3D Printed scaffolds as cancer microenvironment models for drug discovery

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To my Family and Friends

ABSTRACT

Cancer is one of the most common diseases in the modern world and major efforts are made globally to develop new diagnostics and treatments. It originates from a cell which, at some point, has begun to divide and grow uncontrollably. The most common type is breast cancer, and like all other cancers there is a need of more efficient drug therapies. Drug development is an expensive and time-consuming process and in conventional pre-clinical evaluation, drugs are tested on cells grown in 2D followed by experimental studies in animals. Only the drug candidates with best efficacy and safety profiles are allowed to proceed to clinical trials in humans. A major problem is that the pre-clinical test methods most often do not adequately represent the microenvironment in the human body and only a portion of the drugs that show good effect in pre-clinical studies pass the clinical trials and reaches market. Failures in late development mean large losses both financially and in time, and better pre-clinical test methods are needed that can predict more accurate results for safety and efficacy.

The behavior of cancer cells is strongly influenced by the surrounding microenvironment, but today's drug development focuses mainly on the cells themselves and does not sufficiently take this into account. This thesis combines 3D printing and cell biology to develop new and more representative test systems, with the ambition to mimic the tumor microenvironment in three dimensions. By using patient tumor tissue and removing the original cells, we produce a cell-free extracellular matrix scaffold to which standardized reporter breast cancer cell lines are reintroduced. The cell lines grown in the patient derived scaffolds developed more stem cell properties and formed a more heterogeneous cell population compared to 2D cultures. Moreover, the gene expression profile could be linked to clinical data, such as relapse. In an attempt to synthetically mimic the human tumor tissue, we used an alginate-based biomaterial to print 3D scaffolds. Breast cancer cells cultured in the 3D printed scaffolds showed a more similar growth- and gene expression pattern to cells cultured in patient derived scaffolds indicating that we were able to simulate the human tumor microenvironment. Further, we showed that the cells cultured in both patient derived scaffolds and 3D printed scaffolds had a similar response to hypoxic conditions – which is an important factor in tumors. Finally, we also showed that nanocellulose could be used to 3D print and that cells cultured in these scaffolds demonstrated comparable results to cells grown in alginate-based 3D printed scaffolds.

Keywords: 3D Printing, Breast cancer, Biomaterials, Drug development

SAMMANFATTNING PÅ SVENSKA

Cancer är en av de vanligaste sjukdomarna i modern tid och stora ansträngningar görs globalt för att utveckla ny diagnostik och behandlingar. Cancer härstammar från en cell som någon gång har börjat dela sig och växa okontrollerat. Bröstcancer är den vanligaste typen och precis som alla andra cancerformer finns ett stort behov av att utveckla nya och mer effektiva läkemedel. Läkemedelsutveckling är en dyr och tidskrävande process och i konventionellt prekliniskt arbete testas läkemedel på celler odlade i 2D följt av experimentella studier på djur. De läkemedelskandidater med bästa effektmått och säkerhetsprofil tillåts gå vidare till kliniska prövningar på djur och människa. Ett stort problem är dock att de prekliniska testmetoderna oftast inte på ett adekvat sätt representerar mikromiljön i den mänskliga kroppen och endast en bråkdel av de läkemedel som visar god effekt i prekliniska studier går igenom de kliniska prövningarna och når marknaden. Misslyckanden i sen utvecklingsfas innebär stora förluster både ekonomiskt och tidsmässigt och det behövs bättre prekliniska testmetoder som mer säkert efterliknar resultaten av kliniska prövningar.

Cancercellernas egenskaper påverkas betydligt av mikromiljön som omger cellerna, men dagens läkemedelsutveckling fokuserar främst på tumörcellen och tar inte hänsyn till mikromiljöns egenskaper. Denna avhandling kombinerar 3D-printing och cellbiologi för att utveckla nya, mer representativa testsystem, med ambition att efterlikna tumörens mikromiljö. Genom att använda tumörvävnad från patienter och tvätta bort de ursprungliga cellerna producerar vi en cellfri bindvävsstruktur till vilken vi kan återinföra standardiserade reportercellinjer. Cellinjerna som odlades i bindvävsstrukturerna från patienter utvecklade fler stamcellsegenskaper och bildade en mer heterogen cellpopulation jämfört med konventionella 2D-kulturer. Dessutom kunde genuttrycksprofilen kopplas till kliniska data såsom återfall. I ett försök att syntetiskt efterlikna tumörvävnaden använde vi ett alginatbaserat biomaterial för att producera 3D-printade strukturer. Bröstcancerceller som odlades i dessa 3D-utskrifter visade liknande tillväxt- och genuttrycksmönster som celler odlade i bindvävsstrukturer från patienttumörer vilket indikerar att vi kunde simulera den mänskliga tumörens mikromiljö. Vidare så kunde vi visa att celler odlade i bindvävsstrukturer från verkliga patienttumörer och 3D-printade strukturer hade liknande svar på syrefattig miljö – vilket är en viktig parameter i tumörbeteende. Slutligen visade vi även att nanocellulosa kunde användas för 3D-printing av bindvävsstrukturer, och att celler odlade i dessa visade liknande resultat som celler odlade i alginatbaserade 3D-utskrifter.

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 D, **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 Mar;235:119705*

II. Svanström A*, **Rosendahl J***, Salerno S, Leiva MC, Gregersson P, Berglin M, Bogestål Y, Lausmaa J, Oko A, Chinga-Carrasco G, Petronis S, Standoft S, Ståhlberg A, Håkansson J, Landberg G. Optimized alginate-based 3D printed scaffolds as a model of patient derived breast cancer microenvironments in drug discovery, *Biomed Mater. 2021 Jun 25;16(4).*

III. **Rosendahl J***, Svanström A*, Berglin M, Petronis S, Bogestål Y, Stenlund P, Standoft S, Ståhlberg A, Landberg G, Chinga-Carrasco G, Håkansson J. 3D Printed Nanocellulose Scaffolds as a Cancer Cell Culture Model System, *Bioengineering (Basel). 2021 Jul 10;8(7):97.*

IV. Svanström A, **Rosendahl J**, Salerno S, Jonasson E, Håkansson J, Ståhlberg A, Landberg G. The Effect of Hypoxic and Normoxic Culturing Conditions in Different Breast Cancer 3D Model Systems, *Front Bioeng Biotechnol. 2021 Nov 4;9:711977*.

* = equal contribution

Additional publications not part of this thesis:

i. Landberg G, Jonasson E, Gustafsson A, Fitzpatrick P, Isakson P, Karlsson J, Larsson E, Svanström A, Rafnsdottir S, Persson E, Andersson D, **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 Brief. 2020 Jun 16;31:105860*.

ii. Chinga-Carrasco, G., Ehman, N.V., **Pettersson, J**., Vallejos, M.E., Brodin, M.W., Felissia, F.E., Håkansson, J., Area, M.C. Pulping and pretreatment affect the characteristics of bagasse inks for 3D printing. *ACS Sustainable Chem. Eng., 2018, 6 (3), pp 4068–4075*.

iii. Book chapter, Nanocelluloses – Nanotoxicology, Safety Aspects and 3D Bioprinting, Gary Chinga-Carrasco, **Jennifer Rosendahl**, and Julia Catalán; H. Louro, M. J. Silva (eds.), Nanotoxicology in Safety Assessment of Nanomaterials, *Advances in Experimental Medicine and Biology 1357, https://doi.org/10.1007/978-3-030-88071-2_7* (in press to be published 2022)

CONTENT

ABBREVIATIONS

2D	EN: Two Dimensional SV: Tvådimensionell
3D	EN: Three Dimensional SV: Tredimensionell
3DP	3-Dimensional Printing
3DPS	3-Dimensional Printed scaffold
5-FU	5-fluorouracil
CNF	Cellulose nanofibrils
CSC	Cancer stem cell
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ECM	Extracellular Matrix
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
FACS	Fluorescence-activated cell sorting
GAGs	Glycosaminoglycans
HA	Hydroxyapatite
HER2	Human epidermal growth factor receptor 2
IHC	Immuno Histo Chemistry
PCA	Principal component analysis
PDS	Patient-derived scaffold
PEG	Polyethylene glycol

PR	Progesterone Receptor
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
SEM	Scanning Electron Microscopy
TEMPO	2,2,6,6-tetramethylpyperidine-1-oxyl

INTRODUCTION

Cancer is one of the most common diseases in the modern world and out of the different types breast cancer is the most common [1]. In recent years, the understanding of cancer biology has increased within both treatment and prevention (by screening). Breast cancer, like all cancer, is a genetic disease where cells mutate and gain cell dividing properties that let them proliferate at the expense of normal cells [2]. The most common treatments are surgery (to remove the tumor), radiotherapy, and chemotherapy or endocrine treatment [3]. However, even if the same cancer type is treated with the same drug the results commonly differ between patients. This thesis is focused on how cells within the tumor are affected by the surrounding tumor microenvironment and the resulting changes in the cells [3]. In the tests used in early cancer drug development this factor is not accounted for as most of the early drug screenings are performed on 2D cell cultures.

CANCER

Cancer is a collective name for numerous different diseases which can originate from almost anywhere in the body and is further divided into different subgroups. It is one of the most common diseases and is a complex societal challenge and a diagnosis can be devastating for both the patient and his or her relatives. Cancer numbers as a leading cause of death with an annual estimated 19.3 million new cases diagnosed worldwide each year, of which the most common is female breast cancer with an estimated 2.3 million new cases (11.7%) [1]. Only about 1% of all cancers are hereditary and most occurs in somatic cells [4]. Genetic mutations in healthy cells are common in all forms of cancer and the mutated cell will start to divide, grow and spread uncontrollably [4, 5]. Mutations in cancer can be single-nucleotide mutations, substitutions or larger rearrangement, amplifications or deletions [6]. Mutations in cells are common and occur all the time without leading to cancer. There are, in most cases, not just one mutation but rather several that will result in a cancer cell and if these alterations occur in proto-oncogenes and tumor suppressor genes there is a higher risk of cancer cell development [4]. Proto-oncogenes are often involved in pathways that stimulate cellular growth. If a mutation occurs in these genes and results in an oncogene, the output will be gain of function, which will stimulate cell growth, division, and survival [7, 8]. Tumor suppressor genes, on the other hand, are often involved in pathways that inhibit cell division, induce apoptosis, and suppress metastasis. If the mutation instead occurs here, the output will be loss of function in DNA repair,

preventing unrestrained cellular growth and cell cycle checkpoint activation [7-9]. Carcinogenesis, i.e. the transfer of a normal cell into a tumor cell, is mainly dependent on the balance between oncogenes and tumor suppressor genes. The development of breast cancer and its growth is mainly stimulated by hormones and different growth factors [3]. Cancer mutations affect the most essential aspects of cellular function, such as DNA repair, cell cycle, apoptosis, differentiation, cell migration and cell-cell contact [4]. It often displays an invasive behavior, resisting cell death, evading growth suppression, experiencing uncontrolled replication, showing sustained growth, and triggering abnormal angiogenesis [5].

BREAST CANCER

Globally, one out of nine women below age of 75 are diagnosed with breast cancer [10]. In Sweden year 2016, 22518 persons were diagnosed with cancer and of these 34% were women diagnosed with breast cancer [11]. Breast cancer tumors vary greatly in terms of structural and cellular composition and due to this both the prognosis and treatment differs, with different survival rates as a result.

The most common way to categorize tumors is to assess the expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), by immunohistochemistry (IHC) [12]. As shown in Figure 1, all **Luminal A and B cancers** are PR+/ER+ and can be either HER2+ or -. The difference between them is that Luminal A is slower growing which can be explained by low levels (below 14%) of the proliferation marker Ki-67 whilst faster growing Luminal B cancer

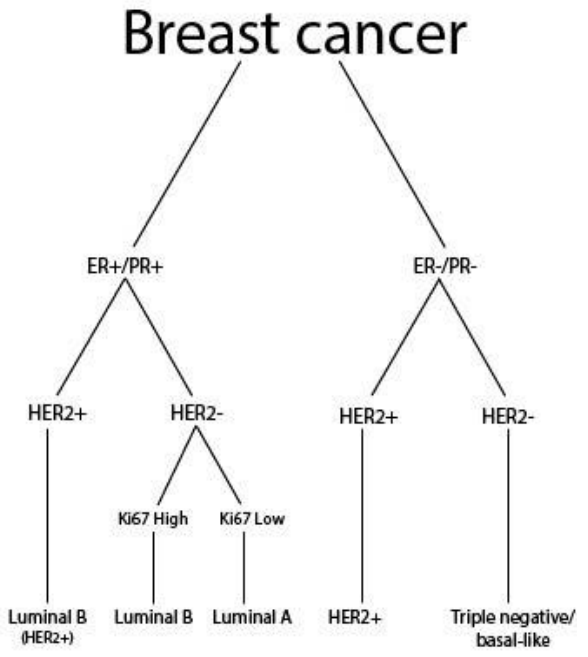

Figure 1 Breast cancer subtypes

has high Ki-67 levels. Approximately 80-85% of all breast cancers are ER+[3]. **Triple-negative/basal-like** cancer is PR-/ER- and HER2- and is usually connected to gene mutation in the *BRCA1* gene. The subtype **HER2+** is a fast growing cancer with an expression profile that is PR-/ER- and HER2+ and can often be treated with targeted therapies (Figure 1) [13]. The cancers are called luminal and basal like due to the fact that ER-positive tumors resemble normal glandular cells, "luminal", while ER-negative tumors are more similar to myoepithelial cells, "basal like". Depending on the subtype, the prognosis will vary. A fast-growing metastatic tumor will result in a worse prognosis than a slow growing non-metastatic tumor [14] (Figure 2).

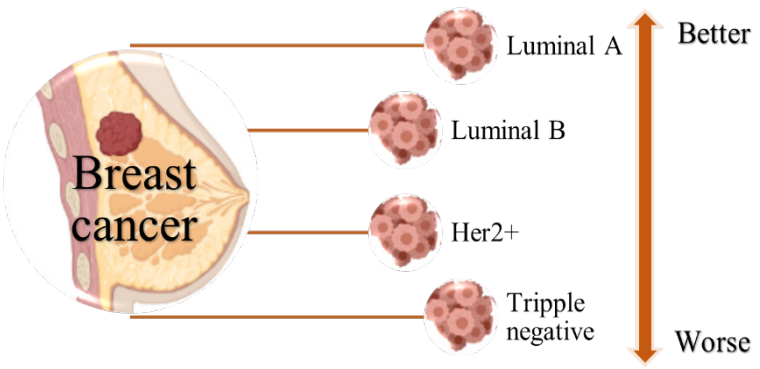

Figure 2 Different kind of breast cancer have different prognosis. Created with Biorender.com

Eight out of ten women survive a breast cancer diagnosis in Sweden [11]. The most common treatments are surgery, chemotherapy, endocrine or cytostatic treatment. Breast cancer treatments are becoming more individualized and precision medicine has taken a huge leap in going from vision to reality, where gene expression profiling helps with choosing the correct treatment regime [15]. Each patient will be evaluated dependent on their age, previous treatments, relapse, aggressiveness of the tumor, possible metastasis etc. If the patient is young, has a high Ki67 or if the cancer has metastasized, chemotherapeutic/cytostatic treatment will most likely be used in conjunction with surgery [3]. For chemotherapeutic treatment in breast- and colorectal cancer, one of the most common drugs are 5-fluorouracil (5-FU), a pyrimidine analog that exerts its anticancer effects through inhibition of thymidylate synthase, which then incorporates its metabolites into DNA and RNA [16, 17]. Another common chemotherapeutic treatment in breast, bile duct and endometrial cancer is doxorubicin (DOX). DOX is most likely dose dependent and inhibits topoisomerase II in low doses. At higher doses, intercalation into the DNA or free radical formation has been explored [18, 19]. Endocrine

treatment is only available for ER+ breast cancer, due to that the endocrine receptor needs to be expressed [3]. The female sex hormone estrogen can bind to the cell nuclei of the tumor through the endocrine receptors and stimulate cell division leading to tumor growth. Endocrine treatment blocks the effect of estrogen or reduces the level of estrogen in the body [3].

To be able to perform research on different alternative treatments for breast cancer, immortalized cell lines are used. In this thesis, we focus on MCF-7 which is ER+/PR+/HER2- [20], T47D which is ER+/PR+/HER2- and MDA-MB-231 which is ER-/PR-/HER2-. These cell lines are widely used in the literature and account for much of the research done on breast cancer [20-23]. MDA-MB-231 show a more infiltrative phenotype compared with the other cell lines [22]. MCF-7 is non-invasive, not very aggressive [24] and has low metastatic potential [25]. T47D is quite similar to MCF7 and represent a luminal A tumor; however, T47D is more susceptible to progesterone [26].

TUMOR HETEROGENITY AFFECTS EXPRESSION OF GENETIC MARKERS

Tumor heterogeneity differs both between patients (inter tumor heterogeneity) and within each tumor (intra tumor heterogeneity) [12]. Apart from the genetic diversity there are several other factors that influence the variability in tumors, including epigenetic variations, microenvironment, cellular differentiation and

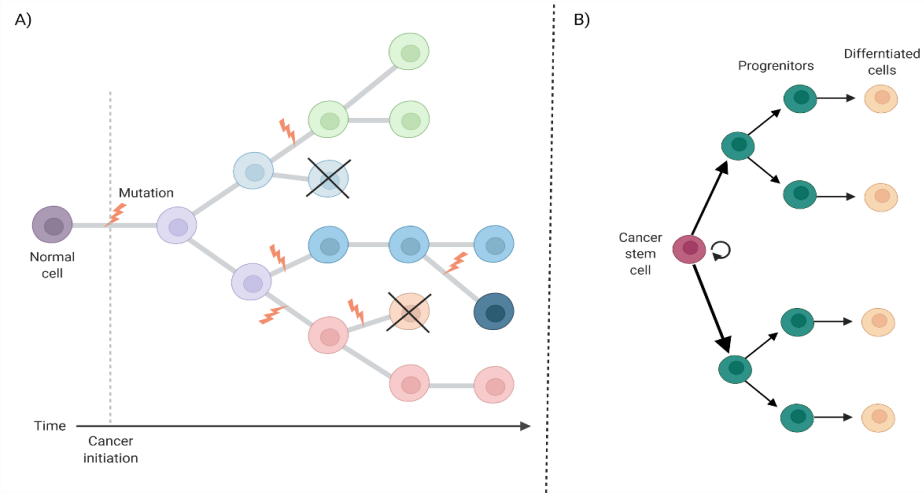

Figure 3 Theories of cancer heterogeneity. (A) Clonal evolution, which explains different sub-populations (clones) of cells. Lightning bolts represents mutations and crossed over cells are non-surviving clones. Adapted from Nowel, 1976. (B) Cancer stem cell theory where a cancer stem cell is self-renewed and gives rise to different types of cells. Adapted from Beck et al. 2013. Created with Biorender.com

gene expression variations [27]. There are two main theories regarding cancer heterogeneity and growth: Clone based or stem cell based. The first theory is based on clonal evolution, which describes how genetic changes can lead to tumor progression [28]. A normal cell which acquires advantageous genetic changes starts to divide and proliferate. The daughter cells will obtain more mutations due to genetic instability and sub-groups will form, so called clones (Figure 3A). The second theory is based on cancer stem cells (CSC), and similar to normal stem cells, CSCs would have the capacity of self-renewing. Cancer stem cells are often discussed as one of the core features of cancer with tumor-initiating capability since it can not only self-renew but also clone itself to form identical daughter cells and give rise to progenitors and subsequent differentiated cells(Figure 3B) [29].

Tumor cells are often characterized by genetic markers, and the ones used in this thesis were markers for CSC (*CD44 ABCG2*), epithelial-mesenchymal transition (EMT) (*SNAI1*, *VIM*, *TWIST*, *MUC1*), proliferation (*MKI67*, *CCNA2*, *ERBB2, CDKN1A*), differentiation (*CDH1*, *ESR1*, *CD24*, *PGR*, *EPCAM*) and pluripotency (*SOX2*, *POU5F1*, *NANOG*). High expression of *CD44, ABCG2* and low *CD24* are linked to tumor-initiation [30, 31], chemotherapy-resistant cells [32] and tumor progression [33]. The EMT is one of the alterations the cells can go through to transfer a non-motile epithelial cell into an invasive mesenchymal phenotype [34]. This phenomenon can often be seen genetically by induction of the mesenchymal marker vimentin (*VIM*), cell surface associated *MUC1* and increase of the transcription factors *SNAIL*, *SLUG* and *TWIST* to downregulate E-cadherin (*CDH1*) [34, 35]. Differentiation of cancer cells implies the maturation of the cell to a phenotype more similar to the tissue it originated from with no renewal capacity [36]. One of the markers related to differentiation is Estrogen receptor 1 (*ESR1),* which is often mutated in primary tumors and is enriched during metastasis and affects cell growth, metabolism, sexual development, gestation and other reproductive functions [37, 38]. Progesterone receptor *(PGR)* has recently been identified as a possible prognostic marker where a higher level of expression correlates to a more poor prognosis [39]. This is also the case for epithelial cell adhesion molecule (*EPCAM)* [40]. Another important factor to monitor is pluripotency, which is the ability for a cell to transfer to other cell lineages in response to the environment [41]. *SOX2*, *POU5F1*, *NANOG* are all transcription factors used as pluripotency markers [42]. Proliferation markers are related to cell division. *MKI67* and Cyclin A2 (*CCNA2*) are both expressed during cell division. Erb-b2 receptor tyrosine kinase 2 (*ERBB2)* (also referred to HER2) is an epidermal growth factor receptor which has been reported to be highly genetically expressed in invasive breast carcinomas. Finally, Cyclin Dependent Kinase 1 (*CDK1A*), which is a cell cycle inhibitor [43]. Even

though there is genetic heterogeneity in breast cancers, it seems like the number of specific signaling pathways activated within each subtype of the disease are limited [44].

MICROENVIROMENT

Each tumor is composed of cells with different characteristics [45] which are all influenced by the complex and dynamic microenvironment surrounding the cells [46, 47]. The microenvironment directly influences the cells to be more or less potent for the different characteristics which gives each tumor an individual complexity, i.e. stem cells, differentiation, pluripotency, proliferation and EMT [8]. The breast tissue is composed of different components, of which the most important parts are mammary epithelial cells, stromal fibroblasts and extracellular matrix (ECM) which prevalently contains collagens, heparin, laminins, glycosaminoglycans (GAGs), elastin and fibronectin forming the microenvironment [48]. The microenvironment surrounding the cells influences the interaction with neighboring cells, both by altering protein expression and by stimulating e.g. communication via exosomes and hormones [49]. Cell-to-cell communication is a complex network of different stimuli, mainly containing growth factors, cytokines, inflammatory mediators and matrix remodeling enzymes [50]. As each tumor has its own eco system that determines the characteristics of the tumor by influencing whether it is malignant or benign, sensitive or insensitive to growth inhibition, capable of avoiding apoptosis/limitless cell division and if it is metastatic and/or invasive, the influence of microenvironment on cells are important [51].

Cancer tissues have different stiffness which can vary from very soft (e.g. lung) to very hard (e.g. bone), even in different areas within the same tumor. Figure 4 illustrates the elastic modulus of different tissues which also represents the whole span of stiffness in breast cancer [48]. Dependent on the level of

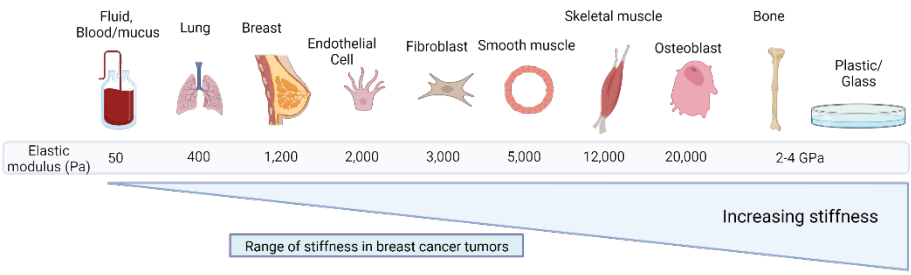

Figure 4 Stiffness in different tissues and cells. Picture adapted from Butcher et al. 2009. Created with biorender.com

stiffness, cells will behave differently which in turn affect cell growth, survival and motility [52]. In breast cancer malignancy, a higher grade of stiffness has been found to correlate with a more poor prognosis [53] which is most likely due to increasing stiffness causes cells to be more proliferative and to differentiate more [52]. This is an important factor for 3D culture systems where 3D printed structures can be modulated for different levels of stiffness whereas organoids and spheres will have a very low grade of stiffness.

Patient Derived scaffolds

To investigate the effect of the microenvironment on cancer cells, patient tumors were decellularized using mild detergents leaving the ECM structure, that we call patient derived scaffolds (PDS). The heterogeneity of the PDSs microenvironment will cause the cells within the scaffold to differentiate in different directions. To monitor this, standardized cancer cell lines (described above) were seeded to the scaffolds and gene expression was assessed for a number of marker genes (described above). The variation in gene expression can be of diagnostic and prognostic value and a possible way to further subgroup the tumors for more precise treatment regimens (Figure 5). These systems also make it possible to test different therapeutic drugs on the patient's own tumor material in the lab before initiating treatment, as well as a tool for drug screening.

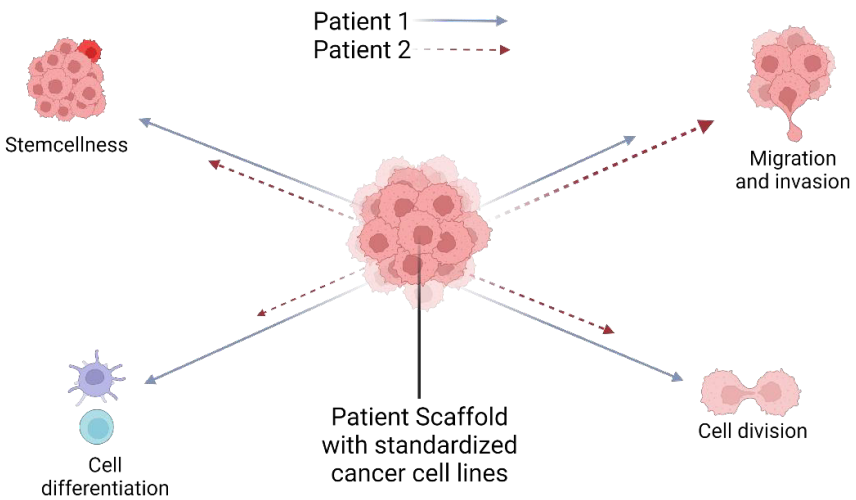

Figure 5 Patient derived scaffold cellularized with standardized cancer cell lines. The individual microenvironment will affect the gene expression of the cells in different directions for each patient resulting in different tumor characteristics for each patient.

Hypoxia

In solid tumors, hypoxic tissue regions with reduced levels of oxygen are often found due to that vascularization development is disordered which leads to an inefficient supply of oxygen to some areas in rapidly growing tumors [54]. If the tissue is not properly vascularized, hypoxia will arise around 100 μm from the blood vessel with a gradient of oxygen level further away [54]. Hypoxia is an important factor as it will influence cell growth rate, metastasis induction, treatment sensitivity, neovascularization [23] and is often associated with poor prognosis [55, 56]. Cancer cells adapt to the hypoxic environment to survive which contributes to a more malignant and aggressive tumor behavior [57]. Such a shift can be due to the cancer cells changing their metabolism from aerobic to anaerobic in hypoxic environment or by production of growth factors that induce angiogenesis [58]. One important issue within cancer treatment is that radiotherapy does not work in hypoxic areas, as radiation treatment requires free radicals from oxygen to destroy target cells [57].

2D VS. 3D

Today, mainly conventional 2D cell cultures in plastic plates are used for cancer research and drug development. However, this system has several disadvantages compared with 3D structures, one of which is making the cells grow in a monolayer as a homogenous cell population. A 3D growth environment allows the cells to grow in a more similar way to their normal growth patterns *in vivo*, including cell heterogeneity [50], migration pattern, motility and metabolism [59]. 3D cell cultures often resemble the *in vivo* situation better compared with 2D concerning factors such as integrin expression, cell migration, cell mechanics [60-63], enrichment of CSCs [64-67] and proliferation [68]. Cells growing in a 3D microenvironment also show different effects regarding treatment with drugs compared with 2D cultures, where cells in a 3D environment are more drug resistant [69-74] which more closely mimics the *in vivo* situation [75-81]. Therefore, drug screening is preferably made in a 3D culture that better represents the tissue and matrix organization *in vivo* compared with 2D [82].

There are several different ways to create a 3D environment for cell cultures: cultured aggregates [83] such as mammosphere [84], micromass [85], spheroids [86] and microfabricated tissues [87, 88], materials 3D printed as scaffolds [89] or cells embedded in gels [90].

3D PRINTING

Bioprinting has modernized the way biology and biomaterials can be combined into three dimensional structures and it has recently been given a lot of attention due to the new design possibilities that is impossible to accomplish using other methods. By varying the design and choice of materials, different parameters such as overall porosity of scaffold controlling, mechanical properties, filament curvature, chemical functionality, *in-vivo* degradation and remodeling among others can be accomplished. It is even possible to do individual customization of the printed parts with alterations in biomechanics, permeability, and degradations rates of the materials [91]. Bioprinting can be used for tissue engineering and to manufacture tissue specific models to create biomimetic organs [92]. By using different biomaterials as 'bioink', it is possible to manipulate the chemical and biological environment to create complex structures [93].

There are different strategies to print materials of which inkjet, stereolithography, laser assisted printing and extrusion-based printing are the most common ones [93-96]. In *Inkjet printing*, also called droplet printing, small droplets are placed in a predetermined pattern similar to a paper ink-printer used to print documents at most offices [97]. It can be used to print either one or several different types of cells in a specific pattern or to print biomaterials [98, 99]. *Stereolithography* uses a selective photo-initiated curing reaction to cross-link liquid polymers to solid structures. Each structure is developed in a layer-by-layer manner and the biomaterial used must be photo-crosslinkable [100]. *Laser assisted printing* uses a laser beam by sending it through a material to form droplets which are then placed in a predetermined pattern on an underlying substrate [96]. Similar to ink jet printing, laser assisted printing can be used to print both biomaterials and cells in a pre-defined pattern [101]. The resolution of laser assisted printing is very good, from sub micrometer scale to micro-scale [102]. *Extrusion based printing*, also called pressure-assisted printing, is a technique that extrudes biomaterial from a nozzle or needle by a coordinated movement in an XYZ-manner. Depending on the material, the needle size and the speed and pressure used, a filament of varying size will be created which is then applied in a layer-by-layer manner to create a 3D structure [103]. In Li *et al*. the advantages and disadvantages of different bioprinting techniques are further reviewed, here summarized in Table 1 [102].

Table 1 Advantages and disadvantages with different bioprinting techniques.

Type of bioprinting process	Advantages	Disadvantages
Inkjet bioprinting	Low cost, high resolution, high speed	Very limited vertical structure, induces stress on cells, limited in materials (only liquid)
Extrusion based bioprinting	Many materials can be used, conditions can be mild, homogenous distribution of cells, low cost	Low resolution, limited mechanical stiffness, slow speed, can induce stress on cells
Laser assisted bioprinting	Nozzle free, non-contact process, precise delivery	Very limited vertical structure, high cost, time consuming, metallic particle contamination as metallic films are required
Stereolithography	Nozzle free, high accuracy	Simultaneous printing of cells not possible, limited selection of biocompatible and biodegradable polymers, toxic residuals, harmful

In this thesis, extrusion-based printing has been used, in which biomaterials are pushed pneumatically by a piston through a syringe in a pre-designed XYZ-pattern. There are many different settings which can be adjusted in an extrusion printer, such as: diameter of needle outlet and design, piston pneumatic pressure, movement speed of needle, temperature of the material, dispersing several materials at the same time, distance from needle to platform, platform temperature, delayed starts and stops, heating curves etc. Altogether, there are a lot of settings that can be adjusted for each material, which makes the process very flexible but also challenging to fully control final scaffold properties

MATERIALS IN EXTRUSION BASED BIOPRINTING

Materials used for bioprinting are called often bioinks and they need certain properties to be suitable for extrusion based bioprinting. Essential properties for *in vitro* systems include dispensability, shear thinning, it should not block the nozzle, quickly solidifying after material dispensing, the layers should be joined between each Z-movement, biocompatible, preferably maintain its volume during 3D printing, have suitable biomechanical properties and have none or a controlled degradation over time [104]. Furthermore, to act as a screening system when using cells, the successfully printed ink should allow the cells to develop properties that resemble *in vivo* like conditions, maintain the cells integrity post-printing as well as expose cell adhesive motifs [94]. Depending on the application, different properties need to be obtained. For example when it comes to tissue engineering, the scaffolding should most likely be degradable over time, whilst for drug screening platforms it might be a disadvantage if the test system degrade with time [68]. There are also different ways of applying cells to the material. Cells can either be mixed in with the material before printing or seeded on the scaffold after printing. The latter puts less constraints on the biomaterial and the printing process, as cells are sensitive to high pressures and temperature variations. This thesis is focused on generating scaffolds aimed at evoking an *in-vivo* like cell response when seeded after printing, making them suitable to be used as *in vitro* drug screening systems.

Inks can be produced from either natural or synthetically derived sources. The most frequently used materials from natural sources are gelatin, fibrin, Matrigel®, collagen, chitosan, nanocellulose and alginate. The most commonly used synthetic sources are polyethylene glycol (PEG), Block-poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Pluronic®) and Poly(N-isopropylacrylamide) [93, 103, 105-109]. In this thesis, focus has been alginates and nanocellulose with different additives.

Modifications

As most biomaterials do not contain all the essential functions from start, there has been a lot of research performed on the subject of acquiring good printability and modifying the materials to comply with the cell culture [110]. One of the approaches has been to mix different biomaterials, e.g. alginate and gelatin [111], alginate and nanocellulose [112] or alginate and collagen [113]. Another important parameter is that some materials will stabilize and stiffen immediately while others need some kind of crosslinking process, such as UV-light, addition of chemicals or a physical process. The crosslinking of the material often makes it shrink, which can be harmful for living cells inside the

biomaterial [104]. An additional way to modify and functionalize the biomaterial is to add different proteins and peptides, either dispersed in or covalently attached to components in the bioink. In this thesis periostin has been used - a secretory adhesion protein upregulated in breast cancer [114] which is required for stem cell maintenance and regulates the infiltration of tumor cells into secondary target colonization [115].

Alginate

Alginates are salt derivates of alginic acids that originate from natural sources, either from the cell wall of marine brown algae or from bacteria [96]. Alginate is a polysaccharide with two building blocks of guluronic acid and mannuronic acid [116]. The parameters that are of interest are M/G ratio (content of mannuronic/guluronic acid), presence of ions (as these are known to influence the viscosity/elastic modulus and shear thinning properties) [117] and the printing abilities of the substrate. Gelation of alginate is often achieved with divalent cations which forms intermolecular bonds between alginate chains. The speed of the physical cross-linking and solidification can be adjusted by changing the concentration of both the alginate and the divalent cations [104]. Alginate is widely used in the 3D printing area, and is known for biocompatibility, solubility, low cost and shear thinning [96, 110, 118].

Nanocellulose

Nanocelluloses can be obtained from different sources of raw material including bacteria, wood, plants and marine sources. Hard- and softwood chemical pulp fibers are most commonly used to produce nanocellulose and is obtained from processing wood pulp with chemical and enzymatic treatments to generate two main types of wood nanocellulose fibers, i.e., cellulose nanofibrils (CNF) and cellulose nanocrystals [108, 119, 120]. Depending on the treatment, the nanocellulose will acquire different properties which are relevant for cell growth, attachment and cytotoxicity. Nanocellulose has proven to be suitable as a material for biomedical purposes due to its biocompatibility and low cytotoxicity [108, 121]. Nanocellulose has the possibility to form gels at low concentrations while still maintaining viscoelastic behavior [122] and has been used for tissue engineering purposes in earlier studies for producing *in vitro* scaffolds for e.g., blood vessels and liver tissue [110].

Hydroxyapatite

Hydroxyapatite (HA) is a naturally occurring mineral form of calcium phosphate and also a natural component of human bone and other calcified structures in the body. HA is widely used in different biomedical applications and dental implants [123]. It has previously been shown to be biocompatible for tissue engineering of bone, with inkjet [124], extrusion-based [104] and laser assisted printing [110]. In breast cancer, there are often traces of microcalcifications that are composed of either calcium oxalate crystals or other hydroxyapatite crystals [125]. Hydroxyapatite has also been shown to be involved in genetic regulation of calcification and tumorigenesis [126].

DRUG SCREENING PLATFORM

Development, screening and testing of new cancer drugs are today performed in different stages, i.e., drug discovery, pre-clinical phase and clinical phase [127]. A lot of effort is put into the pre-clinical phase where screening is made to identify the drug candidates that are considered promising and which ones ought to be discarded. Only 7% of all cancer drugs candidates that have shown effect in pre-clinical phase demonstrate enough efficacy in later stages to reach the market [128, 129]. There is a broad consensus that animal models and 2D cell cultures do not represent the human tumor environment well enough in terms of drug resistance, tumor progression, growth and heterogeneity [82, 128]. As previously described, a 2D culture does not exhibit the same sensitivity to drugs as 3D cultures or the human body does [130]. Xenograft *in vivo* tumors are often more fast growing and therefore they are more likely to respond to anti-proliferative agents [128]. Although animal testing better represents the complex *in vivo* environment, they do not mimic the environment in the human body well enough. They are also time consuming, costly and from an ethical perspective, animal experiments should only be performed when relevant and be in compliance with 3R - "Replace, Reduce, Refine". Together, there is a large need to improve efficiency, predictability and effectiveness of drug screening models in order for the pharmaceutical industry to find new potent drugs in a time and cost-efficient way.

METHODOLOGICAL CONSIDERATIONS

To evaluate cell- and scaffold characteristics, the following methods have been central for this thesis.

qPCR

Gene expression analysis with fluorescence based quantitative PCR (qPCR) uses specially designed small nucleic acid primers, that match a specific gene, to amplify a nucleic acid sequence. The amount of amplified product is quantified by either a fluorescent labeled probe, specific for the targeted gene, or by incorporation of a fluorescent dye in the DNA product [131]. In this thesis, gene expression has been performed as illustrated in Figure 6.

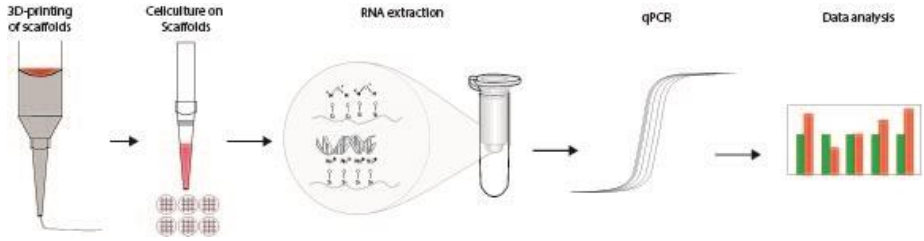

Figure 6 Schematic illustration of gene expression analysis of cells grown in 3D printed scaffolds

Western Blot

Protein analysis by western blot is a complementary method to verify the gene expression analysis on a protein level. Proteins are separated by molecular weight on a gel using electro-current. The proteins are then transferred to a membrane on which they are visualized using specific targeted primary antibodies and secondary chemiluminescence enzymes – so called label antibodies.

Cancer stem cells assays

Holoclone formation is a well-established functional analysis with which stem cell properties can be determined. Cells from different culture systems are seeded on culture plates and depending on the clonality of the cells, distinct colonies are formed, so called holoclones, meroclones and paraclones, where stem cell properties influence the extent of holoclone formation [132-134]. Another method is *mammosphere assay* where the cells are seeded on a non-adherent plate and stem cell properties of the cells will induce mammospheres formation [135]. Mammospheres are cell clusters of cells that have stem

cell/early progenitor activity and are able to survive and start to proliferate as spheres in low concentrations without adhering to the surface of a cell culture plate.

Cell viability/metabolism/proliferation/migration assays

Cell abundance measurements can be performed in different ways. Coloring cells with *Trypan blue* and counting them is one of the most common ways to determine living or dead cells. Trypan blue will enter dead cells, with a disrupted cell membrane, and color the cytosol while living cells will be colorless. *Alamar blue* is a resazurin-based solution that will change color when metabolized (resazurin is reduced to resorufin) in the cell and it is a non-invasive test. *Wound healing assay* is a way to measure proliferation and cell migration. Cells from different culture systems are seeded to 2D culture dishes with a silicon insert and allowed to form a semi-confluence layer. Removing the insert generates a "wound" in the cell culture and proliferation and cell migration can be monitored by taking images of the culture during closure of the "wound". The last method used in this thesis for assessing cell growth properties is *flow cytometry* with which the size of the cells, related to stage of cell division, is quantified.

Viscosity and Mechanical evaluation

The *printability* of a bioink can be evaluated by measuring filament width after printing and comparing that with the nozzle diameter. The outflow of the material can be assessed by performing a cross-test. *Matrix stiffness* (resistance to deformation) of a scaffold/tissue/material can be measured at different hierarchical levels i.e., the macro, micro and nano level. The nano level mainly affects cells on a molecular scale, the micrometer level affects at a cellular level and the macro level mainly affects at a tissue level.

AIM

The overall aim of this thesis was to develop new 3D cell culture systems mimicking the microenvironment of human tumors. For this purpose, we used patient derived scaffolds and 3D-printed scaffolds based on alginate- or nanocellulose.

The specific aims were:

Paper I: To determine the influence of the microenvironment on standardized breast cancer cell lines by analyzing their response and characteristics when grown in cell-free patient-derived scaffolds. To develop an *in vivo* like culturing system for tumor characterization and to evaluate how the drug response is influenced by the microenvironment.

Paper II: To develop a tuneable 3D scaffold mimicking the cellular response to that of patient derived scaffolds by 3D printing and analyzing using gene expression profiling and functional cellular assays.

Paper III: To evaluate the nanocellulose TEMPO-CNF as a 3D printable ink to produce scaffolds for tumor cell culturing.

Paper IV: To determine how hypoxia affects tumor cells in 3D and 2D environments.

RESULTS AND DISCUSSION

This thesis is based on data from four papers. In the first, human tumor tissue was used to develop patient derived scaffolds as a 3D cell culture system. In the following papers, different biomaterials were used to generate 3D printed structures which were analyzed in a combination of functional assays and gene/protein expression analysis to evaluate the microenvironment's effect on cancer cells.

PAPER I - PATIENT-DERIVED SCAFFOLDS UNCOVER BREAST CANCER PROMOTING PROPERTIES OF THE MICROENVIRONMENT

All tumors have their own complex and dynamic microenvironment which will affect the tumor's characteristics and generate individual complexity [45]. In paper I, we aimed to determine the influence of the microenvironment on standardized reporter breast cancer cell lines cultured in cell-free patient-derived scaffolds (PDS). PDSs were generated by washing tumors from patients with mild detergents to remove all cells (de-cellularization), creating a cell-free ECM scaffold. The scaffolds were then repopulated with breast cancer cell lines and due to the maintained microenvironmental properties, the cells developed different characteristics dependent on the original tumor properties (Figure 7).

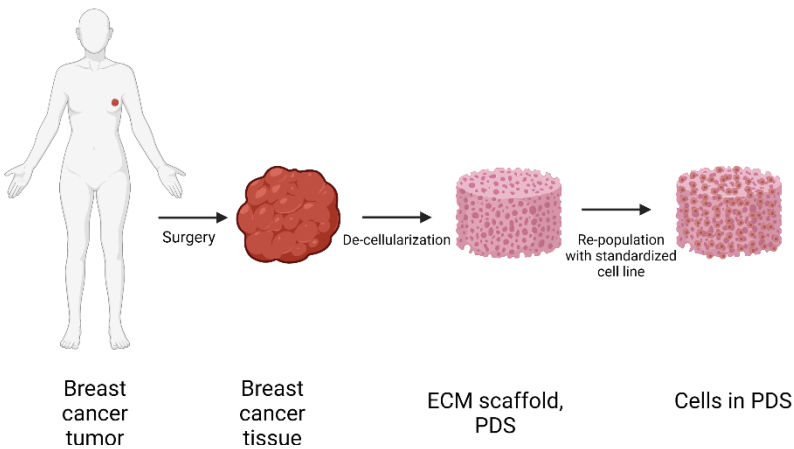

Figure 7 A breast cancer tumor has surgically been removed from patient, washed to remove all cells and cellular materials and repopulated with standardized cell lines. Created with BioRender.com

Characterizations of the PDSs were performed with scanning electron microscopy (SEM) for structural analysis, and with mass spectrometry for protein composition. We could show that the scaffolds preserved the original structure through the decellularization process and that the tumors had structural heterogenous variations within the same tumor as well as between tumors. The protein characterization revealed groupings of proteins that could be linked to tumor grade assessed from the original tumor.

Repopulating the PDSs with two different cell lines, triple negative MDA-MB-231 and ER-positive MCF7 showed that the invasive MDA-MB-231 could infiltrate the PDS while the less invasive MCF7 created nests of tumors cells close to the surface and in structural cavities. Both cell lines showed phenotypic adaption and analysis of the gene expression profile revealed increased expression of markers associated with pluripotency and EMT whereas proliferation and differentiation markers were downregulated compared with 2D cultured cells. Functional studies with increased mammosphere formation and increased tumor take in a xenograft model confirmed stimulation of CSC/EMT in the PDS grown cells. These results are also consistent with previous data on 3D culture models [67, 136, 137]. Time spanning analysis over 21 days using gene expression profiling showed that cells cultured for shorter time periods, 7 days and below, had more similarities with 2D cultured cells while cells grown for 14 and 21 days showed increased expression of EMT and pluripotency markers and decreases in proliferation and differentiation markers which is similar to *in vivo* environments. This demonstrated that cellular adaptation in the scaffolds is a temporal process. Gene expression analysis of cells grown in 46 PDSs further showed that a high expression of *SNAI2* and *VIM* correlated with clinical reoccurrences and relapse indicating that the tumor microenvironment itself carries valuable information that can be linked to clinical information.

RNA sequencing was used for a deeper analysis comparing cells growing in 2D, PDS or xenografts. Principal component analysis (PCA) did not only distinguish the two different cell lines used, but also separated the 2D cultures from the PDS and xenograft suggesting more similarities between the 3D models compared with 2D.

In subsequent studies, to evaluate how cells grown in the scaffolds were affected by drug exposure, endocrine and chemotherapy treatment was added to cells grown in PDSs and 2D. Cells grown in PDSs showed more drug resistance compared with 2D in that higher drug doses had to be used for receiving effect. For endocrine therapies, it was shown that drug resistance was higher in the PDS and genetic response was in accordance with the function of

the drug [138]. For chemotherapies, it was also shown that higher doses of drugs were needed in the 3D cultures and modified the cells' response to the different drug agents [139].

PAPER II - OPTIMIZED ALGINATE-BASED 3D PRINTED SCAFFOLDS AS A MODEL OF PATIENT DERIVED BREAST CANCER MICROENVIRONMENTS IN DRUG DISCOVERY

3D printing has opened up new ways to combine knowledge in material science with biology. In paper II, we aimed to develop a simple tunable 3D printed scaffold mimicking the microenvironment of the PDSs. Biocompatible alginate was printed in 3D scaffold structures with an EnvisionTec extrusion bioplotter. To increase the stiffness, and mimic microcalcification often observed in breast cancer tissue [53, 125], the natural mineral hydroxyapatite was added to the alginate. For functionalization, and to make the scaffolds more similar to breast cancer tissue, the protein periostin (expressed in breast cancer tissue) was also added to the printing material.

Alginates vary in stiffness and shear response in relation to concentration and composition. Depending on the material characteristics, cells will react differently in response to mechanical properties and dimensional features [140, 141]. This was shown by decreased proliferation in response to increased alginate concentration and increment of pluripotency and EMT markers with increasing concentration.

Evaluation of the 3D printed alginate scaffolds, with addition of hydroxyapatite and periostin, with 2D and PDS as references, was performed with qPCR, migratory functional assay, western blot, IHC, holoclone formation and flow cytometry. Gene expression analysis of cells grown in 3D printed scaffolds and PDSs showed similarities separated from the 2D cultured cells. The addition of periostin and hydroxyapatite also affected the expression of EMT and pluripotency markers. It could be argued that Matrigel® would be a more suitable material for generating 3D printed structures; however, Matrigel® has a batch-to-batch difference regarding composition and it is not declared by the manufacturers. This is not preferable when developing tunable bioinks where we want as much control as possible to know which cellular response is caused by which material property.

Cells cultured in 3D printed scaffolds, PDSs and 2D were further treated with the chemotherapeutic drugs 5-FU and DOX, and the effects were evaluated with gene expression analysis. In contrast to the cells in 2D-cultures, cells cultured in 3D printed scaffolds and PDS's showed higher drug resistance, downregulated proliferation and EMT markers, and similar regulations of several genes in response to the drugs.

In summary, both the functional and transcriptional responses of the standardized cells distinguished 3DPS and PDS from 2D cultured cells. The cell responses in a 3D environment were also tunable by the additives HA and periostin and the reporter cells cultured in 3DPS and PDS were shown to respond similarly to cytotoxic drug treatments.

PAPER III - 3D PRINTED NANOCELLULOSE SCAFFOLDS AS A CANCER CELL CULTURE MODEL SYSTEM

Cellulose nanofibrils (CNF) is a promising material which has been studied as gel formulation for wound healing, drug carriers and scaffolds for tissue engineering [142-146]. In paper III, we aimed to study the cellular response of breast cancer cells cultured in 3D printed scaffolds of cellulose nanofibrils pre-treated with 2,2,6,6-tetramethylpyperidine-1-oxyl (TEMPO-CNF). 3D printed scaffolds were produced from 3D printed freeze dried TEMPO-CNF and breast cancer cells were cultured in the same way as in paper I and II. Similar to the microenvironment of PDSs and 3D printed alginate-based gels, the cells in TEMPO-CNF 3D printed scaffolds adapted heterogenous morphologies and grew in multiple layers within the scaffolds. Cells adapted and grew well on the 3D printed TEMPO-CNF scaffolds, also filling out holes and cavities in the material. The gene expression profile of the cancer cells followed the same patterns as in PDSs and alginate-based 3D printed scaffolds with upregulated EMT and stem cell markers which was further functionally verified with holoclone formation assay.

In summary, we developed a 3D printable cellulose nanofibril material that allowed for breast cancer cell lines to attach and grow on. The microenvironment in the material caused the cells to increase the expression of CSC and EMT markers contributed to a heterogenous cell growth, similar to observations in paper I and II, on PDSs and alginate-based 3D printed scaffolds.

PAPER IV - THE EFFECT OF HYPOXIC AND NORMOXIC CULTURING CONDITIONS IN DIFFERENT BREAST CANCER 3D MODEL SYSTEMS

Hypoxia in cancer, often observed in the core of fast-growing tumors [54], is an important factor of tumor development as it will influence cell behavior, growth rate of cells, metastasis, treatment sensitivity and neovascularization [23]. In paper IV, we aimed to study the effect of hypoxic versus normoxic conditions on cancer cells in 3D environments. Cells cultured in 2D, PDS, molded Matrigel® and 3D printed alginate-based scaffolds were exposed to hypoxic and normoxic conditions followed by gene- and protein expression profiling of markers representing cell death, metabolism, differentiation, cell division, invasion, proliferation, angiogenesis, CSC, EMT, pluripotency, hypoxia, and epigenetics [23]. The study showed that there was a resemblance between cells cultured in 3D printed scaffolds and PDS which were separated from 2D and Matrigel® for expression of gene- and protein markers in both hypoxic and normoxic conditions.

CONCLUSION

We have developed 3D cell culture model systems based on patient derived scaffolds and 3D printed scaffolds, respectively. We have, with gene- and protein expression analysis, as well as functional studies, analyzed the 3D cell culture systems and conventional 2D cell cultures and can conclude that our novel 3D systems seems to be more *in vivo*-like compared with 2D.

Paper I – Patient derived scaffolds resemble *in vivo*-like conditions as a cell culture system for breast cancer cells. The microenvironment is unique for each tumor and expression profiling could be correlated to clinical recurrence data.

Paper II – Alginate-based 3D printed hydrogel scaffolds (3DPS) showed similar growth conditions to PDSs. Breast cancer cells cultures in 3DPS had comparable growth- and gene expression patterns with PDS, which were more *in vivo*-like compared with 2D cultured cells.

Paper III – TEMPO-CNF was shown to represent a promising material for 3D printed cell culture systems for cancer cell applications with favorable printing and cell growth properties.

Paper IV – Breast cancer cell lines cultured on alginate-based 3D printed scaffolds closely resembled cells cultured on patient derived scaffold, differing from 2D cultured cells, in both hypoxic and normoxic conditions.

FUTURE PERSPECTIVES

The tumor microenvironment has proven to have a large impact on cancer cell behavior by affecting both treatment response and progression. The main objective of this thesis was initially to try to understand the influence of the breast cancer tumor microenvironments on the cancer cells, and therefrom develop 3D printed, tunable structures that could mimic aspects of the cellular responses in the *in vivo* milieu, and to use the scaffolds as a tool for drug screening.

We have used breast cancer tumors to produce PDSs as a model for investigating how the microenvironment affects tumor cells. A complementary study has been performed on colorectal cancer PDSs in our lab confirming our clinically relevant findings that standardized colorectal cancer cell lines, repopulating the PDS, gained characteristics reflecting clinical data from the patient, like cancer mortality [147]. These findings motivate further generalization of the PDS model for additional cancer types and open up possibilities for personalized diagnostics and prognostics. An advantage of this model is that it is based on tumor material that is surgically removed regardless of whether it will be used for this model or not.

As patient material is limited, 3D printed replications of the PDS in which cancer cells can be cultured, have potential to be used as a high throughput screening model of novel cancer drug candidates [127]. In this thesis, we have evaluated alginate and nanocellulose as base materials for 3D printing of cell culture scaffolds. We will now continue to functionalize these materials, building a material platform to which proteins and other molecules believed to control cell responses can easily be linked. The material can also be fine-tuned by coupling of hydrophobic molecules and peptides controlling viscosity and shear-thinning properties, allowing filaments to be printed with higher resolution. In addition to better control of the porosity of the scaffold, this might also influence the cell response (curvotaxis) to the substrate altering their morphology and gene- and protein expression profiles [148]. It could also be of value to further analyze the PDS protein content with focused mass spectrometric analyses to find additional proteins that can be linked to different aspects of tumor progression and grade, which can then subsequently be linked to the tunable alginate gel.

For evaluation of immune response, PDSs and 3DPSs will be used for evaluation of co-culture of cancer cells and T-cells. The immune response might be more similar to the *in vivo* situation, and for the PDS there might be

immune response information in the scaffold that is stored in the ECM. As for 3D printing of scaffolds, it might be of importance to evaluate the immune response to biomaterials with or without additives, especially for tissue engineering where the biomaterial might trigger an immune response in the body [149]. Evaluation could be performed on genes related to immune response or with FACS (fluorescence-activated cell sorting).

Tumors in regular tissue are vascularized and thereby interacts with other tissues and the supply of oxygen and nutrients through blood perfusion [74]. To make our systems even more *in vivo*-like, PDSs and 3DPs could be cultured in a bioreactor with continuous flow of media. Constant supply of fresh media, in contrast to change of media every 2 to 3 days, might influence the tumor cells to become even more similar to the *in vivo* environment.

The 3D models developed during this thesis have the potential to be important tools for future personalized medicine and drug screening. One important cue for industrialization will be to modify the 3DPs into a high throughput system. With this optimization, these systems have a large potential for the industry to save a lot of time and money, which might result in more efficient and cheaper cancer drugs to the benefit of both our society and the individual patient.

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→

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