The breast cancer microenvironment and cancer cell secretion

- specific effects on cancer progression and subtypes of cancer cells

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Gothenburg 2021

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The breast cancer microenvironment and cancer cell secretion – specific effects on cancer progression and subtypes of cancer cells

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ISBN 978-91-8009-182-4 (PRINT)
ISBN 978-91-8009-183-1 (PDF)

Printed in Borås, Sweden 2021
Printed by Stema Specialtryck AB

Tillägnad mamma och pappa

ABSTRACT

Breast cancer is the cancer form responsible for the most cancer-related deaths among women worldwide, and novel targeted therapies are highly needed. The tumor microenvironment consists of several components, including different cell types, extracellular matrix, oxygen and nutrient gradients and soluble factors that plays a key role in cancer progression. Cancer cell secretion affects tumor characteristics, such as proliferation, migration, invasion and priming of the pre-metastatic niche. In this thesis, we have investigated the effect of tumor microenvironmental-induced secretion by studying hypoxia and the extracellular matrix and the induction of secretion in relation to cancer progression and subpopulations of breast cancer cells. We demonstrated that hypoxia-induced secretion affects the cancer stem cell subpopulation, but in opposing directions depending on estrogen receptor status. Moreover, by developing a novel *in vivo*-like model based on decellularized breast cancer tissue we could show induced changes in reintroduced cell lines in gene expression and cell secretion, both towards a more dedifferentiated cell state compared to monolayer cells. In addition, we demonstrated that one subgroup of decellularized breast cancers induced secretion of proteins such as interlukin-6, chemokine (C-C motif) ligand 2 and plasminogen activator inhibitor 1, all associated with cancer stem cell characteristics and priming of the pre-metastatic niche. This subgroup also included tumors of higher grade and with shorter patient relapse-free survival, further displaying the aggressiveness of these microenvironments. Further, we revealed that the well-known cancer stem cell inducing cytokine interlukin-6 increased after treatment with the hypoxia-induced growth factor progranulin and that interlukin-6 increased the cancer stem cell propagation in a sortilin dependent way. In conclusion, in this thesis we explored the importance of the tumor microenvironment and continued to unravel the complex network of tumor microenvironmental-induced secretion and the significance for breast cancer progression and patient outcome.

Keywords: Breast cancer, cancer microenvironment, secretion, hypoxia

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SAMMANFATTNING PÅ SVENSKA

Bröstcancer är den vanligaste cancerformen hos kvinnor i världen och det finns ett ständigt stort behov för nya läkemedel och behandlingsstrategier. Det är inte bara cancercellerna i sig själva som bidrar till tillväxt av cancer, utan även den miljö som cellerna växer i, den så kallade mikromiljön. Den påverkar många av cancerns egenskaper så som tillväxt, spridning och motståndskraft mot läkemedel. Tumörens mikromiljö består av många olika delar så som olika celltyper, proteiner som bygger upp tumörskelettet, tillväxtfaktorer och andra signalmolekyler. Signalmolekylerna produceras i cellen, men transporteras sedan till utsidan för att signalera till andra celler i närheten, eller andra delar av kroppen. De olika signalmolekylerna påverkar cancern genom att bland annat reglera tillväxt och genom att via blodet cirkulera till andra organ i kroppen för att cancercellerna skall kunna börja växa där. I den här avhandlingen har vi studerat utsöndring av proteiner och hur denna kan påverkas av olika faktorer i mikromiljön. Vi har bland annat visat att låga halter av syre påverkar vissa cancerceller som kallas cancerstamceller. Cancerstamceller har föreslagits vara de cancercellerna som är ansvariga för och kan initiera spridning av cancern samt påverka hur tumören svarar på olika behandlingar. Vi visade här att celler som uttrycker östrogenreceptorn på sin yta utsöndrar proteiner som ökar mängden cancerstamceller och celler som saknar receptorn utsöndrar proteiner som minskar antalet cancerstamceller. Vi har även utvecklat en metod för att kunna studera den unika mikromiljön av specifika tumörer från patienter, genom så kallade cellfria tumörskelett som består av allt från en tumör förutom celler. När vi tillsatte cancerceller till tumörskeletten kunde vi se att de inducerade förändringar i cellernas genuttryck samt utsöndring av olika proteiner. Dessa förändringar påverkades av karaktärsdrag så som grad av den ursprungliga tumören som användes för att genera modellen. Från dessa tumörskelett identifierade vi en grupp av patienter vars tumörer fick cellerna att utsöndra höga mängder av proteinerna IL-6, CCL2 och PAI. Dessa proteiner är sedan tidigare kända för att påverka cancerstamceller och spridningen av cancer. Därefter kunde vi visa att IL-6 påverkar cancerstamcellerna via en receptor på cellernas yta som heter sortilin. Vidare studier krävs, men eventuellt kan receptorn sortilin användas för att designa nya läkemedel för att behandla bröstcancer. Sammanfattningsvis, i denna avhandling har vi visat att mikromiljön i bröstcancer är mycket viktig och avslöjar tidigare dold information om patienter.

LIST OF PAPERS

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

Paper I

Hypoxia-induced secretion stimulates breast cancer stem cell regulatory signalling pathways

Jacobsson H., Harrison H., Hughes É., Persson E., Rhost S., Fitzpatrick P., Gustafsson A., Andersson D., Gregersson P., Magnusson Y., Ståhlberg A., Landberg G.
Mol. Oncol. 2019; 13(8): 1693-1705

Paper II

Patient-derived scaffolds uncover breast cancer promoting properties of the microenvironment

Landberg G., Fitzpatrick P., Isakson P., Jonasson E., Karlsson J., Larsson Lekholm E., Svanstrom A., Rafnsdottir S., Persson E., Gustafsson A., Andersson D., Gregersson P., Magnusson Y., Håkansson J. and Ståhlberg A.
Biomaterials 2020; 235; 119705

Paper III

Patient-derived scaffolds influence secretion profiles in cancer cells mirroring clinical features and breast cancer subtypes

Persson E., Gregersson P., Gustafsson A., Fitzpatrick P., Rhost S., Ståhlberg A., Landberg G.
Manuscript

Paper IV

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

Berger K*., Persson E*, Gregersson P., Jonasson E., Ståhlberg A., Landberg G., Rhost S.

**Equal contribution. Manuscript*

Additional publications not part of this thesis

Paper i

Identification of breast cancer stem cell related genes using functional cellular assays combined with single-cell RNA sequencing in MDA-MB-231 cells

Jonasson E., Ghannoum S., Persson E., Karlsson J., Kroneis T., Larsson E., Landberg G., Ståhlberg A.
Front Genet. 2019; 10:500

Paper ii

Characterization of cell-free breast cancer patient-derived scaffolds using liquid chromatography-mass spectrometry/mass spectrometry data and RNA sequencing data

Landberg G., Jonasson E., Gustafsson A., Fitzpatrick P., Isakson P., Karlsson J., Larsson Lekholm E., Svanstrom A., Rafnsdottir S., Persson E., Andersson D., Rosendahl J., Petronis S., Ranji P., Gregersson P., Magnusson Y., Håkansson J. and Ståhlberg A.
Data brief. 2020; 16(31); 105860

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ABBREVIATIONS

AF	AF38469
BMI	body mass index
BRCA	breast cancer type 1suceptabiity protein
C-X-C	cystein-X-cystein
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial to mesenchymal transition
EphA2	ephrin type-A receptor 2
ER	estrogen receptor
gp130	glycoprotein 130
HDL/Apo A-I	high-density lipoprotein/apolioprotein A-I
HER2	human epidermal growth factor receptor 2
HGF	hepatocyte growth factor
HIF1α	hypoxia-inducible factor 1 alpha
IL12RB2	interleukin 12 receptor beta 2 subunit
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
IL-8	interleukin-8
LM	laminin
LOD	limit of detection
LOX	lysyl oxidase

MMP	matrix metallopeptidase
NK-cells	natural-killer cells
PDS	patient-derived scaffold
PDX	patient-derived xenograft
PEA	proximity extension assay
PLA	proximity ligation assay
PR	progesterone receptor
sIL-6R	secreted interleukin-6 receptor
SLPI	secretory leucocyte protease inhibitor protein
SOM	self-organizing map
TGF-β	transforming growth factor beta
TNFR	tumor necrosis factor receptor
VPS10	vacuolar protein sorting/targeting protein 10

INTRODUCTION

BREAST CANCER

Malignancies were the second most common cause of death in Sweden 2019 [1] and new treatment options and targeted therapies are highly needed. In 2018, breast cancer affected 5% of women under the age of 75 years and was therefore the most common cancer amongst women worldwide [2]. The risk of developing breast cancer increases with age and numerous intrinsic factors including sex, race and genetic background also affect the risk. Mutations in oncogenes, which are genes that contribute to cancer progression when activated, or in suppressor genes, that are genes where loss of function contributes to cancer progression, are drivers of cancer [3]. Approximately 5-10 % of all breast cancer cases are familial, and caused by known inherited genetic factors. The most common mutations in breast cancer are mutations in the tumor suppressor breast cancer type 1 susceptibility protein 1 and 2 (*BRCA1* and *BRCA2*). Mutations in these genes increases the risk of developing breast cancer up to 40-80 %. In addition, mutations in several other genes also increases the risk of developing breast cancer, such as *TP53* and *PTEN*. Additional factors such as body mass index (BMI), environmental factors and consumption of hormonal contraceptives also affects the risk as well as early menarche and late childbearing [4-6].

Breast cancer subtypes

Breast cancer is a heterogeneous disease and is divided into subgroups based on cell origin, growth pattern and expression of hormone receptors and molecular markers. These subgroups have different behaviors and demands individual treatment approaches depending on subgroup properties.

Histological subtypes

The most common breast cancers are carcinomas, which are malignant tumors arising from the epithelial cells surrounding both inner and outer

surfaces in the body. When carcinomas arise in the breast, they usually develop in the milk ducts (ductal carcinomas) or in the lobules (lobular carcinoma) and these two forms account for 90-95 % of all breast cancer cases. In the breast, 18 different invasive cancer types have been identified including medullary carcinoma, mutinous carcinoma and tubular carcinoma and the more rare forms account for only 5-10 % of breast cancers [7, 8]. Carcinoma *in situ* (ductal and lobular) are sometimes treated as breast cancers and are non-invasive tumors in the duct or lobules of the breast. These tumor types can progress to invasive cancers and thereafter act as invasive carcinomas [7, 9]. There are other types of cancers arising in the breast including sarcomas, which arise in the connective tissues in the breast, but these types are extremely rare [10].

Molecular subtypes

Breast cancers are also subdivided based on expression of molecular markers, were the most common ones are estrogen receptor (ER), progesterone receptor (PR) and amplification of human epidermal growth factor receptor 2 (HER2) gene. The expression of these markers are usually assessed by immunohistochemical stainings, chromogenic *in situ* hybridization or fluorescent *in situ* hybridization. Expression levels are strongly correlated with patient outcome and are therefore guiding treatment decisions. PR is the receptor of the hormone progesterone and ER is the nuclear receptor for the estrogen hormones (estrone, estradiol, estriol and estretrol) [11]. ER exists in two forms, ERα and ERβ. ERα is expressed predominantly in sex organs including breast and ovary whereas ERβ is also expressed in breast but can additionally be found in the skin, bone and brain. ERα is mainly responsible for the estrogen signaling in the breast and its expression results in cell growth and differentiation [12, 13]. The HER2 protein is encoded by the *ERBB2* gene and is overexpressed in 20-30 % of breast cancers. HER2 overexpression is associated with a more aggressive cancer and is correlated to poor survival [14].

The expression of molecular markers together with evaluation of growth pattern by the proliferation marker Ki-67 collectively result in molecular subtypes of breast cancer. There are four groups including Luminal A (ER+ and/or PR+, HER2- and Ki-67+ < 14%), Luminal B (ER+ and/or PR+, HER2- and Ki-67 ≥ 14% or ER+ and/or PR+, HER2+ and any Ki-67),

HER2+ (ER-, PR- and HER2+) and basal-like (usually triple-negative) (ER-/PR- and HER2-) [8]. The subclassification of breast cancers based on receptors and growth patterns are involved in decision making regarding treatment options and are strongly linked to aggressiveness and patient prognosis and survival, where Luminal A has the best prognosis and triple negative the worst (Figure 1) [15, 16].

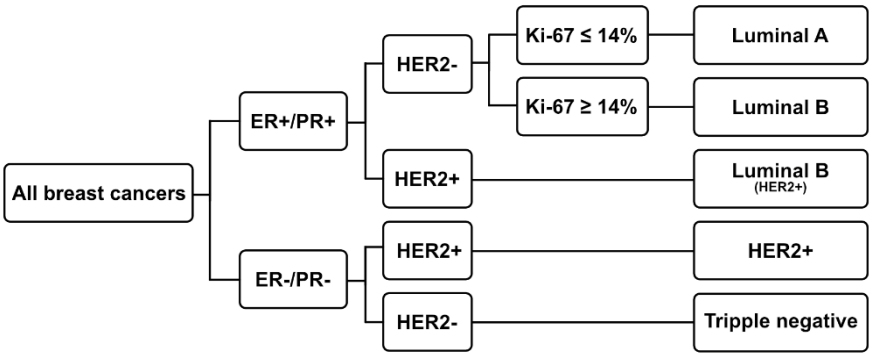

Figure 1. Schematic picture of molecular subtypes of breast cancer.

Histological grade and stage

The histological grading system is classifying invasive breast carcinomas based on the degree of differentiation and how similar the cancer cells are to non-malignant epithelial cells. Histological grade (grade I-III) is based on a scoring system including degree of tubule and gland formation as well as nuclear pleomorphism and mitotic count. The score for each category is then added together and gives each tumor a score from 3-9, were a score of 8 or 9 correspond to a grade III tumor. [17]. Breast cancer stage involves the size of the primary tumor and the presence of cancer cells in one or several of the adjacent lymph nodes or as distant metastasis. Together these parameters describe the stage of the tumor (stage 0-4), where stage 4 involves distant metastasis [18].

Breast cancer treatments

The value of subclassification of breast cancer is related to the importance of treating every patient with the most appropriate strategy. Treatment options includes surgery, radiation, endocrine treatment, treatment targeting HER2 and chemotherapy (Figure 2). Surgery is the primary treatment for all non-metastatic breast cancers with a complete mastectomy or partial mastectomy to remove the tumor. Upon diagnosis, 90% of breast cancer cases are non-metastatic, and the aim with disease treatment is then to eradicate the tumor and prevent it from relapse and spreading in order to cure the patient. For metastatic breast cancers the aim is to prolong life and palliate symptoms [19].

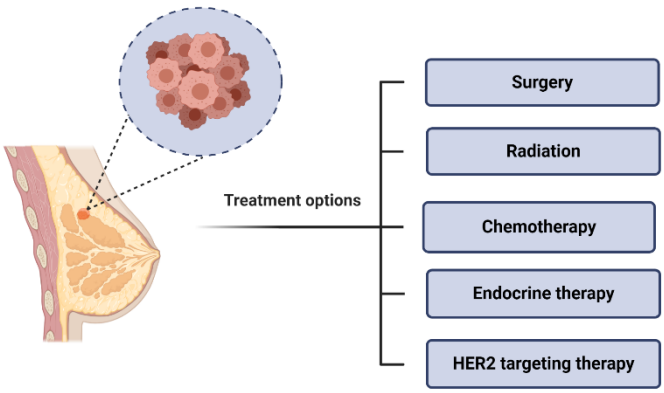

Figure 2. The most commonly used treatments for breast cancer patients. Created with Biorender.com

In ERα+ patients, endocrine therapies are administrated to block the estrogen signaling and thereby prevent proliferation and survival of the estrogen dependent cells. Endocrine therapies include (1) selective estrogen receptor modulators (for example Tamoxifen) that binds to ER and thereby hinders downstream signaling; (2) ER downregulators (for example Fulvestrant) that reduces the levels of ER in the cells and (3) aromatase inhibitors that interfere with estrogen production and thereby reduce estrogen/ER signaling [20]. For patients with HER2 amplifications the monoclonal antibody Trastuzumab (Herceptin) is used to bind to the extracellular part of the HER2 receptor and thereby prevent proliferation and cancer cell survival [21, 22]. For triple negative breast cancer chemotherapy is the only available treatment option, since the tumor is

lacking known targetable receptors. However, chemotherapy in triple negative breast cancer have been suggested to be more effective than in ERα+ cancer, possibly due to higher proliferation [23, 24].

THE CANCER MICROENVIRONMENT

Cancer cells interact with their surroundings and the tumor microenvironment is an important part of tumor development and cancer progression [25-27]. The microenvironment is both complex and dynamic, and changes throughout cancer progression. It affects tumor growth in several steps, from disease initiation to metastasis formation and patient outcome [28]. The tumor microenvironment consists of several components, including different cell types, extra cellular matrix, soluble factors as well as physical properties like oxygen concentration and pH levels. The tumor microenvironment has been shown to affect multiple cellular characteristics such as proliferation, differentiation, invasion and angiogenesis (Figure 3) [29-31].

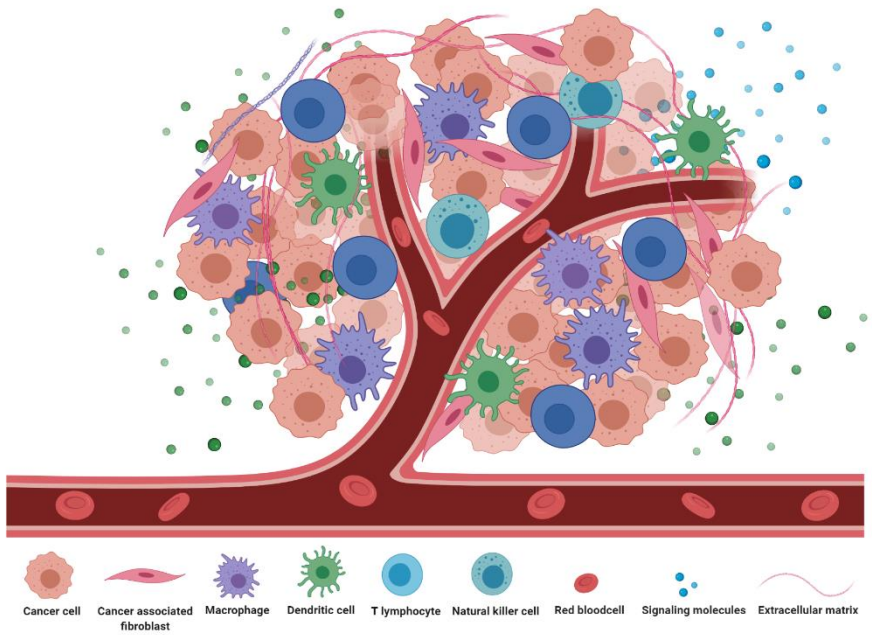

Figure 3. Schematic picture of the cancer microenvironment, including several cell types and extracellular matrix. Created with Biorender.com

Extracellular matrix

In the mammary glands the extracellular matrix consists of the basement membrane and interstitial matrix, and is a complex network of several proteins including collagens, laminins, fibronectin, glycoproteins and proteoglycans. This network contributes with a three-dimensional structure, interactions with cell surface receptors and with biomechanical properties. The biomechanical properties, including matrix stiffness, are correlated to breast cancer progression by increasing focal adhesions and thereby enhancing integrin signaling [32, 33].

Collagens

Collagens account for approximately 30 % of the protein mass in the human body and is a major contributor to the extracellular matrix. Collagens are glycoproteins built up by at least one triple-helix and there are at least 28 collagens, which can be categorized into four subgroups in humans [34, 35]. The crosslinking of collagens contributes to matrix stiffness and biomechanical properties [35]. Dysregulation of collagens are linked to cancer progression, including collagen I and collagen XIII that are associated with tumor invasion, metastasis and poor prognosis for patients [36, 37].

Laminins

Laminins are the most abundant non-collagen proteins of the epithelial extracellular matrix. It consists of three polypeptide chains that can vary between 11 different types. Even though many combinations of these 11 chains could theoretically be possible, only 16 have been found experimentally. The most important laminin function is to interact with receptors in the plasma membrane of cells adjacent to the basement membrane, and through that regulate a number of cellular processes and signaling pathways [38]. Laminins have been suggested to have several different effects on cancer progression. Laminin (LM)-332 is highly expressed in triple negative breast cancers and have been linked to cell migration and invasion [39], while LM-511 has been shown to promote pluripotency of mouse embryonic stem cells *in vitro* [40].

Matrix-bound nanovesicles

Matrix-bound nanovesicles are, similarly to other extra cellular vesicles, small vesicles containing RNA, lipids and proteins secreted from cells. Recent studies have shown that these matrix-bound nanovesicles were embedded within the extracellular matrix [41] and their content differed significantly from other secreted liquid-phase vesicles [42]. Matrix-bound nanovesicles were tightly associated with the collagen network and could only be isolated after strong enzymatic digestion with proteinase K. Previous studies have also shown that the content from isolated matrix-bound nanovesicles have biological effects including macrophage activation and neurite extension in neuroblastoma cells [41]. This research area is still poorly understood, and the effect of matrix-bound nanovesicles in breast cancer is still unknown.

Cell types

Cancer associated fibroblasts

Cancer-associated fibroblasts is a heterogeneous cell population and the most abundant stromal cell type within breast tumors [43, 44]. Their origin is debatable, but recent studies suggests that they emerge from transformed normal fibroblast due to changes induced by tumor cells and the tumor microenvironment [45]. Cancer-associated fibroblast are involved in creating both the tumor microenvironment and the pre-metastatic niche by secretion of several factors with pro-tumorigenic properties including transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), platelet-derived growth factor PDGF and extra cellular matrix-related proteins such as collagen and matrix metallopeptidases (MMPs)[44, 45]. Even though cancer-associated fibroblasts have several characteristics that drive cancer progression, depletion of these cells could be a dangerous approach. Studies have shown that depletion of cancer-associated fibroblasts in a mouse model of pancreatic cancer resulted in poorly differentiated and aggressive tumors and in clinical trials, at best, no effect were seen for this approach [46]. In breast cancer, several subtypes of cancer-associated fibroblasts are proposed and further studies of these

subpopulations and their effect on breast cancer progression are needed [47].

Immune cells

Chronic inflammation is known to promote cancer progression and many cell types, both in the innate and adaptive immune system, have pro-and/or anti-carcinogenic properties [48]. Dendritic cells, macrophages, natural-killer cells (NK-cells) and granulocytes are part of the innate immune system and the first line of defense for the human body [49]. Both NK-cells and granulocytes are involved in targeting cancer by secretion of several granules, cytokines and chemokines [48, 50]. However, as for many immune cell types granulocytes have also been demonstrated to be involved in cancer progression, for example by secreting pro-angiogenic factors. [51]. T-lymphocytes are cells in the adaptive immune system, and is the primary immune cell in targeting cancer [52]. T-lymphocytes is a heterogeneous cell population that target cancer cells via secretion of several cytotoxic factors or by direct interaction with apoptosis inducing receptors [53, 54].

Soluble factors

Soluble factors secreted by cancer cells and other cell types in the cancer microenvironment are affecting cancer formation in many aspects. Secreted factors promote key events in cancer progression including recruitment of cancer-promoting stromal cells, angiogenesis, migration, invasion and resistance to therapeutics. Secreted soluble factors that affects the microenvironment includes, cytokines, chemokines, growth factors and enzymes [27, 55].

Progranulin

Progranulin, also called acrogranin, prostate cancer cell-derived growth factor or proepithelin, is a cysteine-rich, secreted 88kDa glycoprotein encoded by the *GRN* gene. The full-length protein is comprised several domains called granulins. There are seven full-length domains (G, F, B, A, C, D and E) and one half-length domain, paragranulin (p). The cleavage from progranulin into the domains are accomplished by several different

neutrophil secreted proteins such as elastase, MMP-9, MMP-12, MMP14 and proteinase 3. Cleavage of progranulin can result in the 7.5 domains with active biological functions [56-58]. In contrast, high-density lipoprotein/apolioprotein A-I (HDL/Apo A-I) and secretory leucocyte protease inhibitor protein (SLPI) can protect progranulin from cleavage and keep the full-length protein intact (Figure 4) [56].

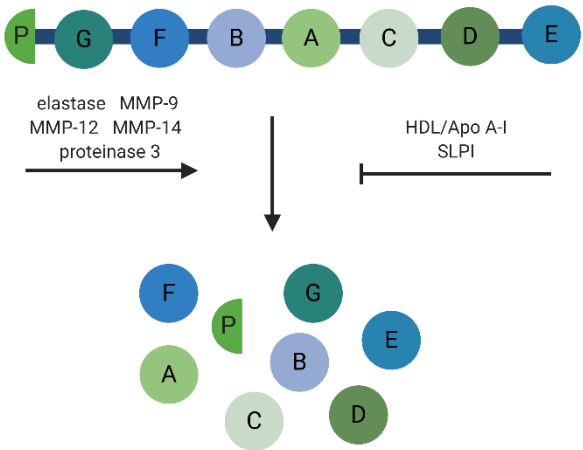

Figure 4. Schematic picture of structure of progranulin and cleavage into the granulin domains. Addapted from [59] Created with Biorender.com

Progranulin has been demonstrated to bind four receptors, sortilin, tumor necrosis factor receptor 1 (TNFR1) and 2 (TNFR2) and ephrin type-A receptor 2 (EphA2). Sortilin is a receptor in the vacuolar protein sorting/targeting protein 10 (VPS10) family that binds the C-terminus of progranulin via a beta propeller structure [56, 57]. The direct binding of progranulin toTNFR1 and TNFR2 has been debated, and previous studies have found opposing results regarding their interaction [60, 61]. However, recent evidence suggest that progranulin do bind TNFR1 and 2 with high affinity [62] and it was proposed that due to complex tertiary structure of progranulin several studies have failed to show these interactions [60]. Progranulin interactions with the TNFR receptors could lead to less inflammation, since it is blocking the signaling between TNFα and the receptors, which has an inflammatory response. Progranulin has also been identified to bind the EphA2 receptor of the receptor tyrosine kinases

family, with similar affinity as to the sortilin receptor, and is thereby suggested to autoregulate the expression of the *GRN* gene [63].

Progranulin is involved in several biological processes including modulation of immune response, growth stimulation, wound healing and neural functions [56]. In cancer, progranulin have been demonstrated to increase the cancer stem cell propagation [57], migration and invasion [64, 65] and high serum levels in metastatic breast cancer patients were associated with worse prognosis and shorter overall survival [66].

Interleukin-6

Interleukin-6 (IL-6) is a 21-26kDa cytokine encoded by the *IL6* gene. IL-6 has pleiotropic effects in the body and is involved in inflammation, stimulation of antibody production in B-cells and angiogenesis [67]. The cytokine is synthesized by numerous cell types and affect cells by binding to several receptors. The classical IL-6 signaling pathway is through binding to the transmembrane IL-6 receptor (IL-6R) which subsequently interacts with the signal transducing receptor glycoprotein 130 (gp130) that initiate a cellular response. Interestingly, the IL-6R is only expressed on hepatocytes and some subgroups of leukocytes, and the signaling through this receptor is suggested to be anti-inflammatory. IL-6 is well-known for the involvement in pro-inflammatory responses and this is suggested to be by trans-signaling through the secreted form of the IL-6R (sIL-6R). The receptor sIL-6R can either be a cleaved form of the IL-6R, were cleavage occur by metalloproteases ADAM10 and ADAM17, or secreted as a sIL-6R translated from a spliced version of mRNA. IL-6 can bind sIL-6R and then interact with gp130 that is expressed on all cells in the body. In this way, IL-6 signaling can occur even though the recipient cell do not express the IL-6R. To control the IL-6 signaling, secreted forms of gp130 also exist, to neutralize the IL-6/sIL-6R complex (Figure 5) [67-69].

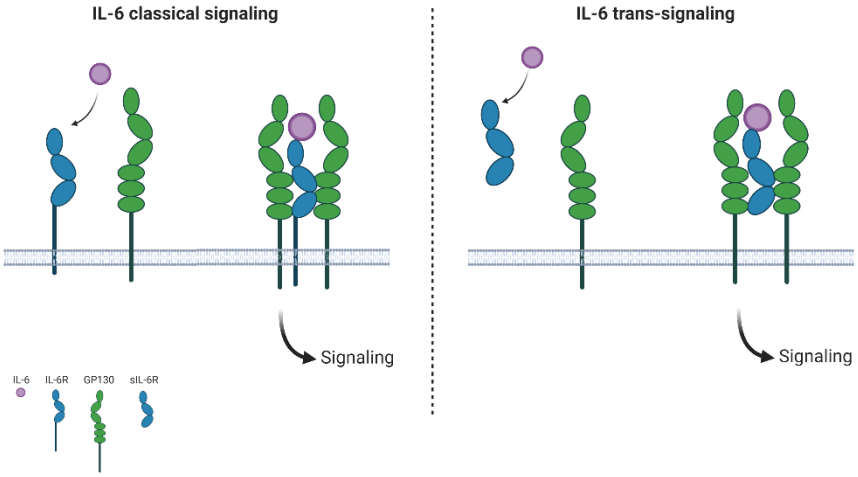

Figure 5. Schematic picture showing two of the IL-6 signaling pathways, the classical signaling pathway and the trans-signaling pathway. Adapted from [68] Created with Biorender.com

Furthermore, IL-6 has also been demonstrated to bind the sortilin receptor with high affinity [70, 71]. Further, IL-6 induces activation of the JAK/STAT3 signaling pathway as well as SHP-2 driven Ras-Raf-MAPK pathway targeting genes related to angiogenesis (*HIF1α* and *VEGF*), epithelial-to-mesenchymal transition (EMT) (*SNAI1, TWIST* and *VIM*) and proliferation (*Bcl-2* and *c-Myc*) [68, 69, 72]. IL-6 have been shown to be involved in several diseases including arthritis, asthma and cancer [73-75]. In breast cancer, IL-6 has been demonstrated to affect cancer stem cell propagation, invasion and metastasis and high serum levels have been associated with poor prognosis and survival [76-79].

Interleukin-8

The interleukin-8 (IL-8) protein, also called CXCL8, is an 8.4 kDa chemokine in the cystein-X-cystein (CXC) family. IL-8 is encoded by the *CXCL8* gene and has two forms, one with 72 amino acids secreted from monocytes and macrophages and one with 77 amino acids secreted from non-immune cells. IL-8 can be present both as a monomer and as a dimer and signal through the receptors CXCR1 and CXCR2 that are present on neutrophils, monocytes and endothelial cells as well as on tumor cells. The

primary pathway induced by IL-8 signaling is PI3-Akt and signaling through this pathway promote cell survival and induces migration and angiogenesis [80]. Secretion of IL-8 has several functions including attracting neutrophils to sites of infection, to clear infected areas of pathogens and to promote angiogenesis [81, 82]. Expression of IL-8 has been associated with several diseases including pulmonary diseases and cancer [83, 84]. In breast cancer, IL-8 has been demonstrated to increase the cancer stem cell subpopulation and high levels of IL-8 in patient serum have been associated with high tumor burden and more aggressive cancers [76, 85].

Hypoxia

An additional factor that contributes to the complexity of the tumor microenvironment is deprivation of oxygen supply in certain areas, which is also referred to as hypoxia. Hypoxia is common in solid tumors and linked to poor survival and high mortality [86-88]. Hypoxia-inducible factor 1 alpha (HIF1α) is the master regulator of cellular hypoxia and is one of two subunits of the transcription factor HIF1. HIF1α is composed of basic helix-loop-helix structures and contains the oxygen dependent domain. During normoxic conditions (21% O_2), HIF1α is rapidly degraded by proteasomes and can therefore not bind to HIF1β and induce transcription of target genes. In hypoxia, HIF1α dimerizes with HIF1β, and together with co-activators bind to targets and induce gene expression related to several processes including angiogenesis, glucose metabolism and proliferation [89, 90]. Studies have shown that 1 to 1.5 % of the genes in the genome is responsive to hypoxia, but it varies distinctly between different cell types [91].

In cancer, hypoxia often arise due to fast proliferative cells that lead to increased oxygen consumption and hypoxic areas. Hypoxia also arises due to abnormal angiogenesis, where blood vessels do not form correctly and results in insufficient transportation of oxygen to the tumor microenvironment [90]. Importantly, hypoxia has been shown to affect cancer properties such as invasion and metastasis as well as to increase the breast cancer stem cell population in ERα+ cancers [30, 92, 93]. Hypoxia has also been associated with the loss of ERα and therefore to a more

aggressive and difficult disease to treat. To reduce ERα-loss by inhibiting HIF1α is suggested to be beneficial in combination with endocrine therapy [94].

SECRETION

Secretion is defined as the process were proteins and vesicles carrying cargos are transported from inside the cell to the outside intercellular space. This process is important for cell-to-cell communication and signaling, and makes it possible for cells to interact, not only with connecting cells, but also with cells on distant sites [95-97]. Secretion affecting the signaling cell itself is called autocrine secretion, secretion affecting cells in proximity to the signaling cells is called paracrine secretion and secretion affecting cells in other parts of the body is called endocrine secretion [96]. Signaling through secretion involves several types of molecules, including RNAs and proteins [95, 98]. All secreted proteins from cells such as enzymes, growth factors and cytokines are collectively called the human secretome, and has important functions for cell and organisms survival. Secretion can be specific for one or several cell types, such as insulin from the β-cells in the pancreas [99] and gut hormones from enteroendocrine cells [100], or more general among multiple cell types.

Secretory pathways

Classical pathway

Secretion occurs in different ways, and the most common is the classical pathway used by almost all eukaryotic cells. This pathway is Golgi-dependent and through the endoplasmic reticulum. Here the protein is synthesized as a precursor protein with a signal peptide guiding it to the endoplasmic reticulum. In the lumen of the endoplasmic reticulum, the signal peptide is cleaved off and the protein is folded and packed into vesicles. The vesicles are then transported to the Golgi, where they are sorted into one of two new vesicle types. The first one is the transport vesicle, where proteins that are continuously secreted from the cell are

sorted. Here, the transport vesicles fuse immediately with the plasma membrane and proteins are released to the extracellular space by exocytosis. The second vesicle type is the secretory vesicles, where the vesicles are fusing with the plasma membrane and secrete its content only upon extracellular stimuli [101, 102].

Non-classical pathways

There are several proteins that are not secreted through the classical pathway, and several types of non-classical secretion are known. Non-classical secretion does not involve the Golgi, instead there are several other types of mechanisms involved. Proteins secreted through non-classical pathways are missing signal peptides and are secreted by transport across plasma membrane lipid pores (Type I), through ATP-binding cassette transporters (Type II), by endocytic compartments that later fuses with the plasma membrane (Type III) or by the endoplasmic reticulum, similar to the classical pathway, but never enters the Golgi before fusing with the plasma membrane (Type IV) [103, 104].

Secretion in cancer

Cell secretion is vital for survival but impaired secretion is involved in many diseases including cancer. The secreted proteome of cancer cells is important for cell signaling and cell-to-cell communication. Cancer cells influence neighboring cells, both by autocrine and paracrine secretion which affects cell characteristics such as proliferation, invasion and metastatic capacity [76, 77, 95, 105, 106].

Metastasis is the major cause of death among cancer patients, and avoiding metastasis or an effective treatment strategy for metastatic cancer would be of great importance. Metastatic cancer means that cancer cells have left the primary site and colonized in one or several other organs in the body [107]. Breast cancer normally metastasizes to the bones, lungs, brain or liver and ERα+ breast cancers usually have later recurrences (after five years) compared to much earlier recurrences for triple negative breast cancers [108]. The metastatic process is not a random event, it is suggested that tumors form pre-metastatic niches at distant sites by cancer cell secretion [109]. Even prior to cancer cell dissemination, cancer cells secrete factors such as nutrients and extracellular matrix to prime the metastatic site and

make a favorable milieu for circulating cancer cells [97, 110, 111]. Primary tumor cells also secrete factors to affect pro-tumorigenic immune cells such as IL-17 to increase neutrophil abundance and lysyl oxidase (LOX) to attract myeloid cells [112, 113].

If factors that are secreted from cancer cells can be identified, they could serve as biomarkers in patient blood and add value in a clinical setting. This could easily be accomplished by non-invasive liquid biopsies which would allow monitoring of disease-progression in real time, and potentially reveal targets for novel therapies [114, 115].

TUMOR HETEROGENEITY

The origin of tumor heterogeneity and cancer growth is intensely debated and has led to multiple theories on cancer progression. The first theory explains tumor heterogeneity by the model of clonal evolution. This model implicates that all cells are equipotent and could potentially drive cancer progression by acquired mutations [116]. Any cell could by this theory acquire genetic alterations resulting in malignant features and cancer progression. The second theory is called the cancer stem cell theory where a subpopulation of cells can drive tumor progression. Here the cancer stem cells, like normal stem cells, have the capacity of self-renewal and dived both symmetrically and asymmetrically giving rise to both new cancer stem cells at the same time as more differentiated cancer cells (Figure 6) [117].

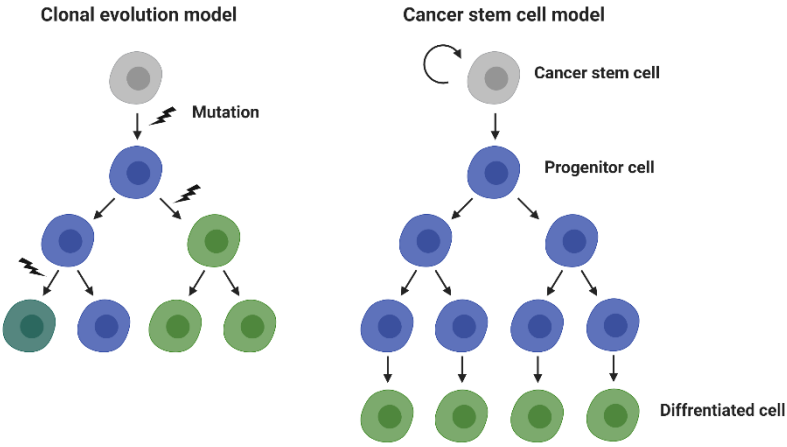

Figure 6. Schematic picture showing two models leading to tumor heterogeneity, the clonal evolution model (left) and the cancer stem cell model (right). Adapted from [116] Created with Biorender.com

Cancer stem cells

The cancer stem cells, or cells with cancer stem cell features, share characteristics with both cancer cells and normal stem cells. Cancer stem cells are defined by their capacity to both self-renew and divide into cells that differentiate [118]. There are no exact markers for breast cancer stem cells but the subgroup is defined by $CD24^{low}/CD44^{high}$, ALDH positive, possess the capacity to grow anchor-independently and to initiate tumors in mouse models [119, 120]. In breast cancer, studies have shown that as few as 100 cancer cells with the phenotype ($CD24^{low/-}/CD44^{+}$) could initiate tumor formation in mice whereas 10 000 cells with other phenotypes failed of tumor initiation in the same model [121]. In breast cancer, cancer stem cells have been demonstrated to be involved in metastasis, recurrence and treatment resistance [122, 123]. There are several assays to assess the cancer stem cell subpopulation, including mammosphere assay, holoclone assay and gene panels assessed by qPCR [120, 124, 125]

The arise of cancer stem cells could be different for different tumor types [126] and the origin and existence of cancer stem cells are highly debated. Cancer stem cells are hypothesized to origin from either normal stem cell or progenitor cells that through acquired mutations or microenvironmental

changes end up as malignant cells but with stem cell features. The second theory find the cancer stem cells to arise from normal somatic cells through acquired mutations and genetic alterations [118]. There are also some evidence regarding plasticity in cancer cells transitioning between a stem cell and non-stem cell state [117].

METHODOLOGY ASPECTS

Tumor model systems

In medical exploration, including cancer research and drug development, several model systems are utilized. Different *in vitro* models, such as the use of established cancer cell lines are commonly used. Cell lines have several advantages including rapid growth rate, supply of unlimited source of material, they are easy to handle and inexpensive. Additionally, with cell lines growing on conventional plastic surfaces it is easy to control the surroundings and keep external parameters constant [127, 128]. However, the drawback with cancer cell lines involves the lack of circulation, *in vivo*-like signaling system, as well as the lack of surrounding stroma and immune system. When using *in vivo* models such as cell-line derived xenografts and patient-derived xenografts (PDX) the advantages are that there is a microenvironment, including a three-dimensional structure, more patient-like circulation and interaction with stromal cells. However, in PDX models, the human stroma is exchanged for mouse stroma already after one to three passages [127]. Even though mouse models are more similar to human tumors, they could be difficult to establish, they are labor intensive and require animal testing which is a limited source and not optimal due to ethical reasons regarding animal testing [127, 128].

Three-dimensional *in vitro* culturing systems

New three-dimensional *in vitro* model systems have been developed to generate more *in vivo*-like models but at the same time avoid animal testing. One commonly used model is the use of Matrigel, a hydrogel for three-dimensional cell growth made from an extract of mouse sarcoma basement membranes. Growth in Matrigel gives the cells the possibility of three-dimensional growth structure and extracellular components relevant for adhesion, signaling and cell to cell communication. However, Matrigel could suffer from batch differences, the composition is not completely defined and it is not derived from a human source [129, 130]. The sphere-forming assay is a selective assay, were cells that are able to grow anchorage independently form spheres on non-adherent plates. This model

have been demonstrated to select for cancer stem cells and also to be more *in vivo*-like regarding nutrient and oxygen gradients. Sphere-forming assays differs from organoid models, where pluripotent or primary cells give rise to a cellular structure that highly resemble the original organ, can be used to investigate cell-to-cell contact, signaling and is often applied in research ranging from developmental biology to cancer research. However drawbacks with this method includes the use of Matrigel, it requires expensive additives to the culturing media and the lack of interactions with a human tumor microenvironment [130]. To be able to study cellular interactions with the tumor microenvironment, several scaffold models have been developed from both synthetic and biological origin. Synthetic scaffolds are created by various materials to create structures that support three-dimensional growth of cell cultures. The cultivations acquire a more heterogeneous cell population compared to two dimensional growth conditions and the synthetic scaffolds are possible to produce in a large scale with well-defined material and physical properties. Nerveless, the synthetically scaffolds lack the presence of signaling molecules and adhesion molecules such as integrins and a proper circulation of nutrients, signaling molecules and removal of cellular waste [131]. Biological scaffolds induces more *in vivo*-like interactions and signaling between cells and the microenvironment, and provides the possibility to investigate patient specific tumor microenvironment heterogeneity [132, 133]. However, biological scaffolds are not well-defined and there is a limited access of these materials. Recently, a method called organ-on-a-chip has been developed, were different cell types and extracellular matrixes can interact and it is also possible to include various physical properties such as hypoxia. This method involves cell contact with extracellular matrix as well as interaction with several cell types and an *in vivo*-like circulation. However, these models are complex and time consuming and today it is not possible to perform high throughput screenings with this model [134].

Protein analysis

When measuring and quantifying proteins in biological samples several approaches are possible. Which method is preferable depends on protein

concentration, sample matrix, cost and time. In this thesis several approaches were utilized.

Proximity extension assay

Proximity Extension Assay (PEA) developed by OLINK (Uppsala, [135]) is an antibody-based assay developed from the Proximity Ligation Assay (PLA). A pair of oligonucleotide-labeled antibodies bind to the target protein and if the antibody pair binds in proximity to each other, the oligonucleotides can hybridize and be amplified and measured by real-time PCR. PEA have been used successfully to measure protein levels in several types of matrixes including blood, saliva, tears and cell media [136-139]. This method makes it possible to detect proteins at low concentrations in complex matrixes and with low sample volumes [140]. We chose to use this method when analyzing conditioned media from cultivations from patient-derived scaffolds as well as for progranulin treated cells, due to the possibility to multi-plex (92 assays per panel), the utilization of small sample volumes (20µl) and because the analysis does not require serum free media. The drawbacks with this method are the pre-defined target panels, even though we analyzed 184 cancer relevant proteins, it is a selection of proteins and interesting key proteins could potentially be missed due to this selection. Another drawback is that it is not possible to get an exact protein concentration with this type of measurements, since no standard curve is used. All protein concentrations are relative, and we chose to normalize it to the total protein secretion.

Western Blot

Western blot is an antibody-based analysis were the sample proteins are charged and separated based on molecular mass in an electrical field. The proteins are thereafter transferred onto a membrane and detected by a primary antibody specific to the target followed by a secondary enzyme labeled antibody specific to the primary antibody. By adding a substrate the signal can be quantified and detected by chemiluminescence. Western blot enables measurement of proteins in a complex matrix, however the method

is a semi-quantitative method and only relative protein concentrations can be measured [141].

Mass spectrometry

Mass spectrometry is a method for determining protein content. It is a semi-quantitative measurement where the ratio of mass-to-charge of ions is analyzed. The ratios are thereafter compared to known mass to charge ratios and by comparing several ratios the original molecule can be identified [142]. Advantages with this method is that it is unbiased, without a pre-defined panel that could potentially limit the number of detected proteins, and it is a method suited for identifying novel biomarkers. However, drawbacks with this method includes that it require large sample volumes (if liquid is analyzed) and complex matrixes, including medium containing serum, could overtake the analysis and masque other results.

Cytokine arrays

A cytokine array, or antibody array, is based on the interaction between antibodies and antigen. The antibodies are immobilized on a membrane and the sample, with the target of interest, is added to the membrane. A secondary enzyme-labeled antibody followed by a substrate is added, and then a signal can be detected corresponding to the amount of protein in the sample. Advantages with a cytokine array is that it is easy to multiplex and thereby measure numerous of targets in the same sample. Drawbacks with this assay includes large sample volumes and pre-defined fixed panels.

Conclusion of protein analysis methods

Included in this thesis are several assays regarding protein measurements. Different approaches are utilized for different aims, and selected based on what is most important in that specific setup or experiment. For analysis of the hypoxia, progranulin or the PDS-induced secretome we utilized the PEA or cytokine arrays (dependent on sample volume) to be able to include

as many targets as possible but without the need of a serum-free medium background. For analysis of few selected targets, including IL-6 and IL-8, we utilized western blot, since no multiplexing were required. When analyzing the protein composition in PDSs, mass spectrometry was used to be able to define as many proteins as possible in the scaffold protein network.

AIM

The purpose and overall aim with this thesis was to uncover the impact of cancer cell secretion on cancer progression and disease outcome for breast cancer patients. We also aimed to delineate how secretion induced by the tumor microenvironment affects subpopulations of cancer cells and how secretion could either be enhanced or blocked, as a therapeutic strategy for treatment of breast cancer.

The specific aims were:

Paper I: In this paper, we aimed to display how a hypoxic environment affects cancer cell secretion and what effect the hypoxic secretome has on cancer stem cell propagation, in both ERα+ and ERα- breast cancer.

Paper II: Here, we aimed to develop an *in vivo*-like culture system based on cell-free patient-derived scaffolds. We also aimed to demonstrate how this *ex vivo* model affects cancer cells and how it can be used to characterize breast cancer subtypes based on the tumor microenvironment.

Paper III: In this paper, we aimed to determine how the secretion profile of breast cancer cell lines was affected by the tumor microenvironment, utilizing the patient-derived scaffold model system. We also aimed to define subgroups of breast cancers based on their induced cancer cell secretion profiles, and how these groups associated with clinical parameters.

Paper IV: Here, we aimed to display how the known cancer stem cell-inducing protein progranulin affect cancer cell secretion and whether the progranulin receptor sortilin is involved in the progranulin-induced secretion and cellular responses.

RESULTS AND DISCUSSION

PAPER I: Hypoxia-induced secretion stimulates breast cancer stem cell regulatory signaling pathways

In paper I we aimed to delineate the effect of hypoxia-induced secretion on breast cancer stem cells, both in ERα+ and ERα- cells and primary cancers from breast cancer patients.

The secretome affects cancer stem cell characteristics differently depending on ERα status

Hypoxia is defined as low oxygen concentration and is known to be involved in tumor progression [90, 143]. Previous results have demonstrated contrasting ERα dependent effects of hypoxia on the cancer stem cell subpopulation, where cells grown in hypoxic cultures increased their cancer stem cell pool in ERα+ cell lines and decreased the pool in ERα- cell lines [30]. Subsequently we hypothesized that this behavior could partially be mediated by an altered protein secretion from the cancer cells. To elucidate the effects of hypoxia-induced secretion on cancer stem cell-like characteristics, conditioned media from the two ERα- cell lines, MDA-MB-231 and MDA-MB-468, and ERα+ cell lines, MCF7 and T47D was used.

Conditioned media was harvested from cells after 48 hours of growth in hypoxic (1% O_2) conditions, while conditioned media from cell cultures grown in normoxic (21% O_2) conditions were used as controls. Thereafter, the cell lines were treated with normoxic and hypoxic conditioned media from both the ERα+ and ERα- cells. Results revealed that that the mammosphere formation increased in all cell types when treated with hypoxic ERα+ conditioned media, while decreased when treated with hypoxic ERα- conditioned media, independent of their own ERα status. These results were further supported by treating MCF7 cells with conditioned media from patient tumors, where conditioned media from ERα+ tumors increased the fraction of cancer stem cells in hypoxia while ERα- tumors decreased the cancer stem cell propagation. Previous studies have shown that hypoxia-induced secretion affects the cancer stem cell

population in gliomas [144], but to our knowledge, the observed ERα dependency in breast cancer have never been demonstrated.

The ERα depending response on cancer stem cell propagation by hypoxia was confirmed with siRNA knockdown of *ESR1* in MCF7 cells and transient expression of ERα in MDA-MB-231 cells. Following knockdown of *ESR1* in MCF7 cells, the mammosphere assay showed a decrease in mammosphere formation compared to scrambled control. An expression of ERα in MDA-MB-231 cells did not result in a decrease in mammosphere formation. Taken together, these results demonstrated that hypoxia-induced secretion is dependent on cells ERα-status and that the secreted factors affect all recipient cell types similarly, independent of their own ERα-status.

Increased pluripotency in cells treated with hypoxic ERα+ conditioned media

To further demonstrate the contrasting effects and to define cell subgroups after treatment with hypoxic media from ERα+ and ERα- cells we used a single-cell approach. MCF7 cells were treated with conditioned media from both MCF7 and MDA-MB-231 cells and the differentiation stage was assessed by gene expression profiling with assays related to pluripotency (*NANOG, POU5F1* and *SOX2*), EMT (*SNAI1* and *VIM*), differentiation (*EPCAM*, *ESR1* and *KRT18*) and proliferation (*CCNA2* and *PCNA*) [120]. Cells treated with hypoxic conditioned media from ERα+ cells had a significantly increased expression of *POU5F1* and decreased expression of *ESR1* compared to cells treated with normoxic conditioned media, suggesting a less differentiated phenotype. Cells treated with hypoxic ERα-conditioned media showed no significantly altered gene expression.

Next, we used self-organizing map (SOM) to define subgroups of single cells with possible association to gene expression profiles. Three SOM groups were formed (SOM1, SOM2 and SOM3) based on gene expression and results demonstrated an overrepresentation of cells treated with hypoxic ERα- and ERα+ conditioned media, in SOM1 and SOM3 respectively. When investigating gene expression profiles in the three SOM groups we discovered increased levels of pluripotency markers in cells in SOM3 and increased levels of differentiation and proliferation markers in SOM1. Cells in SOM2 expressed high levels of the pluripotency markers but also higher

levels of differentiation and proliferation markers compared to SOM3, suggesting SOM2 to include cells in a differentiation stage between cells in SOM1 and SOM2. These results further confirmed our previous data that cells receiving hypoxic conditioned media from ERα+ cells will show a less differentiated phenotype and with enriched cancer stem cell characteristics compared to cells treated with hypoxic media from ERα- cells.

JAK-STAT and related cytokines are involved in the regulation of the contrasting hypoxic response in ERα+ and ERα- breast cancer cells

Moreover, in order to analyze the secreted proteins in the conditioned media, cytokine arrays were performed on hypoxic and normoxic conditioned media from MCF7 and MDA-MB-231 cells. Interestingly, in hypoxic ERα+ conditioned media we observed an overall increase in secreted factors, while ERα- conditioned media showed an overall decrease compared to normoxic control media.

To observe pathway enrichments, string analysis on secreted proteins were performed using the KEGG pathway enrichment. Results revealed the JAK-STAT pathway to be involved in the hypoxic response for both ERα+ and ERα- cell lines. The JAK-STAT pathway have previously been associated with cell migration and cancer progression [145, 146], and the involvement of the JAK-STAT pathway in the observed cancer stem cell propagation was further explored using a panel of JAK-STAT inhibitors. Results showed that in MCF7 cells, both JAK and STAT inhibitors decreased the mammosphere forming capacity after treatment with hypoxic media from ERα+ MCF7 cells. However, for MDA-MB-231 cells only STAT inhibitors could block the decrease after treatment with hypoxic conditioned media from ERα- MDA-MB-231 cells.

When further investigating the differences in cytokine secretion between hypoxic and normoxic conditioned media, interleukin-6 (IL-6) and Interleukin 12 receptor beta 2 subunit (IL12RB2) were found to be the most altered cytokines in hypoxia for ERα+ and ERα- cell lines respectively. IL-6 secretion was significantly decreased from ERα- cells grown in hypoxic conditions. High levels of IL-6 have previously been demonstrated to increase cancer stem cell characteristics, invasion and metastasis, as well as be associated to poor prognosis in breast cancer patients [76, 147, 148]. Our

results suggests that the strikingly decrease in IL-6 secretion from hypoxic ERα- cells affect the cancer stem cell propagation in receiving cells.

MDA-MD-231 sphere formation could be rescued by supplementing IL-6 to the hypoxic media, and addition of IL12RB2 in normoxic media treatment for MCF7 cells mimicked the increase in mammosphere forming capacity seen after growth with ERα+ conditioned media. Western blot analyses further confirmed the downstream target STAT3 to be more phosphorylated after treatment with either IL-6 or IL12RBP. Phosphorylated and activated STAT3 have been demonstrated to be involved in several cancer promoting processes, including migration, invasion and chemoresistance [146, 149]. These findings demonstrate that both IL-6 and IL12RBP are important secreted factors modulating the cancer stem cell response.

Taken together, hypoxic behavior influences breast cancer cells differently depending on ERα status, both in cell lines and patient tumors. Hypoxic responses can spread via secretion and cytokines involved in the JAK-STAT pathways were suggested to be highly responsible for hypoxic alterations in the cancer stem cell propagation.

PAPER II: Patient-derived scaffolds uncover breast cancer promoting properties of the microenvironment

Breast cancer is a complex and heterogeneous disease, which is divided into subgroups based on several histological and molecular markers, including Estrogen receptor (ER), Progesterone receptor (PR) and Ki-67 [150]. All subgroups have varying clinical behaviors and treatment strategies. The crosstalk and dynamic interactions with cancer cells and their surrounding microenvironment is not used in terms of selecting an appropriate treatment, and categorization into subgroups based on the tumor microenvironment are not used in clinics today. Here, in paper II we aimed to elucidate the effect of the cancer microenvironment on cancer cells by establishing a human *in vivo*-like breast cancer model of patient-derived scaffolds (PDS). We also aimed to demonstrate how subgroups of PDSs are linked to clinical parameters.

Establishing and validating the patient-derived scaffold model

To be able to study the impact of the tumor microenvironment on cancer cell lines, we developed a human *in vivo*-like growth system based on patient derived tumor samples. The breast cancer pieces used to create the PDSs were collected directly after surgery or taken frozen from a biobank. In order to create the cell-free PDSs, the pieces were washed with mild detergents for a series of steps and subsequently, by histological stainings and genomic DNA measurements, validated to be cell-free. Further, to evaluate the protein network building up the extracellular matrix after the washing procedure effect we performed collagen stainings. Collagens are the most abundant proteins in the extracellular matrix and important for tumor structure and signaling [35, 36]. Our results showed that after washing, we still have intact collagen structures in the PDSs.

Cell survival, growth and infiltrative behaviors of cells in the PDS-model were tested for two commonly used breast cancer cell lines, MCF7 (ERα+) and MDA-MB-231 (ERα-). Both cell lines grew within the PDSs, demonstrated by positive Ki-67 stainings. Interestingly, the triple negative MDA-MB-231 cells were observed to infiltrate into the PDS while the ERα+ MCF7 cells grew in clusters, closer to the PDS surface. This growth pattern mimic the *in vivo* situation, were triple negative breast cancers show a more infiltrative phenotype [151].

Differences in cells cultivated in PDSs compared to monolayer cells

Next, we further demonstrated the differences in cell lines cultivated in the PDS-model compared to cell lines cultivated in conventional monolayers. We showed that cells in the PDS-model expressed higher levels of genes associated with pluripotency and EMT and had a lower expression of genes related to differentiation and proliferation. This is in line with previously studies, where an increase in EMT and cancer stem cell characteristics have been shown for other 3D-models [133, 152, 153]. These results were confirmed by western blots and functional studies. Cells grown in the PDS-system had a greater mammosphere forming capacity and a higher tumor initiating capacity when injected into mice than cells grown in monolayers.

Breast cancer is a heterogeneous disease and to further demonstrate the heterogeneity of the cells in the PDS-model we used a single cell approach where cells were collected after cultivation in two separate PDSs (n=83, n=85) or grown in a monolayer culture (n=88). An unsupervised clustering method, self-organizing map (SOM), were utilized and three SOM groups were created based on analysis of genes associated to pluripotency (*NANOG POU5F1*), differentiation (*CD24, CDH1* and *EPCAM*), EMT (*SNAI1*) and proliferation (*CCNA2, CCNB2* and *MKI67*) [120]. One group (SOM3) contained a majority of monolayer cells while the other two groups (SOM1 and SOM2) had an equal representation of cells from both PDSs. Monolayer cells highly expressed genes related to differentiation and proliferation. Interestingly, both groups with PDS cells expressed low levels of proliferation associated markers, but SOM1 also expressed high levels of pluripotency markers while SOM2 highly expressed transcriptional markers related to differentiation.

The two SOM groups containing PDSs further demonstrated that the PDS-model in general shift cells to a more stem-like stage and that the cell population are more heterogeneous in the PDS-model. Previous studies have shown that cells grown in a 3D-model resembles cells *in vivo*, compared to monolayer cultures [154].

Thereafter, we investigated how similar cells cultivated in the PDS-model are to cells from xenografts in mice. Xenograft models have been shown to predict patient responses to several drugs but is labor intense and require animal experiments [155, 156]. Xenograft models also lack human

extracellular matrix and fibroblasts. With RNA sequencing we could confirm that cells in the PDS-model are more similar to cells from xenografts in mice then to monolayer cells. These results suggests that with the PDS-model, we can achieve similar cell responses as with xenografts but with a human microenvironment and without the use of animal experiments.

Protein composition of patient-derived scaffolds and correlation to clinical behavior

The tumor microenvironment consist of a complex protein network, including many different types of proteins. In paper II, we wanted to investigate how heterogeneous these protein networks are and if the PDS composition could be linked to clinical behavior. We investigated 15 PDSs with mass spectrometry and found 143 different proteins. Again, using an unsupervised clustering method, SOM, the PDSs were subdivided into two groups based on 38 of these 143 proteins. One of these SOM groups were correlated to low grade tumors and low proliferative cells suggesting that the specific protein composition of these PDSs results in a favorable patient outcome. Previously published data have shown that breast cancer can be divided into relevant clinical subgroups based on their microenvironment [157], our data further support the findings that the ECM protein composition could be clinically relevant.

To further explore the differences in PDS protein composition we used a bioinformatics approach and created a network of all detected PDS proteins as well as proteins they interact with, based on data from Human Protein Reference Database [158]. Interestingly, we found that 12 out of 16 of the central proteins were related to exosome secretion, indicating an important role for protein secretion in establishing the tumor microenvironment and the extracellular matrix. This is in line with previously published data, where exosome secretion have been shown to be important to module the tumor microenvironment and also that micro vesicles could be incorporated into the extracellular matrix [41, 159]. We could also further demonstrate the importance of cancer cell secretion in the PDS-model in paper III, where the PDS-induced secretion was linked to subgroups of microenvironments.

Gene expression in 46 PDSs and correlation to clinical parameters

Every PDS is different, with unique patient-specific characteristics. By using biobanked tumors (n=46) we investigated cellular changes in MCF7 cells in the PDS-model and correlate them to clinical characteristics and patient outcome. Gene expression analysis showed that high expression of the EMT markers *VIM* and *SNAI2* significantly correlated to breast cancer recurrence.

In conclusion, the development of an *in vitro* model with high resemblance to *in vivo* models is highly needed to study the crosstalk between the tumor microenvironment and cancer cells. Today many drugs fail in late stage clinical trials, even though extensive tests has been carried out both in animal and monolayer cultures which is costly to the society [160]. The addition of a novel *in vivo*-like experimental model to the models used today, could lead to fewer late stage drug fails, thus resulting in both economical and ethical benefits. By using this model, malignancy-promoting aspects of the cancer microenvironment as well as patient-specific microenvironments can be assessed and used to further divide breast cancers into clinically relevant subtypes.

PAPER III: Patient-derived scaffolds influence secretion profiles in cancer cells mirroring clinical features and breast cancer subtypes

The breast cancer microenvironment is both complex and dynamic, and influences cancer progression by providing physical properties, such as matrix stiffness as well as containing proteins influencing cell receptor signaling and cell-to-cell communication [26, 27]. In paper III we aimed to demonstrate how the tumor microenvironment can affect cancer cell secretion in both ERα+ and ERα- breast cancer cell lines and how this could affect disease progression. In this study we utilized a newly developed *in vivo*-like growth system where breast cancer samples from patients were decellullarized and repopulated with standardized breast cancer cell lines to investigate how variations in individual microenvironments could affect cancer secretion patterns.

Secretome from PDS cultivations increase the cancer stem cell subpopulation

We have previously showed that the PDS-model shifts cancer cells to increase genetic markers related to cancer stem cells and epithelial to mesenchymal transition (paper II, [161]). We hypothesized that this could partially be due to an altered cancer cell secretion and that the secreted proteome from PDS cultivated cells could have an effect on cancer stem cell propagation.

In paper III, the cancer stem cell subpopulation in monolayer cultures were investigated after treatment with conditioned media from PDS cultures and compared to treatment with media from conventional monolayers. The cancer stem cell subpopulation was assessed based on their ability to avoid anoikis and proliferate anchorage-independently by using the mammosphere assay [124]. Interestingly, the secretome from cells cultivated in the PDS-model significantly increased the mammosphere forming capacity of monolayer MCF7 cells (ERα+, n=3) as well as in two out of three PDSs for MDA-MB-231 cells (ERα-). Importantly, the secretome from one PDS culture with MDA-MB-231 cells decreased the cancer stem cell pool and indicating that subtypes of cancer microenvironments could possibly be favorable for breast cancer patients.

With this limited material it was not possible to draw any conclusions related to clinical characteristics for these PDSs, but the decreased mammosphere formation induced by one of the PDSs for MDA-MB-231 cells highlighted the difference in microenvironmental-induced secretion and its potential to affect cancer stem cell propagation.

Analysis of conditioned media

In order to qualitatively determine the secretome responsible for the increase in stemness and also investigated the link to clinical parameters, we evaluated secretion from PDS cultures as well as monolayer cultivated cells. Both the general differences in secretion between standard monolayer cultured cells and the PDS-model as well as the impact of the patient specific tumor microenvironment has on cancer cell secretion. To achieve this, a proximity extension assay were utilized were 184 proteins were analyzed for all samples.

Normalization strategies

In general, normalization of larger datasets could potentially change the result outcome and the conclusions that would be drawn from these results. In paper III, we considered several different normalization strategies. We considered to normalize to a reference protein, but there are to our knowledge, no secreted protein that could be considered a house-keeping protein and serve as a reference. We considered to normalize to the amount of seeded cells (300 000 cells for each PDS), but then risking higher proliferation in some PDSs compared to others which could result in cell number differences and thereby secretion differences. We also considered to normalize to RNA concentration, however the RNA content in each cell is not equal [162]. Therefore, we decided to normalize to the total protein secretion, to achieve the most unbiased normalization, but still avoiding studying results based on only cell numbers.

Analysis of conditioned media in 2D and PDS

When analyzing the 184 proteins included in the chosen panels there was a clear difference in secretion between monolayer cultured cells and cells cultivated in the PDS-model, for both studied cell lines. For MCF7 cells, 32 proteins were secreted from monolayer cells and 72 proteins were secreted above the limit of detection (LOD) from PDS cultures. For MDA-

MB-231, 84 and 121 proteins were secreted above the LOD for monolayer and PDS cultures respectively. The difference in number of secreted proteins, as well as the concentration, for the two cell lines are consistent with earlier published results demonstrating a higher cytokine content for ERα- breast cancer cells [163]. The high intrinsic levels of protein secretion in MDA-MB-231 in monolayer cultures could explain why the PDSs seem to influence the secretion in this cell line to a less degree than in MCF7s.

Correlation to clinical parameters

When investigating secretion from MCF7 cells grown in 57 PDSs, results showed several proteins that correlated with clinical parameters, including PAI ($p<0.0001$), ADA ($p=0.002$), IL-6 ($p=0.004$) and CXCL1 ($p=0.028$) that was significantly induced from PDSs from high-grade tumors. Interestingly, all these proteins have previously been associated with cancer progression and poor patient outcome in cancer. PAI is a protein normally involved in the plasminogen activator system but have been shown to affect cell adhesion, invasion and migration in breast cancer by modulating collagen crosslinking and fibrin deposition [164, 165]. The chemokine CXCL1 have been shown to affect invasion and migration in breast cancer cells [166]. The enzyme ADA have previously been correlated with both lymph node metastasis and histological grade in breast cancer [167] and the cytokine IL-6 have been associated with an increase of cancer stem cells (as shown in paper IV), invasion and metastasis and high serum levels have been correlated to poor prognosis and disease progression [76, 147, 168]. Taken together, several of the induced secreted factors have previously been linked to breast cancer progression and disease outcome suggesting that the tumor microenvironment provided by the PDS-model could carry unique patient information resulting in patient specific secretomic fingerprints.

Subpopulations of PDSs

To investigate subgroups of PDSs based on protein secretion we utilized an unsupervised clustering method, self-organizing map (SOM). This is a clustering method to visualize and display high dimensional data in a low-dimensional diagram (usually two or three dimensions) and to categorize data into a pre-selected number of groups. In this paper, two SOM-analysis were performed, one for MCF7s and one for MDA-MB-231s. For MDA-

MB-231 no stable SOM-groups could be formed, possibly due to the high intrinsic levels of secreted proteins. Contrary, for MCF7 cells, three stable SOM-groups were formed, SOM1, SOM2 and SOM3.

Next, we investigated which proteins had the greatest impact on subgroup formation in MCF7 by comparing secretion in one group with secretion in the two other groups. For SOM1 group ANG1, CASP8, CCL2, COL1A1, IL-6, IL-6RA, PAI, PLC, PIGF and TNFRSF21were identified as significantly higher secreted compared to the other two SOM-groups. For SOM2, high secretion of ALCAM, BLMH, CHI3L1, CSTB, CTSD, CXCL16, GAL-3, GRN, PDGF-A and PI3 defined the group. However, for SOM3 we found no proteins that were highly secreted in this group, instead SOM3 was defined by low secretion of several proteins including ALCAM, CSTB, CTSD, CXCL16, GRN, IGFBP2, JAM-A, KLK6, PDGF-A and PLC. The low secretion of a majority of the proteins in SOM3 could indicate that the tumor microenvironment is not as active and do not influence the cancer cells as much compared to PDSs in SOM1 and SOM2.

Furthermore, cancer cells and other pro-tumorigenic cells secreted factors that prepare distant sites or organs in the body for circulating cancer cells, and thereby facilitates metastasis formation [110]. Interestingly, when investigating the induced proteins in PDSs in SOM1 we found several proteins associated with metastasis and priming of the pre-metastatic niche including CCL2, IL-6 and PAI [164, 169, 170]. These proteins could be used either as biomarkers in the PDS-model or possibly also in patient serum to predict patient outcome or as targetable key proteins to avoid metastasis formation and cancer progression.

Thereafter, possible correlations of the SOM-groups and clinical parameters were investigated. SOM1 correlated significantly with high-grade tumors ($p=0.05$) and relapse-free survival ($p=0.013$), demonstrating that PDSs from patients in SOM1 have a microenvironment that promote cancer progression and result in a worse disease outcome.

Pathways/processes upregulated in a subgroup of PDSs

To gain more information of possible pathways that could be regulated in subgroups of the PDSs, an analysis was preformed, where the secretion of proteins were correlated to pre-defined processes by OLINK. All proteins

were assigned to one or several different processes including, *apoptosis/cell killing*, *chemotaxis*, *metabolism/autophagy*, *promote tumor immunity*, *suppress tumor immunity*, *vascular and tissue remodeling*, *angiogenesis*, *catabolic processes*, *cell adhesion*, *coagulation*, *inflammatory response*, *MAPK cascade*, *platelet activation*, *proteolysis*, *response to hypoxia*, *response to peptide hormones* and *wound healing*. For all proteins in each pathway/process the PDSs were divided by the median and placed into two groups, high or low secreting PDSs. Next, we investigated if the SOM-groups had an overrepresentation of PDSs with high secretion to conclude if that specific process could be upregulated in that SOM-group. Interestingly, PDSs in SOM1 had a significant overrepresentation of PDSs where the pathways *apoptosis/cell killing* ($p_{SOM1\text{-}SOM2}<0.0001$, $p_{SOM1\text{-}SOM3}<0.0001$), *metabolism/autophagy* ($p_{SOM1\text{-}SOM2}=0.039$, $p_{SOM1\text{-}SOM3}=0.008$), *suppress tumor immunity* ($p_{SOM2}=0.002$, $p_{SOM3}<0.0001$), *vascular and tissue remodeling* ($p_{SOM2}=0.003$, $p_{SOM3}<0.0001$) and *wound healing* ($p_{SOM2}=0.008$, $p_{SOM3}<0.0001$) were suggested to be upregulated. All processes except *promote tumor immunity* and *response to peptide hormones* were significantly lower in SOM3. These results does not show that these processes are downregulated in the PDSs in SOM3, rather that they are possibly not as upregulated as in SOM1 and SOM2.

To conclude, in this paper we demonstrated that breast cancer cell secretion is altered in a three-dimensional *in vivo*-like human growth system compared to in conventional monolayer cultures. We also showed that breast cancer can be subdivided based on tumor microenvironmental-induced secretion. One subgroup of PDSs (SOM1) were shown to correlate with tumors from patients with poor prognosis and cancer cells in these PDSs had an altered secretomic and transcriptomic profile favoring cancer progression. These novel findings highlight the importance of studying secretion and cell-to-cell communication in an *in vivo*-like system and describes how the tumor microenvironment influences the secretion of cytokines and other cell signaling proteins in breast cancer.

PAPER IV: Interleukin-6 induces stem cell propagation through liaison with the sortilin-progranulin axis in breast cancer

Cell to cell communication and signaling are important parts of the tumor microenvironment and cancer progression. Signaling can occur directly via cell-to-cell contact or via secretion of molecules that signal to cells via for example receptor/ligand interaction. Signaling molecules includes cytokines, growth factors and chemokines and have vital functions in the human body.

Progranulin is a growth factor and is known to be upregulated under hypoxic conditions and has been associated with various types of cancer [57, 59, 171] and high serum levels have been correlated with worse prognosis for patients with breast cancer or malign lymphomas [66, 172]. Progranulin and one of its signaling receptors sortilin have previously been shown to be involved in cancer stem cell propagation and in metastasis formation [57]. In paper IV, we aimed to delineate the role of progranulin-induced secretion in both ERα+ and ERα- breast cancer cell lines and the possible correlation to expansion of the cancer stem cell subpopulation.

Analysis of progranulin and sortilin dependent secretome

As we have previously shown, the progranulin cancer stem cell effect is dependent on sortilin [57]. Here we investigated the progranulin induced protein secretion in detail and the cancer stem cell propagating effect of progranulin and downstream events exerts on breast cancer cells. To investigate protein secretion induced by progranulin and to elucidate if this secretion was sortilin dependent, both MCF7 and MDA-MB-231 cells were treated with progranulin, the sortilin inhibitor AF38469 (AF) and a combination of these two. Conditioned media was collected from all samples and analyzed by Proximity Extension Assay. 184 proteins were analyzed in each sample and each protein was normalized to the total secretion of that sample. For MCF7 cells there was an increase in secretion of IL-6, IL-8, CXCL1, CD40, FASL, CSF-1, tPa and TNF after progranulin treatment. Interestingly, IL-6 [76, 168], IL-8 [76, 173], CXCL1 [174] have all been associated with higher prevalence of cancer stem cells and priming of the pre-metastatic niche [97], further demonstrating that progranulin

might have a direct effect on cancer stem cells as well as an indirect effect via inducing secretion of cancer stem cell-promoting cytokines.

Interestingly, several of the progranulin-induced proteins were decreased with the combination treatment of progranulin and the inhibitor AF, such as IL-6 and IL-8, indicating a sortilin dependent secretion of these proteins. For the MDA-MB-231 cells we observed similar results but not as pronounced, possibly due to high cytokine basal levels, which is in line with earlier publications [163]. The higher protein concentration of specific proteins after progranulin/AF treatment could be due to induced secretion in the cell or protein accumulation after competition for the sortilin receptor. Indifferently of why the protein concentration increases, these proteins will have a biological impact and affect nearby or possibly distant cells.

Identified crosstalk between IL-6 and progranulin

Next, MCF7 and MDA-MB-231 cells were treated with increasing concentrations of progranulin to further validating the crosslink to IL-6 and IL-8. Intracellular protein levels were assessed by western blot. For both the studied cell lines, progranulin treatment increased the cell expression of IL-6 and IL-8, demonstrating an increase in protein expression as well as secreted proteins. We could also demonstrate by western blot that the expression of progranulin increased after IL-6 treatment, suggesting a crosstalk between these two proteins. Indeed, previous studies have shown an increase in IL-6 mRNA expression after progranulin treatment in adipose tissue [175]. Further, in both hepatocellular carcinoma [176] and cholangiocarcinoma [177], IL-6 treatment have been shown to increase both progranulin gene expression and protein expression. However, to our knowledge no data have previously demonstrated the possible feed-back loop between IL-6 and progranulin and the implications in breast cancer. Progranulin can be cleaved into granulins and IL-8 have been reported to increase after treatment with progranulin subunit B (GRNB) but not with progranulin in epithelial cell lines [178], but similarly to IL-6, no data have demonstrated the possible link between progranulin and IL-8 in breast cancer.

IL-6 and IL-8 and their effect on breast cancer stem cells

IL-6 have recently been identified as a ligand to the progranulin receptor sortilin [70]. Here we found that progranulin-induced IL-6 secretion via sortilin and that a crosstalk between progranulin and IL-6 exists in breast cancer. In order to determine whether both IL-6 and IL-8 bind directly to sortilin, a competitive fluorescent polarization assay was performed. In this assay, cytokines were competing with the known sortilin ligand neurotensin for the binding to sortilin. Results, using this competitive binding assay demonstrated that IL-6 binds to sortilin with an IC50 of 11.2 μM and that IL-8 does not bind to the sortilin receptor (at least not to the neurotensin binding site), suggesting a direct and indirect cancer stem cell effect for IL-6 and only indirect effect for IL-8.

Furthermore, IL-6 and IL-8 are well-known to affect the cancer stem cell propagation [76, 147, 179] and induced secretion of these cytokines could partially be responsible for the increase in the cancer stem cell subpopulation after progranulin treatment. Here, we could confirm a dose dependent increase of cancer stem cells after 48h of treatment with both IL-6 and IL-8 using both MCF7 and MDA-MB-231 cells. Next, we investigated if the binding to sortilin affected the cancer stem cell inducing capacity of IL-6 and IL-8. As expected, IL-6 induced an increase of the cancer stem cell population that could be blocked by the sortilin inhibitor AF in both MCF7 and MDA-MB-231, while the increased cancer stem cell pool by IL-8 could not be blocked by inhibiting sortilin. These results further demonstrated that IL-6 bind to sortilin and subsequently induced activation of cancer stem cells. Contrary, the IL-8 induced cancer stem cell propagation could not be blocked by the sortilin inhibitor, thereby suggesting that the cancer stem cell inducing capacity of IL-8 is not sortilin dependent. However, IL-8 could have an indirect effect on cancer stem cells via sortilin by increasing IL-6 secretion and thereby increase cancer stem cell propagation in a sortilin dependent way.

Correlation of progranulin and IL-6 in an in vivo-like three-dimensional model

To further investigate the crosstalk between IL-6, IL-8 and progranulin we utilized a three-dimensional *in vivo*-like breast cancer model where breast cancer samples from patients are decellularized and repopulated with standardized breast cancer cell lines [161]. The PDS-model have been

demonstrated to induce stemness by gene expression profiles and cancer cell secretion ([161], Persson *et al*, paper III).

When comparing patient-derived scaffolds (PDS) cultured cells to monolayer cells, new analyzes of previously published RNA sequencing data [180] showed an increase in expression of progranulin, IL-6, IL-8 for MDA-MB-231 cells and IL-6 and IL-8 for MCF7 cells in the PDS-system, further supporting the increased stemness in the PDS-model. The PDS-model have been shown to increase cell stemness. The IL-6 receptor IL-6R and the IL-6 signal transducer receptor GP130 were also upregulated in the PDS culture for both MCF7 and MDA-MB-231 cells, suggesting a possibility for an increase in autocrine secretion. In addition to the NGS dataset, we used a dataset with secretion measurements from 57 and 53 PDSs, cultivated with MCF7 and MDA-MB-231 cells respectively (paper III). Results from these PDS cultures demonstrated that IL-6 and progranulin secretion correlated significantly in both MCF7 ($p<0.001$) and MDA-MB-231 cells ($p=0.023$), further indicating that there is a crosstalk between these proteins. Studying secretion and cell to cell communication in an in vivo-like growth system could be beneficial, and the increased levels of progranulin associated proteins and receptors indicates that this three-dimensional model could be utilized to better understand the progranulin-sortilin signaling.

Taken together, these results demonstrated that progranulin increased cell secretion of many proteins, several of them dependent on sortilin binding. It has previously been shown that progranulin increase metastasis formation via sortilin, but here we also show a progranulin-dependent increase of secreted proteins such as CXCL1, IL-6 and IL-8, which also have been shown to be involved in metastasis and priming of the pre-metastatic niche, suggesting an additive overall increased risk for metastasis with high progranulin levels. Inhibiting the binding of progranulin and IL-6 to sortilin could potentially decrease cancer stem cells and reduce metastasis. A sortilin targeted therapy in combination with existing therapies could potentially be beneficial for breast cancer patients.

SUMMARY RESULTS AND DISCUSSION

Breast cancer is a heterogeneous disease and the tumor microenvironment highly contributes to the diversity in cancer and cancer progression. In this thesis we have demonstrated the impact of the cancer microenvironment components on cancer cell secretion and how this influence the composition of cancer cell subpopulations. The four papers included in this thesis have collectively lead to a greater understanding of the importance of tumor microenvironmental-induced effects on cells phenotypes, protein/gene expression and secretion patterns.

In paper I we demonstrated an ERα dependent hypoxic-induced secretion response in breast cancer. Hypoxia has previously been shown to affect the propagation of cancer stem cells in opposing directions for breast cancer cell lines depending on ERα status [30], and in paper I we further showed that this is at least partially mediated through secretion. One of the hypoxic-induced factors, specific for ERα+ cells were the growth factor progranulin. We have previously shown that progranulin affects the cancer stem cell propagation and metastasis formation in a sortilin dependent manner [57], suggesting that this protein can, at least partially, mimic the hypoxic response. In paper IV we could show that progranulin might have a direct and indirect effect on the cancer stem cell subpopulation, since it induces secretion of IL-6 and IL-8, both cytokines well-known for their cancer stem cell propagating effect. We also demonstrated that progranulin increased the secretion of several proteins involved in the formation of the pre-metastatic niche. When taking results from paper I and paper IV into consideration, hypoxia induces several cytokines that could affect stemness and metastasis formation. These proteins could also increase secretion of other potent cytokines regarding cancer stem cell propagation resulting in a chain of events promoting metastasis and disease progression.

In paper II, we developed an *in vivo*-like three-dimensional model to enable the study of cancer cell line changes in a three-dimensional culture system and simultaneously investigated the heterogeneity and patient characteristics of cancer microenvironments. We demonstrated that cell lines changed their expression profile when cultivated in patient-derived scaffolds (PDSs) and that these profiles correlated with clinical parameters of the original tumors that the samples were derived from. The development

of *in vivo*-like methods to study cell behavior is of great importance since this could provide results more similar to clinical behaviors and there is also a possibility to decrease the use of animals with new improved *in vitro*-models. To further study cell changes in the PDS-model, we investigated cell secretion in relation to tumor microenvironment heterogeneity in paper III. We demonstrated a significant difference between cell line secretion in monolayer cultures and PDS cultures, and since we previously showed that cell gene expression is more similar to *in vivo* cells we hypothesized that the secretion could also be more *in vivo*-like. Furthermore, we demonstrated the importance of studying secretion and cell-to-cell communication in an *in vivo*-like model where several secreted factors could potentially be used as biomarkers or partially explain changes in cell behavior when cultivated in a three-dimensional model. Interestingly, we showed that cells grown in PDSs from high-grade tumors and tumors with lymph node metastasis secreted higher levels of IL-6 than others. This highly induced secretion of IL-6 could, based on results in paper IV, increase the cancer stem cell propagation.

Taken together, the tumor microenvironment highly affects breast cancer heterogeneity and induced secretion could be used as both potential biomarkers or as targetable key proteins mediating cancer progression and be involved in disease outcome. The four articles included in this thesis collectively show the potential of the tumor microenvironment as a factor to divide breast cancers into potentially clinical relevant subgroups, as well as a target for cancer treatment.

FUTURE PERSPECTIVES

The tumor microenvironment and induced secretion have been shown to affect cancer cell behavior and disease progression. In this thesis we highlighted the importance of including microenvironmental factors when investigating cancer behavior and possible treatment strategies.

We utilized a newly developed *in vivo*-like culturing system, where the patient-specific tumor microenvironment could be evaluated. We used breast cancer tissue samples to establish this model, but the method could be used on several other tumor types. This opens up for patient-specific microenvironmental studies in additional cancer types. In future research, the PDS-model could potentially serve as a drug-screening tool, both to screen for novel compounds but also to test existing drugs and the efficacy for specific patients. Further, the use of this method could lead to improved personalized medicine, and drugs could be screened in the PDS-model after the tumor has been removed by surgery. However, additional research is needed to evaluate if treatment responses in PDSs correlate to treatment responses in patients. In addition, it would be interesting to evaluate correlations between secreted proteins induced by a specific patient PDS to levels in the corresponding patients serum.

We have also demonstrated that secreted factors induced by components of the microenvironment, including extracellular matrix and hypoxia, have specific effects on cancer cell subpopulations. These secreted factors could potentially serve as biomarkers for aggressive disease or act as targets for treatment. We identified IL-6 to be secreted by cells in high-grade tumors and tumors with lymph node metastasis, as well as having an effect on the cancer stem cell subpopulation via the receptor sortilin and/or the JAK-STAT signaling pathway. Cancer stem cells are known to be tumor-initiating and treatment resistant. To further investigate sortilin and sortilin inhibition in relation to IL-6 and progranulin secretion in patients would be of great importance. In addition, their effect on cancer stem cells and their potential role as an additional therapeutic strategy needs to be further investigated.

Taken together, in this thesis we have started to unwind the complex network interactions between unique tumor microenvironments and its

influence on cell secretion. In future research, the tumor microenvironmental effect on cancer cells needs to be further evaluated and possibly also included when dividing breast cancers in to subgroups to achieve the most appropriate treatment for breast cancer patients.

ACKNOWLEDGEMENT

There are so many people I would like to say thank you to, which have helped and supported me throughout my PhD-studies!

First, I would like to thank my supervisor Göran for providing such an inspiring work environment and always being super interested in any new results and guiding me during my PhD. I also want to thank you for helping me grow as a researcher and to believe in my own abilities. I would also like to thank my co-supervisor Anders for sharing your passion for research and all your helpful discussions and feedback throughout these years.

I want to say thank you to all current and previous members of Landberg group. To Pernilla for all your help in the lab and all fun discussions during manuscript drafting. Your encouragement and endless supply of liquorice have helped during the most stressful times! To Sara, for all the work you do to support the people around you. Your knowledge and kindness shine through in everything you do! To Anna for always being there when I needed someone to talk to, for your honesty, endless support and pep-talk. What would I have done without you and our discussions about everything and nothing! To Karoline for all your help and all discussions about research and everything else, your generosity and kindness are truly inspiring! To Paul for all your support and your singing! Thank you for always sharing your knowledge! To Simona, Elena, Mamen, Jennifer, Andreas, Ylva, Susan, Gabrielle and Svanheidur for all discussions and fun social activities, you have all made it fun to come to work!

I want to thank Emma, Soheila, Lisa, Parmida, Daniel, Gustav, Stefan, Malin, Christoffer, Pernilla and all other members of Ståhlberg and Åhman groups, for valuable collaborations and fun lunch discussions. To all other colleagues and friends at Sahlgrenska Cancer Center, for making such a fun and collaborative work environment. A special thank you to Kristell, Emil, Marta, Maryam and Elin for all fun office-times and for your encouragement and support during these years.

To my friends. To Sara, you are like a sister to me and I truly appreciate your honesty and support, through my PhD-education but also through everything else in life. To my ”Lunda-gäng”. Alisa, Ida, Kajsa and Rickard. To talk to you about research and work when things have been tough as

well as just laughing and hanging out are so important to me! None of this would have been as fun without you!

Sist men absolut inte minst vill jag rikta ett stort tack till min familj, utan er hade detta aldrig varit möjligt. Till mina föräldrar som alltid stöttar och tror på mig. Till min mormor och morfar som alltid uppmuntrar mig till att göra det jag tror på och att gå min egen väg. Till mina bröder Anton och Albin för att ni alltid finns vid min sida och gör allting roligare. Till mina syskonbarn Malte, August och Harry som utan att egentligen veta om det stöttat mig genom att bara vara och påminna mig om att ibland är det okej att bara ta det lugnt och titta på Pokémon.

REFRENCES

[1]. Socialstyrelsen. *Statistik om dödsorsaker 2019*. 2020 [cited 2020 20-11-30]; Art.nr: 2020-6-6798]. Available from: https://www.socialstyrelsen.se/globalassets/sharepoint-dokument/artikelkatalog/statistik/2020-6-6798.pdf.
[2]. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2018. **68**(6): p. 394-424.
[3]. Osborne, C., P. Wilson, and D. Tripathy, *Oncogenes and Tumor Suppressor Genes in Breast Cancer: Potential Diagnostic and Therapeutic Applications.* 2004. **9**(4): p. 361-377.
[4]. Kamińska, M., et al., *Breast cancer risk factors.* Przeglad menopauzalny = Menopause review, 2015. **14**(3): p. 196-202.
[5]. Fasching, P.A., et al., *Breast Cancer Risk - Genes, Environment and Clinics.* Geburtshilfe und Frauenheilkunde, 2011. **71**(12): p. 1056-1066.
[6]. Fackenthal, J.D. and O.I. Olopade, *Breast cancer risk associated with BRCA1 and BRCA2 in diverse populations.* Nature Reviews Cancer, 2007. **7**(12): p. 937-948.
[7]. Sharma, G.N., et al., *Various types and management of breast cancer: an overview.* Journal of advanced pharmaceutical technology & research, 2010. **1**(2): p. 109-126.
[8]. Guiu, S., et al., *Molecular subclasses of breast cancer: how do we define them? The IMPAKT 2012 Working Group Statement.* Ann Oncol, 2012. **23**(12): p. 2997-3006.
[9]. van Seijen, M., et al., *Ductal carcinoma in situ: to treat or not to treat, that is the question.* British journal of cancer, 2019. **121**(4): p. 285-292.
[10]. Pencavel, T.D. and A. Hayes, *Breast sarcoma – a review of diagnosis and management.* International Journal of Surgery, 2009. **7**(1): p. 20-23.
[11]. Fuentes, N. and P. Silveyra, *Estrogen receptor signaling mechanisms.* Advances in protein chemistry and structural biology, 2019. **116**: p. 135-170.
[12]. Deroo, B.J. and K.S. Korach, *Estrogen receptors and human disease.* The Journal of clinical investigation, 2006. **116**(3): p. 561-570.
[13]. Omoto, Y. and H. Iwase, *Clinical significance of estrogen receptor β in breast and prostate cancer from biological aspects.* Cancer science, 2015. **106**(4): p. 337-343.
[14]. Mitri, Z., T. Constantine, and R. O'Regan, *The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in*

Therapy. Chemotherapy research and practice, 2012. **2012**: p. 743193-743193.
[15]. Rouzier, R., et al., *Breast cancer molecular subtypes respond differently to preoperative chemotherapy.* Clin Cancer Res, 2005. **11**(16): p. 5678-85.
[16]. Eliyatkın, N., et al., *Molecular Classification of Breast Carcinoma: From Traditional, Old-Fashioned Way to A New Age, and A New Way.* The journal of breast health, 2015. **11**(2): p. 59-66.
[17]. Rakha, E.A., et al., *Breast cancer prognostic classification in the molecular era: the role of histological grade.* Breast Cancer Research, 2010. **12**(4): p. 207.
[18]. Giuliano, A.E., et al., *Breast Cancer-Major changes in the American Joint Committee on Cancer eighth edition cancer staging manual.* CA Cancer J Clin, 2017. **67**(4): p. 290-303.
[19]. Waks, A.G. and E.P. Winer, *Breast Cancer Treatment: A Review.* Jama, 2019. **321**(3): p. 288-300.
[20]. Osborne, C.K. and R. Schiff, *Mechanisms of endocrine resistance in breast cancer.* Annual review of medicine, 2011. **62**: p. 233-247.
[21]. Hudis, C.A., *Trastuzumab - Mechanism of action and use in clinical practice.* New England Journal of Medicine, 2007. **357**(1): p. 39-51.
[22]. Piccart-Gebhart, M.J., et al., *Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer.* N Engl J Med, 2005. **353**(16): p. 1659-72.
[23]. Wahba, H.A. and H.A. El-Hadaad, *Current approaches in treatment of triple-negative breast cancer.* Cancer biology & medicine, 2015. **12**(2): p. 106-116.
[24]. Lønning, P.E., *Poor-prognosis estrogen receptor- positive disease: present and future clinical solutions.* Therapeutic advances in medical oncology, 2012. **4**(3): p. 127-137.
[25]. Korkaya, H., S. Liu, and M.S. Wicha, *Breast cancer stem cells, cytokine networks, and the tumor microenvironment.* J Clin Invest, 2011. **121**(10): p. 3804-9.
[26]. Oskarsson, T., *Extracellular matrix components in breast cancer progression and metastasis.* Breast, 2013. **22 Suppl 2**: p. S66-72.
[27]. Soysal, S.D., A. Tzankov, and S.E. Muenst, *Role of the Tumor Microenvironment in Breast Cancer.* Pathobiology, 2015. **82**(3-4): p. 142-52.
[28]. Quail, D.F. and J.A. Joyce, *Microenvironmental regulation of tumor progression and metastasis.* Nature medicine, 2013. **19**(11): p. 1423-1437.
[29]. Mittal, K., J. Ebos, and B. Rini, *Angiogenesis and the tumor microenvironment: vascular endothelial growth factor and beyond.* Seminars in oncology, 2014. **41**(2): p. 235-251.

[30]. Harrison, H., et al., *Contrasting hypoxic effects on breast cancer stem cell hierarchy is dependent on ER-α status.* Cancer Res, 2013. **73**(4): p. 1420-33.
[31]. Acerbi, I., et al., *Human breast cancer invasion and aggression correlates with ECM stiffening and immune cell infiltration.* Integrative biology : quantitative biosciences from nano to macro, 2015. **7**(10): p. 1120-1134.
[32]. Hu, G., L. Li, and W. Xu, *Extracellular matrix in mammary gland development and breast cancer progression.* Frontiers in Laboratory Medicine, 2017. **1**(1): p. 36-39.
[33]. Maziveyi, M. and S.K. Alahari, *Cell matrix adhesions in cancer: The proteins that form the glue.* Oncotarget, 2017. **8**(29): p. 48471-48487.
[34]. Xu, S., et al., *The role of collagen in cancer: from bench to bedside.* Journal of Translational Medicine, 2019. **17**(1): p. 309.
[35]. Insua-Rodríguez, J. and T. Oskarsson, *The extracellular matrix in breast cancer.* Adv Drug Deliv Rev, 2016. **97**: p. 41-55.
[36]. Liu, J., et al., *Collagen 1A1 (COL1A1) promotes metastasis of breast cancer and is a potential therapeutic target.* Discov Med, 2018. **25**(139): p. 211-223.
[37]. Zhang, H., et al., *Membrane associated collagen XIII promotes cancer metastasis and enhances anoikis resistance.* Breast Cancer Res, 2018. **20**(1): p. 116.
[38]. Aumailley, M., *The laminin family.* Cell adhesion & migration, 2013. **7**(1): p. 48-55.
[39]. Kwon, S.Y., et al., *Laminin 332 expression in breast carcinoma.* Appl Immunohistochem Mol Morphol, 2012. **20**(2): p. 159-64.
[40]. Domogatskaya, A., et al., *Laminin-511 but Not -332, -111, or -411 Enables Mouse Embryonic Stem Cell Self-Renewal In Vitro.* 2008. **26**(11): p. 2800-2809.
[41]. Huleihel, L., et al., *Matrix-bound nanovesicles within ECM bioscaffolds.* Science Advances, 2016. **2**(6): p. e1600502.
[42]. Hussey, G.S., et al., *Lipidomics and RNA sequencing reveal a novel subpopulation of nanovesicle within extracellular matrix biomaterials.* 2020. **6**(12): p. eaay4361.
[43]. Liu, T., et al., *Cancer-associated fibroblasts: an emerging target of anti-cancer immunotherapy.* Journal of Hematology & Oncology, 2019. **12**(1): p. 86.
[44]. Busch, S., et al., *Cellular organization and molecular differentiation model of breast cancer-associated fibroblasts.* Molecular Cancer, 2017. **16**(1): p. 73.
[45]. Lappano, R., et al., *Cancer associated fibroblasts: role in breast cancer and potential as therapeutic targets.* Expert Opinion on Therapeutic Targets, 2020. **24**(6): p. 559-572.

[46]. Pereira, B.A., et al., *CAF Subpopulations: A New Reservoir of Stromal Targets in Pancreatic Cancer.* Trends Cancer, 2019. **5**(11): p. 724-741.
[47]. Salimifard, S., et al., *Cancer associated fibroblasts as novel promising therapeutic targets in breast cancer.* Pathol Res Pract, 2020. **216**(5): p. 152915.
[48]. DeNardo, D.G. and L.M. Coussens, *Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression.* Breast Cancer Research, 2007. **9**(4): p. 212.
[49]. Shihab, I., et al., *Understanding the Role of Innate Immune Cells and Identifying Genes in Breast Cancer Microenvironment.* Cancers, 2020. **12**(8): p. 2226.
[50]. Galli, F., et al., *Relevance of immune cell and tumor microenvironment imaging in the new era of immunotherapy.* Journal of Experimental & Clinical Cancer Research, 2020. **39**(1): p. 89.
[51]. Soto-Perez-de-Celis, E., et al., *Tumor-Associated Neutrophils in Breast Cancer Subtypes.* Asian Pacific journal of cancer prevention : APJCP, 2017. **18**(10): p. 2689-2693.
[52]. Speiser, D.E., P.-C. Ho, and G. Verdeil, *Regulatory circuits of T cell function in cancer.* Nature Reviews Immunology, 2016. **16**(10): p. 599-611.
[53]. Martínez-Lostao, L., A. Anel, and J. Pardo, *How Do Cytotoxic Lymphocytes Kill Cancer Cells?* 2015. **21**(22): p. 5047-5056.
[54]. Disis, M.L., H. Bernhard, and E.M. Jaffee, *Use of tumour-responsive T cells as cancer treatment.* Lancet (London, England), 2009. **373**(9664): p. 673-683.
[55]. Pontiggia, O., et al., *The tumor microenvironment modulates tamoxifen resistance in breast cancer: a role for soluble stromal factors and fibronectin through β1 integrin.* Breast Cancer Research and Treatment, 2012. **133**(2): p. 459-471.
[56]. Arechavaleta-Velasco, F., et al., *Progranulin and its biological effects in cancer.* Medical oncology (Northwood, London, England), 2017. **34**(12): p. 194-194.
[57]. Rhost, S., et al., *Sortilin inhibition limits secretion-induced progranulin-dependent breast cancer progression and cancer stem cell expansion.* Breast Cancer Res, 2018. **20**(1): p. 137.
[58]. Cenik, B., et al., *Progranulin: a proteolytically processed protein at the crossroads of inflammation and neurodegeneration.* The Journal of biological chemistry, 2012. **287**(39): p. 32298-32306.
[59]. Demorrow, S., *Progranulin: a novel regulator of gastrointestinal cancer progression.* Transl Gastrointest Cancer, 2013. **2**(3): p. 145-151.

[60]. Jian, J., et al., *Progranulin directly binds to the CRD2 and CRD3 of TNFR extracellular domains.* FEBS Letters, 2013. **587**(21): p. 3428-3436.
[61]. Chen, X., et al., *Progranulin does not bind tumor necrosis factor (TNF) receptors and is not a direct regulator of TNF-dependent signaling or bioactivity in immune or neuronal cells.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2013. **33**(21): p. 9202-9213.
[62]. Hessman, C.L., et al., *YB-1 interferes with tnfα–tnfr binding and modulates progranulin-mediated inhibition of TNFα signaling.* International Journal of Molecular Sciences, 2020. **21**(19): p. 1-17.
[63]. Neill, T., et al., *EphA2 is a functional receptor for the growth factor progranulin.* The Journal of cell biology, 2016. **215**(5): p. 687-703.
[64]. Tangkeangsirisin, W. and G. Serrero, *PC cell-derived growth factor (PCDGF/GP88, progranulin) stimulates migration, invasiveness and VEGF expression in breast cancer cells.* Carcinogenesis, 2004. **25**(9): p. 1587-92.
[65]. Yang, D., et al., *Progranulin promotes colorectal cancer proliferation and angiogenesis through TNFR2/Akt and ERK signaling pathways.* American journal of cancer research, 2015. **5**(10): p. 3085-3097.
[66]. Tkaczuk, K.H.R., et al., *Association of Serum Progranulin Levels With Disease Progression, Therapy Response and Survival in Patients With Metastatic Breast Cancer.* Clinical Breast Cancer, 2020. **20**(3): p. 220-227.
[67]. Tanaka, T., M. Narazaki, and T. Kishimoto, *IL-6 in inflammation, immunity, and disease.* Cold Spring Harbor perspectives in biology, 2014. **6**(10): p. a016295-a016295.
[68]. Rose-John, S., *IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6.* International journal of biological sciences, 2012. **8**(9): p. 1237-1247.
[69]. Wolf, J., S. Rose-John, and C. Garbers, *Interleukin-6 and its receptors: A highly regulated and dynamic system.* Cytokine, 2014. **70**(1): p. 11-20.
[70]. Yabe-Wada, T., et al., *TLR signals posttranscriptionally regulate the cytokine trafficking mediator sortilin.* Scientific Reports, 2016. **6**(1): p. 26566.
[71]. Mortensen, M.B., et al., *Targeting sortilin in immune cells reduces proinflammatory cytokines and atherosclerosis.* The Journal of clinical investigation, 2014. **124**(12): p. 5317-5322.
[72]. Méndez-García, L.A., et al., *Breast Cancer Metastasis: Are Cytokines Important Players During Its Development and Progression?* J Interferon Cytokine Res, 2019. **39**(1): p. 39-55.

[73]. Hunter, C.A. and S.A. Jones, *IL-6 as a keystone cytokine in health and disease.* Nature Immunology, 2015. **16**(5): p. 448-457.
[74]. Jevnikar, Z., et al., *Epithelial IL-6 trans-signaling defines a new asthma phenotype with increased airway inflammation.* Journal of Allergy and Clinical Immunology, 2019. **143**(2): p. 577-590.
[75]. Johnson, D.E., R.A. O'Keefe, and J.R. Grandis, *Targeting the IL-6/JAK/STAT3 signalling axis in cancer.* Nature Reviews Clinical Oncology, 2018. **15**(4): p. 234-248.
[76]. Ortiz-Montero, P., A. Londoño-Vallejo, and J.P. Vernot, *Senescence-associated IL-6 and IL-8 cytokines induce a self- and cross-reinforced senescence/inflammatory milieu strengthening tumorigenic capabilities in the MCF-7 breast cancer cell line.* Cell Commun Signal, 2017. **15**(1): p. 17.
[77]. Iliopoulos, D., et al., *Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion.* Proc Natl Acad Sci U S A, 2011. **108**(4): p. 1397-402.
[78]. Salgado, R., et al., *Circulating interleukin-6 predicts survival in patients with metastatic breast cancer.* Int J Cancer, 2003. **103**(5): p. 642-6.
[79]. Jin, K., N.B. Pandey, and A.S. Popel, *Simultaneous blockade of IL-6 and CCL5 signaling for synergistic inhibition of triple-negative breast cancer growth and metastasis.* Breast Cancer Research, 2018. **20**(1): p. 54.
[80]. Liu, Q., et al., *The CXCL8-CXCR1/2 pathways in cancer.* Cytokine & Growth Factor Reviews, 2016. **31**: p. 61-71.
[81]. Nasser, M.W., et al., *Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer.* J Immunol, 2009. **183**(5): p. 3425-32.
[82]. Heidemann, J., et al., *Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2.* J Biol Chem, 2003. **278**(10): p. 8508-15.
[83]. Gilowska, I., *[CXCL8 (interleukin 8)--the key inflammatory mediator in chronic obstructive pulmonary disease?].* Postepy Hig Med Dosw (Online), 2014. **68**: p. 842-50.
[84]. Bakouny, Z. and T.K. Choueiri, *IL-8 and cancer prognosis on immunotherapy.* Nature Medicine, 2020. **26**(5): p. 650-651.
[85]. Benoy, I.H., et al., *Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival.* Clin Cancer Res, 2004. **10**(21): p. 7157-62.
[86]. Najafi, M., et al., *Hypoxia in solid tumors: a key promoter of cancer stem cell (CSC) resistance.* Journal of Cancer Research and Clinical Oncology, 2020. **146**(1): p. 19-31.

[87]. Jing, X., et al., *Role of hypoxia in cancer therapy by regulating the tumor microenvironment.* Molecular Cancer, 2019. **18**(1): p. 157.
[88]. Campbell, E.J., et al., *Activation of the hypoxia pathway in breast cancer tissue and patient survival are inversely associated with tumor ascorbate levels.* BMC Cancer, 2019. **19**(1): p. 307.
[89]. Ziello, J.E., I.S. Jovin, and Y. Huang, *Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia.* The Yale journal of biology and medicine, 2007. **80**(2): p. 51-60.
[90]. Gilkes, D.M. and G.L. Semenza, *Role of hypoxia-inducible factors in breast cancer metastasis.* Future oncology (London, England), 2013. **9**(11): p. 1623-1636.
[91]. Denko, N.C., et al., *Investigating hypoxic tumor physiology through gene expression patterns.* Oncogene, 2003. **22**(37): p. 5907-14.
[92]. Hoffmann, C., et al., *Hypoxia promotes breast cancer cell invasion through HIF-1α-mediated up-regulation of the invadopodial actin bundling protein CSRP2.* Scientific Reports, 2018. **8**(1): p. 10191.
[93]. Liu, Z.-J., G.L. Semenza, and H.-F. Zhang, *Hypoxia-inducible factor 1 and breast cancer metastasis.* Journal of Zhejiang University. Science. B, 2015. **16**(1): p. 32-43.
[94]. Padró, M., et al., *Genome-independent hypoxic repression of estrogen receptor alpha in breast cancer cells.* BMC cancer, 2017. **17**(1): p. 203-203.
[95]. Uhlén, M., et al., *The human secretome.* 2019. **12**(609): p. eaaz0274.
[96]. GM, C. *The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Signaling Molecules and Their Receptors*. 2000 [cited 2020 20-11-25]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK9924/.
[97]. Paltridge, J.L., L. Belle, and Y. Khew-Goodall, *The secretome in cancer progression.* Biochim Biophys Acta, 2013. **1834**(11): p. 2233-41.
[98]. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nature Cell Biology, 2007. **9**(6): p. 654-659.
[99]. Fu, Z., E.R. Gilbert, and D. Liu, *Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes.* Current diabetes reviews, 2013. **9**(1): p. 25-53.
[100]. Gribble, F.M. and F. Reimann, *Function and mechanisms of enteroendocrine cells and gut hormones in metabolism.* Nature Reviews Endocrinology, 2019. **15**(4): p. 226-237.
[101]. Viotti, C., *ER to Golgi-Dependent Protein Secretion: The Conventional Pathway*, in *Unconventional Protein Secretion: Methods and Protocols*, A. Pompa and F. De Marchis, Editors. 2016, Springer New York: New York, NY. p. 3-29.

[102]. Lodish H, B.A., Zipursky SL. *Molecular Cell Biology. 4th edition. New York: W. H. Freeman; 2000. Section 17.3, Overview of the Secretory Pathway*. 2000 [cited 2020 2020-11-26]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK21471/.
[103]. Dimou, E. and W. Nickel, *Unconventional mechanisms of eukaryotic protein secretion.* Curr Biol, 2018. **28**(8): p. R406-r410.
[104]. Kim, J., H.Y. Gee, and M.G. Lee, *Unconventional protein secretion – new insights into the pathogenesis and therapeutic targets of human diseases.* Journal of Cell Science, 2018. **131**(12): p. jcs213686.
[105]. Roswall, P., et al., *Microenvironmental control of breast cancer subtype elicited through paracrine platelet-derived growth factor-CC signaling.* Nat Med, 2018. **24**(4): p. 463-473.
[106]. Gregori, J., et al., *Enhancing the Biological Relevance of Secretome-Based Proteomics by Linking Tumor Cell Proliferation and Protein Secretion.* Journal of Proteome Research, 2014. **13**(8): p. 3706-3721.
[107]. Redig, A.J. and S.S. McAllister, *Breast cancer as a systemic disease: a view of metastasis.* Journal of internal medicine, 2013. **274**(2): p. 113-126.
[108]. Jin, X. and P. Mu, *Targeting Breast Cancer Metastasis.* Breast cancer : basic and clinical research, 2015. **9**(Suppl 1): p. 23-34.
[109]. Kaplan, R.N., et al., *VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche.* Nature, 2005. **438**(7069): p. 820-7.
[110]. Peinado, H., et al., *Pre-metastatic niches: organ-specific homes for metastases.* Nat Rev Cancer, 2017. **17**(5): p. 302-317.
[111]. Doglioni, G., S. Parik, and S.-M. Fendt, *Interactions in the (Pre)metastatic Niche Support Metastasis Formation.* 2019. **9**(219).
[112]. Coffelt, S.B., et al., *IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis.* Nature, 2015. **522**(7556): p. 345-348.
[113]. Erler, J.T., et al., *Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche.* Cancer Cell, 2009. **15**(1): p. 35-44.
[114]. Loke, S.Y. and A.S.G. Lee, *The future of blood-based biomarkers for the early detection of breast cancer.* European Journal of Cancer, 2018. **92**: p. 54-68.
[115]. Hanash, S.M., C.S. Baik, and O. Kallioniemi, *Emerging molecular biomarkers—blood-based strategies to detect and monitor cancer.* Nature Reviews Clinical Oncology, 2011. **8**(3): p. 142-150.
[116]. Beck, B. and C. Blanpain, *Unravelling cancer stem cell potential.* Nature Reviews Cancer, 2013. **13**(10): p. 727-738.

[117]. Kreso, A. and J.E. Dick, *Evolution of the cancer stem cell model.* Cell Stem Cell, 2014. **14**(3): p. 275-91.
[118]. Yu, Z., et al., *Cancer stem cells.* Int J Biochem Cell Biol, 2012. **44**(12): p. 2144-51.
[119]. Zhou, J., et al., *Stem Cells and Cellular Origins of Breast Cancer: Updates in the Rationale, Controversies, and Therapeutic Implications.* Frontiers in oncology, 2019. **9**: p. 820-820.
[120]. Akrap, N., et al., *Identification of Distinct Breast Cancer Stem Cell Populations Based on Single-Cell Analyses of Functionally Enriched Stem and Progenitor Pools.* Stem cell reports, 2016. **6**(1): p. 121-136.
[121]. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(7): p. 3983-3988.
[122]. Elbaiomy, M.A., et al., *Clinical Impact of Breast Cancer Stem Cells in Metastatic Breast Cancer Patients.* Journal of oncology, 2020. **2020**: p. 2561726-2561726.
[123]. Zhao, J., *Cancer stem cells and chemoresistance: The smartest survives the raid.* Pharmacology & therapeutics, 2016. **160**: p. 145-158.
[124]. Shaw, F.L., et al., *A detailed mammosphere assay protocol for the quantification of breast stem cell activity.* J Mammary Gland Biol Neoplasia, 2012. **17**(2): p. 111-7.
[125]. Beaver, C.M., A. Ahmed, and J.R. Masters, *Clonogenicity: holoclones and meroclones contain stem cells.* PloS one, 2014. **9**(2): p. e89834-e89834.
[126]. Hanahan, D. and Robert A. Weinberg, *Hallmarks of Cancer: The Next Generation.* Cell, 2011. **144**(5): p. 646-674.
[127]. Mirabelli, P., L. Coppola, and M. Salvatore, *Cancer Cell Lines Are Useful Model Systems for Medical Research.* Cancers, 2019. **11**(8): p. 1098.
[128]. Wilding, J.L. and W.F. Bodmer, *Cancer cell lines for drug discovery and development.* Cancer Res, 2014. **74**(9): p. 2377-84.
[129]. Lv, D., et al., *Three-dimensional cell culture: A powerful tool in tumor research and drug discovery.* Oncology letters, 2017. **14**(6): p. 6999-7010.
[130]. Langhans, S.A., *Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning.* Front Pharmacol, 2018. **9**: p. 6.
[131]. Knight, E. and S. Przyborski, *Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro.* 2015. **227**(6): p. 746-756.
[132]. D'Angelo, E., et al., *Patient-Derived Scaffolds of Colorectal Cancer Metastases as an Organotypic 3D Model of the Liver Metastatic Microenvironment.* Cancers (Basel), 2020. **12**(2).

[133]. Dunne, L.W., et al., *Human decellularized adipose tissue scaffold as a model for breast cancer cell growth and drug treatments.* Biomaterials, 2014. **35**(18): p. 4940-9.
[134]. Sontheimer-Phelps, A., B.A. Hassell, and D.E. Ingber, *Modelling cancer in microfluidic human organs-on-chips.* Nature Reviews Cancer, 2019. **19**(2): p. 65-81.
[135]. *OLINK*. [cited 2020 2020-09-04]; Available from: https://www.olink.com/.
[136]. Ali, A.S., et al., *Candidate protein biomarkers in pancreatic neuroendocrine neoplasms grade 3.* Scientific Reports, 2020. **10**(1): p. 10639.
[137]. Sundberg, I., et al., *Daytime melatonin levels in saliva are associated with inflammatory markers and anxiety disorders.* Psychoneuroendocrinology, 2020. **112**: p. 104514.
[138]. Lindgren, K.E., et al., *Differences in secretome in culture media when comparing blastocysts and arrested embryos using multiplex proximity assay.* Upsala Journal of Medical Sciences, 2018. **123**(3): p. 143-152.
[139]. Csősz, É., et al., *Wound-Healing Markers Revealed by Proximity Extension Assay in Tears of Patients following Glaucoma Surgery.* Int J Mol Sci, 2018. **19**(12).
[140]. Lundberg, M., et al., *Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood.* Nucleic Acids Research, 2011. **39**(15): p. e102-e102.
[141]. Ghosh, R., J.E. Gilda, and A.V. Gomes, *The necessity of and strategies for improving confidence in the accuracy of western blots.* Expert review of proteomics, 2014. **11**(5): p. 549-560.
[142]. Pitt, J.J., *Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry.* The Clinical biochemist. Reviews, 2009. **30**(1): p. 19-34.
[143]. Semenza, G.L., *The hypoxic tumor microenvironment: A driving force for breast cancer progression.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2016. **1863**(3): p. 382-391.
[144]. Almiron Bonnin, D.A., et al., *Secretion-mediated STAT3 activation promotes self-renewal of glioma stem-like cells during hypoxia.* Oncogene, 2018. **37**(8): p. 1107-1118.
[145]. Khanna, P., et al., *GRAMD1B regulates cell migration in breast cancer cells through JAK/STAT and Akt signaling.* Scientific Reports, 2018. **8**(1): p. 9511.
[146]. Ma, J.-h., L. Qin, and X. Li, *Role of STAT3 signaling pathway in breast cancer.* Cell Communication and Signaling, 2020. **18**(1): p. 33.

[147]. Dethlefsen, C., G. Højfeldt, and P. Hojman, *The role of intratumoral and systemic IL-6 in breast cancer.* Breast Cancer Research and Treatment, 2013. **138**(3): p. 657-664.
[148]. Masjedi, A., et al., *The significant role of interleukin-6 and its signaling pathway in the immunopathogenesis and treatment of breast cancer.* Biomed Pharmacother, 2018. **108**: p. 1415-1424.
[149]. Qin, J.-J., et al., *STAT3 as a potential therapeutic target in triple negative breast cancer: a systematic review.* Journal of Experimental & Clinical Cancer Research, 2019. **38**(1): p. 195.
[150]. de Ruijter, T.C., et al., *Characteristics of triple-negative breast cancer.* Journal of Cancer Research and Clinical Oncology, 2011. **137**(2): p. 183-192.
[151]. Sporikova, Z., et al., *Genetic Markers in Triple-Negative Breast Cancer.* Clinical Breast Cancer, 2018. **18**(5): p. e841-e850.
[152]. Fischbach, C., et al., *Engineering tumors with 3D scaffolds.* Nature Methods, 2007. **4**(10): p. 855-860.
[153]. Chen, L., et al., *The enhancement of cancer stem cell properties of MCF-7 cells in 3D collagen scaffolds for modeling of cancer and anti-cancer drugs.* Biomaterials, 2012. **33**(5): p. 1437-1444.
[154]. Myungjin Lee, J., et al., *A three-dimensional microenvironment alters protein expression and chemosensitivity of epithelial ovarian cancer cells in vitro.* Laboratory Investigation, 2013. **93**(5): p. 528-542.
[155]. Tentler, J.J., et al., *Patient-derived tumour xenografts as models for oncology drug development.* Nature Reviews Clinical Oncology, 2012. **9**(6): p. 338-350.
[156]. Hidalgo, M., et al., *Patient-derived xenograft models: an emerging platform for translational cancer research.* Cancer Discov, 2014. **4**(9): p. 998-1013.
[157]. Bergamaschi, A., et al., *Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome.* J Pathol, 2008. **214**(3): p. 357-67.
[158]. Keshava Prasad, T.S., et al., *Human Protein Reference Database—2009 update.* Nucleic Acids Research, 2009. **37**(suppl_1): p. D767-D772.
[159]. Li, I. and B.Y. Nabet, *Exosomes in the tumor microenvironment as mediators of cancer therapy resistance.* Molecular cancer, 2019. **18**(1): p. 32-32.
[160]. Villasante, A. and G. Vunjak-Novakovic, *Tissue-engineered models of human tumors for cancer research.* Expert opinion on drug discovery, 2015. **10**(3): p. 257-268.
[161]. Landberg, G., et al., *Patient-derived scaffolds uncover breast cancer promoting properties of the microenvironment.* Biomaterials, 2019. **235**: p. 119705.

[162]. Karlsson, J., et al., *Transcriptomic Characterization of the Human Cell Cycle in Individual Unsynchronized Cells.* Journal of Molecular Biology, 2017. **429**(24): p. 3909-3924.
[163]. Chavey, C., et al., *Oestrogen receptor negative breast cancers exhibit high cytokine content.* Breast cancer research : BCR, 2007. **9**(1): p. R15-R15.
[164]. Wei, X., et al., *Tumor-secreted PAI-1 promotes breast cancer metastasis via the induction of adipocyte-derived collagen remodeling.* Cell Commun Signal, 2019. **17**(1): p. 58.
[165]. Li, S., et al., *Plasminogen activator inhibitor-1 in cancer research.* Biomed Pharmacother, 2018. **105**: p. 83-94.
[166]. Yang, C., et al., *CXCL1 stimulates migration and invasion in ER-negative breast cancer cells via activation of the ERK/MMP2/9 signaling axis.* International journal of oncology, 2019. **55**(3): p. 684-696.
[167]. Aghaei, M., et al., *Adenosine deaminase activity in the serum and malignant tumors of breast cancer: the assessment of isoenzyme ADA1 and ADA2 activities.* Clin Biochem, 2005. **38**(10): p. 887-91.
[168]. Ravishankaran, P. and R. Karunanithi, *Clinical significance of preoperative serum interleukin-6 and C-reactive protein level in breast cancer patients.* World journal of surgical oncology, 2011. **9**: p. 18-18.
[169]. Jing, B., et al., *IL6/STAT3 Signaling Orchestrates Premetastatic Niche Formation and Immunosuppressive Traits in Lung.* Cancer Res, 2020. **80**(4): p. 784-797.
[170]. Qian, B.Z., et al., *CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis.* Nature, 2011. **475**(7355): p. 222-5.
[171]. He, Z. and A. Bateman, *Progranulin (granulin-epithelin precursor, PC-cell-derived growth factor, acrogranin) mediates tissue repair and tumorigenesis.* J Mol Med (Berl), 2003. **81**(10): p. 600-12.
[172]. Yamamoto, Y., et al., *Association between increased serum GP88 (progranulin) concentrations and prognosis in patients with malignant lymphomas.* Clin Chim Acta, 2017. **473**: p. 139-146.
[173]. Singh, J.K., et al., *Recent advances reveal IL-8 signaling as a potential key to targeting breast cancer stem cells.* Breast Cancer Research, 2013. **15**(4): p. 210.
[174]. Wang, N., et al., *CXCL1 derived from tumor-associated macrophages promotes breast cancer metastasis via activating NF-κB/SOX4 signaling.* Cell Death & Disease, 2018. **9**(9): p. 880.
[175]. Matsubara, T., et al., *PGRN is a key adipokine mediating high fat diet-induced insulin resistance and obesity through IL-6 in adipose tissue.* Cell Metab, 2012. **15**(1): p. 38-50.
[176]. Liu, F., et al., *Interleukin-6-stimulated progranulin expression contributes to the malignancy of hepatocellular carcinoma cells by activating mTOR signaling.* Scientific Reports, 2016. **6**: p. 21260.

[177]. Frampton, G., et al., *Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism.* Gut, 2012. **61**(2): p. 268-277.
[178]. Zhu, J., et al., *Conversion of proepithelin to epithelins: roles of SLPI and elastase in host defense and wound repair.* Cell, 2002. **111**(6): p. 867-78.
[179]. Chen, W., Y. Qin, and S. Liu, *Cytokines, breast cancer stem cells (BCSCs) and chemoresistance.* Clinical and translational medicine, 2018. **7**(1): p. 27-27.
[180]. Jonasson, E., et al., *Identification of Breast Cancer Stem Cell Related Genes Using Functional Cellular Assays Combined With Single-Cell RNA Sequencing in MDA-MB-231 Cells.* 2019. **10**(500).