Patient-derived scaffolds as a 3D model for breast cancer

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Cover illustration: Heatmap of scaffold data by Stefan Filges

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“Jag skall bara”- Alfons Åberg

ABSTRACT

Breast cancer is the most common cancer form in women worldwide. Many patients will have recurrent disease and more efficient targeted therapies are needed. The tumor microenvironment is a heterogenous complex mix of cells and components influencing critical cancer processes including progression, signaling and invasion. In this thesis, we established an *in vivo*-like 3D cell culture platform using decellularized patient-derived scaffolds (PDS) to facilitate cell-cell and cell-microenvironment interactions similar to the situation *in vivo*. The PDS model was extensively evaluated and characterized showing that PDS cultures induced changes in gene and protein expression in cancer cell lines after 21 days of growth. Cancer cell lines growing in PDSs enriched for cells with cancer stem cell properties and decreased cells proliferation compared to monolayer cultures. These findings were corroborated by transcriptomic data from 3 cell lines growing in large PDS cohorts, also showing that genes related to epithelial-to-mesenchymal transition were heavily influenced by properties of individual scaffolds. Innate clinico-pathological characteristics including grade, histological subtype, lymph node metastasis of the cancers, as well as disease-free survival of patients could be identified by gene expression changes in adapting cancer cell lines. The suitability of the PDS model as a drug testing platform was evaluated using well-known endocrine therapies and a CDK4/6 inhibitor which demonstrated that PDS growth induced different cellular phenotypes in response to the drugs compared to 2D cultures, but also presented similarities to other 3D assays. A qPCR-based screen of cancer cell lines growing in PDSs that were treated with chemotherapies identified drug fingerprints that also could be linked to clinical properties of the original tumors, supporting the use of PDSs as a personalized drug testing platform and a prognostic tool *in vitro* that potentially could be utilized as a treatment prediction option for patients. Finally, the proteomic composition of PDSs was delineated using liquid chromatography-mass spectrometry/mass spectrometry identifying subtypes of PDSs based on their relative protein expression which was associated to clinical properties of the PDSs as well as presented important upregulated processes in the PDS model that could be potential therapeutic targets. In conclusion, this thesis elaborated the impact of cancer microenvironments and identified important properties influencing different subtypes of cell-free scaffolds.

Key words: Breast cancer, patient-derived scaffolds, 3D model, endocrine therapy, chemotherapy, cancer stem cells, drug testing

SAMMANFATTNING PÅ SVENSKA

Bröstcancer kan delas in i flera undergrupper med olika sjukdomsförlopp. För några subtyper finns det fler behandlingsalternativ medan subtyper av tumörer som saknar receptorer att angripa har färre behandlingsmöjligheter. Många patienter återfaller i sjukdom och det finns ett stort behov för bättre och mer effektiva behandlingar. Bröstcancer består ofta av olika sorters celler som tillsammans med de omgivande cellerna från stödvävnaden bidrar till uppbyggnaden av deras omgivning, den så kallade tumörmikromiljön. Mikromiljön i sin tur påverkar cellernas tillväxt genom att skapa gradienter av näring och syre samt influerar spridningen av cancern. Genom att påverka tillgängligheten av läkemedel till cellerna så kan även mikromiljön influera hur cellerna svarar på behandlingen samt göra dem mer motståndskraftiga. I denna avhandling har vi använt en metod som baseras på tumörer från bröstcancerpatienter där cellerna har tvättats bort. Tumörens kvarvarande scaffold består av olika proteiner och strukturer som skiljer sig både inom och mellan enskilda tumörer. Denna variation i struktur, hårdhet och komponenter påverkar cancercellinjers beteende när de odlas i dessa 3D-miljöer över tid, på både genuttrycks- och proteinnivå, samt hur de svarade på olika typer av behandling. Genuttrycksanalyser i ett större antal scaffolds påvisade att olika cancercellinjer reagerade på olika sätt i de enskilda mikromiljöerna, men att alla ökade uttryck av markörer relaterade till den tumörinitierande subpopulationen av cancerstamceller samt minskade sin spridningsförmåga. Behandling av scaffolds med konventionella hormonbehandlingar ökade mängden cancerstamceller och visade att celler reagerar annorlunda än när de odlas i cellkulturplattor. Genom att jämföra kliniska data om tumörerna samt de patienter som gav upphov till dem kunde vi finna att cancercellernas genuttryck influerades av egenskaper från de olika scaffoldsen, vilket kunde kopplas till hur cancercellinjerna svarade på kemoterapibehandling. Analys av proteininnehållet i scaffoldsen visade att tumörernas sammansättning är olika och till viss del relaterat till vart tumörerna bildades i bröstet. Andra delar av mikromiljön var mer beroende av tumörens grad och patientens ålder. Vi identifierade även flera proteiner som var högre uttryckta i tumörer från patienter med senare återkommande sjukdom, samt tumörer från patienter med sämre överlevnad. Sammanfattningsvis har vi i denna avhandling påvisat stor variation i den cellfria mikromiljön mellan olika bröstcancrar, samt deras olika förmåga att påverka beteendet i cancerceller vilket starkt indikerar att mikromiljön är ett viktigt mål för framtida behandlingsstrategier.

LIST OF PAPERS

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

I. Landberg G, Fitzpatrick P, Isakson P, Jonasson E, Karlsson J, Larsson E, Svanström A, Rafnsdottir S, Persson E, Gustafsson A, Andersson A, Rosendahl J, Petronis S, Ranji P, Gregersson P, Magnusson Y, Håkansson J, Ståhlberg A. Patient-derived scaffolds uncover breast cancer promoting properties of the microenvironment. Biomaterials 2020; 235; 119705

II. Gustafsson A, Garre E, Leiva MC, Salerno S, Ståhlberg A, Landberg G. Patient-derived scaffolds as a drug-testing platform for endocrine therapies in breast cancer. Scientific Reports 2021; 11; 13334

III. Garre E*, Gustafsson A*, Leiva MC, Håkansson J, Ståhlberg A, Kovacs A, Landberg G. Breast cancer patient-derived scaffolds can expose unique individual cancer progressing properties of the cancer microenvironment associated with clinical characteristics. Cancers 2022; 14; 2172. *Authors contributed equally

IV. Leiva MC*, Gustafsson A*, Garre E*, Ståhlberg A, Landberg G. Breast cancer patient-derived scaffolds representing individual cancer microenvironments influence chemotherapy responses in breast cancer cell lines consistent with clinical features. *Authors contributed equally (*Manuscript*)

V. Gustafsson A, Jonasson E, Ståhlberg A, Landberg G. Proteomics of cell-free patient-derived scaffolds from breast cancer identify clinically relevant imprinted proteins and cancer-progressing properties. (*Manuscript*)

Additional publications not part of this thesis

i. Jacobsson H, Harrison H, Hughes É, Persson E, Rhost S, Fitzpatrick P, Gustafsson A, Andersson D, Gregersson P, Magnusson Y, Ståhlberg A, Landberg G. Hypoxia-induced secretion stimulates breast cancer stem cell regulatory signaling pathways. Molecular Oncology 2019; 13; 8

ii. Landberg G, Jonasson E*, Gustafsson A*, Fitzpatrick P, Isakson P, Karlsson J, Larsson E, Svanström A, Rafnsdottir S, Persson E, Andersson A, Rosendahl J, Petronis S, Ranji P, Gregersson P, Magnusson Y, Håkansson J, Ståhlberg A. Characterization of cell-free breast cancer patient-derived scaffolds using liquid chromatography-mass spectrometry/mass spectrometry data and RNA sequencing data. Data in Brief 2020; 31; 105860. *Authors contributed equally

iii. Leiva MC, Garre E, Gustafsson A, Svanström A, Bogestål Y, Håkansson J, Ståhlberg A, Landberg G. Breast cancer patient-derived scaffolds as a tool to monitor chemotherapy responses in human tumor microenvironments. Journal of Cellular Physiology 2020; 236; 6

iv. Persson E, Gregersson P, Gustafsson A, Fitzpatrick P, Rhost S, Ståhlberg A, Landberg G. Patient-derived scaffolds influence secretion profiles in cancer cells mirroring clinical features and breast cancer subtypes. Cell Communication and signaling 2021; 19; 66

v. Popular scientific article. Gustafsson A, Jonasson E, Landberg G, Ståhlberg A. Nytt testsystem för bröstcancer avslöjar hur tumörens omgivning påverkar cancercellerna. Onkologi i Sverige 2020; 3; 20

ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
4-OHT	(Z)-4-Hydroxytamoxifen
5-FU	5- fluorouracil
CSC	Cancer stem cell
CDK	Cyclin-dependent kinases
DMEM	Dulbecco modified Eagle's medium
EMT	Epithelial-to-mesenchymal transition
ECM	Extracellular matrix
ERα	Estrogen receptor alpha
HER2	Human epidermal receptor 2
IC_{50}	Half-max inhibitory concentrations
LC	Liquid chromatography
MMP	Matrix-metalloproteinases
MS	Mass spectrometry
TMA	Tissue micro array
TME	Tumor microenvironment
TMT	Tandem mass tags
TF	Transcription factor
PCA	Principle component analysis
PDS	Patient-derived scaffold
PDX	Patient-derived xenograft
qPCR	Quantitative polymerase chain reaction

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INTRODUCTION

Cancer

Cancers develop when normal cells acquire malignant features and grow uncontrollably. In 2020, approximately 19.2 million people would receive a new cancer diagnosis around the world according to World Health Organization (WHO), making battling cancer one of the most important health challenges of this century[1]. There are more than hundred types of cancers that can arise from different tissues. Most cancers including breast, colon and ovarian cancers are formed by epithelial cells, while sarcomas are formed in bones as well as soft tissues and fibrous tissues. Non-solid cancers that originate in the blood are called leukaemia and are characterised by elevated levels of white blood cells which outcompetes the normal cells in the blood, but there are also myeloma derived from plasma cells, melanoma originating from melanocytes, brain and spinal cord tumors as well as neuroendocrine tumors[1]. The malignant transformation of cells to cancer cells are summarized as 14 hallmarks of cancer by Hanahan and Weinberg including invasion and metastasis, enabling replicative immortality, and inducing angiogenesis (Figure 1)[2].

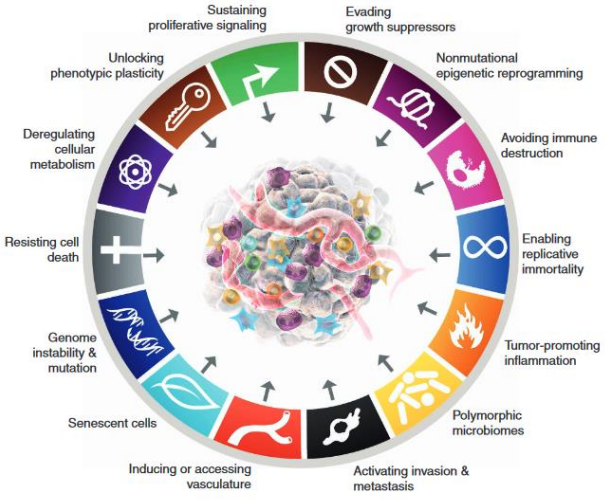

Figure 1. Hallmarks of Cancer. Schematic figure of the 14 suggested hallmarks of cancer from Hanahan[3].

To this list several features has been added over the years such as avoiding immune destruction, tumor-promoting inflammation, deregulating cellular metabolism, genome instability & mutation, unlocking phenotypic plasticity, polymorphic microbiomes, non-mutational epigenetic programming, and senescent cells[3].

Breast development

The breast is a complex endocrine organ that develop during embryogenesis. The dynamic development of the breast occurs in distinct stages during a woman's life and the processes involves local and paracrine signaling. Under the influence of increasing estrogen levels and other hormones during puberty, mammary epithelial cells inside the terminal end buds start to proliferate and differentiate to invade the surrounding stroma to fill the breast fat pads (Figure 2)[4]. The mature breast is ordered into branching network of ducts that join at the nipple with blood and lymph vessels, surrounded by stromal cells[5]. During pregnancy, progesterone and prolactin caused the formation of alveoli, which secretes milk during lactation. The mammary glands are dynamic structures that must be able to undergo morphologic changes during different stages in life and repeatedly fall back to its initial structure[6]. Many tightly regulated pathways are necessary to achieve this high degree of plasticity and dysregulation in this fine-tuned system can result in cancer[7].

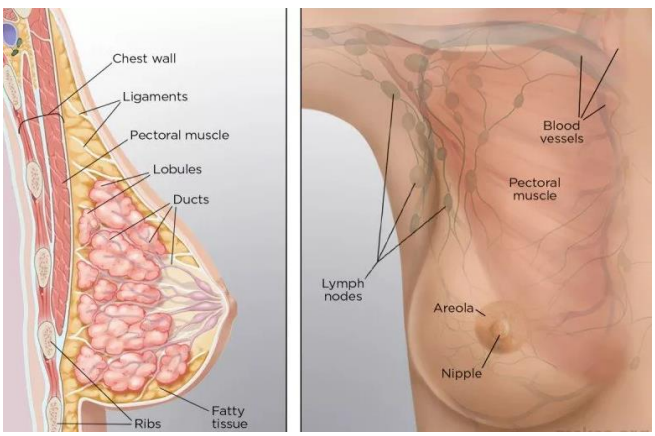

Figure 2. Anatomy of the breast. Schematic illustration of the glandular tissue including lobules and ducts. Fat tissues permeate the areas between glandular and stromal tissue consisting of supportive-, connective tissue and blood vessels. Adapted from mskcc.org.

Breast cancer development

1 in 8 women will develop breast cancer during their life time and globally 685,000 women dies from this disease each year[1]. Risk factors breast cancer are early menarche, late childbearing, high body-mass-index, and alcohol consumption. Breast cancer is characterized by large genomic instability leading to vast inter and intra-tumor heterogeneity[3]. Breast cancer have around 40 known recurrent driver aberrations that contributes to tumor progression and formation of the tumor microenvironment (TME), such as mutations and amplification of oncogenes *ERBB2* and *MYC, PIK3CA* or in tumor suppressor genes such as *BRCA1/2* and *P53,* and the gene *RB1* encoding retinoblastoma protein (RB)[8]. Development of breast cancer starts by epithelial cells in the ductal lining or lobules dividing uncontrollably after acquiring mutations in onco- or tumor suppressor genes (Figure 3). In the pre-invasive lesions, the cancer cells are restricted in the ducts or lobules and are characterized as hyperplasia. Ducts and lobules can also be filled with hyperchromatic polarized cells with high proliferation which is instead referred to as atypical hyperplasia that may not progress into carcinoma but increases the likelihood of developing the disease by 4-fold in women. After complete filling of the duct or lobules, its characterized as ductal/lobular carcinoma *in situ* (DCIS/LCIS)[9]. In ductal or lobular invasive carcinoma (IDC/ILC), the cells evade signals to undergo apoptosis as they lose their cell-cell contact and invade surrounding tissues. Cancer cells can also enter the blood stream or lymphatic system to go to distant sites to metastasize and colonize other tissues, which is considered the most malignant characteristic of any cancer[10]. Invasive breast cancer or metastases can affect vital organs such as the brain and liver, as well as airways which can result in death. Further, the general risk of recurrent breast cancer disease by metastasis or secondary cancer is 30% after treatment[11,12].

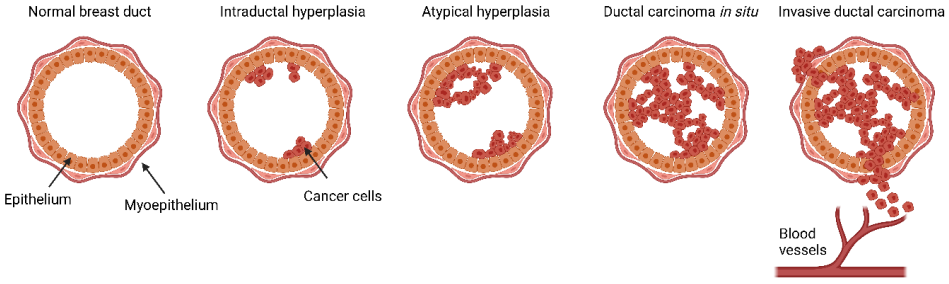

Figure 3. The different stages of breast cancer development illustrated in the ducts. Cancer cells increases proliferation in the mammary ducts which can cause ductal hyperplasia as well as to atypical ductal hyperplasia. This leads to ductal carcinoma *in situ* (DCIS) which is recognized as the filling of the mammary duct with cancer cells. Once tumor cells escape through the myoepithelium and cells leaves the ducts and spreads, the cancer is classified as invasive ductal carcinoma (IDC). Image was based on [9] and created using Biorender.com.

Diagnosis and classification

Primary testing for patients includes ultrasound, mammography, and biopsy that sometimes is complemented with magnetic resonance imaging (MRI). Increased mammography screenings have led to earlier detection rates, which together with improved hormonal treatments has increased the overall 5-year survival rate of breast cancer patients[13,14].

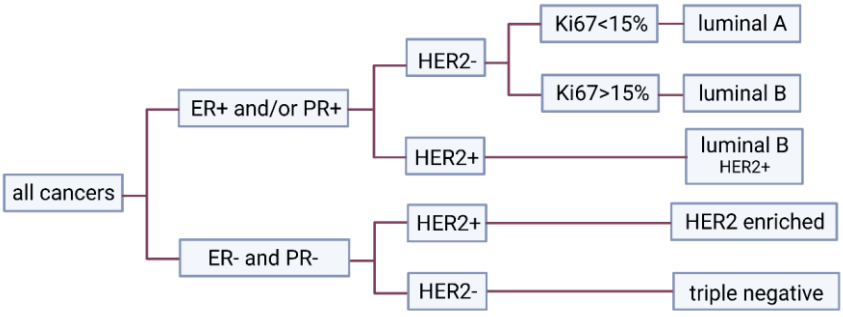

Figure 4. Molecular subclassifications of breast cancer based on protein and RNA expression of markers. Based on data from [5] and [15]. Illustration is created using Biorender.com.

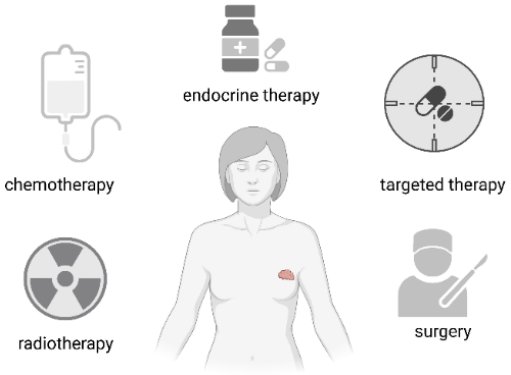

Figure 5. Therapeutic options for breast cancer treatment. Figure is created using Biorender.

Breast cancer is subdivided into four intrinsic types: Luminal A (50-60%), Luminal B (10-20%), triple negative/basal-like (10-15%) and HER2-enriched (15-20%) based on molecular expression patterns. Each subtype is defined by expression of progesterone receptor (PR), estrogen receptor α (ERα), human epidermal growth factor receptor 2 (HER2, encoded by the *ERBB2* gene) as well as proliferation marker Ki67 which is analysed through immunohistochemistry protein evaluation and fluorescent *in situ* hybridization (FISH), summarized in Figure 4[5,15]. These classifications are used to guide treatment decisions, in combination with tumor-node-metastasis staging, histological type, age, size of the tumor and, grade (BRE)[16]. Genomic panels such as MammaPrint, Pam50 or Oncotype DX can further guide treatment decisions regarding chemotherapy as well as predict recurrence risk in patients based on a selection of markers. Inherited mutations such as *BRCA1/2* status also guide treatment decisions accounting for approximately 5-10% of breast cancers[17]. Treatment plans are created by multidisciplinary teams that consider previously described characteristics, as well as general health of the patient. The primary treatment for breast cancer is commonly surgery which can be mastectomy or breast-conserving surgery (Figure 5). Many patients also benefit from radiation to reduce risk of refractory disease after surgerty[16].

Therapies against breast cancer

Endocrine therapy

70-80% of all breast cancer tumors are ERα-positive and most will be treated with endocrine therapies[18]. Binding of estrogen to ERα causes conformational changes in the receptor promoting dimerization and translocation to the nucleus where it activates transcription of the estrogen responsive elements. Estrogen is primarily produced by the ovaries in pre-menopausal women and sometimes ovarian ablation or suppression using medicine can be applied in ERα-positive cancers to reduce the amount of hormone in patients during endocrine treatment[19]. One of the most common endocrine therapies is tamoxifen, a selective estrogen receptor modulator that competitively binds to ERα and thereby inhibit downstream estrogen signaling inducing proliferative arrest and forcing tumor cells to undergo apoptosis limiting disease progression (Figure 6)[20]. Tamoxifen is used in pre-and postmenopausal women over a 5–10-year period and drastically reduces recurrence and mortality in breast cancer patients[21]. Fulvestrant, another widely used endocrine therapy, selectively binds to ERα causing its degradation and limiting ERα signaling. Fulvestrant can be used as a first-line treatment and monotreatment to treat ERα-positive locally advanced or metastatic cancer in post-menopausal women increasing disease-free survival[22]. Post-menopausal women mainly produces estrogen from mesenchymal cells in fat tissues[19]. These patients can also be treated with aromatase inhibitors (such as anastrozole, letrozole and exemestane) to stop cancer progression by inhibiting production of estrogen by blocking conversion of androstenedione/testosterone in the peripheral tissues into estrogen/estradiol (Figure 5). Unfortunately, many patients receiving endocrine therapies will have recurrent disease. In addition, treatment side effects contribute to poor treatment adherence leading earlier relapses[23]. Thus, predicting which patients that are having a poor prognosis with increased need for disease monitoring and extended treatments from those with a better prognosis could also reduce over-treatment.

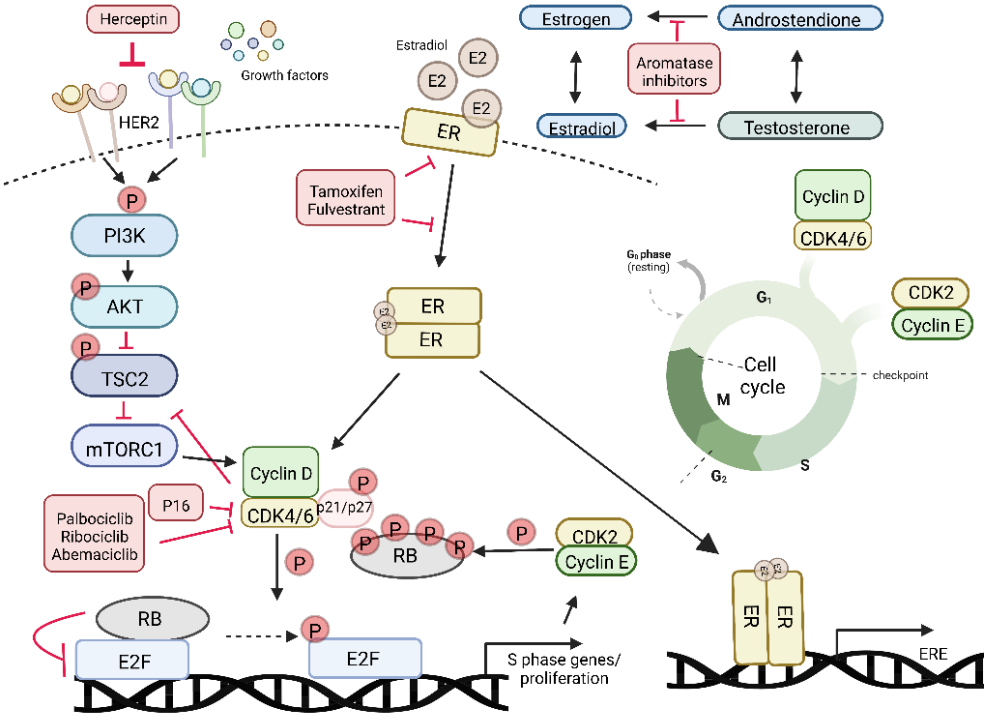

Figure 6. Schematic figure illustrating estrogen receptor signaling and downstream pathways and drug targets. E2; estradiol, ER; estrogen receptor, ERE; Estrogen response elements, CDK; cyclin dependent kinase, RB; RB. Adapted from [24]. Figure created using Biorender.com

Chemotherapies

Prescence of lymph node metastasis, size, grade, metastasis, and HER2-status of the cancers will be considered before administrating chemotherapies to patients. Patients can be treated with chemotherapy prior to surgery to reduce tumor size as well as post-surgery to reduce the risk of recurrence. Chemotherapies are developed to cause damages in the DNA of rapidly proliferating cells through different mechanisms in a non-specific manner. Common therapies for breast cancer include intercalating compounds such as anthracyclines (including doxorubicin and epirubicin). When cells take up the drugs, anthracyclines are being inserted to the DNA inhibiting replication and transcription resulting in cell death. Anti-metabolites (such as 5-flourouracil) restrict the access to building blocks of the DNA or RNA by competing with them or inhibiting their synthesis which leads to impaired cell metabolism and

protein synthesis. Taxanes (including paclitaxel and docetaxel) disrupt the cytoskeleton by inhibiting the function of microtubules that are crucial for cellular processes as migration and cell division[25]. Alkylating agents (such as cyclophosphamide, and carboplatin) are not specific to the cell cycle and react with the DNA which leads to crosslinking and cell death. Toxic effects of chemotherapy often limit its use in patients. These drugs often cause nausea, loss of hair and mouth sore, compromised immune system, cognitive problems, and fatigue among others. Over time patients often develop infertility, bone thinning and heart damage and the risk of side effects among elderly is greater than in younger patients. Chemotherapy is the main option for triple negative/basal like cancers lacking targetable receptors[26,27] but despite its life prolonging effect in patients, relapse is still common after chemotherapy. Also, fewer than 30% of the triple negative patients will have a complete response and the risk of recurrence is higher within the first 10 years after diagnosis compared to other breast cancer types demonstrating a great need to develop better personalized therapies for this subgroup of cancers[12].

Targeted therapies

Other therapeutic approaches include monoclonal antibodies such as Herceptin/trastuzumab targeting HER2 proteins (Figure 6). HER2 protein is a kinase that is encoded by the *ERBB2* gene, which is often overexpressed in 15-20% of breast cancers. Novel monoclonal antibodies conjugated with chemotherapy agents such as Kadcyla or Enhertu can also be used on this breast cancer subtype delivering the drug directly to the cancer cells with HER2 expression[28,29]. The kinase function of HER2 can be targeted by kinase inhibitors which has showed potential in clinical trials and several compounds have been approved for treatment of metastatic breast cancer by the FDA as combination therapies[30]. Targeting kinases involved in the regulations of the cell cycle using small molecules such as palbociclib, ribociclib and ademaciclin can also prevent tumor progression (Figure 6). CDK4/6 inhibitors, such as palbociclib, induce inhibition of cyclin dependent kinase 4 and 6 (CDK4/6) blocking proceeding through G1 to S phase by preventing phosphorylation of RB resulting in reversible proliferative arrest[31]. Palbociclib treatment significantly increases disease-free survival in breast cancer patients with metastatic disease through combination with fulvestrant or aromatase inhibitors but can also be used as a monotherapy[32,33]. Unfortunately, some patients do not respond to palbociclib treatment or develop resistance.

Patients with known *BRCA1/2* mutations have cancer cells with impaired DNA damage repair mechanisms that can be targeted with PARP inhibitors. PARP inhibitors contribute to further destabilise the DNA which kills the cancer cells[17]. Due to the high genomic instability in high grade triple negative cancers, PARP inhibitors have also recently been tested in these tumors with some success. Further, novel therapy Trodelvy has shown to prolong disease-free survival and overall survival drastically in metastatic triple negative patients compared to chemotherapy. Trodelvy is a monoclonal antibody recognizing trophoblast cell-surface antigen 2 that is overexpressed in most breast cancers, conjugated with a topoisomerase I inhibitor. This treatment shows great potential and an increasing number of clinical trials are emerging, adding to the few options available for these patients[34].

The previously described monoclonal antibodies are considered targeted immunotherapies. Immunotherapies are drugs or treatments that help the patient's immune system to identify and kill cancer cells. Immune check point blockade is another class of immune therapy based on monoclonal antibodies targeting specific pathways or receptors, such as PD-1/PD-L1 as well as CTLA-4 that inhibit the signaling of T cell activation. These antibodies remove the suppressive signals and promote the killing of cancer cells expressing these markers[35]. Some of these drugs can be combined with chemotherapy and has showed potential in clinical trials as well as in other cancers[36]. Another emerging immunotherapy approach is engineering patients own T cells to express chimeric antigen receptor (CAR-T cells) that promotes killing of cancer cells with specific antigens. However, estimation of proper dosage is difficult in patients and can lead to autoimmune responses damaging organs or even result in death[37]. To summarize, there are several anti-cancer agents and therapeutic approaches to treat breast cancer, but to the price of severe side effects and treatment resistance. Development of more patient-specific treatments and drug testing models would aid in personalizing cancer therapy reducing overtreatment in breast cancer patients, which could increase quality of life and have cost beneficial effects for the society.

Tumor heterogeneity

Epithelial-to-mesenchymal-transition

Metastases are responsible for most cancer-related deaths[38]. Epithelial-to-mesenchymal transition (EMT) is a critical mechanism of which tumors gain metastatic features (Figure 7). Epithelial cells become de-polarized and lose their cell-to-cell contact and adhesion to the basal lamina and become mesenchymal by changing their morphology to fibroblastic-like elongated cells. Mesenchymal cells can then be transported to distant sites through entering the blood stream or lymphatic system and thereafter undergo the reversed process mesenchymal-to-epithelial transition (MET) to regain an epithelial phenotype and undergo tumorigenesis at a secondary site[39,40]. EMT leads to enhanced mobility, invasion, and resistance towards apoptotic stimuli through transcriptional factors such as *SNAI1*, *SLUG/SNAI2* and *TWIST*. EMT also is greatly dependent on characteristics of the tumor microenvironment (TME) to enable migration of cells such as stiffness.

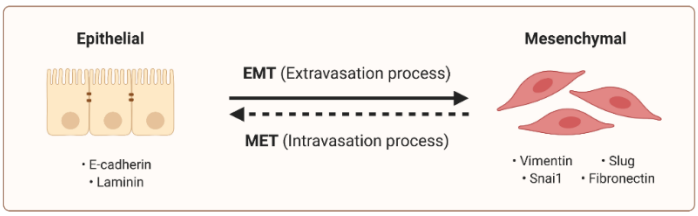

Figure 7. Schematic illustration of epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET). Created using Biorender.com

Clonal evolution and the cancer stem cell model

Tumor heterogeneity is commonly described by two separate models: the clonal evolution theory and the cancer stem cell (CSC) model (Figure 8). The stochastic clonal evolution theory suggests that any cell can acquire genetic changes over time that would eventually lead to tumor formation, also leading to vast cancer cell heterogeneity[41]. Whereas, the CSC model explain many clinical observations for treatment resistance and metastasis, suggesting that a subset of cells have more potential to initiate tumorigenesis[41]. CSC, like normal stem cells are able to self-renew, divide asymmetrically but also

symmetrically. Thus, CSC can also form new CSCs as well as differentiated cells[42].

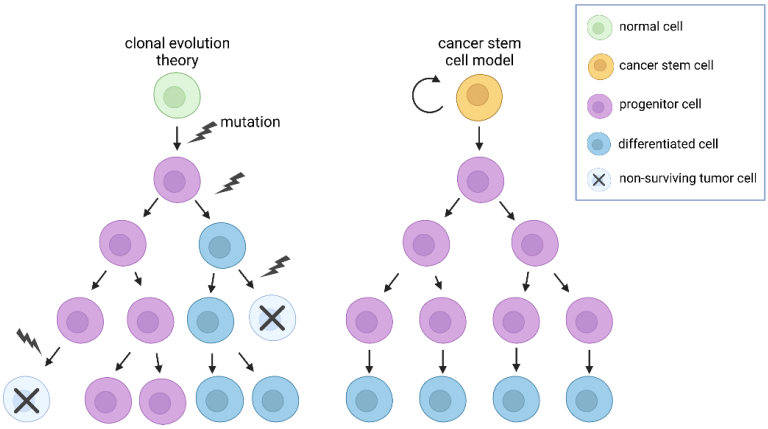

Figure 8. Schematic figure illustration the two models explaining tumor heterogeneity. The clonal evolution theory (left) and cancer stem cell model (right). Adapted from [42] and created with Biorender.com

Cancer stem cells

Cells with CSC properties have been found in most cancers and have common features with stem cells, such as the capability to self-renew and give rise to progenitor cells and differentiated cells (Figure 8)[13]. CSCs are defined based on several characteristics, such as being low-proliferative and able to initiate tumorigenesis and metastasis, survive anchorage-independent growth as well as inducing resistance to treatment and radiation. Breast CSC can be characterised by the ratio of surface expression markers $CD44^{high}/CD24^{low}$ as well as increased aldehyde dehydrogenase (ALDH) expression and ABC drug transporter expression[4], and for lacking ERα-expression even in ERα-positive tumors[43]. This subpopulation is evidently contributing to malignant features in breast cancer but is not targeted by conventional therapies. As described, EMT plays a critical role in metastasis, and several pluripotency genes that are upregulated in CSCs are direct targets of EMT-related genes. Activation of EMT processes can also induce CSC properties in cells suggesting a plasticity between these cellular states[44,45]. CSC are believed to reside in certain niches in the TME, suggesting that developing anti-cancer therapies targeting the TME, as well as EMT would impact the propagation of CSC which could have beneficial outcome in inhibiting tumor progression and metastatic events.

Tumor microenvironment

Properties in the TME such as low pH and oxygen levels (hypoxia) contribute to genomic instability causing mutagenesis and DNA damage in cancer cells demonstrating that properties of the TME itself can induce malignant features in cancer cells[46]. The TME has shown to be important in treatment resistance, tumor progression and formation of metastasis (Figure 6)[47]. The TME is composed of a variety of cells including immune-, endothelial cells and fibroblasts. These heterogeneous cell types interact with each other through signaling mediated by hormones, cytokines, and growth factors, but also with their surrounding TME through dynamic and mechanical interactions[48]. Each tumor has a unique architecture and composition, but it is not well-defined which components in the TME contribute to malignant phenotypes. Therefore, delineating which components in the TME that contributes to a more malignant behaviour, such as the CSC niche, are of great interest for therapeutic targeting. However, many of these critical aspects of the TME are difficult to monitor *in vitro* requiring better model systems.

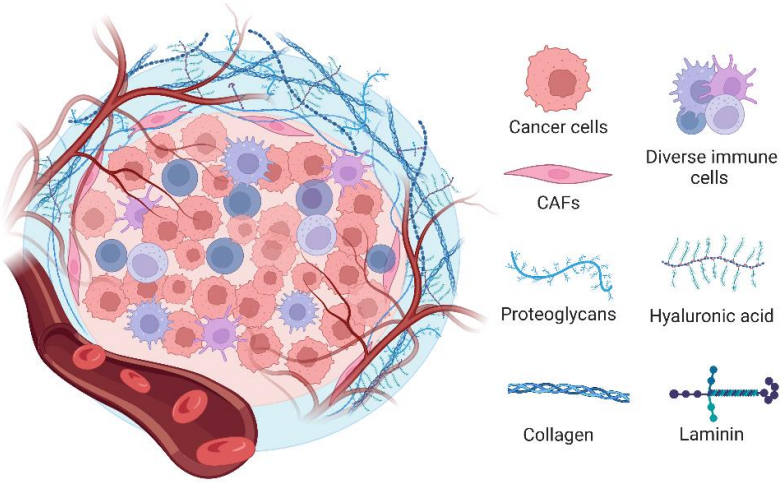

Figure 9. Schematic figure of the tumor microenvironment including different cell types and components of the extracellular matrix. CAFs; Cancer associated fibroblasts. Created with Biorender.com

The extracellular matrix

A key component of the TME is the extracellular matrix (ECM) that is secreted by cells such as fibroblasts. The ECM is an intricate network consisting of water, proteins, polysaccharides, minerals, and adhesion proteins providing physical scaffolding and barrier for the cells. The ECM influences the dispersion of signaling molecules, facilitates cell-cell contacts but also influences drug accessibility to the cancer cells through alterations in stiffness[49,29]. Remodelling of the ECM is a tightly regulated process that is essential in tumorigenesis, and dysfunctional ECM have been linked to facilitating metastasis and tumor growth. The most abundant group of proteins in the ECM are collagens forming long fibers, making up nearly 30% of the total protein mass in the human body[13]. Tumor stiffness is greatly influenced by collagen abundance, as well as their post-translational modifications[50]. There are a total of 28 different types of collagens in the human body, with different abundance in various tissues[51]. Collagen disposition in several cancer forms have been identified as a prognostic factor for lymph node metastasis, clinical stage, invasion and differentiation[52]. Collagens in the body become less organized and the turnover decreases with age[53]. Laminins are another large group of anchoring glycoproteins connecting cell surfaces to the basal lamina influencing cell signaling, migration and differentiation of cells. The loss of laminins in cancers have been shown to promote tumor growth and invasion[54,55]. Other matricellular proteins such as Periostin (POSTN) serves a more regulatory role than structural as it binds to integrins on cells and mediates responses to tissue damages[56]. Periostin is overexpressed in several cancer forms including lung, breast, colon and ovarian cancers and have shown to be promoting as well as inhibiting proliferation of cells depending on tissue type suggesting a dual role in tumeriogenesis[57].

Cancer models

Until now, most cancer research is conducted using cancer cell lines cultured in monolayers on plastic dishes causing selective pressure for highly proliferative cells (Figure 7). Monolayer cultures offer great opportunities to perform high-throughput studies, are versatile in various cell assays, cheap and can be expanded to large amounts in a short time-period. However, monolayer cultures poorly resemble the complexity and structure of tumor tissues

implying the need for better model systems to study critical cancer-related processes such as EMT.

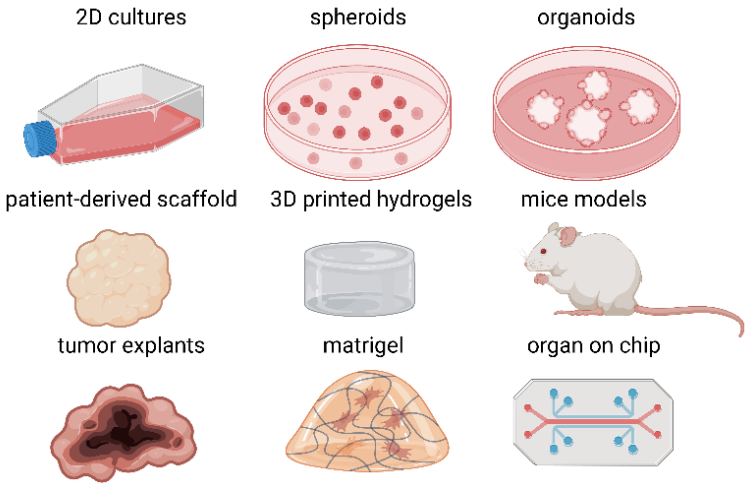

Figure 10. Illustration of different model systems for cancer research.

Immunocompromised mice models are frequently used in cancer research following studies in monolayer cultures and can be gene edited to present different genotypes and phenotypes. Xenograft models can be used to study tumor take, and metastatic disease as a measurement for aggressiveness and stemness of cancer cells in response to different treatments or gene editing therapies. Patient-derived xenograft (PDX) models have shown potential to induce similar treatment responses and retain molecular profiling similar to corresponding patients[58]. However, studies have shown that the stroma of patient tumors is exchanged to mice stroma after three passages and the tumor take-rate is limited[59]. In addition, mice models are expensive, labor intensive and present ethical issues and therefore also limit the source of materials. 3D growth influences cellular behaviors and by growing cells as spheroids in non-adherent conditions cells characteristics can be altered. Spheres enrich for cells with CSC properties, but their shape also leads to the formation of gradients of oxygen as well as nutrients more similar to *in vivo*[60]. Spheroids can be made from cell lines or tumor cells to provide information about cellular heterogeneity but lack the information from interactions with the TME.

Organoid cultures have become a popular approach to resemble 3D-growth *in vitro* and can either be derived from pluripotent stem cell cultures or from primary cells from different tissues. Organoid cultures are often referred to as "mini-organs" showing great resemblance to the original tissues and in drug responses in PDX models[61-63]. The method requires Matrigel and additives to the media. However, organoids are also extremely sensitive to variations in growth conditions and dependent on consistency between laboratory personnel to be able to generate reproducible results and besides, and lack human TME interactions. Matrigel is popular commercial option to model 3D growth *in vitro* and provides physical interactions impacting cellular phenotypes. Matrigel consists of ECM derived from mouse sarcoma models which provides biological activity, but with large batch-to-batch variation without tumor specific architectures. 3D-printed hydrogels provide reproducibility and allow high-throughput screening and have the potential to be engineered to contain components relevant for the experiments[64]. Yet again, 3D-printed tumors lack the patient-specific architectures of real tumors. Tumors cultured as sections in media, called explants, provide cell and TME specific information. Recent improvements increasing longevity of cells are being made[65] for different cancer types, but cell viability is often very limited making them difficult to maintain experimentally[66].

To provide physical cues and interactions, patient-derived scaffolds (PDS) can be created from solid tumors using detergent-washing procedures, which remove the cells but leave the surrounding tumor structure intact. Patient-derived scaffolds can be re-populated and infiltrated with standardized cancer cell lines, mimicking *in vivo*-like growth conditions for adapting cancer cell lines[67-69]. The method is relatively cheap, and easy to operate in the laboratory but requires access to patient materials which can be limiting. Engineering technology "organs-on-chips" models can be combined with cell lines and tissues that are molded into glass or polymers being surrounded by channels to mimic human physiology by providing a bridge between *in vitro* and *in vivo* using microfluidic systems[70]. There are currently no standard operating procedures in this field, and it is expensive as well as technically complicated to implement in a laboratory setting. But the method provides the possibility to improve *in vitro* drug screening by removing metabolites, cellular waste as well as simulate signaling in a way that is not possible with other current methods[71].

METHODS

Mammosphere assay

Self-renewal and anchorage-independent growth are two key CSC characteristics. These features are utilized in the sphere-forming assay to enrich for cells with CSC properties[4]. The mammosphere assay was first developed to isolate neuronal stem cells but has since been applied cancer cell lines and cancer cells from many other cancer forms including breast, sarcoma, prostate, and colon cancer[72-74]. This assay is relatively time-consuming and labour intensive but can be upscaled and automated to generate larger amounts of data which can be utilized for drug screening *in vitro* to identify compounds targeting the CSC population. This method was used in Paper II for evaluating sphere forming capacity of cells after drug treatments in 2D and PDSs[75,76].

Flow cytometry

Flow cytometry is used for many applications, such as sorting or detecting cells based on different cellular morphology and surface markers. Flow cytometry is based on the principle of passing single cells in a liquid stream in front of a single or multiple lasers carrying the cells so that they can be detected and counted. Flow cytometry was applied in Paper II to assess the distribution of cells in each phase of the cell cycle after palbociclib treatment using propidium iodide staining. Propidium iodide is an intercalating agent that permeates the membranes staining dead cells as well as binds to the DNA. This property of propidium iodide can be used to quantify the amount of DNA in cells, which is dependent on their phase in the cell cycle. Fluorescence-activated cell sorting (FACS) is a special type of flow cytometry providing the possibility to sort single cells based on their unique fluorescent and light scattering patterns or based on the labelling with antibodies that are coupled to fluorescent dyes. For FACS a ring of electrical charge is placed around the stream of cells and based on the fluorescent intensity from the cells the currents are changed, and the droplet breaks from the stream and can be sorted into 96-well plates[77]. This method can be used to study cellular heterogeneity and was used in Paper I to isolate cells for single-cell qPCR.

Protein analysis

Different methods can be applied to quantify and detect the expression of proteins. Western blot is a classical analysis technique to detect and quantify protein expression and was used in both Paper I and II. Protein lysates from samples are separated based on molecular mass using an electrical field and thereafter transferred onto membranes where they can be detected with different primary antibodies. Secondary antibodies labelled with enzymes, e.g., horseradish peroxidase allows amplification of the signals and chemiluminescent detection by addition of substrates. This method provides high sensitivity and specificity but is heavily dependent on the quality of antibodies and optimization of conditions which is a time-consuming process when only one or few proteins can be studied at the time[78].

Immunohistochemistry is another antibody-based method that identify and quantify protein expression in tissues or cell pellets and was applied in Paper I to assess expression of stromal POSTN and ALDH1 in tissue micro arrays (TMA). In Paper I and II the presence of cells was evaluated following decellularization and recellularization of scaffolds by histological staining with hematoxylin and eosin (H&E). Hematoxylin stains the nuclei in a blue color whereas eosin stains the cytoplasm and extracellular space pink. In histology, other dyes such as Picro-Sirius Red can be used to detect the presence and structure of collagens and was used in Paper I to visualize the integrity of the ECMs after decellularization compared to unwashed tumor[69].

Mass spectrometry (MS) was utilized in Paper I and Paper V, and the method offers global unbiased analysis of the proteomic composition of materials and tissues[79]. The method is very sensitive and specific, which makes it suitable for diagnostic purposes or to identify novel biomarkers since it can be applied to serum, solid tissues, blood, or cells. Samples are homogenized, reduced, alkylated, and converted into charged ions from the neutral molecules. Each sample can be chemically labeled with tags, such as isobaric tandem mass tags (TMT) before samples are pooled and fractioned using liquid chromatography (LC). The MS ions from each set are separated based on mass to charge ratio (m/z) of molecules in a sample, where m represents mass in Daltons and z is the amount of charge on the ion. The m of each fragment/peptide decides its speed through the machine. The relative abundance for the ions can then be compared against peaks on a mass spectrum representing the relative

abundance for each ratio (m/z)[80]. Two mass spectrometers can be coupled together to improve the fragmentation of samples and increase the identification of ions with similar m/z ratios and is referred to as MS/MS. Data analyses can then identify unique peptides and quantify the relative abundance of the TMT-tags for each sample. The unique TMT-tags for each sample permits multiplexing with several samples per run that can be compared to the same reference pools making mass spectrometry an important tool in personalized medicine where much data can be extracted from little material. However, mass spectrometry experiments are expensive and requires trained personnel as well as often bioinformatical knowledge to analyze the generated data.

Gene expression analysis

Identifying transcriptional changes by quantifying mRNA allow rapid and precise assessment of physiological changes as a response to altered environments or drug treatments and can provide insight into potential targets and important molecular pathways[81]. A standardized method for measuring RNA levels is the fluorescence-based quantitative polymerase chain reaction (qPCR), which was used in paper I-IV. qPCR is a robust analytical tool with high reproducibility using optimized assays and is a simple, quick, as well as cost-effective. The method can be used to analyse expression of single genes, or several genes in a gene panel. mRNA can be extracted from cells, tissues, or formalin-fixed, paraffin-embedded materials. Thereafter complementary strand DNA (cDNA) from the mRNA template is generated using reverse transcriptase enzymes. During the qPCR specific primers allow for amplification of the target gene of interest. At the same time, a fluorescent dye binds to the formed double-stranded cDNA and by quantifying the absorbance the relative amount of the transcript in each sample can be analysed. qPCR is based on relative quantification, and to find the optimal normalization genes in the dataset different algorithms such as NormFinder or GeNorm can be applied to identify the genes with least variation in the dataset in programs such as GenEx (MultiID). Subpopulations of cells will often be missed when performing bulk PCR since the level of transcript will be an average of the entire population. Therefore, single-cell qPCR can be applied to study cellular heterogeneity, and this was used in paper I to compare cells from two PDSs and one 2D culture. For single-cell qPCR, cells are often directly lysed in specific lysis buffer to avoid losses from RNA extraction followed by reverse

transcription of the mRNA of the lysed sample. To increase the number of transcripts, the cDNA can be preamplified using a pool of selected primers. This is followed by a standard qPCR protocol[77]. However, depending on the expression level of the gene of interest, the number of transcripts per cell can be below or close to the limit of detection and be difficult to measure.

Unlike qPCR, RNA sequencing is not limited to the numbers of tested assays but is an unbiased, global analysis that can provide novel targets. RNA sequencing can be used to quantify differential gene expression between samples and was used in Paper I to compare 2D, PDS and xenograft cultures. This method can also be used to examine alternative mRNA splicing, gene fusions as well as variation in gene expression over a period. RNA sequencing covers a large dynamic range, is sensitive and accurate[82]. RNA is extracted from samples, transcribed into cDNA, and amplified. Amplified cDNA is purified, fragmented, and labelled with sample-specific index sequences to allow for pooling of several samples into one sequencing library pool. The libraries can be sequenced using platforms such as Illumina. Sequencing is widely used in research today but is still expensive, limiting the number of samples that can be analyzed, and is less sensitive than qPCR, and the data analysis often require bioinformatical skills.

Online tools for data analysis

Analyzing data from the omics-era often demands advanced analytical skills and programming experience. To aid in the analyses of large datasets, a multitude of online tools have become available. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (string-db.org) is well-established for protein-protein interactions based on experimental data, computational prediction as well as published texts from more than 5000 species. The tool also provides enrichment analysis such as Gene Ontology, Reactome Pathways and KEGG and visualized protein-protein interaction networks[83]. Metascape (metascape.org) is another web-based tool that can be used for gene annotation, gene list analysis as well as enrichment analysis for both gene and protein lists to aid people without programming background[84]. Protein annotation through evolutionary relationship (PANTHER; panther.org) protein annotation database combines annotations of ontology, gene function and different pathways through evolutionary relationship[85].

Patient material

The work in this thesis is based on human materials from several breast cancer patient cohorts as well as numerous freshly harvested samples. In paper I, a TMA was used that was stained for POSTN and ALDH1 including 455 breast cancer patients from an original study (SBII:2) in which the patients were diagnosed between 1984 and 1991. The study was approved by the Regional Ethics committee in Lund and Linköping for Lund University, Sweden (DNR: LU 240–01 and was over time complemented from Linköping DNR: 01–134 as well as DNR: LU 2015–350). Verbal informed consent was obtained from all patients and approved by the Ethics committee. TMAs are created from cancers materials in paraffin blocks. Each cancer block is sectioned and stained with H&E to identify which areas that are appropriate to extract for the TMA. From the cancers small core biopsies will be transferred into new paraffin blocks, melted into one block that can be sectioned by a microtome and slices can be mounted onto glass slides and stained using immunohistochemistry[86]. The additional cancer tissues that were used in the thesis work were taken from Sahlgrenska University hospital and was approved by the Västra Götaland Regional Ethics committee (DNR: 515-12 and T972-18). For the mass spectrometry analyses in Paper I, 15 fresh cancer tissues were used, and 21 additional cancer tissues were used in Paper II. Informed written consent was received from all patients donating fresh materials. For the frozen cancer tissues that were included in Paper I (n=46), and from the larger cohort that was used in different experiments for Paper III-V, the informed consent was waived by the Ethics committee. The samples were collected directly after surgery between 1980-1999 and stored in -140°C and patients were monitored until 2012.

AIM

The overall aim and purpose of this thesis was to elucidate the influence of cell-free breast cancer microenvironments on cancer cell lines using patient-derived scaffolds as a 3D model as well as examine its impact on propagation of subpopulations of cancer cells. We also aimed to identify subgroups of patient-derived scaffolds with similar behaviors and evaluate potential prognostic value of the model.
The specific aims were:

Paper I. To develop an *in vivo*-like 3D growth platform based on decellularised primary breast cancers as well as identify potential subgroups of breast cancer defined by the microenvironment and evaluate the varying impact on adapting cancer cell lines.

Paper II. To use the patient-derived scaffold model for cancer drug testing with a focus on endocrine therapies and CDK4/6 inhibition. We also aimed to optimize the patient-derived scaffold model to generate more functional samples from each cancer as well as compare scaffolds to other relevant 3D-based cell culture platforms.

Paper III. To expand the patient-derived scaffold analyses and include different breast cancer cell lines and larger cohorts of patients in order to relate the findings to clinical characteristics of the original disease.

Paper IV. To study how different breast cancer microenvironments in the form of patient-derived scaffolds influence the cancer cell responses to chemotherapy treatment and relate the findings to clinical parameters in order to reveal subgroups of breast cancers with similar drug response patterns and ultimately treatment predictive information.

Paper V. To delineate the protein composition of cell-free tumors microenvironments and based on that identify potential subgroups of patient-derived scaffolds, as well as important processes and components in the microenvironments that could serve as potential targets in drug development.

RESULT AND DISCUSSION

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

Breast cancer is a highly heterogenous disease and traditional 2D cultures poorly mimics the complexity of dynamic cell-microenvironment interactions occurring *in vivo* that impact cellular behaviors. The effect of the surrounding tissues and presence of potential cancer subgroups with varying microenvironments is poorly considered when treatment options are decided in the clinics today. The main hypothesis in this thesis is that a 3D model originating from human cancers would provide the essential connections to the cancer microenvironment and would provide a more *in vivo*-like growth platform. Therefore, in Paper I we aimed to develop such a model by decellularizing primary breast cancers and demonstrate the microenvironmental effects on cancer cell lines by growing them in the scaffolds. We performed extensive validation of the model using mass spectrometry and compared induced gene expression changes in cells to other model systems. Gene expression- and protein analyses together with functional assays were applied to demonstrate an enrichment of the CSC pool after PDS-growth. We also compared clinical properties of the tumors to impact behaviors in the cells growing in PDSs and demonstrated that the model retains patient specific features *in vitro*.

Patient-derived scaffolds sustain cell growth and infiltration of adapting cancer cell lines

Donor cells were removed from the tumors by detergent washing followed by validation by H&E staining as well as DNA content analyses and maintained integrity of the tissues was confirmed by collagen staining. Collagen proteins are highly abundant structural proteins and important components of the ECM[50]. Structural inter-PDS as well as intra-PDS variability was visualized by scanning electron microscopy (SEM) supporting that individual microenvironments indeed are heterogenous. When standardized cancer cell lines MCF7 (ERα-positive) or MDA-MB-231 (ERα-negative/triple negative) were cultured in the complex scaffold environment, the triple negative cell line infiltrated the tissues and presented both endothelial and fibroblast-like cellular morphologies, while the MCF7 cells more often formed clusters closer to the

surface. Interestingly, MDA-MB-231 cell line is more proliferative, and present more mesenchymal properties while also harboring a p53 mutation, thereby mimicking the more aggressive phenotype of triple negative breast cancers[87,88].

Patient-derived scaffolds display structural and compositional heterogeneity reflecting clinical properties

LC-MS/MS analyses of 15 PDSs showed that scaffolds also expressed different levels of the imprinted proteins, which could be associated with tumor grade as well as cancer cell Ki67 levels suggesting that clinical properties of the tumors is reflected in their composition. Importantly, we showed that decellularized scaffolds contained more than ECM proteins. Multiple studies refer to decellularised tumor models solely as ECM-scaffolds, that could lead to underestimation of important properties of the tumor microenvironment that is mediated through other proteins[89-91]. A protein-protein interaction network displayed that many of the detected proteins were related to secretion, immune response as well as metabolism, indicating that these processes are important for the construction of the microenvironment. To sustain the findings of the relevance of the PDS composition as a representation of the original TME, periostin was chosen as a representative protein. High levels of periostin were associated with low proliferation and grade from the MS-analyses and immuno-histological staining of the protein in TMAs from many breast cancers confirmed that high periostin was negatively associated with high Ki67 levels in cancer cells. An anti-migratory role of periostin was supported by observations in tumor sphere assays, where treatment with recombinant periostin inhibited invasion of MDA-MB-231 to the surrounding matrigel, compared to controls.

Adaptation of cancer cells to the surrounding microenvironments influences their phenotypes compared towards more pluripotency enriched populations

Cancer cells growing in PDSs showed an enriched population with CSC phenotype by increasing sphere formation and higher tumor-take in immunocompromised mice, compared to monolayer cultures and in line with studies from decellularised breast cancers[92]. qPCR analyses showed that PDSs increased expression of pluripotency genes but decreased expression of

proliferation and differentiation-related genes which was supported by western blot analyses. Growing PDSs for different times periods showed that this phenotype was distinct after 14-21 days of growth, and PDSs grown for shorter time clustered with 2D cultures and was characterized by high proliferation and differentiation and lower expression of pluripotency and CSC markers, showing that PDSs induce a cell plasticity over the cultivation time or potentially select for certain cell populations. This observation suggests that cells need time to adapt and invade their surrounding tissues, and it should be taken into consideration when designing and comparing 3D model experiments. Single-cell qPCR showed that PDS growth also induced cellular heterogeneity and displayed a plasticity in their differentiation state while 2D cultures a more homogenous phenotype. Instead, scaffolds induced more similar, but not overlapping gene expression profiles to 3D-printed hydrogels and xenograft cultures from mice for both MCF7 and MDA-MB-231 cells strengthening the *in vivo* resemblance of the model.

Clinical characteristics of the patient-derived scaffolds can be translated into adapting cells

Microarray data and tumor derived ECM-gene signatures have shown prognostic potential, suggesting that evaluating the gene expression induced by individual microenvironments could reveal clinical properties in different cancer types including breast[93-95]. Here, self-organizing map analyses of qPCR data from 46 PDS cultures demonstrated that there was variation between PDSs from patients with different recurrence status. Therefore, the recurrence-free survival in patients were stratified in Kaplain-Meier plots and identified that PDSs inducing lower expression of EMT markers *VIM* and *SNAI2* were coming from patients with a worse outcome, linking observations in the PDSs to clinical properties of the original tumors. A following study in our group showed that cancer cell line secretion patterns were also altered in PDSs with different recurrence-free survival status as well as gene expression of EMT markers, supporting that malignant properties of the cancer microenvironments can influence growing cancer cells[96].

In conclusion, we developed a 3D model that could simulate breast cancer microenvironment properties *in vitro* showing high *in vivo* resemblance. The development of better 3D models could reduce costs in early drug development and reduce the number of animal experiments[97]. Moreover, PDSs could also display patient heterogeneity *in vitro*, which could be used to assess the benefits of treatments in certain subtypes of tumors, or present novel disease subtypes based on their cell-free microenvironmental characteristics that could be studied using this model.

Paper II: Patient-derived scaffolds as a drug-testing platform for endocrine therapies in breast cancer

Connections between cancer cells and their adjacent microenvironments stimulate responses within the cells which are provided by 3D growth but limited in 2D cultures and can impact responses to drugs, causing a need for novel growth systems that can provide potential barriers for therapies as well as provide essential interactions to the microenvironment. The main hypothesis in Paper II was that the PDS model would be a more physiologically relevant drug testing platform than 2D cultures. The aim of this paper was to evaluate the drug testing potential of the PDS model using well-established endocrine therapies tamoxifen and fulvestrant but also the CDK4/6 inhibitor palbociclib. We optimized the model and performed expression analyses by qPCR of genes representative of relevant tumor biological processes including differentiation, proliferation, EMT, pluripotency and CSC, as well as functional mammosphere assays to evaluate the presence of cells with CSC characteristics after drug treatment in both 2D and 3D conditions. Drug responses in PDSs were compared to other model systems by gene expression analyses showing that several other 3D models were able to induce similar behaviors to PDSs for fulvestrant and palbociclib, but not 4-OHT.

The PDS model can be optimized to generate more functional replicates from each cancer tissue

Here, we adapted the decellularization process described in Paper I to include a sectioning step to get more biological replicates from each tumor, since the amount of patient material is limited. We succeeded reducing the thickness to 150 µm, increasing the number of replicates from 3-4 pieces from each tumor like most scaffold-protocols to instead get up to more than 70 pieces from large tumors allowing us to perform multiple analyses on the same tissues[69,90,92]. The thinner slices induced similar gene expression changes as larger PDSs with little variation between the replicates, except for the EMT gene *TWIST* that may be more dependent on spatial cues than the other markers. Histological sections confirmed that MCF7 (and MDA-MB-231, unpublished) infiltrated the tissues well.

Cells growing in PDSs become more resistant to therapies compared to monolayer cultures

2D and PDS MCF7 cultures were treated with tamoxifen derivate 4OHT, fulvestrant as well as palbociclib. The IC_{50} values for PDS cultures were evaluated by LDH assay, RNA- and DNA measurements whereas variation in gene expression between the drug concentrations and the two culture conditions were assessed by qPCR. IC_{50}-values that worked for most samples were chosen since we observed variation in toxicity in this small cohort of PDSs. Both 4-OHT and fulvestrant treatment inhibit proliferation and induce cell death in ERα-positive cells, while palbociclib stop cells from proliferating by blocking the cell cycle[31,98]. In line with earlier reports of cancer cell lines requiring higher drug concentrations in 3D, PDS cultures needed increased concentrations of 100x for 4OHT and 20x for fulvestrant to achieve cell death as well as altering gene expression profiles of the cells[99,100]. The same concentration of palbociclib appeared to be efficient for both 2D and 3D to inhibit cellular growth as well as generate distinct effects in the qPCR analyses.

Endocrine therapies in PDS cultures further enrich for CSC-properties in cells

We hypothesized that endocrine therapies which are known to increase the fraction of CSC would further enrich or select for these cells with CSC properties in the PDS model[101,102]. 4OHT treatment induced little effect in PDS cultures on the gene expression of cancer-related markers except for a few of them, whereas 2D cultures induced more variation in gene expression compared to controls without treatment. Fulvestrant treated PDSs induced distinct gene expression profiles in all gene families but 2D cultures induced more pronounced effects to the drug in many of the same genes. In 2D, fulvestrant induced or selected for cells with high expression of pluripotency genes *POU5F1*, *NANOG* and *NEAT1*, but this effect was probably masked in PDSs since the levels were already increased by PDS-growth. Both drug treatments induced more expression of *SOX2* in PDS cultures, which was also confirmed on protein level, and interestingly in numerous studies higher levels of *SOX2* have been linked to endocrine resistance in breast cancer[101,102]. 4-OHT treated 2D cultures did not induce more mammospheres or enhance their ability to self-renew, in contrast to previous reports[101]. 4-OHT treated cells from PDSs even reduced the mammosphere forming capacity but enriched for

cells that could self-renew. Fulvestrant treated cells did not induce any significant changes in primary sphere formation, whereas both 2D and PDS cultures induced more self-renewal, with a pronounced variation between cells from different PDS cultures. On the other hand, palbociclib treatments in both growth models heavily decreased expression of proliferation markers, and 2D cultures also altered the expression of several other genes that was not observed in PDSs. Mammosphere assays clearly validated that palbociclib has a reversible effect, as PDS-derived cells formed many large spheres when the growth-restriction caused by 3D-matrices were removed. These cells were however not able to self-renew, demonstrating the need for complementary CSC readouts for compounds that also target proliferation growing in 3D models[31].

The selection of 3D growth platform influences drug response in cells depending on properties of the treatment

We demonstrated in Paper I that PDSs induced more similar gene expression profiles to 3D-printed hydrogels and xenograft models than 2D cultures. Here, to examine the relevance of the PDS model, we compared PDSs to 3D-printed hydrogels, matrigel and gelatin sponges to stimulate 3D growth. The largest variation between cell culture conditions was observed in the untreated samples, but importantly all 3D models induced more expression of pluripotency genes with varying degree, compared to 2D cultures. The different 3D models induced similar gene expression changes in response to fulvestrant as well as palbociclib. 4OHT treatment induced more similar behaviors between cells grown in *in vivo*-derived materials PDS and matrigel compared to the other models, suggesting that not only 3D conformation but also the heterogenous composition of the substrates influence the cells response to the drug. In addition, findings by our colleges supported that cells drug responses to cytotoxic drugs such as doxorubicin and 5-flourouracil are depending on properties of their growth substrate demonstrated by analyses of 2D, PDSs, as well as 3D-printed hydrogels[64]. MCF7 cells responded differently to doxorubicin in all three platforms but PDSs and 3D-printed hydrogels were more similar in response to 5-FU, supported by similar observations using colon cancer cell lines in colon cancer PDSs[64,103]. Importantly, an unpublished study from our group using a large PDS cohort substantiated a vast PDS-variability in response to 4OHT, fulvestrant and palbociclib in both MCF7 as well as T-47D cancer cells that were associated

with several clinical properties of the tumors. These findings support the relevance of using PDSs to model patient-variability in response to these treatments *in vitro* which cannot be replicated by other 3D models lacking tumor-specific features, for example matrigel.

In summary, our results from Paper II show that PDSs could be used as a 3D model for drug testing and cellular responses in scaffolds varies from conventional 2D cultures. This study highlights the importance of complementing 2D studies with 3D growth platforms since they give different results depending on the drugs mechanism of action, and PDSs treated with 4OHT or fulvestrant further induced propagation of cells with CSC properties. Together these data suggest that the PDS platform could also be used to validate novel compounds.

Paper III: Breast cancer patient-derived scaffolds can expose unique individual cancer progressing properties of the cancer microenvironment associated with clinical characteristics

Breast cancer is today distinguished into several molecular subtypes, each one with different treatment options[25]. However, better classification of cancers is needed to identify and predict which patients will benefit from more extensive disease monitoring or pro-longed treatments. In paper I, we observed inter-PDS variability in gene expression that were associated with patients' recurrence-free survival-status. Therefore, we hypothesized that by including a larger PDS cohort we could uncover PDS specific properties of tumor microenvironment that induce common behaviors in cells growing therein. The aim of Paper III was to further explore this variability between individual PDSs and relate it to clinical characteristics of the original tumor and substantiate the prognostic value of the PDS model. We performed a qPCR-based screen including 14 genes from cancer-related processes in 110 unique PDSs from biobanked tumors. PDS were recellularised with ERα-positive MCF7, and T-47D, and 89 PDSs were also grown with ERα-negative MDA-MB-231. We observed cell-line dependent changes in the genes, as well as variation between scaffold cultures. MCF7 induced more changes related to clinical characteristics of the PDSs and, the pluripotency marker *NANOG* was evaluated to be an independent prognostic marker for disease-free survival in our model. PDSs were stratified based on ERα-status which resulted in stronger and novel associations between induced gene expression patters and clinical properties suggesting an intricate relationship between hormone receptor status and tumor microenvironmental properties.

Induced gene expression changes were dependent on cell lines as well as on properties of the individual scaffolds

Each PDS slice was able to sustain growth for all three cell lines. PDSs induced a substantial variability in gene expression between the cancer cell lines, compared to 2D cultures but also displayed vast differences between individual scaffolds. Importantly, in line with our observations in Paper I, we observed common behaviors for all cell lines and PDS cultures such as increased expression of pluripotency markers, as well as decreased expression of proliferation markers. Of the three cell lines, T-47D induced the most variation between individual PDSs. Most of the variation between all three cell lines

were observed in expression of CSC and EMT genes, while most variation within PDS cultures with T-47D and MDA-MB-231 was observed in the pluripotency genes.

Clinical cancer properties impact PDS culture gene expression

Using statistical analyses, we showed that differential gene expression changes in the ERα-positive cell lines were significantly linked to the PDSs tumor grade, ERα-, PR-status as well as lymph node metastasis whereas MDA-MB-231 did not induce any associations, and most significant links were related to changes in the expression of EMT markers. We also observed that PDSs from cancer patients with a poor-disease free survival induced higher expression of *NANOG* in MCF7 cells than those from patients with a better prognosis, which corroborated findings in human breast cancer tissues[104]. Lymph node metastasis is considered as a prognostic tool for cancer aggressiveness, and we found that PDSs coming from patients with associated lymph node metastasis increased levels of *FOSL1* which has also been observed *in situ* for breast as well as, head and neck cancer patients[105,106]. The EMT program is highly important in initiation of tumorigenesis as well as CSC propagation suggesting that malignant properties of these cancer microenvironments influence cellular phenotypes which could be detected in the PDS model.

ER*α*-status of the tumors influence the phenotype of the cancer cell lines growing in the scaffolds

Cancer cells ERα-status has massively influence properties of breast cancers, including the tumor microenvironment[98]. Here in Paper III, we saw additional associations between gene expression profiles and clinical properties of the original tumors when the ERα-status were matched in the PDS and the cancer cell line. MDA-MB-231 are known to have a more mesenchymal phenotype, and when grown in ERα-negative PDSs we observed differential expression of primarily EMT and pluripotency genes between PDSs that could be significantly associated with patients' disease-free survival status. Interestingly, when PDSs were stratified based on their ERα-expression, levels of *SOX2* in MDA-MB-231 cells were significantly linked to disease-free survival for both ERα-positive and negative PDSs, but in opposite directions. The expansion of CSCs in breast cancer has been associated with the estrogen receptor, and one potential mechanism could be by expression of *SOX2* that

have been reported to be dependent on ERα through regulation by ERα-downstream target miR-140[107]. Together, these observations suggest a potentially important role of ERα in mediating the imprinted information from PDSs and larger PDS cohort including more ERα-negative tumors should be analyzed to further elucidate this relationship.

In summary, we found large inter-PDS variability that were more noticeable in T-47D than MCF7 and MDA-MB-231 cells, despite that they were grown in the same tumor tissues. We identified differential gene expression patterns in PDSs that could be significantly associated to properties of the original tumors, supporting our earlier findings in Paper I indicating that characteristics of the tumor microenvironment could potentially be imprinted into adapting cancer cell lines in the PDS model. These observations could potentially help us to make predictions using the PDS model about unknown parameters of cancers taken directly from surgery, such as the survival-prognosis of the patients. In addition, by including methods like RNA sequencing on larger PDS cohorts we could extract even more relevant data from the PDS cultures as well as possibly identify novel prognostic markers in the model.

Paper IV: Breast cancer patient-derived scaffolds representing individual cancer microenvironments influence chemotherapy responses in breast cancer cell lines consistent with clinical features

Paper IV focused on monitoring the inter-PDS variability in drug responses in a large PDS cohort based on the observation of large variability between individual PDSs in earlier studies. For this paper, we utilized tumors from the same cohort as Paper III. 64 PDSs repopulated with MCF7 and 72 with MDA-MB-231 were treated with 5-flourouracil (5-FU) and doxorubicin (DOX). Drug concentrations used in this paper were evaluated in a previous study by Leiva *et al* using a smaller PDS cohort. In line with our observations for endocrine therapies in Paper II, cells growing in PDSs required higher doses than 2D to achieve cytotoxic properties[100]. The study also showed that 2D and PDS gene expression profiles were vastly different for both MCF7 and MDA-MB-231 cells in response to the therapies. Here in Paper IV, drug-induced gene expression patterns in cancer-related markers were evaluated by qPCR after drug treatment. To analyze the effect of the drug without taking the substantial effects of the tumor microenvironment into account that was described in Paper III, we subtracted expression values of the PDS controls from the corresponding treated sample, creating drug fingerprints.

Chemotherapy treatment in patient-derived scaffolds induce general drug responses in the adapting MCF7 cells, but also present inter-PDS variability

We showed by comparing RNA levels from PDSs after treatment, that cells growing in certain microenvironments were more sensitive to the drugs which was demonstrated by large differences in the RNA expression. High correlation between 5-FU and DOX treated scaffolds RNA levels showed a mutual sensitivity towards both treatments which varied extensively among PDSs. Using a panel of 24 genes involved in relevant cancer processes, we identified common behaviors for both drugs such as reduced levels of proliferation markers as well as expression of individual genes from several gene families and increased expression of CSC-related genes. One of the main differences between drug induced gene expression profiles was observed in the pluripotency markers that were elevated after 5-FU treatment but heavily reduced in DOX treated samples.

Scaffolds induce similar changes in differentiation and EMT genes after 5-FU and DOX treatment

Most inter-PDS variability in the drug fingerprints was observed in the EMT genes after both treatments but was most notable in DOX treated samples. The EMT program is heavily influenced by signaling from the tumor microenvironment and can mediate drug resistance mechanisms, which could contribute to the observed gene expression changes between PDSs after treatments[108]. Correlation analyses showed that PDSs induced similar expression changes after 5-FU treatment within the differentiation genes but also the EMT genes. Markers from CSC, drug resistance and apoptosis gene families were also commonly regulated in the PDSs by the drug treatment. Similar associations after DOX treatment but weaker than with 5-FU were primarily found in differentiation as well as CSC-related genes. Comparing drug fingerprints for both 5-FU and DOX for each PDS culture we could see that PDSs similarly influenced drug response in genes involved in differentiation and EMT processes.

Unique properties of the tumor microenvironment influence cells response to chemotherapy treatment affecting similar process but in a PDS-dependent manner

When we stratified the PDS cohort based on clinical characteristics of the original tumor, similar to Paper III, we found that several markers in MCF7 cells were differentially expressed in PDSs with different ERα-status, grade, PR-status and recurrence-status after both 5-FU and DOX treatment. Interestingly, presence of lymph node metastasis was the variable with most clinical associations with gene expression changes in MCF7 growing in PDSs after 5-FU and DOX treatment, whereas only one gene, *FOSL1*, showed significant associations to this parameter in untreated PDSs. These data indicate that we can identify different associations between gene expression pattern and clinical properties of the original tumor from the PDS model by adding drug treatments compared to untreated PDSs.

Malignant properties of cancers can be translated into drug treated cancer cell lines

More predictive markers in cancer could aid clinicians to make more informed decisions about patient's risk profiles that could lead to more disease monitoring and longer treatments in high-risk patients. Using our model, we found that PDSs coming from more malignant cancers from patients with shorter disease-free survival induced different gene expression patterns in cells after treatment compared to those with better prognosis, which was more obvious after DOX than 5-FU treatment in MCF7 cells. These were different from what we observed in untreated PDSs in Paper III and substantiate that adding drug treatments *in vitro* to patient-materials could add prognostic value. Based on our observations as well as unpublished findings, we hypothesize that more malignant TME could potentially promote "less treatment responsive" PDS cultures. Observations from our colleges in colon cancer PDSs also corroborate this hypothesis, showing that patients with a poor prognosis induced less gene expression changes after 5-FU treatment[103]. This hypothesis however needs to be further evaluated, preferentially using single-cell studies to evaluate how certain subpopulations are enriched or selected for in individual PDSs after therapeutic treatment.

MDA-MB-231 cultures corroborate that 5-FU and DOX therapy induce different gene expression patterns dependent on properties of the PDS

Like MCF7 cells, MDA-MB-231 cells decreased proliferation and heavily increased expression of pluripotency and CSC markers compared to monolayer cultures, but no common behaviors induced by PDSs could be identified using correlation analyses between MDA-MB-231 and MCF7 cells for either treatment. Suggesting that the PDS does not induce similar phenotypes in the cells with different ERα-status, in line with the findings in Paper III where we observed different behaviors between cancer cell lines. This may in part be explained by observations in colon cancer where 5-FU treatment promotes stemness through p53 and the WNT/β-catenin pathway which is important for regulation of stem cell homeostasis[109]. MCF7 express wild type p53 whereas MDA-MB-231 harbors a p53-mutation.

DOX treatment induced most differential and stronger response in MDA-MB-231 than 5-FU, displaying large inter-PDS variability in EMT-, pluripotency as well as CSC markers. The DOX treatment substantially reduced the expression of several pluripotency markers as well as proliferation genes, and the expression of several EMT markers were shifted. Compared to MCF7, we found that fewer genes were commonly regulated by PDSs for the two treatments. Several markers were however related to clinical properties of the tumors, but for 5-FU primarily to grade and lymph node metastasis for DOX. Changes in the expression of the differentiation gene *EPCAM* after DOX treatment between PDS cultures could be associated with the patient's disease-free survival, while no links were observed in MDA-MB-231 untreated PDS in Paper III, unless the PDSs were stratified based on their ERα-expression.

In summary, these data show that drug treatments can uncover properties in the scaffolds that was not detectable in untreated samples in Paper III using our gene panel. MDA-MB-231 in the PDS model poorly uncovered prognostic data but still demonstrated pronounced effects after DOX treatment such as pronounced downregulation of pluripotency genes similar to MCF7 cells suggesting that this cell line still can be used to represent drug-relevant information in the model. More ERα-negative PDSs should be included to elucidate potential influence of the ERα-status of PDS and cancer cell lines in terms of chemotherapy treatments in future experiments. An RNA sequencing approach could also be applied to drug treated PDS to identify new potential markers that could be evaluated as prognostic markers in the model to make predictions about drug response in new cancers from patients with unknown risk profiles.

Paper V: Proteomics of cell-free patient-derived scaffolds from breast cancer identify clinically relevant imprinted proteins and cancer-progressing properties

Throughout papers I-IV, PDS cultures have shown to promote tumor-specific properties into the adapting cancer cell lines to different degrees. Additionally, we found in Paper I that the protein composition of PDSs could be linked to proliferative capacity of cancer cells as well as histological grade, proposing links between clinical properties of the tumors and the PDS composition. Therefore, in this project the aim was to identify subgroups of cell-free PDSs based on their proteomic composition as well as to identify key processes related to cancer properties. To address the question, we performed a large scale global proteomic analyses on 63 cell-free PDSs created from frozen tumors from a breast biobank, and 8 cell-free normal breast tissues.

Breast cancer PDSs are enriched in proteins related to metabolism

In this study we were able to detect more than 1800 proteins across all 8 TMT sets for the samples compared to 143 proteins from 15 PDSs in Paper I, which is explained by improved MS detection and sample preparations at the Proteomic Core Facility (GU). In theory, 1-2 µg peptides were analyzed per 20 fractions in the second dataset leading to a total 20-40 µg peptide, compared to analyzing 1-2 µg altogether for the first dataset, leading to more hits. The two datasets also showed different protein profiles of the PDSs where the first MS-dataset presented a large proportion of ECM-related proteins, whereas the second MS-dataset were most enriched in proteins related to metabolism, showing that signs of cancer cells metabolic profiles are imprinted into the scaffolds[3]. The differences in ECM-protein expression can be explained by that the peptide quantification for the first paper was set to a minimum of 2 peptides and therefore more biased towards high abundant peptides including ECM proteins. Whereas for the second analyses the quantification limit was set to 1 peptide allowing the identification of smaller and lower abundance proteins, leading to a smaller percentage of ECM proteins in total. Surprisingly, most proteins were annotated as intracellular in our model. Intracellular proteins were also detected in Paper I and have been documented in several decellularised models from various cancer types from tissues that were purified with even harsher methods[97,110,111]. Together, these observations propose that intracellular proteins are a part of the tumor scaffolds and should be considered

when designing novel *in vivo*-like 3D models to resemble the tumor microenvironment.

Scaffolds were separated into clusters based on their expression of proteins mainly related to secretion-, immune- and metabolic processes but also ECM- as well as localization-related pathways

Secretion-, immune- and metabolic processes were identified in Paper I to be important biological processes in the cell-free cancer microenvironments. In Paper V, we corroborated these findings but also adding pathways such as ECM organization and cellular transport. Besides, we showed that the processes were differentially upregulated in different subtypes of PDSs which was also significantly associated with the corresponding tumors histological subtype. Our findings indicated that ductal cancers expressed higher levels of proteins related to metabolism compared to lobular cancers. Lobular cancers that are less common than ductal cancers, were enriched in proteins related to the ECM and various immune processes. These data support findings from breast tumors and cancer cells suggesting that lobular cancers could potentially be more suitable for immunotherapies[112,113]. Interestingly, from the hundreds of proteins in each cluster, the expression of 25 proteins from various cellular processes were sufficient to distinguish each PDS cluster from each other in principle component analyses. These data propose that the expression levels of the identified proteins could be related to important properties in regulating the tumor microenvironment that should be further investigated.

Decellularised normal tissues present other characteristics than cancer scaffolds

Notably, we found that proteomic profiles of decellularised normal tissues were heavily distinguished from tumor PDSs, expressing a relatively lower protein abundance. These observations supported findings in Paper I, where we showed that cancer scaffolds included more proteins and were richer in nitrogen and oxygen composition compared to normal tissues which were higher in carbons since fat, one of the main components of normal tissues, contains more longer carbon chains. Cell-free normal tissues also induce dissimilar gene expression changes in adapting cancer cell lines compared to cancer PDSs (unpublished), suggesting that cell-free normal tissues and cancers microenvironments are different, which should be considered if cell-

free normal tissues would be used as experimental controls for cancer scaffolds.

Members of two proteins families were differentially expressed between PDSs from patients that developed recurrences compared to PDSs from patients with a better prognosis

A large proportion of the PDSs in the cohort came from patients with known disease recurrence and based on results from Paper I, III and IV where PDS cultures induced different gene expression profiles that were correlated to the recurrence/disease-free survival status of the corresponding patients we hypothesized that these PDSs would also present different proteomic profiles. Here in Paper V, we identified numerous proteins that were differentially expressed between PDSs from patients with or without disease recurrence, including several proteins that were indicated to have prognostic potential, although that needs to be further validated (unpublished). We focused on two protein families that were enriched in the PDSs coming from patients with recurrence, 8 intermediate filament proteins from the keratin family, as well as 6 members from the solute carrier protein-family. Numerous proteins in these two families have already been identified as biomarkers and to have prognostic value in several cancer types, moreover keratins are already used by clinicians as a diagnostic marker of epithelial cancers, and our findings in cell-free breast cancers substantiate the prognostic role of these proteins[114-116].

PDS clusters based on the expression of ECM-related proteins were associated with tumor grade and patients' age

Properties of the ECM influence cellular behaviors such as migration and invasion which are important in the EMT processes and development of metastasis[117]. In the previous papers, we have demonstrated that EMT markers were heavily altered by growth in various cancer microenvironments, and in Paper V we showed that the PDS microenvironments expressed different levels of ECM-related proteins. Here, transcription factor (TF) analyses showed that many of the expression of proteins in our analyses, and particularly the ECM-proteins appeared to be regulated by EMT-related TFs, which could be one potential link between the composition of PDSs influencing the regulation of EMT-genes in growing cells that we could quantify by qPCR.

We performed clustering of the PDS cohort based on their expression of ECM-related proteins, which resulted in some overlap to the PDS clusters established based on all proteins. Interestingly, the ECM-clusters were instead strongly associated with patient's age and tumor grade. PDSs from high grade cancers expressed lower levels of ECM proteins suggesting a less stable microenvironment in the scaffolds. This could possibly result in fewer cell-ECM connections when cancer cell lines are growing in high-grade PDSs, which could potentially have contributed to the variation in gene expression changes between high and low-grade PDS cultures we observed in Paper III and IV. We also established novel links showing that several collagens in our dataset decreased with patient's age. Collagens are known to influence drug resistance as well as treatment effectiveness based on its role in the tumor microenvironment through regulation of drug adsorption and accessibility by altering the density of tumors. This data indicated that age-dependent alterations of the ECM-composition should be considered when using the PDS model but suggest that age of patients should also further elaborated in cancer research in general. Aging is characterized by decreasing stem cell levels, and the stem cell maintenance is affected by the properties of the ECM in tissues[118]. The relatively lower levels of ECM protein in PDSs from older patients could potentially also influence the propagation of CSC sharing many properties with stem cells and should be further investigated in the PDS model.

In summary, the tumor scaffolds are a net result of the events that have occurred between cancer-, stromal- and immune cells, through physical interactions or secretion contributing to tumorigenesis, and formation of the tumor scaffold. This study demonstrated variability in relative protein abundance in cell-free breast cancers that likely contributes together with other properties to the induced gene expression profiles in Papers I-IV. However, transcriptomic, and proteomic profiles of PDSs need to be further compared to describe exactly which proteins influence the observed gene variations from the other studies, as well as evaluate additional properties of the tumors contributing to the inter-PDS variability. We believe that this study could provide data to create better 3D models of breast cancer, as well as highlight the variability in the composition of the tumor microenvironment that needs to be considered when modelling the disease *in vitro.*

FINAL CONCLUSION

In this thesis, we have demonstrated that PDS generated from decellularised primary breast cancers support cancer cell growth and infiltration and can be used for monitoring the activity of specific cancer microenvironments. PDS-adapted cancer cell lines showed altered morphology with increased heterogeneity and enrichment for CSC properties more similar to the *in vivo* situation. Gene expression analyses of cancer cells growing in PDSs indicated resemblance to cancer cells growing as xenografts in mice, as well as similarity to other 3D culture systems compared to monolayer cultures. The data highlight the relevance and usefulness of the PDS model and illustrate that specific cancer microenvironments induced variability in the growing cells affected by properties of the individual scaffolds. The expression of EMT genes was particularly influenced by characteristics from individual microenvironments and displayed large variation between scaffolds both with and without drug treatments. Several distinct groups of PDS with similar capacities could also be identified and were often associated with clinical parameters of the original cancer including disease recurrence. By treating PDS cultures with two front-line endocrine therapies and one CDK4/6 inhibitor we showed that PDS cultures induced different drug responses than monolayer cultures. We also exemplified with two front-line chemotherapy compounds how the PDS model could reveal *in vivo* mimicking data by introducing PDS-variation, as well as showing potential prognostic values.

Proteomic analyses of the cell-free scaffolds further illustrated that the protein composition of the scaffolds varied and contained ECM-related proteins and other extracellular proteins as well as intracellular proteins. We also found that proteins related to various cellular processes were present in the scaffold microenvironments which were likely produced by immune cells and other stromal cell types in the cancer. Interestingly, the protein compositions of the scaffolds were related to clinical findings supporting that the analyses can reveal key proteins imprinted in the cancer microenvironment influencing cancer progressing properties. The data can be used to identify novel cancer drug targets, biomarkers or key components that will be essential in human-mimicking synthetic 3D growth models.

FUTURE PERSPECTIVES

This thesis has provided extensive evidence that breast cancer microenvironments have major influences on cancer cell behaviors including their response to drugs. We provided a detailed characterization and functional testing of the PDS model. However, additional experiments could be performed to further characterize scaffolds and identify more properties in the microenvironments that impact the adapting cancer cell lines.

Further outlining of the model

The relationship between PDS induced gene expression profiles in cancer cell lines should be further detailed by comparing global RNA sequencing profiles of PDS cultures to the original donor cells from the corresponding patients. The transcriptomic data from the cells could be analyzed together with proteomic data of the scaffolds, linking innate properties of the original cancer as well as stromal cell behaviors to further outline important cellular processes influenced by individual microenvironments. More complexity can be added to the systems by also analyzing post-translational modification of the proteins in the PDSs such as glycoproteomics. Glycoproteomics is emerging in the field of cancer research. Identifying common glycosylation patterns of proteins within tumors adds an additional layer of heterogeneity to the model that can be combined with earlier described analyses to further classify scaffolds[119]. Also, protein phosphorylation is another critical post-translational modification that regulates protein function, and phosphorylation analyses through commercially available assays or mass spectrometry could potentially be applied to PDS cultures to study protein function, as well as identify potential dysregulated cellular signaling pathways[120]. Further, much of the work in this thesis have focused on inter-PDS variability but we did also observe structural intra-PDS variability by SEM in Paper I which could be further evaluated by spatial transcriptomics to study how specific compartments in the tumor microenvironment, such as potential CSC niches influence cancer cell line behaviors in restricted areas of the PDSs[121].

Applications of the model

By further characterization of the model, innate properties of individual PDSs could be utilized in generating more efficient translational preclinical models. For example, members of our group are evaluating co-cultures with immune cells together with cancer cell lines in the PDSs which could be used to test immunotherapies. By testing different drugs in more relevant PDS microenvironments, such as for example "immune-cold" and "immune-hot"-PDSs, could potentially help us to better identify which patients that would benefit from the specific therapies[9]. Other examples could be to use PDSs from patients with different age-spans in our studies to elaborate on age-specific effects from the cancer microenvironments in e.g., drug testing, since we showed in Paper V that the age of patients was significantly associated with relative abundance of ECM-related proteins. For drug testing purposes, donor cancer cells from tumors could be used to form organoids in parallel to scaffold cultures, providing both cellular and tumor environmental aspects to patient-specific toxicity profiles and drug responses. Scaffold cultures, and potentially also organoid cultures, could provide a suitable middle-step between *in vitro* and *in vivo* mice experiments and would limit, but not replace the numbers of animals needed which would reduce costs and be in line with the 3R principle (replace, reduce, and refine). To further decrease the use of animal models, properties from PDSs could also be reproduced *in vitro* by including relevant proteins identified from human cancer scaffolds into 3D-printed synthetic tumor models, to mimic different PDS-subtypes that could be mass-produced and used for high-throughput drug screening. Modelling the *in vivo*-situation and patient heterogeneity in an early pre-clinical stage in drug development could potentially be beneficial in increasing the success rate for later clinical trials.

Vascularization and circulation are tumor properties that are difficult to model *in vitro* but are nevertheless important modulators of highly relevant microenvironmental properties such hypoxia, pH as well as distribution of nutrients. To better represent the physiology in breast cancer, the PDS model could potentially be coupled to microfluidic systems using microengineering that would regulate biochemical properties impacting distribution of secreted factors from the cells, as well as drug availability in the model. This approach has also been proposed for organoids and supported by experiments using kidney organoids which led to an enriched endothelial progenitor pool and

formation of vascular networks into the surrounding lumen[122,123]. Interestingly, Zheng *et al* showed that collagen scaffolds could be engineered to support microfluidic vessels which also impacted the cells metabolism by being able to control nutrient supply by altering perfusion rate in the model[124], indicating that this technology could potentially be adopted to the PDS model or to 3D-printed tumors.

Patient-derived scaffolds as prognostic models with treatment prediction properties

All characterization and functional testing contribute to an increased understanding and robustness of the PDS model in various of preclinical testing regimes, which could be of benefit to patients. A goal for this model is to be used as an *ex vivo* treatment prediction platform as well as prognostic-tool to further risk-stratify patients based on the imprinted information in the tumor microenvironments.

Growth in the PDS model induced alterations in genes, such as several EMT-markers expressed by the cancer cell lines that could be related to clinical properties of the original tumors. However, other genes than the ones from our selected gene panel are likely also influenced by PDS growth and global RNA sequencing could potentially provide additional relevant genes to analyze in the model. A global RNA screening could also increase the chances of identifying genes in the PDS model that could potentially serve as prognostic markers for disease-free survival or recurrence in patients. Such analyses if preferably performed using multiple cancer cell lines as well as being validated in several larger PDS cohorts. In addition, individual proteins or protein panels that would be identified in PDSs could potentially also serve as prognostic markers to provide information about the aggressiveness of the tumor microenvironment in patients. In a clinical setting, the expression levels of such proteins could be quantified by traditional immunohistochemistry or other well-established approaches, such as proximity extension assays (Olink) that can be combined with qPCR or sequencing. These prognostic genes- or protein-panels, based on breast PDSs, could be commercially developed, and standardized to be used in a clinical setting to benefit patient characterization using the PDS model. Patients with a worse predicted risk prognosis based on analyses using the PDS model could for example be more extensively monitored after surgery using less invasive approaches. One example of a non-

invasive approach is through measurements of circulating tumor DNA by liquid biopsies from patient blood, which could be used to reveal potential disease progression leading to earlier interventions. For drug treatment prediction purposes, additional PDS cohorts needs to be evaluated with therapeutic compounds to generate PDS drug fingerprints, as well as clarify links between the PDS drug fingerprints to tumor properties for each therapy. Subsequently, new cancers could be screened in the PDS model after surgery to guide the clinical treatment strategy. In future research, the inter-PDS variability and potential subgroups of microenvironments that was elaborated during this thesis work could ultimately contribute to personalized medicine.

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