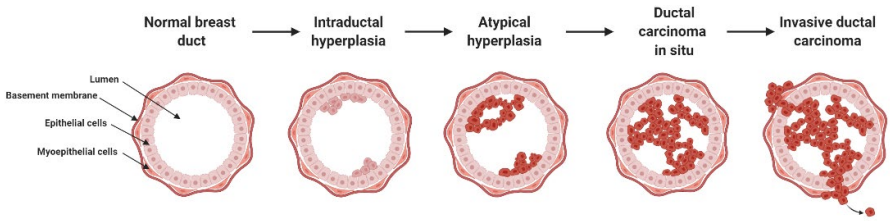

Figure 3. Breast cancer progression. The tumor progresses from normal cells going through alterations leading to elevated proliferation of the cells (hyperplasia). Through additional increases in proliferation, as well as downregulation of apoptosis and a more abnormal morphology, the tumor gradually continue to progress leading to atypical hyperplasia. Proceeding further, we have carcinoma *in situ*, where the cells are still growing inside the boundary of the tumor. Upregulation of markers related to angiogenesis, epithelial mesenchymal transition and extracellular matrix-remodeling direct the tumor into an invasive carcinoma. Image adapted from [11] and created with BioRender.com.

Breast cancer classification and subtypes

Breast cancer is a highly heterogeneous disease that can be divided into various subtypes. The subtypes are defined using different classification systems that are based on histology or the molecular basis of the tumor [14]. These subtypes are used as both prognostic markers (predicting the likely outcome of the disease) and treatment-predictive indicators (probability to benefit from a certain treatment) to help decide treatment strategies for the patients [15-17].

Histological classification primarily divides breast cancer into ductal or lobular carcinoma *in situ*, the precursor of breast cancer, or invasive carcinoma, where ductal carcinoma is the most common [17].

Another classification of breast cancer utilizes prognostic markers to characterize the tumor stage or tumor node metastasis (TNM), depending on tumor size, lymph node status and metastatic spread, or the tumor grade [18]. Tumor grade is divided into high or low grade based on how well differentiated the cells are and how fast the cells grow [19]. A high-grade

tumor is defined as poorly differentiated and have a high expression of the cellular proliferation marker Ki67.

Classification by immunohistochemistry (IHC) is carried out through examining protein expression levels, especially the status of the commonly used biomarkers estrogen receptor alpha (ERα), the progesterone receptor (PR) and human epidermal receptor 2 (HER2).

Molecular subtype classification was first proposed in year 2000 by Perou and Sørlie, and are based on gene and protein expression data and epithelial cell origin (PAN50: a 50 gene expression signature) [15, 16, 20]. The molecular subtypes are summarized in Table 1 and include the ERα-positive luminal tumors, which are further separated into luminal A and luminal B tumors, the HER2-enriched and basal-like (most often triple negative for ERα, PR and HER2) breast cancer.

Table 1. Molecular subtypes of breast cancer. Based on data from [5, 15, 21-25].

Subtype	Hormone status	Grade/outcome	Proliferation (Ki67)
Luminal A (50-60%)	High ER and PR expression, low HER2	Low grade, good outcome	Low proliferation
Luminal B (10-20%)	ER and PR expression (lower than Luminal A), variable HER2 expression	Higher grade, poor survival compared to luminal A	Higher expression of proliferative genes compared to Luminal A
HER2 (ERBB2 overexpressing) (15-20%)	Low or no ER and PR expression	High grade, often aggressive, intermediate prognosis (but respond well to HER2-targeted therapies)	High proliferation
Basal-like (10-15%)	Often triple negative (no ER, PR or HER2)	High grade, poor outcome (only 20% respond to chemotherapy)	High proliferation

Breast cancer treatment

Standard treatments for breast cancer patients are usually breast surgery, either breast conserving or removal of the entire breast, which in some cases also are followed by radiotherapy. More specific, systemic treatments are also given, such as chemotherapy, endocrine treatment or targeted therapy (*e.g.* small molecular inhibitors and antibody-based therapy) [16, 26]. These treatments can be given as neoadjuvant treatment, prior to surgery, to shrink the tumor or as a response-indicator. Alternatively, they

are given after surgery, alone or in combinations, depending on tumor burden or subtype, to prevent recurrence and prolong survival. Patient prognosis and treatment options differ widely depending on the molecular subtype of the tumor, having to take into account the presence or absence of hormone receptors, grade, lymph node status and gene expression of specific markers [16, 27]. Individualization of therapy have become more common, directing the treatment towards the biology of the tumor and to reduce adverse side-effects and prevent resistance, by rewiring of signaling pathways, which usually arises from conventional therapy where all patients receive the same treatment [5, 19].

Endocrine therapy

Approximately 70% of all tumors express ERα and are treated with endocrine adjuvant therapy, such as tamoxifen or aromatase inhibitors [28]. In the breast, ERα is the predominant estrogen receptor and is used in clinical setting for treatment decisions, as ERß levels have been shown to vary when it comes to treatment response [29]. The hormone estrogen binds to its main receptors ERα and ERß and induce the expression of PR expression, activated by progesterone.

Tamoxifen is a selective estrogen receptor modulator (SERM), functioning by competing with estrogen for the binding to the ER, blocking the effect of estrogen (estrogen antagonist). Tamoxifen is effective against metastatic breast cancer and are often given as adjuvant therapy up to 5-10 years after surgery, depending on the risk of relapse. Tamoxifen also function as an ER agonist in the bone, by increasing bone mineralization through decreasing low-density lipoprotein (LDL) cholesterol levels [28]. However, despite tamoxifen's success in improving the survival of the hormone receptor positive patient group, many patients experience tumor relapse or therapy resistance [26].

Another way of blocking the estrogen receptor is by the use of selective estrogen receptor degrader (SERD), such as fulvestrant [19]. Fulvestrant compete for estrogen receptor as estrogen antagonist, but its binding causes targeting of the ER for destruction by the immune system.

Aromatase inhibitors like anastrozole or letrozole are blocking estrogen production by inhibiting the enzyme aromatase that normally converts testosterone to estradiol [26, 28].

Monoclonal antibody-targeted therapy

HER2 positive tumors are tumors with an ERBB2 amplification or HER2 overexpression, which represent 15-20% of all breast cancers. These patients usually respond well to monoclonal antibody treatment targeting the receptor HER2, such as Trastuzumab (Herceptin) [19, 30]. ERBB2 is a proto-oncogene that when amplified, it causes the activation of the HER2 pathway. This activates proliferation, cell survival, metastasis through various pathways [5].

Chemotherapy

There are several types of chemotherapy drugs available, such as microtubule stabilizers (Docetaxel, Pacliaxel), anti-metabolic factors (5-FU, fluorouracil), DNA intercalators (cisplatin, doxorubicin) and CDK4 and CDK6 inhibitors (Palbociclib) [19]. The different types can be used alone or in combinations with each other, and also together with other types of therapy, such as endocrine therapy or HER2 targeted therapy to improve effectiveness and patient survival.

In addition, 15% of all breast cancers are basal-like tumors. The majority of basal-like tumors are triple negative, lacking ERα, PR and HER2. These tumors are difficult to treat, as they lack effective drug targets [23]. The standard treatment for this subtype is still chemotherapy, but patients often have a shortened disease-free and overall survival rate, and an increased risk of developing distant metastases compared to other forms of the disease. Metastases accounts for more than 90% of cancer-related deaths [31]. Therefore, there is a great need to develop treatments for triple negative breast cancers and identify subgroups that respond to specific therapeutic agents [32, 33].

In conclusion, there is an increasing need to identify biomarkers and key mediators involved in breast cancer progression, to be able to distinguish subgroups of breast cancer patients which will benefit from specific treatments [34].

The tumor microenvironment and breast cancer heterogeneity

As mentioned earlier, breast cancer is a very heterogeneous disease, where functional and phenotypical differences can be seen both between tumors and within the tumor itself [21, 35]. These variations are due to several factors, including various genetic and epigenetic alterations, the surrounding tumor microenvironment, as well as the presence of a small subpopulation of tumor cells, called cancer stem cells (CSCs), which potentially could lead to therapy resistance, cancer progression, metastasis or even tumor relapse [36, 37]. Moreover, cells such as fibroblasts and immune cells that are present in the tumor microenvironment communicate with each other and the surrounding tumor niche, secreting a range of different cytokines and growth factors, promoting tumor growth and metastasis (Figure 4) [38, 39].

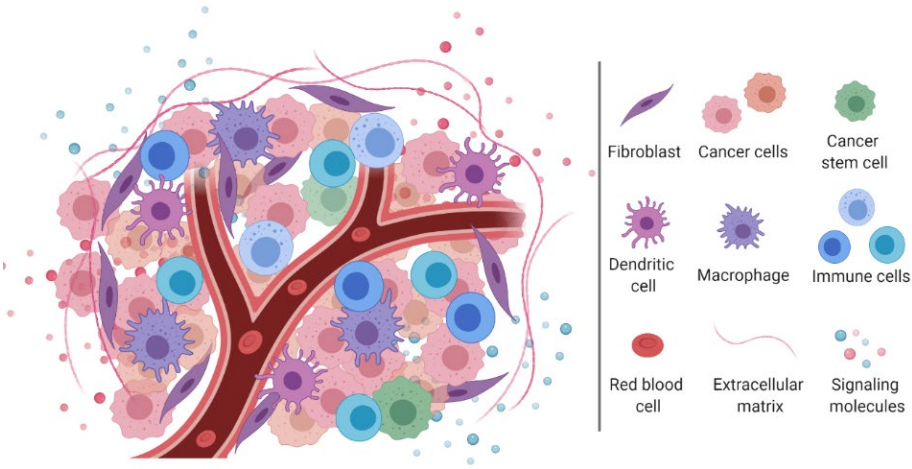

Figure 4. Components of the tumor microenvironment. The tumor microenvironment consists of various cell types (*e.g.* fibroblasts and immune cells) and extracellular matrix components (*e.g.* collagens, laminins and fibronectin). The cells in the tumor microenvironment communicate through paracrine signaling mediated by various cytokines and growth factors, which modifies cellular processes leading to tumor progression. Image based on [38] and created with BioRender.com.

Further, low oxygen levels (hypoxia) play a major role in cancer progression, as it has been linked to poor overall patient survival and a more malignant tumor phenotype [40]. Areas of hypoxia are present in many solid tumors and potentially in the metastatic sites, suggesting that hypoxia influence cancer cells including the CSCs. The hypothesis of the origin of CSCs starts with a single stem cell that have acquired different mutations, or from cells that are more differentiated, and acquire stem cell traits during tumor progression [17, 41]. CSCs have characteristics in common with normal stem cells, including the ability to self-renew, give rise to progenitor cells or cells that are more differentiated, and share common signaling pathways, such as the Notch, Wnt and Hedgehog pathways [35, 36, 42, 43]. Furthermore, CSC are characterized as possessing anchor-independent growth abilities, being low proliferative, acquire tumor-initiating capacities and metastatic potential and express some common surface markers, including the $CD44^{+}/CD24^{-/low}$ and $ALDH^{high}$ phenotypes (although no universal maker for CSCs have been identified) [44-47]. Importantly, CSCs have been described as resistant to radio- and chemotherapy, and may therefore contribute to cancer relapse [38, 42]. These CSCs have therefore been proposed as a promising target for treating breast cancer [43]. CSCs can be identified by different methods, including functional assay such as tumor sphere formation and *in vivo* xenograft assays, as well as specific cell surface markers and Hoechst staining/side-population sorting (drug resistance in CSCs) [42, 48].

The clonal evolution theory and the hierarchal CSC model

There are two current models explaining the cause of cancer and intra-tumor heterogeneity (Figure 5) [49, 50]. The classical hypothesis, the clonal evolution theory, is based on clonal expansion, where a single cell has gone through random genetic mutations providing it with growth advantages [50, 51]. Through clonal expansion of that cell or clones with a more dominant and aggressive phenotype, it will eventually give rise to a tumor. A heterogeneous cell population then appears when some of the cells acquire properties that are advantageous on their own, possibly due to influences from the tumor microenvironment.

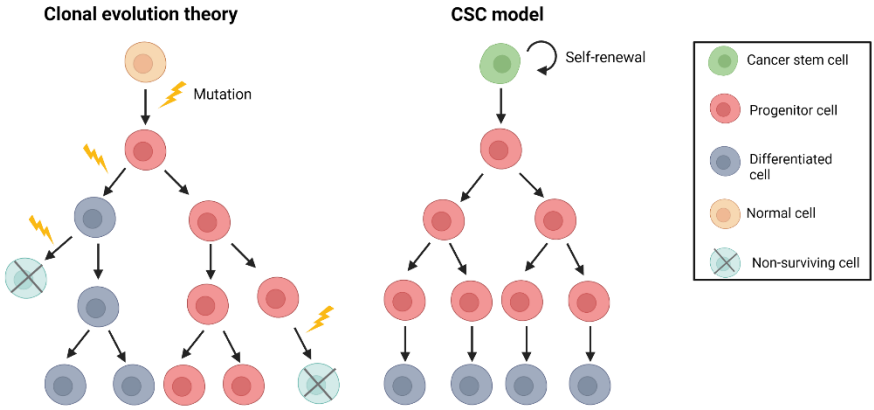

Figure 5. Tumor heterogeneity models. Clonal evolution theory vs cancer stem cell model. Adapted from [52, 53]. Image created with BioRender.com. CSC: cancer stem cell.

The CSC hypothesis, on the other hand, explains tumor heterogeneity through a hierarchical cellular organization [37]. In this organization, a small proportion of the cancer cells have the ability to sustain tumor growth and generate heterogeneity through differentiation. This provides the cells with stem cell properties, such as tumor-initiating capacity and metastatic potential [46]. It has been shown in immune-deficient mice that only a small subset of the cancer cells are able to proliferate and cause tumor growth, regenerating the original tumor [46, 54]. In order for the CSCs to maintain the CSC pool, they undergo symmetric division, but also asymmetric division to generate cells with low tumorigenic potential. Moreover, some researchers have proposed a plastic CSC model, explaining how some non-CSCs can retrieve their CSC phenotype through dedifferentiation [46]. Other researchers explain this through the induction of epithelial mesenchymal transition (EMT) [46, 55]. EMT is normally seen in embryonic development, where epithelial cells gain properties, making them more mesenchymal and fibroblast-like. In cancer, this enables the cells to leave the primary tumor, enter the circulation and metastasize at distant sites [56]. CSCs may be created by a single stem cell that have acquired different mutations, or from cells that are more differentiated and acquire stem cell traits during tumor progression. The CSCs have been described as resistant to radiotherapy and chemotherapy, and may thus contribute to cancer relapse after surgery and treatment [42, 50].

The tumor heterogeneity and progression might be explained by a combination of the two theories, and also in combination with

microenvironmental influences, as none of them explains how the tumor formation is initiated [50].

Progranulin

Progranulin, also known as PC cell-derived growth factor or granulin-epithelin precursor, is a cysteine-rich, heavily glycosylated, autocrine growth factor involved in various biological processes, such as wound healing, tumorigenesis, inflammation, as well as various neurological diseases [57-59]. This 88-kDa glycoprotein can be cleaved by neutrophil elastase, proteases and different matrix metallopeptidases to produce different biologically active peptide domains of about 6 kDa (Figure 6) [60, 61]. These granulins are named from para-granulin, which is a half-length domain, to granulin G, F, B, A, C, D and E, ordered from the N-terminus of progranulin to the C-terminus [58]. The interest in progranulin has emerged over the last few years, with publications demonstrating an overexpression of progranulin in a range of different cancer types, as well as being associated with poor prognosis and survival, suggesting that progranulin may be a relevant predictive and prognostic biomarker in various types of cancer [58].

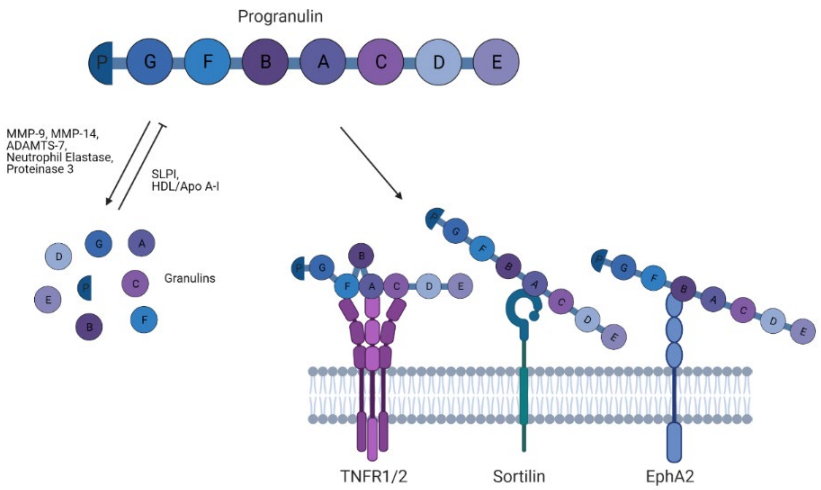

Figure 6. Progranulin and its associated receptors. Progranulin and its respective domains are shown as circles. Cleavage of progranulin by proteolytic processing (*e.g.* by MMP-9, MMP-14, ADAMTS-7, NE or PR3) produces biologically active granulins that are thought to be involved in inflammation. The full-length progranulin protein has been shown to have an anti-inflammatory effect by binding to tumor necrosis factor receptors (TNFR1/2), and has also been shown to bind to Ephrin type-A receptor 2 (EphA2) and sortilin. Illustration created in BioRender.com, adapted from [58].

Progranulin binds to the sortilin receptor, also called neurotensin receptor-3, to mediate progranulin internalization. Sortilin is a member of the vacuolar protein sorting 10 protein (Vps10p) domain receptor family, mostly expressed in neurons to regulate neuronal function and viability, as well as in other tissues and cell types, such as B-lymphocytes, metabolic tissues and solid tumors [62, 63]. Sortilin has multiple roles in cellular transport and signaling, both intracellularly and as a cell surface receptor, involved in targeting and sorting proteins to different fates [63, 64]. Consequently, sortilin is involved in tumorigenesis, cardiovascular and metabolic diseases and neurological disorders, such as dementia, and have been proposed as a potential drug target for these diseases [62, 65].

Furthermore, progranulin binds to tumor necrosis factor (TNF) receptor 1 and 2, highlighting its importance in the immune response [57, 66]. More recently, progranulin has also been shown to bind to the newly identified receptor Ephrin type-A receptor 2 (EphA2) [67].

IL-6

Cytokines and chemokines are secreted by various cells in the tumor microenvironment, creating a link between inflammation and cancer [68, 69]. This type of inflammation is disrupting the balance in the tumor microenvironment, between cytokines, chemokines, transcriptional factors and reactive oxygen species, leading to tumor growth and cancer progression [70].

IL-6 is an inflammatory cytokine produced and secreted by numerous cells and is involved in the regulation of B- and T-cell activation, growth and differentiation, as well as in recruiting neutrophils [68, 71, 72]. In addition, IL-6 is thought to be involved in tumorigenesis and resistance to cancer therapy by regulating signaling pathways important for tumor development and progression [73, 74]. High IL-6 levels in serum and tissue have been detected in various types of cancer, including breast cancer, and are correlated with poor prognosis, advanced disease, metastasis and worse response to therapy [68, 72, 74]. Moreover, IL-6 as well as its receptor have been shown to play a role in proliferation and expansion of the CSC pool in several types of cancer, as well as to induce EMT, cell migration and invasion, leading to metastasis formation [73, 74].

IL-6 binds to the IL-6 receptor (IL-6R) and forms a complex that associates with a signal transducing receptor glycoprotein 130 (gp130, expressed on most cells) on the receiving cell, called classical signaling (Figure 7, left) [71, 75]. However, binding can also occur through the soluble form of the IL-6 receptor (sIL-6R), so called trans-signaling, where gp130 is activated

without a membrane bound IL-6 receptor (Figure 7, right) [71, 75]. Trans-signaling is thought to activate more pro-inflammatory pathways and to be more important in cancer [74]. Signaling pathways activated by IL-6 are the Janus tyrosine family kinase (JAK) and the signaling transducer and activator of transcription (STAT) pathway (JAK-STAT pathway), the extracellular signal-regulated kinase 1 and 2 (ERK1/2), mitogen-activated protein kinase (MAPK) pathway (ERK1/2-MAPK pathway), as well as the phosphoinositide 3-kinase (PI3-K) pathway [70, 71, 76].

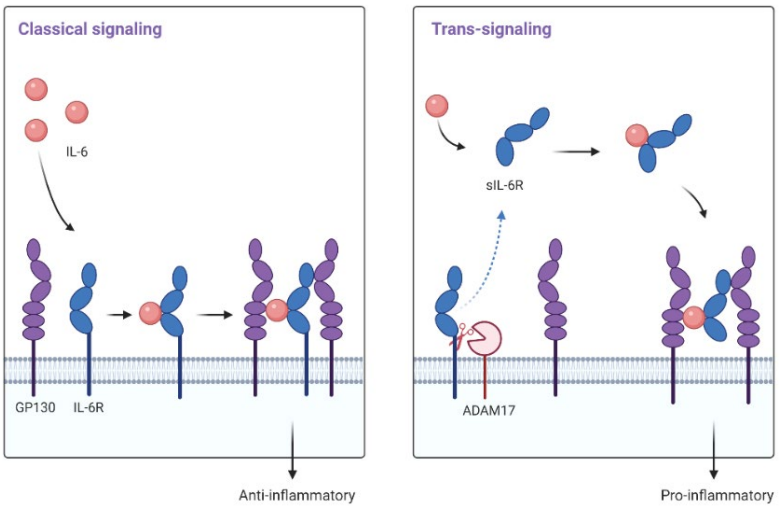

Fig. 7. IL-6 signaling. In the classical signaling pathway, IL-6 binds to IL-6R and recruits pg130 for cell signal initiation, causing an anti-inflammatory response. Trans-signaling occurs when IL-6 binds to the soluble form of the IL-6 receptor (sIL-6R), which is cleaved by metalloproteases (ADAM10 or 17) or created from alternative splicing of the mRNA. This binding complex then interacts with gp130 and activates pro-inflammatory signaling pathways. Image adapted from [74] and created with BioRender.com.

IL-8

An additional pro-inflammatory cytokine, IL-8, also called chemokine (C-X-C motif) ligand 8 (CXCL8), recruits inflammatory neutrophils and is involved in the promotion of angiogenesis by synthesis of matrix metalloproteases [68, 77]. IL-8 is produced by many different cell types, including various cancer cells. [68, 77]. High levels of IL-8 are associated with tumor size, stage, drug resistance, angiogenesis and infiltration in several cancer types, including metastatic breast cancer [68, 77].

IL-8 binds to the receptors IL-8RA (CXCR1) and IL-8RB (CXCR2) and stimulates pathways, including PI3-K/Akt and MAPK/ERK [68, 77].

Hypoxia

Solid tumors, such as most breast cancers, often contain regions with low oxygen levels (hypoxia), due to insufficient vascularization, and is a part of the intricate tumor microenvironment [39, 78, 79]. Patients with these tumors often have a poor prognosis, showing more malignant and treatment-resistant properties due to the tumor cells adapting to the lower levels of oxygen, controlled by hypoxia inducible factors (HIFs) [31, 79, 80]. HIFs, often called master transcriptional regulators of the hypoxic response, are involved in numerous processes, such as inducing proliferation, EMT, metastasis, survival, angiogenesis, invasion and metastasis, pH regulation, changes in metabolism and glucose uptake, as well as the maintenance of stem cells [39, 81]. In addition, HIFs have been proposed as a potential therapy target for cancer [78, 79].

HIF1 is a heterodimer consisting of an α-subunit that is only expressed at low oxygen concentrations (oxygen sensitive), and a β-subunit, which is constitutively active [39]. The transcription factor HIF1α is universally expressed in the body, while the other isoforms, HIF2α and HIF3α, are only expressed in some tissues and at varying oxygen levels [79]. Under normoxic conditions (21% O_2), HIF1 has almost no activity, due to low levels of HIF1α. When oxygen is present, oxygen-sensitizing enzymes (hydroxylases) facilitates HIF1α hydroxylation on the oxygen-dependent-degradation domain on HIF1α, leading to ubiquitination and degradation [79]. During hypoxia, HIF1α is stabilized and translocated to the nucleus, where it forms a dimer with HIF1β. The HIF1 complex then bind to different hypoxic response elements (HREs), and interacts with various co-activators to regulate transcription of different target genes.

Aims

The overall aim within the project is to elucidate the role of the microenvironmentally induced autocrine growth factor progranulin and its associated receptor sortilin in breast CSC propagation and prognosis prediction. Limiting breast cancer progression by targeting sortilin could be a potential therapeutic strategy for the treatment of patients with breast tumors having elevated progranulin and sortilin expression.

The more specific aims for each paper are:

Paper I: To determine the influence of progranulin and its receptor sortilin on breast cancer propagation and CSC expansion using functional assays and *in vivo* mouse models.

Paper II: To identify the prognostic value of progranulin and sortilin tumor expression in premenopausal breast cancer patients using a unique randomized tissue microarray cohort with long follow-up.

Paper III: To identify and characterize progranulin-induced secreted compounds that affect CSC activity and determine the clinical relevance of these factors, using functional assays, protein expression analysis, as well as an *in vivo*-like culturing system.

Paper IV: To delineate the role of cleaved progranulin peptides on CSC expansion and their reliance on sortilin receptor binding.

Paper V: To determine the sortilin down-modulating effect of various CADA molecules and identify the most potent candidate inhibiting progranulin-induced CSC propagation that can be selected for further studies *in vivo*.

Methods

Tumor model systems

In vitro models

In this thesis, we have used various established cancer cell lines, including the ERα positive cell lines MCF7 and T47D and the ERα negative cell lines CAL-120, MDA-MB-231 and MDA-MB-468, as well as the non-malignant breast epithelial cell line MCF10a as a control. Established cell lines are commonly used for studying disease mechanisms, as they are inexpensive, easy to grow and maintain in cell culture and experiments can be performed with high throughput [82, 83]. In addition, cell lines allow the identification of active substances and make it easy to control external factors, permitting higher reproducibility [82]. One of the drawbacks of using cell lines for studying tumor development and progression is that these models do not fully resemble real life situations, as they usually do not include surrounding factors (*e.g.* environmental stimuli influencing tumor growth). Moreover, growing cells on plastic can also induce a selective pressure on the cells, altering gene expression and cell phenotype. For drug development, it is therefore important to add optimal model systems in the validation phase to remove less suitable candidates [82, 83].

In vivo animal models

It is difficult to mimic real life situations *in vitro* using only molecular techniques and cell culturing. For that reason, we included mouse models in our study. When working with animals, you always have to keep in mind the principles of the 3Rs: *Replacement*, *Reduction* and *Refinement*, concerning the optimization of animal welfare and minimizing the use of animals in research. Ethics permissions for the use of animal models were obtained by the Research Animal Ethics Committee in Gothenburg.

Mice are suitable models for research on various human diseases due to their similar anatomy and physiology, where 99% of the genes in mice have a human homolog [84]. Mice are relatively small, easy to house and have a short reproduction time. Nevertheless, they have a higher metabolic rate than humans have, and might require a higher drug concentration to get the same biological effect.

For the animal studies performed in this thesis, we used luciferase-tagged breast cancer cells that were injected subcutaneously into NOD/SCID (non-obese diabetic/severe combined immunodeficiency) gamma mice. These

mice are immunocompromised, meaning that they lack functional T- and B-lymphocytes, as well as having reduced NK cell and macrophage functions [85]. Assessments of tumor growth and metastasis were performed using an *In Vivo* Imaging Software (IVIS) whole body imager based on luciferase expression from stably transfected cell lines.

The use of cell line derived xenografts or patient derived xenografts (PDX), where you graft cell lines or tumor cells into immunodeficient mice, are commonly used for studying cancer [86, 87]. These models more accurately mimic human tumors and correlate better with treatment response in patients than *in vitro* models [88]. However, these models can be very time consuming to develop, and since these mice models do not have functional immune cells, it is not possible to study immune responses [89]. Recently, humanization of mouse models, using tumor infiltrating lymphocytes (TILs) and inflammatory cytokines (*e.g.* IL-2) have been developed, as well as the use of genetically engineered mouse models with intact immune systems, where you can study disease progression more accurately [90, 91].

In vivo-like 3D models

To try to minimize the use of animals when performing experiments, various *in vivo*-like 3D models have been developed, generating similar cell responses and even reflect clinical features of the original tumors [92, 93]. These models make it possible to answer important scientific questions related to human health and biology without the use of time-consuming and costly animal experiments.

Matrigel is often used to create a 3D model, as it provides the cells with a 3D environment and ECM components important for cell adhesion and signaling. However, matrigel is a hydrogel with an undefined concentration and content, derived from mouse sarcoma basement membranes, making it hard to reproduce experiments [94].

More recently, various *in vitro* 3D tumor models have been developed, using tissues from different organs and tumors, such as scaffolds derived from cells, tissues or even bioprinted [95]. These models support the growth of cell cultures on 3D structures to study the interaction with the tumor microenvironment. They preserve cell interactions with the ECM in the tumor microenvironment and are implied to generate more robust data for predicting patient outcome and treatment responses [95]. In addition, they can be used for drug screening and provide information about the role of ECM remodeling and malignant properties of the cells affected by the microenvironment during cancer progression [96-98]. In our research group, we have developed *in vivo*-like 3D model systems based on cell-free

patient-derived scaffolds (PDSs) for breast cancer, and more recently also for colorectal cancers that can be used as drug-testing platforms [93, 99, 100].

Experimental methods

Functional cancer stem cell enriching assays

To study CSC characteristics in cells, we performed *in vitro* mammosphere formation assays, to study non-adherent conditions of cells, originating from the neurosphere assay developed in the early 1990s [101]. Here, researchers were able to culture and identify cells with stem cell properties from the adult brain, where only cells surviving anoikis resistance were able to form spheres [47, 101, 102]. These cells have the ability to differentiate and self-renew [101]. The mammosphere formation assay is a relatively time-consuming and slightly complicated assay that involves specific culturing requirements and training to perform and analyze correctly. However, compared to tumor-initiating studies in mice, this assay is an effective way to assess CSC activity in cell lines and tumors.

Gene expression analysis (qPCR)

To measure gene expression in cells or tissues, various methods can be used, including real-time quantitative polymerase chain reaction (qPCR), microarray analysis, hybridization-based assays and various sequencing techniques. qPCR is the most common technique for gene expression measurement, studying biomarkers and validating microarray data [103].

In Paper I, real-time qPCR was performed on single cells or on bulk-level with various gene-specific primers for key regulators or markers for differentiation, proliferation and pluripotency, to define the existence of subpopulations of cells. RNA from cells were extracted, followed by reverse transcription of RNA to complementary DNA (cDNA). Single cell analysis requires cell sorting and preamplification [104]. qPCR on cDNA samples were run with different gene-specific primers using a thermal amplification cycle program with SYBR green detection system, followed by gene expression data analysis on mRNA levels using GenEx™ (MultiID).

Protein analysis

In this thesis, we performed western blots for protein expression of various markers, validation and pathway analysis. Western blots are routinely used in various research fields and has multiple applications, including

protein abundance, protein-protein interaction, cellular location, kinase activity, post-translational modification (phosphorylation, glycosylation) [105]. It is a method used to separate proteins based on their molecular mass, using an electrical field. Proteins are then transferred onto a nitrocellulose membrane and proteins can be detected by the use of specific primary antibodies (binding to antigens or proteins directly). Primary antibody incubation is followed by staining with a secondary antibody labelled with *e.g.* the enzyme horseradish peroxidase (HRP), allowing signal amplification and detection by chemiluminescence [105]. This allows the detection of relative protein concentration at relatively high sensitivity and specificity. However, western blots are relatively time-consuming, as you generally study one protein at a time, and you need to know which protein you are looking for and have adequate antibodies.

Other assays to study protein expression are ELISAs (enzyme-linked immunosorbent assays), where you also look at only one protein at a time. ELISAs require a smaller volume and can be more sensitive than western blots. However, it is not possible to see differences in size of the proteins, *e.g.* if there are isoforms present. Protein arrays, on the other hand, allow you to study multiple target proteins in a single sample. Various protein targets or pathways can be identified and later verified using western blot. Mass spectrometry is another way of studying protein expression at high throughput. Mass spectrometry is expensive and requires a lot of equipment and large sample volumes, but can be used to confirm antibody specificity and determine protein interaction after immune-precipitation. In addition, it is semi-quantitative and can uncover post-translational modifications and detect isoforms. Proximity extension assays (PEAs) are commonly used for cell media and different body fluids. The PEA is based on a set of DNA oligo-labeled antibodies (probes) that when in proximity to their target proteins, they will bind and hybridize. Using a DNA polymerase, the probes are extended and you can then perform pre-amplification of the probes and quantify the levels using a qPCR detection system. This offers a high throughput and requires only a small sample volume. On the other hand, you need to use defined panels and can only get relative protein values.

Patient data and analysis

Tissue microarrays (TMAs) are often used to study patient data, where you can analyze protein expression and potential biomarkers in relation to well-defined patient subgroups. TMAs are a collection of multiple tumor tissue cores, allowing the study of tumors from many different patients at the same time. IHC staining can be used to assess hormone status and proliferation status of patients, as well as detection of a biomarker of interest. These biomarkers can potentially be used as prognostic markers or treatment-predictive indicators for various patients.

In Paper II, we used a TMA from a randomized clinical trial including 444 premenopausal breast cancer patients. These patients were diagnosed between 1984-1991 and received either two years of adjuvant tamoxifen treatment (n=212) or no systemic treatment (n=232). All patients were followed up for up to 30 years. This study was approved by the Ethics Committees of Linköping and Lund Universities in Sweden. Data for patient follow up were taken from the Swedish Causes of Death register. The TMAs were stained for progranulin and sortilin tissue expression to study their relation to other clinical markers and patient outcome. The survival analysis includes time to an event data, *e.g.* time from initial breast cancer diagnosis to disease-specific death. In this thesis, we study breast cancer-specific survival (BCSS).

Kaplan-Meier survival curves are commonly used to estimate the survival probability of an individual over time, providing a summary of the data, as well as an estimation of the median survival time [106]. Hazard ratio (HR) measures the relative survival between two groups (the risk of surviving) [106]. A HR equal to one means that there is no difference in survival between the two groups, while HR above one means an increased mortality (less likely to survive at an indicated time). Moreover, performing multivariable analyses are central, as they take into consideration other factors (covariates) that might affect patient prognosis (survival) [107]. The Cox proportional hazards (CPH) model is a regression model analyzing survival time data in relation to the effects of a set of covariates.

Results and discussion

Paper I - Sortilin inhibition limits secretion-induced progranulin-dependent breast cancer progression and cancer stem cell expansion

Cell-to-cell communication and signaling through secretion of various cytokines and growth factors in the tumor microenvironment can drive tumor progression and influence treatment responses. Similarly, hypoxia is common in solid tumors like breast cancer and influence cancer cells and their secretion. The main hypothesis within this project is that hypoxia induces secretion of proteins and growth factors in the tumor cells, spreading a CSC propagating signal to its surrounding microenvironment. CSCs are thought to be important drivers of tumor progression and treatment resistance [38, 108]. Published data from our research group demonstrates that hypoxia increases the amount of CSCs in ERα positive breast cancer whereas it in contrast decreased the CSC-fraction in ERα negative disease [41, 109]. For that reason, targeting this aggressive subpopulation of cancer cells could be an appealing therapy to improve patient outcome. In this paper, we aimed to elucidate the role of the secreted factor progranulin and its receptor sortilin in breast cancer propagation. Different *in vitro and in vivo* relevant conditions were used to validate breast CSC expansion mediated by progranulin, through its receptor sortilin.

A hypoxic tumor microenvironment induces secretion of components stimulating cancer stem cell activation

In order to study the influence of the microenvironment, and more specifically how a hypoxic environment induce secretion and affect breast CSC activity, we treated different breast cancer cell lines with conditioned media from ERα positive breast cancer cells or primary breast cancer explants cultured in hypoxia. We then examined the mammosphere forming potential of the cell lines treated with the hypoxic conditioned media. Results showed an increase in the mammosphere forming capacity of both ERα positive and ERα negative cell lines with hypoxic conditioned media compared to cells treated with normoxic conditioned media, suggesting that the hypoxic-induced secreted microenvironment from the ERα positive conditioned media induced CSC propagation in various breast cancer cell lines. In contrast, the ERα negative hypoxic condition media has

been shown to decrease the mammosphere forming capacity of the cells, independent on hormone status [41, 109].

Progranulin influences cancer stem cell propagation both *in vitro* and *in vivo* by inducing mammosphere formation and metastasis formation in mice

To define secreted factors within the conditioned media resulting in CSC spreading, a cytokine screen using conditioned media from hypoxic ERα positive breast cancer cells (T47D and MCF7) was performed. From this screen a total of 507 cytokines and proteins were examined and we identified progranulin as being significantly upregulated in the hypoxic ERα positive conditioned media. Indeed, progranulin turned out to be one of the main mediators of CSC activation, shown by an increased mammosphere forming capacity in both progranulin-treated MCF7 and MDA-MB-231 cells. This implies that progranulin is driving CSC propagation in both ERα negative and positive breast cancer.

Next, we assessed if progranulin could influence breast cancer growth and progression *in vivo* by performing repetitive injections of progranulin in tumor-bearing mice using a luciferase-tagged MDA-MB 231 (or T47D) breast cancer cell line xenograft. After 3 weeks of treatment during xenograft growth, there were no significant difference in the tumor burden between progranulin-injected mice and the vehicle control. However, a significant increase in lung metastasis in mice subjected to progranulin injections were observed, demonstrating that progranulin induce tumor progression and mediate a more metastasizing cellular subtype. In addition, when studying tumor initiation, cells pretreated with progranulin were injected into mice in a serial dilution format and required a lower concentration of cells for tumor initiation, suggesting a higher CSC frequency in these cells.

Progranulin is acting through the receptor sortilin

Previously, researchers have observed an increase in extracellular levels of progranulin in the brain after inhibiting sortilin, suggesting that sortilin regulates brain progranulin levels and can be used to treat dementia caused by progranulin haploinsufficiency [110, 111]. Sortilin has been reported to be overexpressed in various cancer types, including melanoma, chronic lymphocytic leukemia (CLL), breast and ovarian carcinomas [62]. Sortilin overexpression is linked to proliferation, migration and invasion in these cancer types, and anti-sortilin antibodies have been proposed as a

therapy option inducing apoptosis in CLL [62]. In prostate cancer, reports have shown that progranulin binds to sortilin, leading to progranulin internalization and degradation, suppressing progranulin-induced proliferation, migration and anchor-independent growth [112]. However, in ovarian carcinoma, sortilin knockdown induces apoptosis and reduces proliferation [113], implying that most studies regarding sortilin in cancer link high levels of sortilin with bad prognosis. To illuminate the role of sortilin in progranulin-induced breast cancer spreading we assessed several ways of targeting sortilin with the aim to inhibit the progranulin induced CSC propagation *in vitro*, using different breast cancer cell lines. By targeting sortilin using small interfering RNA (siRNA) for sortilin, a sortilin degrader (1-[2-(2-tert-butyl-5-methylphenoxy)-ethyl]-3-methyl-piperidine, termed MPEP) or by pharmacological inhibition of sortilin using a small sortilin-binding compound (AF38469 [114]), we were able to inhibit the progranulin mediated mammosphere forming capacity of MDA-MB 231 cells. Similar results could be observed using MCF7, T47D and CAL-120 cell lines.

To test if sortilin inhibition had the same effect on the progranulin-induced cancer progression *in vivo*, we used a more potent part of the progranulin protein, named granulin A, as it had a more pronounced effect *in vivo* compared to full-length progranulin. Importantly, results showed that the granulin A-induced increase in lung metastasis could be blocked by AF38469.

Combined, these results demonstrate that sortilin is a functional receptor of progranulin and is responsible for driving progranulin-induced breast CSC propagation *in vitro*, leading to tumor progression and metastasis, as shown *in vivo*.

Paper II - Tumor co-expression of progranulin and sortilin as a prognostic biomarker in breast cancer

Each year, almost 1400 women in Sweden dies from breast cancer [2]. The survival rate from breast cancer is relatively high. Nonetheless, many patients experience therapy resistance and tumor relapse. As breast cancer is the most common cancer in women, with more than 8000 diagnosed in Sweden every year, early detection and identification of robust clinical markers are central to increase treatment efficiency and patient survival. In Paper I, we established the role of progranulin and sortilin on breast CSC propagation, tumor progression and metastasis [115]. In paper II, we determined the clinical impact of progranulin and sortilin tumor expression in breast cancer and investigated if these markers can be used as prognostic or treatment-predictive biomarkers for breast cancer patients.

To evaluate whether progranulin and sortilin tumor expression could be used as biomarkers in breast cancer, we analyzed protein expression data from a TMA consisting of 560 premenopausal breast cancer patients with long follow-up time. These patients were randomized and given either two years of tamoxifen treatment or no adjuvant therapy. The samples were stained for progranulin and sortilin tumor tissue expression using IHC, then scored for high or low expression and evaluated in relation to various clinical markers and patient outcomes. In this breast cancer cohort, more than 70% of the patients were positive for ERα, which is representative for the whole population of breast cancer patients [28].

Progranulin and sortilin scoring and association with clinical parameters

Progranulin and sortilin tissue expression were evaluated by IHC, using specific antibodies for progranulin and sortilin. Tissue microarrays from 444 patients with good enough material and clinical data were successfully stained and selected for further analysis. The staining for each patient were then given a score 1-4, where l-2 were seen as a low expression of the markers and 3-4 as high expression of the markers. In this cohort, 50% of the patients were categorized as having high sortilin tissue expression, and the other 50% as having low expression. For progranulin, 66% were categorized in the high expression group, and 34% in the low expression group.

Interestingly, there was a positive correlation with the expression of progranulin and sortilin, where patients with high expression of progranulin also tended to have a higher expression of sortilin. In addition, high progranulin expression significantly correlated with high grade, the proliferation marker Ki67 and the hypoxic marker HIF1α, all associated with CSC characteristics, tumor aggressiveness, resistance and formation of the pre-metastatic niche in solid cancers [19, 116, 117]. This indicates that tumors with high expression of progranulin tend to be more aggressive.

Further, ERα positive tumors had lower expression of progranulin than ERα negative tumors. This correlates with results from other researchers [118], as well as from Paper I where secretion of progranulin were higher in MDA-MB-231 than in MCF7 cells. In contrast, sortilin expression was higher in ERα positive tumors, and high sortilin expression correlated negatively with age.

Patients with high co-expression of progranulin and sortilin have worse breast cancer-specific survival

In addition to the clinical relevance for progranulin as a prognostic and predictive biomarkers in various types of cancer [58, 119-121], sortilin has been shown to be overexpressed various cancer types, including prostate, ovarian and breast cancers [62]. As recent work in our lab (Paper I), as well as that other researchers have reported sortilin as a functional receptor for progranulin and its link to more clinically aggressive properties [112, 115, 122], we set out to determine if a combination of both progranulin and sortilin tumor expression were related to BCSS in our patient cohort. Dual tissue expression of progranulin and sortilin could be scored in 395 of the 444 tumors, where 20% of these were double high, expressing both high progranulin and high sortilin levels. When comparing the double high group against tumors with variable expression levels of both progranulin and sortilin, we observed a significantly decreased BCSS in the double high group compared to the mixed expression group for all patients in the cohort. In addition, this effect was also observed when looking at patients not receiving initial tamoxifen treatment, providing prognostic information independent of treatment interference.

Only 14% of the patients had high levels of progranulin and low levels of sortilin, showing that most of the patients with high progranulin levels also had high sortilin levels. When analyzing the groups with various expression levels of sortilin and progranulin separately, the difference in BCSS between double high and high single progranulin expression was not

significant. However, when using multivariate analysis and studying the high progranulin group alone, we detected that high sortilin expression in this group was related to lower BCSS. This implies that sortilin adds prognostic information on survival when combined with progranulin and that there is a link between progranulin and sortilin expression. We therefore hypothesize that targeting the progranulin-sortilin axis may be a therapeutic option for this patient group with high co-expression of progranulin and sortilin.

To further strengthen our results, we performed multivariate CPH regression analysis adjusting for known prognostic factors, such as histological grade, tumor size, age, lymph node status, ERα status and treatment, in addition to progranulin and sortilin score. In these analyses, we identified co-expression progranulin and sortilin, together with high tumor grade and lymph node positivity as independent prognostic covariates, associated with high risk factors giving a lower BCSS.

Furthermore, when analyzing the ERα positive breast cancer patients separately to study the tamoxifen treatment effect, our results indicates that progranulin expression is not associated with tamoxifen resistance. These results are in contrast to previous reports suggesting a link between progranulin expression and tamoxifen resistance in ERα positive breast cancer patients [123]. However, patients in this cohort only received tamoxifen treatment for two years, opposed to the now recommended five to ten years [124, 125], implying that further studies are needed in order to determine the role of progranulin in tamoxifen treatment resistance. In addition, any following treatment decisions after the initial trial start are not taken into consideration, which may have an impact on the results. To further validate and strengthen our results, an external cohort should be added to make sure the model can be applied to other datasets before the use in clinical practice. Off note, this cohort only included premenopausal breast cancer patients and therefore may not be representative of the whole population.

Collectively, these results suggest that co-expression of progranulin and sortilin defines a highly malignant subgroup of breast cancer patients, and that targeting progranulin through its receptor sortilin could be a potential novel breast cancer therapeutic approach in addition to conventional treatment strategies.

Paper III - Interleukin-6 induces stem cell propagation through liaison with the sortilin-progranulin axis in breast cancer

The tumor microenvironment plays an important role in tumor progression and therapy resistance, where cell-to-cell communication is affected by secretion of signaling molecules and interactions with the surrounding tumor stroma [38, 126]. To further study the effect of progranulin on cancer cells, we aimed to elucidate the role of progranulin-induced secretion of cytokines and growth factors on the CSC population in breast cancer.

Progranulin-induced secretion in breast cancer cells

Our research group is focusing on the crosstalk between the tumor microenvironment and the cancer cells, including the impact of cell secretion. In this study, we intended to explore how progranulin affects secretion from our breast cancer cell lines. In addition, we examined if the enhancing effects on the CSC population caused by progranulin-induced secretion could be prevented by the use of sortilin modulators. This was accomplished by analyzing conditioned media from breast cancer cell lines treated with progranulin, the sortilin inhibitor AF38469, or a combination of both, using a proximity extension assay (PEA) performed by OLINK at SciLifeLab in Uppsala. Panels containing cardiovascular and immuno-oncology markers were chosen for this high throughput multiplex assay due to their relevance and involvement in tumor biology. This allowed us to study almost 200 different proteins. The secretion screen revealed distinct secretion profiles between the treatments in MCF7 and MDA-MB-231 cells. In particular, progranulin treatment increased the secretion of IL-6 and IL-8, as well as other cytokines involved in inflammation, metastasis and CSC formation, including tumor necrosis factor (TNF), CXCL1, Fas ligand (FASL) [116, 127-133]. Secretion profiles and cytokines induced by the other treatment combinations should also be studied in more detail.

Crosstalk between progranulin and IL-6 expression in breast cancer cells

Further, to validate if progranulin-induced secretion of IL-6 and IL-8 also affected the internal expression of these interleukins. Western blot analysis on cell lysates confirmed that progranulin treatment induced protein expression of both IL-6 and IL-8 in a dose-dependent manner. In

line with this, other researchers have observed that treatment with IL-6 in liver and bile duct cancer increased the expression of progranulin *in vitro*, as well as that progranulin-induced production of IL-6 in adipose tissue [134-136]. However, to our knowledge, no feedback loop have been documented in the same system. Importantly, treating breast cancer cell lines with increasing concentrations of IL-6 or IL-8 elevated the levels of progranulin in the cells in a dose-dependent manner, suggesting a crosstalk between the expression of progranulin and IL-6, and IL-8.

IL-6-induced CSC propagation and its dependence on sortilin

IL-6 and IL-8 have both been associated with the induction of CSC characteristics in cancer [71, 137]. Therefore, we wanted to explore if IL-6 and IL-8 affect the amount of CSCs in MCF7 and MDA-MB-231 breast cancer cells. When treating the cells with recombinant IL-6 or IL-8, we observed an increase in mammosphere formation, similar to what we could see with progranulin. This indicates that both IL-6 and IL-8 mediate breast CSC expansion in breast cancer.

Interestingly, sortilin has also been suggested as a high-affinity receptor for IL-6 in immune cells [64, 138]. Using a FPA, we were able to show that IL-6 outcompeted the fluorescently labeled neurotensin bound to the soluble sortilin receptor, confirming this interaction. However, IL-8 did not outcompete neurotensin, hence did not bind to sortilin at this binding site. Next, we explored if the CSC effect caused by the cytokine treatment was dependent on, or regulated by the binding to sortilin. Results showed that only the IL-6-induced mammosphere formation in MCF7 and MDA-MB-231 cells were dependent on sortilin, using the sortilin-binding molecule, AF38469. Although, more studies are needed to evaluate if the IL-6- and progranulin-induced sphere formation acts directly or indirectly via sortilin, or if there are other pathways and mechanisms involved.

Correlation between progranulin and IL-6 in an in vivo-like model system

Primary derived scaffolds (PDS models) are emerging as a model to study the influence of the tumor microenvironment on various types of cancer, by mimicking the growth of the cells in a more *in vivo*-like situation [93, 95, 97]. A previous study form our group which included RNA sequencing where we compared cells grown in the PDS model with cells derived from *in vivo*-like xenografts, as well as normal 2D growth conditions [93].

Analysis revealed that cells grown in PDS cultures were more similar to *in vivo* conditions compared to 2D. The PDS model contains intact ECM components and signaling molecules, and has properties associated with the clinical parameters of the original tumor [93]. The cells grown on PDSs have a genomic profile, as well as a proteomic profile of secreted proteins showing involvement in, among others, differentiation, EMT and stemness markers compared to cells grown as conventional 2D cultures [93, 95, 139]. In addition, when studying the data in more detail, we observed an upregulation of IL-6, IL-8 and GRN (progranulin) messenger RNA (mRNA) expression in the PDS system, as well as the receptors SORT1 (sortilin) and IL-6 receptors (IL-6R and GP130) compared to 2D. This confirms the importance of these genes in association with CSC characteristics and involvement in the priming of the pre-metastatic niche [59, 133].

Further, when looking more specifically at the secretion induced by cells grown in the PDS model, we observed that cells grown in specific PDSs mimicked clinical features of the original tumor [92]. Focusing on the correlation between secretions of relevant molecules in this study, we observed a positive correlation between IL-6 and progranulin in both MCF7 and MDA-MB-231 cells. In MCF7 cells, there was a positive correlation between IL-6 and IL-8, while this correlation was negative in the MDA-MB-231 cell line. In the MDA-MB-231 cell line, there was also a negative correlation between IL-8 and progranulin. Interestingly, MDA-MB-231 cells grown in PDSs had a higher basal secretion, which is consistent with other studies [140].

Paper IV - Granulin peptide domains induce breast cancer stem cell propagation via sortilin

The balance between progranulin and its peptide domains are regulated by naturally occurring proteases and protease inhibitors [60]. As of today, an understanding about the role of the eight progranulin cleaved peptide domains, including their function and binding partners, are still modest [60, 141].

The enzyme human neutrophil elastase cleaves progranulin into smaller fragments

In Paper I, we showed that granulin A was an active progranulin domain affecting CSC activity in several breast cancer cell lines. This suggests that some of the granulin domains themselves are important mediators of the progranulin effect. In this part of the project, we aim to better define how progranulin is cleaved and delineate the CSC functions of the individual peptide domains compared to the full-length progranulin.

The growth factor progranulin is thought to have anti-inflammatory properties, while the granulin domains are thought to be pro-inflammatory, suggesting that they might have contrasting functions in the cells [141]. Cleavage of progranulin occurs naturally by several enzymes, such as elastase, and can be blocked by various protease inhibitors, thereby controlling the balance between progranulin and the granulin domains [60, 141]. In this study, we hypothesize that this balance might be lost during the formation of breast cancer. Western blot analysis confirmed that progranulin could be cleaved into smaller peptide fragments by human neutrophil elastase. Further, progranulin cleavage was blocked by adding the protease inhibitor secretory leukocyte protease inhibitor (SLPI), leaving the full-length progranulin intact. Moreover, sphere formation in MCF7 cells treated with progranulin or elastase-cleaved progranulin revealed an additive effect of the cleaved peptides compared to full-length progranulin, suggesting that the small peptide fragments indeed have potent CSC activity.

Cleaved progranulin peptide domains increase breast cancer stem cell activity

To delineate which of the granulin peptides that were responsible for affecting the mammosphere-inducing ability of the cells, we treated breast cancer cell lines with individual peptide domains synthesized according to

their amino acid sequences. We also included the small C-terminal part of progranulin, as it has been shown to be required for progranulins binding to sortilin and may have an effect of its own [111]. Results showed that of the eight peptides testes, only para-granulin, granulin A, granulin C and the C-terminal part of progranulin induced mammosphere formation, suggesting that these granulin domains indeed have tumor-progressing properties.

Progranulin domains binds to sortilin

To identify the exact mechanism on how individual granulin domains induce CSC propagation, we investigated the role of the progranulin receptor sortilin on these domains. From our FPA, we were able to show that para-granulin, granulin A and the C-terminal part of progranulin bind to sortilin at the same site as progranulin, with the C-terminal part of progranulin showing the highest binding potency. This is in line with data published by other researchers [110, 111]. Interestingly, these sortilin-binding domains were also the granulins capable of inducing CSC activity *in vitro*. Distinctively, granulin C induced sphere formation in vitro, but did not seem to bind to sortilin (data not shown). Moreover, treatment with a sortilin degrader effectively reduced the peptide-induced mammosphere increase in MCF7, seen with para-granulin, granulin A and C-terminal part of progranulin. Although, further studies are required to identify and evaluate other binding partners or binding at different sites on sortilin.

In summary, the individual granulin peptides para-granulin, granulin A and the C-terminal part of progranulin induce CSC growth in a sortilin-dependent manner in breast cancer.

Paper V - Reduction of progranulin-induced breast cancer stem cell propagation by sortilin-targeting cyclotriazadisulfonamide (CADA) compounds

We have demonstrated that by restricting the binding of progranulin to sortilin, we could block progranulin-induced mammosphere formation *in vitro*, as well as granulin A-induced lung metastasis in an *in vivo* xenograft model (Paper I) [115]. In addition, clinical data proposes progranulin and sortilin as prognostic markers in breast cancer and other cancer types (Paper II) [58, 122, 142]. Subsequently, sortilin could be a potential novel drug target for breast cancer.

In order to block the interaction between progranulin and sortilin we used the antiviral agent cyclotriazadisulfonamide (CADA). CADA has been shown to down-regulate cluster of differentiation 4 (CD4) expression in T-cell lines and peripheral blood mononuclear cells, resulting in a marked inhibition of the human immunodeficiency virus (HIV) entry to the host cell [143]. Importantly, CADA also modulates sortilin expression by binding to its signal peptide and inhibits the co-translational translocation of sortilin to the lumen of the endoplasmic reticulum, giving a 50% reduction in sortilin protein expression [144]. Therefore, in collaboration with the inventors of the CADA molecules, we aimed to identify CADA analogs that have a more than 50% downregulation of sortilin expression and could effectively reduce breast cancer stem cell propagation induced by progranulin.

CADA molecules down-modulate sortilin in breast cancer cell lines

A range of synthesized compound similar to CADA were tested for cellular toxicity, looking at cell viability/proliferation and for potency, in both breast cancer cell lines and HEK239 cells. In addition, the compounds ability to downregulate sortilin expression were also tested in two different breast cancer cell lines, one ERα positive (MCF7) and one ERα negative (MDA-MB-231). Seven of these compounds were included in Paper V. Results showed that out of the seven CADA compounds; compounds **2**, **5** and **6** were the most potent analogs, efficiently downregulating the expression of sortilin in both the ERα positive breast cancer cell line MCF7 and the ERα negative cell line MDA-MB-231.

CADA molecules reduce progranulin-induced CSC propagation in breast cancer cell lines

The most potent sortilin down-modulating compounds were further tested for their ability to reduce progranulin-induced CSC propagation. We chose a concentration of 1 µM of the compounds, due to their good down-modulatory effect on sortilin expression, while at the same time showing no significant reduction in cell viability. Importantly, several of the CADA compounds showed a significant inhibition on the progranulin-induced mammosphere formation, independent on hormone status.

The most potent sortilin-down-modulators were found to be compound **2**, **5** and **6**, where compound **2** was selected as the top candidate, due to the slightly higher toxicity seen with compounds **5** and **6**, especially in the HEK293 cell line. Results also showed that less sortilin expression on its own did not lead to a reduction in the mammosphere forming capacity of the cells, suggesting that the sphere inhibitory effect is due to the progranulin stimulation being blocked.

Conclusively, based on flow cytometry data on sortilin expression and toxicity data on HEK293 cells, together with the western blot protein expression data on the breast cancer cell lines, substance **2** was identified as the most selective compound showing least toxicity. Furthermore, compound **2** efficiently blocked progranulin-induced breast CSC expansion *in vitro*, independent on ERα status, and we are therefore selecting this compound for further optimization and upcoming *in vivo* studies.

Conclusions

Breast cancer is a very heterogeneous disease, containing different cell populations interacting in complex networks consisting of various secretion systems and components in the surrounding tumor microenvironment. It is necessary to understand the molecular mechanisms and signaling pathways involved in cancer progression and metastasis formation, and more evidence are emerging that the CSC niche and the tumor microenvironment are partly responsible for this effect. In this thesis, we have identified several markers and potential pathways that are important for the maintenance of the CSC population in breast cancer. These factors can potentially be used as biomarkers for cancer progression and treatment prediction or as possible future drug targets in order to improve existing treatment approaches.

More specifically:

Paper I: Here, we identified progranulin as a hypoxia-induced secreted factor influencing breast CSC propagation *in vitro*, as well as promoting tumor progression and mediating a more metastasizing cellular subtype *in vivo*. In addition, by targeting the progranulin receptor sortilin, through small sortilin-binding molecules or by degradation, we could demonstrate that sortilin is a functional receptor of progranulin in breast cancer and is responsible for driving the progranulin induced breast CSC propagation *in vitro* and *in vivo*.

Paper II: We found that elevated progranulin and sortilin tumor protein levels are associated with unfavorable clinical prognosis and poor outcomes in breast cancer patients. In summary, these results suggest that high co-expression of progranulin and sortilin defines a highly malignant subgroup of breast cancer patients and can be used as a prognostic biomarker in breast cancer patients.

Paper III: Here, we further described how progranulin induced secretion of cytokines involved in CSC propagation. In conclusion, the interplay between IL-6 and the progranulin-sortilin axis in breast cancer is responsible for driving CSC activity and are linked to aggressive features in breast cancer.

Paper IV: During different biological circumstances, progranulin can be cleaved into smaller peptide fragments by human neutrophil elastase. In this paper, we demonstrated that specific granulin peptide domains induce

CSC propagation in breast cancer and that these peptides bind and act through sortilin in a similar manner as progranulin.

Paper V: In this paper, we identified chemical compounds (CADA compounds) with high potency towards down-modulating sortilin protein expression and subsequently reducing progranulin-induced CSC activity. These CADA compounds were highly selective with low cellular toxicity at optimal sortilin down-modulating conditions and can be further evaluated as therapeutic compounds in relevant *in vivo* models.

Taken together, targeting progranulin through its receptor sortilin could be a potential novel breast cancer therapeutic approach, used as a combination therapy together with chemotherapy or other conventional subtype-based treatments.

Future perspectives

This thesis has provided substantial evidence for the involvement of progranulin and sortilin in breast CSC propagation and patient outcome. These findings are supported by studies from other researchers, highlighting the importance of targeting the progranulin-sortilin axis as a potential treatment strategy for cancer patients expressing high levels of progranulin or sortilin. However, more experiments are needed to further elucidate the mechanisms and pathways involved.

Sortilin targeted therapy in breast cancer

Neutralizing antibodies for progranulin are currently under preclinical development for treating cancers, such as lung and breast cancer [141]. A different therapeutic approach could be by targeting the progranulin receptor sortilin, as sortilin overexpression is seen in various types of cancers compared to normal cells, and has been shown to play a role in CSC activity, differentiation, EMT and invasion [62]. In cancer, anti-sortilin antibody treatment has been shown to induce apoptosis in leukemic cells without affecting normal cells [145], and in pancreatic cancer, sortilin knockdown by siRNA or inhibition by AF38469, obstructs pancreatic cancer cell adhesion and invasion [146]. Moreover, our studies have shown that, *in vivo*, AF38469 blocks the progranulin-induced lung metastasis in mice [115]. This implies that targeting sortilin may be a therapeutic option to treat various types of cancer, and might be worth exploring further [62]. Moreover, sortilin is also a receptor for the neuronal growth factor neurotensin. In several types of cancer, sortilin has been shown to induce neurotensin-mediated cancer cell growth and proliferation, emphasizing the need to study the role of neurotensin in breast cancer [62].

Currently, in collaboration with SciLifeLab drug discovery and development platform, external chemists, Sortina Pharma AB, GU Venture and the Grants and Innovation Office at the University of Gothenburg, we are identifying and developing new sortilin targeted compounds, such as small molecule sortilin inhibitors and sortilin-targeting antibodies. We aim to identify sortilin-targeted drugs for treatment of patients with imbalance in the progranulin-sortilin signaling pathway in order to reduce the progranulin-induced CSC spreading and metastasis formation. Novel synthesized compounds are tested using our sortilin-binding FPA setup. Potential sortilin-binding compounds are further validated using the

mammosphere assay, as well as additional functional assays *in vitro*, before validation of top candidates *in vivo*. Our long-term goal is to develop a sortilin-based drug for clinical trials. We anticipate that the developed drug will probably not eradicate tumor cells itself, but will limit the fraction of CSCs that in the long term will suppress the tumor and metastasis formation by limiting the repopulation capacity of the tumor (Figure 9). These drugs can be used as an adjuvant therapy in combination with other cancer-specific targeting drugs such as tamoxifen.

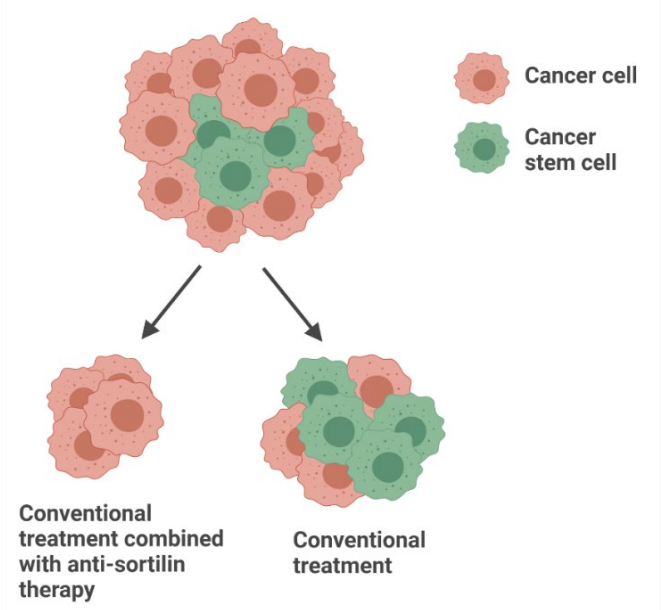

Figure 9: Cancer stem cell-targeted therapy. Progranulin or sortilin could be an attractive target for breast cancer therapy, targeting the resistant cancer stem cells. Combined with conventional treatment to prevent resistance and cancer recurrence. Image adapted from [147] and created with BioRender.com.

Further, sortilin also exists naturally in a soluble form, termed soluble sortilin (sSortilin), derived from transmembrane sortilin cleaved by various proteases [62]. In neuroendocrine tumors and colon cancer, sSortilin have been shown to be involved in tumorigenesis and cancer progression through the activation of focal adhesion kinase (FAK)-Scr signaling, through interaction with an unidentified receptor on the target cells, leading to FAK-Scr phosphorylation [62]. This phosphorylation avtivates pathways, including Akt and PI3K that are involved in cell survival, migration and tumor invasion [62]. In addition, sSortilin leads to loss of cellular matrix contact due to actin microfilament modifications in the plasma membrane

in some cancers [62]. More investigations are needed to determine the importance of sSortilin in breast cancer and its role as a potential plasma biomarker for various diseases [62].

Besides sortilin, additional receptors are known to bind to progranulin, such as the inflammatory receptors TNF receptor 1 and 2, enlightening progranulins role in inflammation, as well as EphA2 that are expressed in several types of cancer and are associated with cancer formation and progression [148]. As these receptors also bind to the growth factor progranulin, we aim to further investigate the TNF receptors and EphA2 to delineate their role in breast cancer and involvement in triggering cellular plasticity.

The PDS model as a tool to improve personalized medicine

As mentioned in the methods section, the PDS model can be used as a drug-screening tool for more patient-specific studies to investigate the therapeutic effect by novel compounds and drug combinations, as well as performing efficacy and toxicity studies on cancer cells growing in a patient-specific tumor microenvironment.

In this project, we aim to use the PDS model to combine sortilin-targeting drugs with chemotherapy, as well as in combination with endocrine treatment, providing us with the possibility to evaluate treatment efficiency and responses in tumor microenvironments derived from individual patients. Here, we can also correlate the PDS response with liquid biopsies from the patients, where you use the serum of patients to study secreted proteins or even circulating tumor DNA (ctDNA) looking for altered genes or identification of biomarkers, such as progranulin, sortilin and IL-6.

The role of the progranulin peptides

As progranulin is known to be cleaved into smaller peptide domains, we aimed to delineate the role of the individual progranulin peptides on CSC activity in breast cancer (Paper IV). Little is known about the functions and mechanisms regulated by these individual peptide domains in cancer, and seems to be dependent on the cell type tested [60, 149]. In epithelial cells, granulin A and B lead to growth inhibition and, in contrast, granulin D has shown proliferative effects in glioma cells [60]. Some studies even suggest that epithelial cells induce production of IL-8 in response to granulin B stimulation [60]. This could indicate that the increase in IL-8 secretion described in Paper III is due to progranulin being cleaved by

proteinases in the cells, although further investigations are needed in order to determine this.

In Paper IV, we demonstrated that in breast cancer, some of these cleaved progranulin peptides also bind to sortilin, and granulin A induced lung metastasis in *in vivo* breast xenograft models (Paper I) [115]. However, more mechanistic data and knowledge about pathways regulated by the individual granulin peptides are needed. Interestingly, Zhang and colleagues are developing antibodies specifically targeting the individual granulin peptides, making it possible to study the individual peptides in more detail [150]. Notably, as progranulin is cleaved by various proteases in the cells, it is not always a complete cleavage into the individual granulins, as various lengths and combinations of the peptides are possible. These combinations of peptide lengths can also have an effect on the cells and need to be further investigated.

Progranulin and sortilin relevance in other cancer types

Progranulin and sortilin expression are thought to be elevated not only in breast cancer, but also in other cancer types, such as prostate and pancreatic cancers, melanoma, as well as kidney and lung carcinomas, and are linked to more aggressive clinical phenotypes [58, 62]. To further support our findings and to expand our research field, we will incorporate other cancer types to our research methods, in order to explore the progranulin-sortilin axis and their relevance in other types of cancer.

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