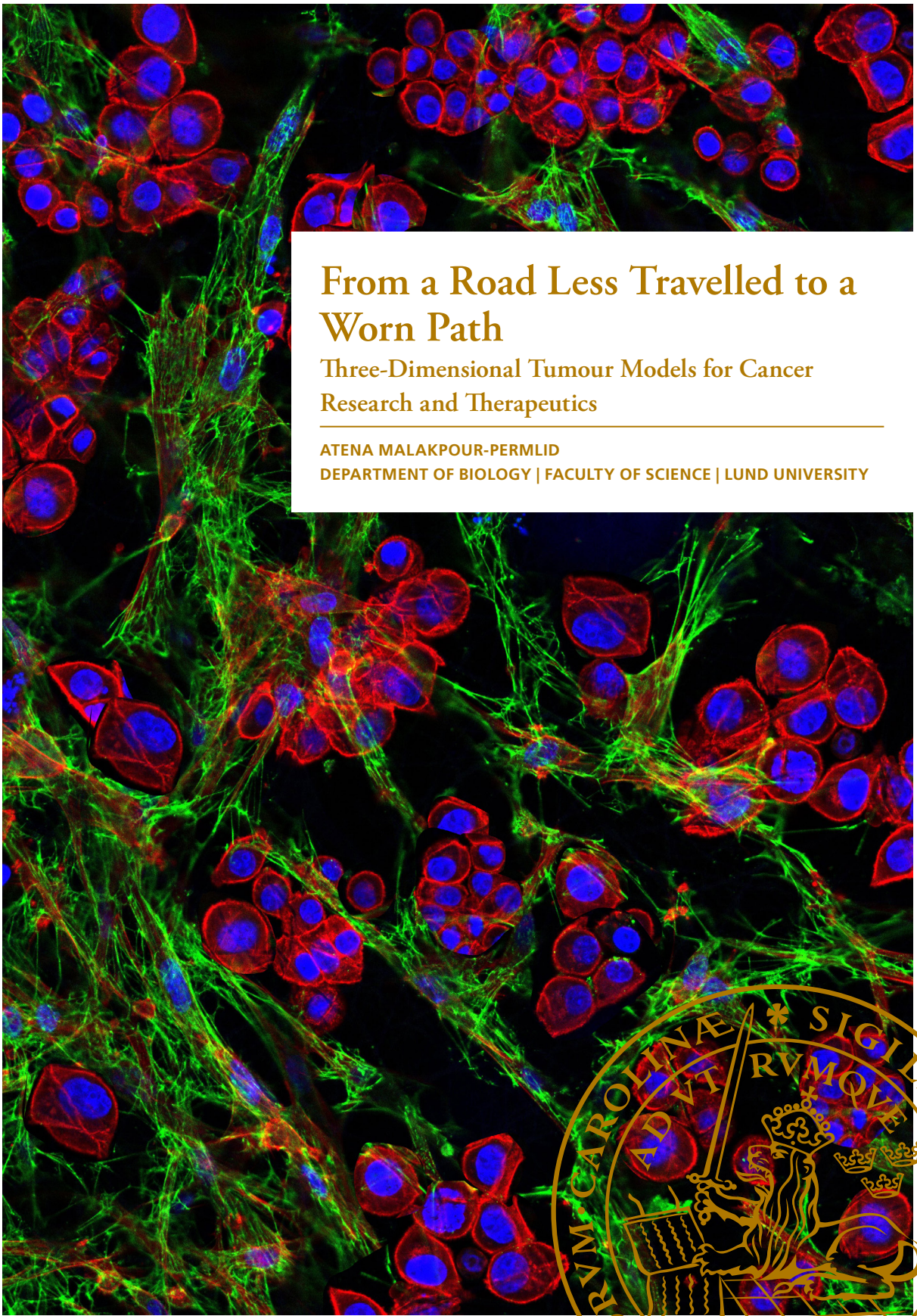
From a Road Less Travelled to a Worn Path: Three-Dimensional Tumour Models for Cancer Research and Therapeutics

Malakpour Permlid, Atena

2020



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Three-Dimensional Tumour Models for Cancer Research and Therapeutics

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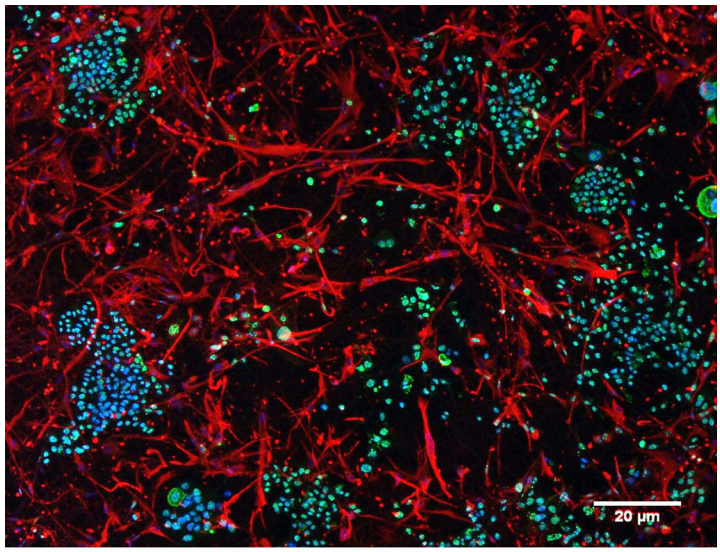
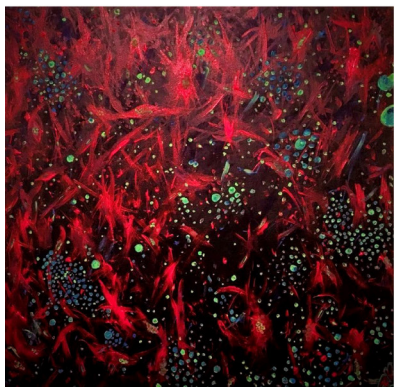
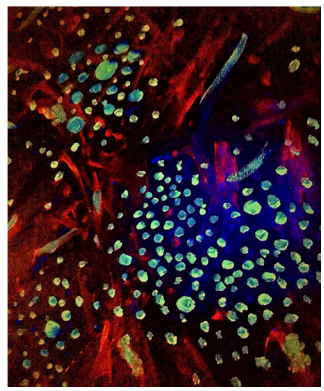
DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY







Flourescence microscopy image of JIMT-1 breast cancer cells and human dermal fibroblasts (HDFs) in 3D co-culture *in vitro* model for 1 week (Top). The cells are stained with cell specific markers to distinguish the HDFs from the JIMT-1 cells. Fibroblasts are stained with vimentin (red), JIMT-1 cells are stained with CD44 (green), and cell nuclei are stained with bisbenzimide (blue). When co-cultured in 3D scaffolds, the JIMT-1 cancer cells grow in irregular tight aggregations surrounded and in direct contact with alongated HDFs in a manner which resembles *in vivo* breast tumour histology. The image was voted as the image of the month on May 2017 by the European Association for Cancer Research (EACR) (photo by Atena Malakpour-permlid).

Two artworks painted with black light acrylic paints on canvas (Bottom). The painting on the left has 50×50×2 cm dimensions. The artist Rasha Kakati from Beirut was inspired by the top photomicrograph of co-cultured cells. The painting was presented at an art exhibition Canvas AUBFM at the American University of Beirut (AUB). Canvas AUBFM is an initiative by medical students at AUB that celebrates art and medicine, shares artwork related to medicine, and raises funds as donations for cancer treatment admissions. The aim is to increase awareness of cancer disease, shed light on the importance of the patients and survivors experience, highlight the role of art in building empathy and compassion, and the significance of scientific research by intertwining the nature of art and medicine.

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LUND
UNIVERSITY

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From a Road Less Travelled to a Worn Path: Three-Dimensional Tumour Models for Cancer Research and Therapeutics

Abstract

Cancer is the second leading cause of death among both men and women worldwide. During the recent years, three-dimensional (3D) tumour models have gained increasing interest as a pre-clinical platform for screening of compounds for potential use in cancer therapy. It is becoming recognized that two-dimensional (2D) cell culturing, in which cells are grown on physiologically irrelevant flat surfaces, are not reliable tools for investigating chemo sensitivity of anti-cancer drug candidates. Therefore, the emerging miniaturized 3D tumour models in vitro are better representatives of the human tumour growing in the in vivo microenvironment. We have established a complex 3D human tumour outside the body using randomly oriented highly porous electrospun polycaprolactone (PCL) fibres, which mimic the collagen structure of the extracellular matrix (ECM). The data show that mono-cultures of cancer cells grow as dense multi-cellular spheroids in the biocompatible 3D scaffolds, while normal cells show spread-out and elongated morphology. When co-cultured, JIMT-1 breast cancer cells and human dermal fibroblasts (HDFs) show a growth pattern similar to what is found in a tumour with the cancer cells growing in tight clusters surrounded by the fibroblasts. When grown in mono-culture or co-culture, the cells grow in the entire depth of the 3D PCL network. In addition, we characterized the proteins deposited by the cells in the 3D scaffolds incubated in the absence or presence of transforming growth factor-β1 (TGF-β1), a tumour promoting cytokine. The data show that the fibrous ECM proteins fibronectin, collagen I, and laminin are deposited throughout the depth of 3D structure. TGF-β1 treatment did not have a significant effect on protein deposition but significantly modulated the activity of matrix metalloproteinases and the level of interleukine-6 cytokines in the medium of our 3D culture. In TGF-β1-treated co-cultures, the cancer cells changed the growth pattern from tight clusters to be spread out along elongated HDFs. The 3D human tumour in vitro was utilized for evaluation of efficacy of two anti-cancer compounds; a well-known anti-cancer drug paclitaxel and an experimental salinomycin analogue (SAEC). The experiments were performed in hypoxia and normoxia. Paclitaxel treatment was more toxic to the cancer cells while the SAEC was more toxic to the HDFs in normoxia and hypoxia. Furthermore, we fabricated and validated a 96-well plates with 3D PCL fibre network as a high-throughput screening (HTS)-based assay. We compared the toxicity of paclitaxel and SAEC in 2D and 3D under normoxic and hypoxic condition. The data show that the 96-well 3D system is a cost-efficient tool that can be used for assessing of new potential chemotherapeutic drugs in an HTS manner. Thus, we have “proof of principle” that our tailor-designed human tumour outside the body has structure similar to a tumour in the body and that it can be used for investigation of chemical toxicity.

Key words 3D tumour models, scaffold-based cultures, cancer cells, fibroblasts, electrospun scaffolds, extracellular matrix, tumour microenvironment, high-throughput screening, Animal-free, Donor herd horse serum.

Classification system and/or index terms (if any)

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2020

Front cover: Florescence confocal image of JIMT-1 breast cancer cells and human dermal fibroblast (HDF) co-culture in 3D PCL electrospun scaffold after 3 days of incubation. The 3D cultures were fixed in 3.7 % formaldehyde and stained to visualize F-actin (red), fibronectin (green) and cell nuclei (blue) (photo by Atena Malakpour-Permlid).

Back cover: Florescence image of JIMT-1 and HDF co-culture in 3D PCL electrospun scaffold (photo by Atena Malakpour-Permlid). Also, artworks painted on canvas using black light acrylic paint. The artist was inspired by the microscopic image of co-cultured cells captured by the fluorescence microscope (paintings by Rasha Kakati from Beirut).

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To my dear Mathias

and to all the strong women out there!
May we know them.
May we be them.
May we raise them.

Don’t be satisfied with stories, how things have gone with others. ***Unfold your own myth***...

Persian poet, theologian and Sufi mystic

Rumi (1207–1273)

Table of Contents

List of Scientific Papers

This doctoral thesis is based on the work presented in the following published scientific paper and manuscripts referred to with the Roman numerals (I-IV) throughout the thesis.

Paper I. **Atena Malakpour-Permlid**, Plaurent Roci, Elina Fredlund, Felicia Fält, Emil Ölund, Fredrik Johansson, and Stina Oredsson (2019). Unique Animal Friendly 3D Culturing of Human Cancer and Normal Cells. *Toxicology In Vitro 60*, 51-60. *

Paper II. **Atena Malakpour-Permlid**, Cecilia Hegardt, Fredrik Johansson, and Stina Oredsson (2020). Three-Dimensional Co-Culture Model of Cancer Cells and Fibroblasts for Evaluation of Anti-Cancer Compounds. Submitted.

Paper III. **Atena Malakpour-Permlid,** Irina Buzzi, Cecilia Hegardt, Fredrik Johansson, and Stina Oredsson (2020). Identification of Extracellular Matrix Proteins Secreted by Human Dermal Fibroblasts Cultured in 3D Electrospun Scaffolds. Submitted.

Paper IV. **Atena Malakpour-Permlid** and Stina Oredsson (2020). A Novel 3D Polycaprolactone-Based High Throughput System for Evaluation of Toxicity in Normoxia and Hypoxia. Submitted.

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The Contributions to the Papers

Paper I.

Together with my co-authors, I designed and planned the study. I have done the plasma modification of the PCL scaffolds and performed the hydrophilicity assay, routine-cultured cells, and seeded them in the 3D scaffolds. Also, I have performed AlamarBlue™ assays, scanning electron/confocal microscopy, and cryosectioning. I have drawn the figures, wrote the first draft of the paper, and finalized it after input from the co-authors. All authors reviewed and approved the submitted manuscript.

Paper II.

I initiated the design of the biological experiments with co-authors. I routine-cultured the cell lines, seeded cells in the 3D scaffolds carried out the biological experiments, and data analysis. I have performed mono- and co-culture experiments and treatments, AlamarBlue™ assays, staining, confocal microscopy, and cryosectioning. I have drawn the figures, wrote the first draft of the paper and finalized it. All authors reviewed and approved the submitted manuscript.

Paper III.

I designed and planned the study with the co-authors. I have performed part of the biological experiments of culturing cells in the 3D scaffolds. I have done part of the staining and all the confocal microscopy imaging. I also have drawn the figures and graphs, analysed the data, and wrote the first draft of the paper and finalized it. All authors reviewed and approved the submitted manuscript.

Paper IV.

I designed and planned the study with the co-author. I was part of performing the biological experiments and drug screening using the MTT assay. I have done the confocal imaging and electron microscopy. I also have drawn the dose-response graphs and analysed the data. Stina Oredsson wrote the first draft of the paper and the authors finalized it together. Both authors reviewed and approved the submitted manuscript.

List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
3Rs	Three Rs (replacement, reduction, and refinement)
α-SMA	alpha-Smooth muscle actin
CAF	Cancer-associated fibroblast
DC	Dendritic cells
ECM	Extracellular matrix
FAP	Fibroblast activation protein
FBS	Fetal bovine serum
DAPI	4',6-Dimidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DHHS	Donor herd horse serum
ER	Estrogen receptor
FDA	Food and Drug Administration
HDF	Human dermal fibroblast
HER2	Human epidermal growth factor receptor 2
HIF-1	Hypoxia inducible factor 1
HTS	High throughput screening
IL-6	Interleukin 6
ICC	Immunocytochemistry
MHC-I	Major histocompatibility class I
NK	Natural killer
PCL	Polycaprolactone
PLA	Polylactic acid
PR	Progesterone receptor
SEM	Scanning electron microscope
TGF-β1	Transforming growth factor-beta1
TME	Tumour microenvironment
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Preface

Techniques to incorporate cells into an artificial three-dimensional (3D) matrix have been introduced to the scientific world not long ago. In this doctoral thesis, we had the chance to explore scaffold-based 3D models for developing a complex *in vitro* tumour model outside the human body. We also wanted to know if these models can be used as a therapeutic tool for selection of candidate anti-cancer drugs and for evaluation of drug efficacy. Despite the tremendous potential of 3D models to better screen chemicals, it is still a long way before we know whether these models can be used first hand as an alternative *in vitro* pre-clinical tumour model or to replace animal models in future. The abuse and suffering of animals in research laboratories does not need to continue and we should all break out of our comfort zone and apply 3D *in vitro* strategies to be able to stop the cruelty and neglect towards animals. Hereby, I would like to acknowledge the contribution and sacrifices of billions of animals to excellence in research and studies of human disease prevention, treatment, and cure.

The work presented here was carried out at the Department of Biology, Faculty of Science at Lund University from 2016 to 2020. The thesis book you are holding is divided into two main parts, where the first part provides the background scientific literature related to the research field and the second part presents the research contributions to this field in the form of peer-reviewed published scientific papers and submitted manuscripts. I hope you enjoy reading this thesis as much as I enjoyed working on this research project for several years. I also wish the work in this doctoral research will help this exciting new research area to move a small step forward in animal free cancer research and contribute to advances in cancer treatment and patient's well-being.

Atena Malakpour-Permlid
Lund, Spring 2020

Popular Science Summary

One of the great challenges of medical science is the development of efficient anti-cancer drugs. The long journey of the drug development process consists of three main stages; discovery, pre-clinical screening, and clinical trials. All stages have to be completed before a drug can reach the patients. Presently, there is a high attrition rate in the process of developing new drugs for cancer treatment and the cost of the drugs is enormous. This is partly due to limitations of current pre-clinical models which do not efficiently provide relevant data. It is imperative that new test models for drug discovery are developed which are easy to use and with more realistic features.

Nowadays, a large part of cancer research experiments is conducted using cancer cell lines that grow in plastic vessels. As they grow in a thin layer where no cell grows on top of another cell, these cultures are called two dimensional (2D). Usually, only one type of cell is present in these 2D cultures. 2D cell cultures have been used extensively and greatly contributed to our understanding of cancer cell biology, but they have many drawbacks. Although convenient to use and well accepted, the 2D cultures do not resemble how cells grow in the human body. One of the most obvious differences between a human tumour and a 2D cancer cell culture is the lack of complex three-dimensional (3D) structures.

A tumour does not only consist of cancer cells but also of different types of normal cells that influence various aspects of tumour biology. They all grow connected and intertwined in a 3D microenvironment. In addition, all the cell types communicate with and regulate each other. Therefore, standard 2D cultures can hardly represent the essential physiology of the tumour in the human body. It is not possible to fully mimic the complexity of a tumour formed in the human body in a cell culture vessel. However, some aspects of the tumour tissue are possible to recreate in a cell culture, e.g. the addition of extracellular matrix (ECM), which is the substance that surrounds and supports the cancer cells. In order to use a test model which can maintain the actual tumour environment, animals, mainly mice, are transplanted with human tumours. These non-humane animal experiments cause animal suffering and abuse. The animal experiments are costly and ethically questionable. They are also not truly representative of human physiology, causing the results to be questionable to some degree for understanding the impact of drugs on humans. Besides 2D cell culturing, animal experiments contribute to the high failure rate when developing new cancer drugs. Therefore, it is imperative to find simple and

cost-efficient cell culture models which will provide relevant data. This will reduce the number of animal experiments that are necessary before the potential drugs go through clinical testing.

As mentioned above, tumours not only consist of cancer cells but also of different types of normal cells such as immune cells, supporting cells called fibroblasts, and cells of blood vessels. Supporting structures such as ECM proteins, and cell signalling molecules such as cytokines and hormones are also part of a tumour. This complexity and heterogeneity have not been considered in traditional 2D models of cell culturing. 3D culture models with increased heterogeneity similar to a human tumour will therefore contribute to obtaining data on clinically relevant concentrations of potential drugs and to minimize side effects of drug treatment. To address this, we have developed a miniaturized human 3D tumour that grows outside the body. We predict that it can be used to obtain cost-effective and more relevant data during the early development of cancer drug treatments. The basis of the 3D system in this thesis is a network of electrospun polycaprolactone (PCL) fibres, which mimics the network of supporting collagen fibres that are part of the extracellular matrix (ECM) (**Paper I**). We showed that cancer and normal cells thrive and grow into the biocompatible PCL fibre scaffold. The cancer cells grow in tight clusters while the normal cells have spread out morphology where they attached to each other and to the fibres. When cancer cells and normal fibroblast were seeded together the cells kept their shape and self-organized into realistic tumours with the cancer cells growing in tight clusters surrounded by fibroblasts. We have shown "proof of principle" that our tailor-designed tumour outside the body indeed can be used to investigate anti-cancer drug toxicity (**Paper II**). However, continued validation work, where we compare our results with established *in vivo* data, will further strengthen the use of this model.

Cancer cells are actively and constantly interacting with each other, with normal cells in their vicinity, and with the ECM through different signalling molecules that mediate the process of tumour formation. In a 3D tumour model, it is important that these interactions can be mimicked. Therefore, we studied the composition of the ECM formed by the cells and signalling molecules secreted by the cells in our 3D tumour model (**Paper III**). Overall, we observed that the different building stones of the ECM found in a tumour were deposited into the PCL fibre scaffold and signalling molecules found in a tumour were detected in the medium of the cultures.

Many standard drug testing methods are based on using flat 96-well plates, which is called a high-throughput screening (HTS) method because of the large number of tests that can be made simultaneously. However, this is still 2D cell culturing and there is a need for development of 3D HTS methods. In the final part of my project, we created a 96-well plate with 3D PCL fibres incorporated in the well. (**Paper IV**). The 3D 96-well plates can be produced in any standard laboratory setting and requires only minimum levels of expertise. The 3D 96-well plates were used to assess toxicity of two anti-cancer drugs on cancer and normal cells and we compared

the toxicity in 2D and 3D. Our data show that our 3D PCL-based 96-well assay is a cost-efficient tool for rapid toxicity testing of compounds.

In conclusion, this research work aimed at reducing the number of animal experiments in cancer research by creating a model of a complex human 3D tumours *in vitro*, which can be used to study cancer development and progression as well as for efficient testing of potential anti-cancer drugs.

Populärvetenskaplig Sammanfattning

Den medicinska vetenskapen står inför stora utmaningar. En är att utveckla effektiva cancerbehandlingar. Att utveckla nya cellgifter är en långdragen process som delas upp i tre steg: 1. forskning och utveckling; 2. pre-kliniska studier; 3. kliniska studier. Innan ett nytt läkemedel kan nå ut till patienterna måste alla stegen gås igenom med godkända resultat. För närvarande faller en stor del av alla nya läkemedelskandidater bort i processen och kostnaden för att ta fram ett nytt läkemedel är mycket stor. En del av problemet är att de tidiga försök man gör för att testa nya läkemedel inte ger tillräckligt bra data. Det är mycket viktigt att ta fram nya försöksmodeller som är lätta att använda och som ger bättre, mer relevanta resultat.

De försöksmodeller som oftast används är cancerceller som växer i plastbehållare. Eftersom cellerna växer i tunna lager där ingen cell växer ovanpå någon annan cell kallas de här cellodlingarna tvådimensionella (2D). I vanliga fall består cellodlingen bara av en enda typ av celler. Cellodlingar har använts i stor omfattning för att nå grundläggande förståelse för cancercellers biologi, men de har stora nackdelar. Även om de är används ofta inom cancerforskning och är praktiska att använda, så är cellernas miljö inte alls lik miljön inuti människokroppen. En av de tydligaste skillnaderna mellan en tumör i kroppen och 2D-cellodlingar är bristen på komplexa tre-dimensionella (3D) strukturer i 2D. En cancertumör består av en blandning av cancerceller och normala celler. De normala cellerna påverkar flera olika tumöregenskaper. Alla celler i en tumör växer tätt hoplänkade och kommunicerar med och reglerar varandra. Detta gör att 2D-cellodlingar med enbart cancerceller inte speglar flera nödvändiga aspekter av en tumörers fysiologi i en tumör i en plastbehållare, men det går att komma flera steg närmare. Tex kan man tillsätta extracellulär matrix (ECM) i plastbehållarna. ECM är en 3D-struktur som omger och stöttar cellerna i en tumör.

Ett annat sätt att närma sig miljön i människokroppen är att använda djurförsök. Då planterar man in tumörer från människa i djur, övervägande möss. Djuren lider, och försöken är dyra och etiskt olämpliga. Resultat från djurförsök är dessutom inte alltid möjliga att överföra till människor, något som bidrar till den stora andel läkemedelskandidater som inte tar sig genom processen. Det är viktigt att skapa enkla och billiga cellodlingsmodeller som återspeglar människokroppens funktioner och som ger relevanta försöksdata. Detta kommer att reducera antalet djurförsök som fortfarande är nödvändiga inför kliniska tester.

Som nämndes tidigare består tumörer inte bara av cancerceller, utan även av många typer av normala friska celler såsom immunceller, stödjande celler som kallas fibroblaster och celler från blodkärl. Dessutom ingår stödjande strukturer såsom ECM-proteiner och olika signaleringsmolekyler såsom cytokiner och hormoner. Den här komplexa strukturen återspeglas inte i 2D-cellodlingarna. Att utveckla 3D-cellodlingar där stora delar av den komplexa tumörmiljön återskapas är viktigt för få relevanta resultat från försök med läkemedelskandidater, något som skulle göra det snabbare och enklare att få fram hur läkemedel fungerar.

Vi har utvecklat en miniatyrtumör utanför kroppen som liknar en mänsklig cancertumör. Vi förutspår att den kommer att bidra med relevanta resultat och att den kommer att sänka kostnaderna i de första stegen av läkemedelsutvecklingen. Grunden till miniatyrtumören är ett nätverk av tunna plastfibrer av polycaprolacton (PCL), en slags plast. Fibrerna härmar det nätverk av stödjande strukturer som finns i en tumör (**Artikel I**). Vi visade att både cancerceller och friska celler trivdes i och växte in i fibernätverket. Cellerna formerade sig spontant till en typisk tumör med cancerceller i täta grupper omgivna av de stödjande fibroblasterna. Vi visade också att vår skräddarsydda tumör i 3D kan användas för att undersöka läkemedelseffekter (**Artikel II**). Det behövs dock fortsatt validering gentemot kända resultat från människor för att ytterligare visa att miniatyrtumören är en bra försöksmodell.

Cancerceller interagerar ständigt och aktivt med varandra, med normala celler och med ECM med hjälp av olika signalmolekyler som styr bildandet av tumören. I en 3D-tumör utanför kroppen är det viktigt att dessa olika aspekter finns med. Därför har vi undersökt vilka ECM-komponenter som bildas när vi sår ut cancerceller och fibroblaster i PCL-nätverket (**Artikel III**). Vi såg att olika ECM-komponenterna avlagrades på PCL-nätverket och cellerna frisätter olika signalsubstanser. Vår 3D-tumör utanför kroppen uppvisar många likheter med en tumör i kroppen.

Många standardtest för läkemedel är baserade på användning av en flat cellodlingsplatta med 96 hål där celler odlas. Detta kallas high-throughput screening (HTS) eftersom man samtidigt kan göra många test. Detta är emellertid 2D-cellodling och det behövs möjligheter att göra 3D-tester som HTS. I sista delen av mitt projekt skapade vi en 96-hålsplatta med PCL-nätverk i hålen (**Artikel IV**). Det är lätt att tillverka denna 3D 96-hålsplatta och de är inte speciellt dyra. Vi använde 3D 96-hålsplattor för att utvärdera giftigheten av två anti-cancersubstanser på cancerceller och fibroblaster och vi jämförde med resultat erhållna från odling av cellerna i flata 96-hålsplattor. Våra resultat visar att 3D 96-hålsplattan är ett kostnadseffektivt och snabbt sätt att få relevanta resultat vid testning av läkemedel.

Sammanfattningsvis siktar detta projekt på att utveckla en 3D-tumör utanför kroppen som kan bidraga till att reducera antalet djurförsök inom cancerforskningen och som kan användas både för att studera tumörutveckling och för att testa nya cellgiftskandidater.

چکیده علمی

یکی از چالش های بزرگ علم پزشکی تولید داروهای ضد سرطان موثر است که برای طراحی و تولید انها مسیری بس طولانی و پر هزینه طی میشود تا به بازار عرضه و در نهایت به دست بیماران برسد. این پروسه شامل سه مرحله اصلی; کشف اولیه دارو، غربالگری پیش بالینی و آزمایشات بالینی بر روی بیماران است. در حال حاضر، روند تولید داروهای ضد سرطان جدید اکثرا با درصد بالایی از شکست مواجه شده (حدود ۹۰ درصد) و به مرحله نهایی نمیرسند. بخش عمده ای از این عدم موفقیت به عدم کارایی و محدودیت مدلهای پیش بالینی موجود در مرحله غربالگری اولیه دارو در آزمایشات اولیه بر میگردد. از این رو، برای رفع این مشکل، یافتن مدل های آزمایشگاهی جایگزین با شرایط کنترل شده و واقع گرایانه تر مورد نیاز است. مدل های کاشت سلولی نوین میتوانند بر محدودیت های مدل های کاشت سلولی سنتی غلبه کرده و جایگزین مناسبی برای روش های سنتی گردند تا غربالگری پیش بالینی را برای کشف داروهای ضد سرطان جدید بهبود بخشند.

امروزه، آزمایشات تحقیقاتی سرطان و بررسی مکانیسم های عملکرد دارویی با استفاده از بانک های سلولی و کشت سلولهای سرطانی انسان در فلاسکهای پلاستیکی امکان پذیر است. از آنجا که سلولها به صورت یک لایه نازک رشد می کنند و هیچ سلولی بر روی دیگری رشد نمی کند، این محیط های کشت دو بعدی تک لایه نامیده می شوند. علاوه بر این، معمولاً فقط یک نوع سلول در محیط های کشت دو بعدی رشد داده میشوند. اگرچه مدلهای رایج دو بعدی همواره ابزاری ارزشمند برای درک کلی ما از زیست شناسی سرطان و کشف یافته های فراوانی به شمار می ایند، اما اشکالات ذاتی فراوانی دارند. رشد سلولی در مدل های کاشت سنتی شباهتی به رشد سلول ها در بدن انسان ندارند. ما واقفیم که سلولهای تومور در بدن انسان در محیط پیچیده سه بعدی رشد میکنند. یکی از بارز ترین تفاوتها بین تومور رشد کرده در بدن انسان و مدل های کشت دو بعدی، فقدان بستر پیچیده سه بعدی است تا بتواند شرایطی فراهم کند که سلولهای مختلف به صورت پیچیده و سه بعدی با هم در ارتباط باشند. تومورها نه تنها از سلولهای سرطانی بلکه از انواع سلولهای طبیعی تشکیل شده است که جنبه های مختلف زیست شناسی تومور انسان را تحت تأثیر قرار می دهند. بنابراین، محیط های کشت دو بعدی نمیتوانند نمایانگر فیزیولوژی واقعی و درست تومور در بدن انسان باشند و بازسازی کامل پیچیدگی تومور انسان در محیط های دو بعدی امکان پذیر نیست. با این حال، افزودن برخی از جنبه های ساده بافت سلولی برای رشد در شرایط آزمایشگاهی امکان پذیر است، که به طور عمده میتوان به محیط فیزیکی سه بعدی بافت سرطانی یعنی ماتریکس بین سلولی و سایر سلولهای میان بافتی اشاره کرد. محیط کشت سه بعدی به سلولها این اجازه را میدهد که بتوانند در همه جهات رشد کنند، درست مانند شرایطی که در محیط واقعی بدن انسان فراهم است. از محیطهای کشت سه بعدی برای مطالعات طولانی مدت و نشان دادن اثرات طولانی مدت داروها نیز میتوان استفاده کرد.

روشهای آزمایشی دیگری که در تحقیقات پیش بالینی سرطان مورد استفاده قرار می گیرد استفاده از حیوانات و عمدتا موش ها است. روش معمول انجام تحقیقات سرطانی در بدن حیوانات بدین صورت است که سلولهای سرطانی انسان به بدن حیوان تزریق شده، پس از مدتی تومور شکل گرفته و داروهای ضد سرطان بر روی حیوان تست میشود. این آزمایشات غیر انسانی و ظالمانه بر روی حیوانات آزمایشگاهی که باعث رنج و آزار انها می شود، نه تنها پرهزینه بوده و از نظر اخلاقی سوال برانگیز است، بلکه چنین مدل های حیوانی کاملا منعکس کننده فیزیولوژی بدن انسان نیستند و منجر به نتایج تحقیقاتی غالباً گمراه کننده میشوند. بنابراین،

ساخت و استفاده از مدلهای ساده و مقرون به صرفه کشت سلولی برای انتخاب و تست داروهای ضد سرطان بخصوص قبل از آزمایش بر روی حیوانات ضروری است، که پیش شرط پیشرفت در آزمایشات بالینی تلقی میشود. به علاوه، این امر باعث کاهش استفاده از آزمایشات حیوانی قبل از انجام آزمایشات بالینی داروهای احتمالی می شود.

همانطور که در بالا ذکر شد، تومورها نه تنها از سلولهای سرطانی تشکیل شده اند بلکه ترکیب پیچیده و ناهمگونی از انواع مختلف سلولهای سرطانی و طبیعی مانند سلولهای ایمنی، فیبروبلاست ها، سلولهای اندوتلیال عروق خونی و همچنین ماتریکسی سرشار از پروتئین ها، سیتوکین ها و هورمون ها هستند. این پیچیدگی و ناهمگونی در مدلهای دو بعدی کشت سلول در نظر گرفته نشده است. . به همین منظور ما با استفاده از سیستم سه بعدی کاشت سلولی، تومورهای سه بعدی مینیاتوری در خارج از بدن انسان تولید و شبیه سازی کرده ایم و پیش بینی میکنیم که این مدل بتواند برای به دست آوردن داده های پیش بالینی مقرون به صرفه و قابل اطمینان تر تست داروهای ضد سرطان جدید مورد استفاده قرار گیرد. اساس سیستم سه بعدی در این پروژه تحقیقاتی، شبکه ای از الیاف پلیمری الکتروریسی پلیمرپلی کاپرولاکتون (PCL) است که مشابه شبکه فیبر کلاژن موجود در ماتریکس بین سلولی بافت بدن انسان عمل می کند (**مقاله اول**). نتایج بدست امده نشان داده است که سلولهای سرطانی و سالم در این محیط سه بعدی سازگار شده، رشد کرده و ساختاری مشابه به تومورهای تشکیل شده در بدن بیمار تولید میکنند. تومورها در بدن انسان به صورت کلونی هایی از سلولهای سرطانی سفت و کوچک هستند که توسط سلولهای نرمال فیبروبلاست احاطه شده اند. ما با "اثبات اصل" نشان داده ایم که تومور طراحی شده ما در خارج از بدن انسان با استفاده از کشت همزمان انواع سلولها در محیط سه بعدی می تواند برای بررسی اثرات ضد سرطانی داروها استفاده شود (**مقاله دوم**). با این حال، در ادامه میتوان نتایج به دست امده از این سیستم سه بعدی را با داده هایی که از آزمایشات حیوانی بدست امده است مقایسه کرد که در نهایت استفاده از مدل های سه بعدی را بیشتر تقویت خواهد کرد.

سلول های سرطانی به کمک مولکول های پیام رسان مختلفی در بافت بین سلولی به طور فعال و مداوم با سلول های طبیعی و ماتریکس خارج سلولی در ارتباط و تعامل هستند که به روند تومورزایی کمک می کنند. بازسازی این تعاملات بین سلولی در مدل های تومور سه بعدی حائز اهمیت است. بنابراین، جنبه های دیگری از محیط اطراف تومور مانند ترشح و تولید پروتئین های مختلف ماتریکس خارج سلولی و همچنین ترشح سیتوکین ها در سیستم سه بعدی در این تحقیق مورد مطالعه قرار گرفته است (**مقاله سوم**). به طور کلی، مشاهده کردیم که انواع پروتین های مختلف ماتریکس بین سلولی در محیط کاشت سه بعدی تولید و ترشح شده اند و همچنین این فرایند با طولانی تر شدن زمان آزمایش افزایش یافت.

بسیاری از روشهای استاندارد آزمایش دارو مبتنی بر استفاده از میکروپلیتهای ۹۶ چاهکی است. از انجا که این متد امکان آزمایشهای متعددی را به طور همزمان مقدور میسازد، روش غربالگری با بازده بالا (HTS) نامیده می شود. با این حال، این آزمایشات به صورت دو بعدی انجام میشود و توسعه آزمایشات دارویی کارآمد در محیط سه بعدی مورد نیاز است. در قسمت آخر پروژه من، ما میکروپلیت ۹۶ چاهکی منحصر به فردی مبتنی بر غربالگری با کارایی بالا و مقرون به صرفه ای ساخته ایم که به کمک ان میتوان ۹۶ تومور کوچک را در خارج از بدن انسان به طور همزمان بررسی کرد (**مقاله چهارم**). این مدل تحقیقاتی را می توان در هر محیط استاندارد آزمایشگاهی و با حداقل سطح تخصص ساخت و استفاده کرد. این روش سه بعدی کارآمد به کمک شبکه سه بعدی پلیمر PCL در چاهک های آب گریز میکروپلیت ۹۶ چاهکی ساخته شده است. غربالگری دارویی کارآمد با استفاده از صفحات میکروتیتر تکنیکی است که به غربالگری سریع و کارآمد هزاران دارو به طور همزمان و کشف موثرترین دارو کمک خواهد کرد. علاوه بر این، از این روش برای ارزیابی اثرات ضد سرطانی دو دارو بر روی سلولهای سرطانی و طبیعی در محیط کشت های دو بعدی و سه بعدی استفاده شد. از آنجا که بافت تومورها به اندازه سایر اعضای بدن اکسیژن کافی ندارند، ما در طول آزمایشات از سطح اکسیژن طبیعی (نورموکسی) و سطح اکسیژن بسیار پایین (هیپوکسی) استفاده کردیم. نتایج بدست امده نشان داده است که میکروپلیت ۹۶ چاهکی سه بعدی ما ابزاری

مقرون به صرفه و با کارایی بالا است که میتواند برای غربالگری سریع داروها در مرحله پیش بالینی مورد استفاده قرار گیرد.

به طور کلی، این کار تحقیقاتی به کمک بازسازی تومورهای سه بعدی پیچیده انسانی در خارج از بدن و تحت شرایط مشابه فیزیولوژی بدن انسان به کاهش استفاده از آزمایشات حیوانی در تحقیقات سرطانی کمک کرده و میتواند برای مطالعه روند تومورزایی و پیشرفت سرطان در سطوح سلولی و مولکولی مورد استفاده قرار گیرد. همچنین غربالگری کارآمد داروهای ضد سرطانی به یافتن دوز مناسب دارو، کاهش عوارض جانبی ناشی از شیمی درمانی و افزایش طول عمر بیماران سرطانی کمک خواهد کرد.

Abstract

Cancer is the second leading cause of death among both men and women worldwide. During the recent years, three-dimensional (3D) tumour models have gained increasing interest as a pre-clinical platform for screening of compounds for potential use in cancer therapy. It is becoming recognized that two-dimensional (2D) cell culturing, in which cells are grown on physiologically irrelevant flat surfaces, are not reliable tools for investigating chemo sensitivity of anti-cancer drug candidates. Therefore, the emerging miniaturized 3D tumour models *in vitro* are better representatives of the human tumour growing in the *in vivo* microenvironment. We have established a complex 3D human tumour outside the body using randomly oriented highly porous electrospun polycaprolactone (PCL) fibres, which mimic the collagen structure of the extracellular matrix (ECM). The data show that mono-cultures of cancer cells grow as dense multi-cellular spheroids in the biocompatible 3D scaffolds, while normal cells show spread-out and elongated morphology. When co-cultured, JIMT-1 breast cancer cells and human dermal fibroblasts (HDFs) show a growth pattern similar to what is found in a tumour with the cancer cells growing in tight clusters surrounded by the fibroblasts. When grown in mono-culture or co-culture, the cells grow in the entire depth of the 3D PCL network. In addition, we characterized the proteins deposited by the cells in the 3D scaffolds incubated in the absence or presence of transforming growth factor-β1 (TGF-β1), a tumour promoting cytokine. The data show that the fibrous ECM proteins fibronectin, collagen I, and laminin are deposited throughout the depth of 3D structure. TGF-β1 treatment did not have a significant effect on protein deposition but significantly modulated the activity of matrix metalloproteinases and the level of interleukine-6 cytokines in the medium of our 3D culture. In TGF-β1-treated co-cultures, the cancer cells changed the growth pattern from tight clusters to be spread out along elongated HDFs. The 3D human tumour *in vitro* was utilized for evaluation of efficacy of two anti-cancer compounds; a well-known anti-cancer drug paclitaxel and an experimental salinomycin analogue (SAEC). The experiments were performed in hypoxia and normoxia. Paclitaxel treatment was more toxic to the cancer cells while the SAEC was more toxic to the HDFs in normoxia and hypoxia. Furthermore, we fabricated and validated a 96-well plates with 3D PCL fibre network as a high throughput screening (HTS)-based assay. We compared the toxicity of paclitaxel and SAEC in 2D and 3D under normoxic and hypoxic condition. The data show that the 96-well 3D system is a cost-efficient tool that can be used for assessing of new potential chemotherapeutic drugs in an HTS

manner. Thus, we have “proof of principle” that our tailor-designed human tumour outside the body has structure similar to a tumour in the body and that it can be used for investigation of chemical toxicity.

Introduction

1 Cancer

The development of cancer evolves through a complex succession of events where a normal cell is transformed into a malignant one, which usually occurs in a step-wise manner over many decades. During this multistep process, the genome of a normal cell acquires mutations in proto-oncogenes, tumour-suppressor genes, and other genes that control, directly or indirectly, cell proliferation, and cell death (Hahn and Weinberg, 2002). Dominant oncogenic activation triggers the positive stimulation of cell proliferation. Mutations in proto-oncogenes are able to transform normal cells to cancer cells because their normal cellular function such as regulating the synthesis and repair of DNA will be disrupted. In contrast, tumour suppressor genes function as negative regulators of cellular growth. Mutations in tumour-suppressor genes usually lead to complete loss of gene functions and increase the genomic instability. Mutations in a single oncogene or tumour-suppressor gene are insufficient to give rise to cancer (Pedraza-Fariña, 2007). Successive rounds and combinations of genetic errors result in a neoplastic phenotype (*e.g.* uncontrolled growth) and hence the formation of an abnormal mass of tissue.

Environmental factors play a critical role in inducing human cancer. In fact, multiple external exposures combined with internal genetic changes are the driving force in initiating tumour development. These external factors namely; aging, family history, smoking, alcohol consumption, sunlight, ionizing radiation, chemical exposure, and diet can result in genetic alterations and hence increase the risk of different types of human cancers (Parsa, 2012). It has been suggested that only 5-10 % of all cancer cases can be attributed to inherited genetic mutations, whereas the remaining 90-95 % have their roots in the environment and lifestyle factors (Anand et al., 2008). Therefore, it seems that cancer to some degree might be a preventable disease where lifestyle changes is a key factor for reducing the incidence and mortality of cancer.

1.1 Key Facts on Cancer

Cancer is the second most common cause of death worldwide after cardiovascular diseases (Wang et al., 2016). Despite the progress in the oncology field over the past decades, incidence and cancer deaths are on the rise. The World Health Organization

(WHO, 2019) estimation of mortality and global death due to various diseases has been updated recently (Fig. 1). According to WHO, it is predicted that global cancer deaths will increase by 45 % in high-income countries and more than 80 % in low-income countries between 2008 and 2030. By 2030, it is projected that there will be ~26 million new cancer cases and 17 million cancer deaths per year. This global increase in cancer is believed to be attributed to the growth and aging of populations and an increased exposure to behavioural risk factors, particularly smoking, unhealthy diet, and physical inactivity (Thun et al., 2010).

The most frequently diagnosed cancers by sex vary considerably across countries (Benny, 2015). The most commonly diagnosed cancer among men is lung cancer in most parts of Eastern Europe and Asia and prostate cancer in North America, Australia, Western and Northern Europe, and South America. Among women, the most frequently diagnosed cancer is breast cancer in most parts of the world, which is also the leading cause of cancer death in women worldwide (Jemal et al., 2010). Genetic and molecular disparities between males and females contribute to differences in the incidence of a variety of cancers (Kim et al., 2018). Moreover, the mortality of cancer from various cancer types is reported to be greater in men than in women due to sex specific differences such as sex hormones (Siegel et al., 2016).

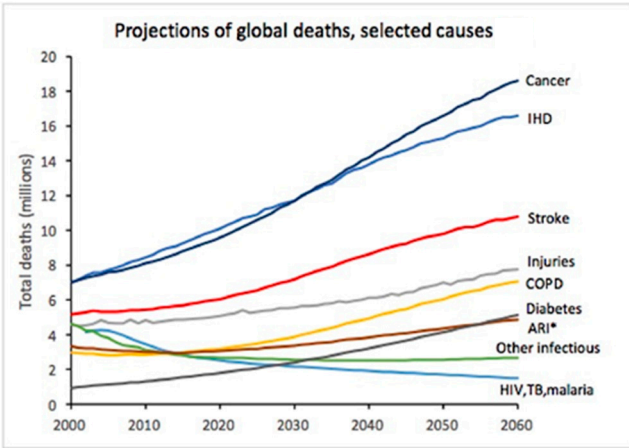

Figure 1. Projected trends in global death rates attributed to different causes, 2000-2060. At the global level, the total projected deaths by cancer are rising with time because of population growth and ageing. It is estimated that cancer will surpass ischemic heart disease as the leading cause of death worldwide by 2040 (WHO, 2019).

* IHD: ischemic heart disease. COPD: chronic obstructive pulmonary disease. ARI: acute respiratory infection (mainly pneumonia). TB: tuberculosis.

1.2 Cancer Classification

There are over one hundred different kinds of cancers affecting nearly every part of the body. Cancers are typically classified using pathological criteria that rely heavily on the tissue site of origin in the body such as lung, breast, or colon cancer. Malignant tumours are divided into three main types: carcinomas, sarcomas, and leukemias/ lymphomas. Carcinomas are the most commonly diagnosed cancer types, which constitute approximately 90 % of all human cancers. They are malignancies of epithelial cells that cover the surface of the body and line the internal organs. Sarcomas are rare in humans and are tumours of connective tissues such as muscles, fat, and bones. Leukemia and lymphomas comprise 8 % of all human cancers and are malignancies of the blood and lymphatic systems (Cooper et al., 2008). Tumours are further classified into different sub-types. For example, breast cancer is a carcinoma with the majority of them deriving from epithelial cells of the ducts (ductal carcinoma) or lobules (lobular carcinoma) of the breast. Histologically, breast cancer can be categorized into *in situ* carcinoma and invasive (infiltrating) carcinoma (Malhotra et al., 2010) and thus be sub-classified as ductal carcinoma *in situ* (DCIS), lobular carcinoma *in situ* (LCIS), infiltrating ductal carcinoma (IDC), and invasive lobular carcinoma (ILC) (Fig. 2). Ductal carcinoma *in situ* is considerably more common and heterogeneous than its lobular carcinoma *in situ* counterpart. IDC is considered the most common subtype accounting for 70–80 % of all invasive breast cancers (Malhotra et al., 2010). Complications in the morphological classification of breast carcinoma *i.e.,* the observation that approximately one-third of IDCs display mixed morphological features, have led to classification of breast cancer based on the receptor status: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Gruver et al., 2011). Through molecular analysis of breast cancers with gene expression profiling, Perou and Sorlie showed that breast cancer could be sub-classified into different subtypes (Fragomeni et al., 2018). According to the St. Gallen consensus 2011, molecular subtypes of breast cancer can be classified into Luminal A and B (ER+/PR+/HER2), HER2-overexpressing (ER-/PR-/HER2+), and triple negative (ER-/PR-/HER2-), or basal like breast cancers (Fig. 2) (Setyawati et al., 2018). Available molecular characteristics of cancer will provide earlier diagnostics, better prognostics, and tailored therapy and should ultimately improve outcome for patients affected by this disease.

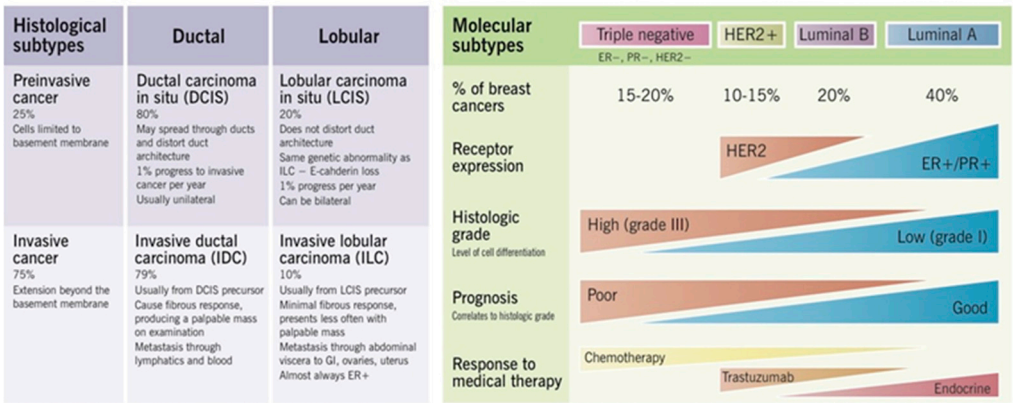

Figure 2. Classification of breast cancer into histological and molecular subtypes. Histological classification shows the heterogeneity in breast cancer based on the morphology and architectural features. Molecular classification is identified by the intrinsic molecular subtypes of breast cancer, which have important implications in breast cancer therapy (McMaster Pathophysiology review: www.pathophys.org by Eric Wong, Jenna Rebelo and Sultan Chaudry).

1.3 Cancer Cell Lines

To be able to study human cancer biology, experimental model systems are required. The commonly used models to study human cancer include human cancer cell lines, patient-derived xenografts, and genetically engineered mouse models, but by far the most commonly used models remain cancer cell lines (Van Staveren et al., 2009). Due to the difficulty of access to clinical tumour samples, *in vitro* human cell line models will remain an important resource for cancer research. Therefore, cancer cell lines are often used as an alternative to primary cells in order to study biological characteristics of cells. They provide a pure population of cells which is a valuable source for biological studies since it ensures consistent and reproducible results (Kaur and Dufour, 2012). The well-known HeLa cell line is the first continuous human cancer cell line derived from a patient with cervical cancer and it was established in 1951 (Scherer and Syverton, 1952). With the advance of high-throughput genome characterization technologies, a large number of different cancer cell lines have now been systematically characterized (Li et al., 2017). These pre-clinical models are particularly helpful in the prediction of anti-cancer drug response. Also, they contribute to improving and developing our understanding of mechanisms of drug actions (Niu and Wang, 2015).

1.4 Cancer Heterogeneity

Tumour development is a multi-step and very complex process, which is best described by common features defined as the hallmarks of cancer by Hanahan and Weinberg (2000). They suggested six essential hallmarks describing cancer progression including sustained proliferation, evasion of growth suppressors and immune cells, resistance to cell death, triggering of angiogenesis, and invasion and metastasis. A decade later, the same authors (2011) updated the tumour features by adding four new hallmarks: deregulated cell energetics, immune destruction avoidance, genome instability, and mutation and tumour-promoting inflammation (Fig. 3). The existence of subpopulations of cancer cells with various genotypes and phenotypes within a tumour, or between tumours of the same histopathological subtype (intra- and inter-tumour, respectively) are acknowledged as tumour heterogeneity (Fisher et al., 2013). However, this view of cancer cells being the foundation of tumour growth and progression has been criticized by other researchers as reductionistic. Pietrasa and Östman (2010) have proposed that many of the hallmarks of cancer are supported and activated by different stromal cells and the extracellular matrix (ECM). Indeed, tumours are not only a mass of cancer cells. They are composed of a highly heterogeneous mix of cellular and non-cellular components, such as endothelial cells, fibroblasts, infiltrating immune cells, and a complex ECM. These different cell types are actively and constantly interacting with cancer cells and each other and together form the tumour microenvironment (TME) (Chen et al., 2015, Hanahan and Weinberg, 2000). Apart from the heterogeneous populations of cancer cells, several studies demonstrate that the components of the TME, such as the cancer-associated fibroblasts (CAFs), are also a heterogeneous population of cells that may vary between and within each tumour (Runa et al., 2017, Sugimoto et al., 2006). The combination of cancer cell and TME heterogeneity results in a highly complex and multifaceted disease state (Felipe De Sousa et al., 2013). Therefore, in order to fully understand and target the process of tumour development, it is necessary to consider the molecular and cellular heterogeneity within the TME.

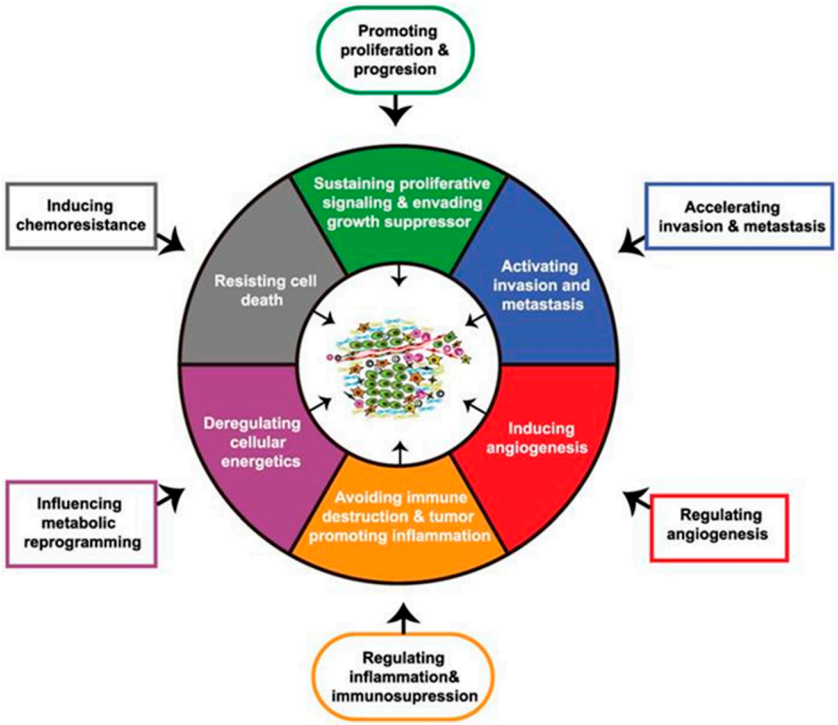

Figure 3. Hallmarks of cancer regulated by the tumour microenvironment. This illustration shows the six hallmarks of cancer originally proposed by Hanahan and Weinberg in 2000. A decade later they highlighted the importance and contribution of the tumour microenvironment in the biology of many tumours (Hanahan and Weinberg, 2011; Sun et al., 2018).

2 The Tumour Microenvironment

In the 1980s, Mina Bissell's lab was one of the pioneers in investigating the influence of the tissue microenvironment, particularly the ECM, on the dynamic reciprocity between tumour cells and the surrounding stromal cells (Bissell, 2007). The normal tissue microenvironment consists of tissue-specific cells and complex ECM with soluble factors, such as growth factors, hormones, and cytokines, and insoluble factors, such as fibrous ECM proteins, formed mainly by stromal cells (Kim, 2005; Rijal and Li, 2016). The ECM provides physical support and biochemical and biomechanical cues with a unique composition for each tissue that is essential for tissue homeostasis, differentiation, and morphogenesis. However, there are compelling differences between the organization and biochemical features of normal epithelial ECM and that of fibrotic tissues and tumours (Fig. 4) (Frantz et al., 2010).

Human tumours are extremely disorganized and contain a heterogenous bulk of numerous cells types whose behaviours are determined by the physical and biological factors that create the TME (Lyssiotis and Kimmelman, 2017; Thoma et al., 2014). The "seed and soil" theory published by Stephan Paget (1889) proposed that the tumour cells "seed" co-evolves with the surrounding stroma "soil" through substantial constant crosstalk. It has recently been accepted that malignancies are not only driven by genetic mutations that have occurred in normal cells but can also be triggered by the continuous remodelling of the microenvironment around the cells (Imparato et al., 2015). Also, the adjacent stromal cells in the TME contribute in many ways to pro-tumourigenic biology of cancer cells during the course of the multistep cancer development (Hanahan and Weinberg, 2011). Molecular components of the dynamic tissue microenvironment are constantly remodelled through enzymatic or non-enzymatic modifications. It involves the regular breakdown of ECM proteins by proteases, mainly by the family of matrix metalloproteinases (MMPs) (Bosman and Stamenkovic, 2003; Frantz et al., 2010). Therefore, the malignant cells are able to create their own TME and the reciprocal interactions between cancer cells and the TME mediates the process of tumourigenesis. The main cellular and molecular components of modified tissues and their function in cancer development will be briefly described as follows:

2.1 Molecular and Structural Composition of ECM

The main macromolecular composition of ECM is fibrillar proteins such as collagens, elastin, and other adhesive proteins such as fibronectins and laminins as well as proteoglycans that form a hydrated gel (Fig. 5). Collagen is the most prominent and abundant fibrillar ECM protein secreted by fibroblasts and it facilitate various cellular functions such as cell adhesion, migration, and chemotaxis contributing to the structural framework of tissues (Rozario and DeSimone, 2010). There are many types of collagens forming a fibrous meshwork serving as a scaffold for the cells (Franz et al., 2010). Fibronectin is another important ECM fibrillar glycoprotein secreted by fibroblasts that supports cell attachment, growth, migration, and differentiation (Pankov and Yamada, 2002). Epithelial cells interact and link with the adjacent matrix scaffolding proteins through fibronectin binding to the integrin cell surface receptors, which are considered the most important class of adhesion receptors (Bosman and Stamenkovic, 2003). Proteoglycans like hyaluronic acid fill the bulk of the ECM space and contribute to tissue buffering, hydration, and force/pressure resistance properties. Hyaluronic acid or *hyaluronan* is a polysaccharide that exist in high concentrations in the ECM of several tissues such as skin and brain. These hydrophilic molecules can bind to cell surface proteins, notably CD44, and regulate hydration and the physical properties of the ECM (Necas et al., 2008). Furthermore, within the ECM there are other soluble factors such as cytokines, growth factors, and hormones secreted by both stromal

and cancer cells which stimulate cell proliferation, survival, morphology, and differentiation (Sainio and Järveläinen, 2014; Wang et al., 2017).

The tumour stroma is characterized as much stiffer and denser than healthy tissue due to increased and abnormal deposition of collagen and excessive remodelling of ECM by fibroblasts (Poltavets et al., 2018). It has been reported that the ECM of a tumour provides a local hypoxic/acidic condition that favours the survival of cancer cells compared to normal cells (Wang et al., 2017). Furthermore, disorganized ECM in the tumour deregulates stromal cells and facilitates angiogenesis and inflammation (Hui et al., 2015).

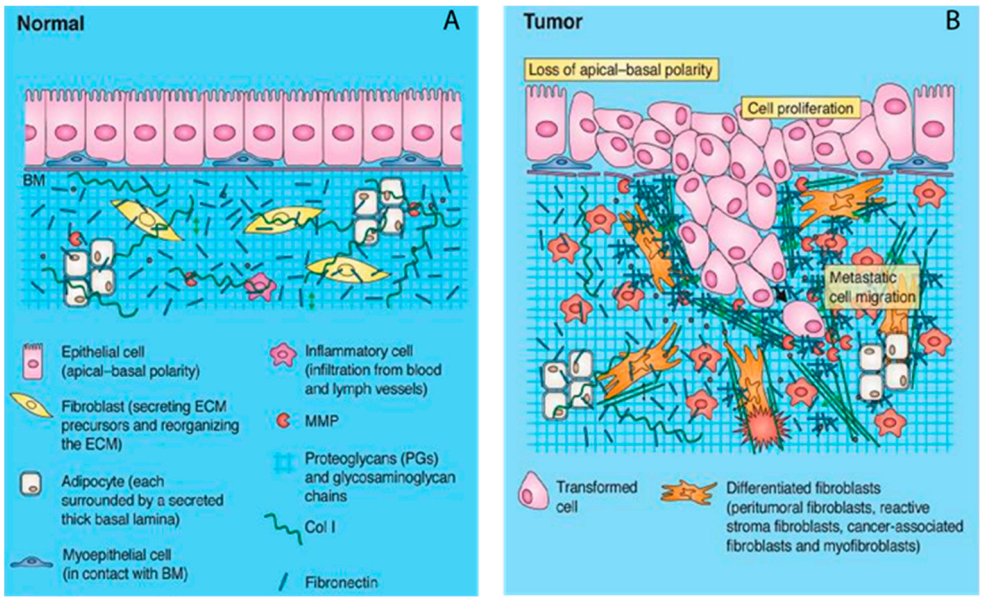

Figure 4. The microenvironment contributes to tumour progression. Schematic illustration of normal (A) and tumour (B) tissue structures encompassing stromal cells and extracellular components (adapted from Franz et al., 2010). The organized normal tissue structure changes drastically during the process of carcinogenesis due to cytoskeletal reorganization and activation of stromal cells. The increased presence of immune cells and fibroblasts results in a higher deposition of ECM component, which increases the density and stiffness of the tissue. The curly collagen fibres in the normal ECM microenvironment change into elongated collagen bundles around tumour cells, which support tumour migration and invasion (Franz et al., 2010).

2.2 Cells of the Tumour Microenvironment

According to Balkwill et al. (2012) the non-malignant cells of the TME can comprise more than 50 % of the tumour bulk. Apart from malignant cells, the TME contains other resident and infiltrating host cells e.g. fibroblasts, pericytes, adipocytes, and endothelial cells as well as infiltrating immune cells such as T lymphocytes and natural killer (NK) cells. Cancer cells are able to recruit the stromal cells from the surrounding endogenous stroma and partake in extensive communication with them which can promotes tumourigenesis (Bussard et al., 2016) (Fig. 5). Two main set of normal stromal cells, CAFs and immune cells, in the complex mechanisms of tumour progression will be discussed briefly here.

2.2.1 Cancer-Associated Fibroblasts

Fibroblasts are the non-vascular, non-epithelial, and non-inflammatory cells of the connective tissue and their biological function is the deposition of ECM to regulate inflammation, homeostasis, and differentiation of adjacent epithelia and furthermore they are involved in the process of wound healing (Kalluri and Zeisberg, 2006). Fibroblasts produce and secrete most of the ECM proteins found in connective tissue such as several types of collagen and fibronectin in a tissue-specific manner (Frantz et al., 2010). They are also the main source of ECM modulating enzymes such as MMPs, which have a prominent role in ECM homeostasis (Kalluri and Zeisberg, 2006). They are involved in the process of wound healing and show increased activity at the sites of chronic inflammation and fibrosis (Kalluri and Zeisberg, 2006). After the normal process of wound repair, the number of activated fibroblasts decreases drastically and fibroblasts retain the resting mode of the healthy tissue. However, there is increasing evidence that CAFs remain constantly active in the TME and contribute to the progression of tumour phenotypes, such as increased cancer cell proliferation, angiogenesis, invasion, and metastasis (Frantz et al., 2010, Kalluri and Zeisberg, 2006). CAFs have shown to have higher levels of ECM deposition and proliferate faster than normal and healthy fibroblasts *in vitro* (Müller and Rodemann, 1991). Several studies that used activated fibroblasts isolated from patients with breast cancer and malignant melanoma have demonstrated their increased proliferation rate compared to normal fibroblasts indicating their role in cancer initiation and promotion (Schor et al., 1986). CAFs are the most abundant cell population in tumour stroma and are found in various proportions in the TME of different solid tumours (Hanahan and Weinberg, 2011). In breast carcinomas, about 80 % of stromal fibroblasts are believed to have acquired the modified phenotypic features of CAFs (Sappino et al., 1988). Several studies demonstrated a direct involvement of CAFs in the initiation of breast cancer *in vivo* through secretion of growth factors and cytokines such as overexpression of transforming growth factor beta (TGF-β) (Kuperwasser et al., 2004; Mazzocca et al., 2010; Orimo et al., 2005). Direct, specific and reciprocal communication between CAFs and cancer cells is said to promote cancer

progression during tumourigenesis. However, the details of how CAFs promote cancer progression are not yet fully understood. The precise origin of CAFs in tumours is still under debate. Several theories have been postulated regarding the origins of CAFs from different kinds of cells such as quiescent fibroblasts residing in the host tissue, epithelial cells through epithelial-mesenchymal transition, and trans-differentiation of endothelial cells (Le Bleu and Kalluri, 2018; Shiga et al., 2015). Bartoschek et al. (2018) has employed single-cell RNA sequencing to define at least three distinct breast CAFs sub-populations with different origins. It also has been suggested that CAFs can be recruited to the tumour from a distant source such as two types of stem cells in bone marrow: the bone mesenchymal stem cells and hematopoietic stem cells (Shiga et al., 2015). The difficulty in identifying their biological origin might explain the heterogeneity, plasticity, and lack of specific markers for CAFs. One such somewhat inconsistent marker of fibroblasts is α-smooth muscle actin (α-SMA), which is often used for identifying fibroblasts (Sun et al, 2016) and its expression frequently is seen in activated CAFs (Patel and Singh, 2020). In breast cancer, four different sub-sets of CAFs were found based on different markers (Costa et al., 2018). They have characterized that one of the sub-sets of breast cancer have α-SMA negative fibroblasts.

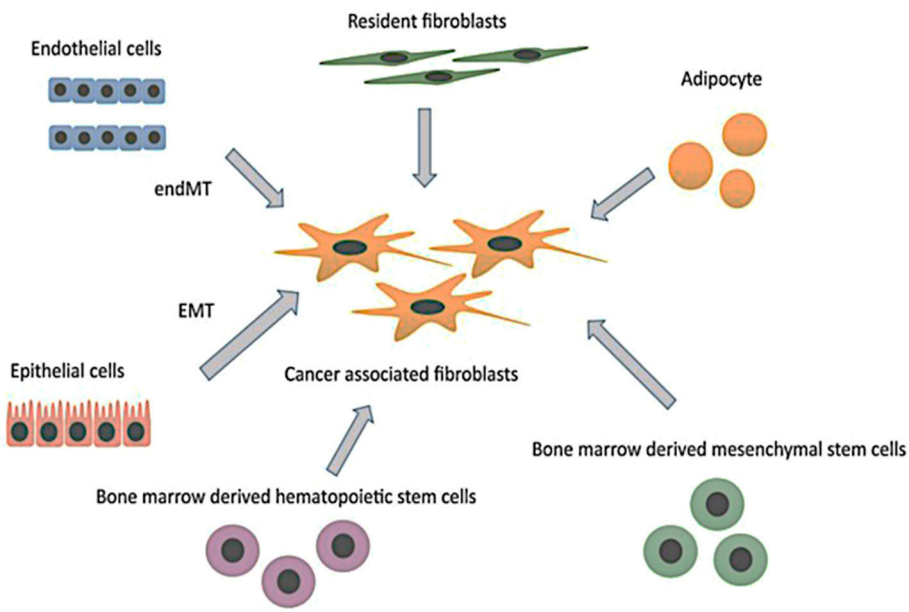

Figure 5. Heterogeneous origin of cancer-associated fibroblasts presents in the tumour microenvironment. A variety of different cell types are considered alternative sources for CAFs including resident normal fibroblasts, endothelial, and epithelial cells, bone marrow-derived hematopoietic stem cells, and mesenchymal stem cells (Shiga et al., 2015).

2.2.2 Tumour-Associated Immune Cells

Although the main function of the immune system is to protect the host from environmental agents such as infectious pathogens and chemicals, immune cells have been shown to function in tumour-antagonizing and tumour-promoting ways (Hanahan and Weinberg, 2011). Innate and adaptive immune response cells that contribute to tumourigenesis found in the TME include macrophages, dendritic cells (DCs), NK cells, and T cells (Bussard et al., 2016, Gonzalez et al., 2018). During the course of wound healing and infection, the function of immune cells will be halted after wound repair and pathogen eradication. However, the immune cells recruited to the sites of tumourigenesis tend to persist at the early stage of activation, which has been associated with tumour development. Production of tumour promoting cytokines and chemokines by immune cells and activation of different signalling pathways in pre-malignant cells can also stimulate tumour growth and survival (Grivennikov et al., 2010). Tumour-associated macrophages and cytotoxic T cells are the most frequent type of immune cells within the tumour microenvironment (Grivennikov et al., 2010). It is suggested that NK cells can also infiltrate the tumour stroma and a high level of NK cells are associated with good prognosis in many solid cancers (Balkwill et al., 2012). DCs are professional antigen presenting cells, however, their function in tumours appears to be impaired (Veglia and Gabrilovich, 2017).

NK cells comprise between 5-20 % of peripheral blood lymphocytes. Morphologically, NK cells contain large granules in their cytoplasm and therefore, they can be distinguished from B and T cells due to their larger size (Timonen et al., 1979). NK cells are part of the innate immune system with well-documented rapid anti-tumour effect. Typically, immune cells recognize the major histocompatibility complex class I (MHC-I) presented on the infected or malignant cell surface, triggering cytokine release and cell death (Wu and Lanier, 2003). In healthy tissue, the binding of NK cells to MHC-I molecules on the normal cells inhibits NK cell activation and regulates immune system homeostasis. However, NK cells have a unique activation mechanism by targeting unhealthy cells with down-regulated or lack of MHC-I expression, which triggers programmed cancer cell death in the tumour microenvironment (Gonzalez et al., 2018; Wu and Lanier, 2003).

2.3 Hypoxia in the Tumour Microenvironment

The TME of all solid tumours is strongly associated with a reduced and inadequate level of oxygen molecules (O_2) called hypoxia. Normal cells require sufficient supply of nutrients and O_2 provided by blood vessels for survival. However, due to abnormally dense stroma, poor, and dysfunctional vascularization there is limited diffusion of O_2 in tumours giving rise to hypoxic conditions (Krogh, 1922). The

transcriptional activator hypoxia inducible factor 1 alpha (HIF-1α) gene is the master regulator of oxygen homeostasis in cells. The HIF-1α gene is constitutively expressed at low levels and the protein product constantly degraded in healthy tissue (Semenza, 2010). However, O_2 deficiency in hypoxic regions results in up-regulation of HIF-1α gene expression resulting in an increased level of HIF-1α protein. HIF-1α influences not only the cancer cells, but also the surrounding stromal cells and the ECM, which promotes cancer progression, metastasis and therapeutic resistance (Muz et al., 2015; Petrova et al., 2018). Considering the paramount effect of TME on cancer initiation and progression, the development of novel pre-clinical models that more accurately resemble the TME in combination with new designs of treatment strategies with efficient targeting of the stromal components seems crucial (Roma-Rodrigues et al., 2019).

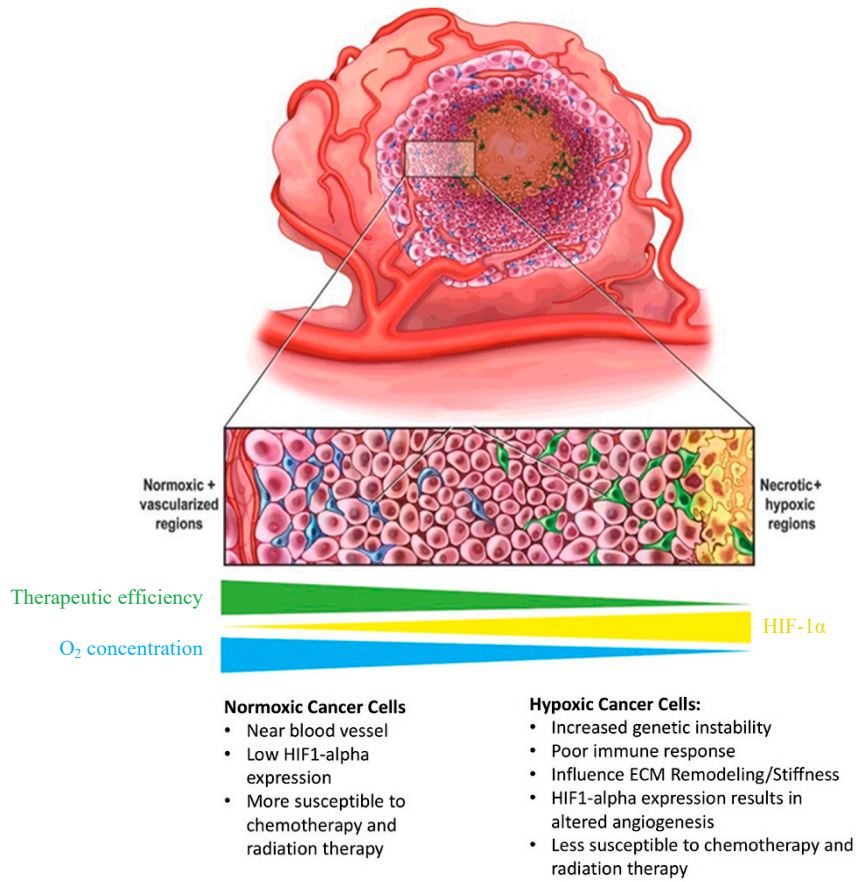

Figure 6. Hypoxia in the tumour microenvironment. After initial tumour growth, the tumour microenvironment may experience a gradual change in oxygenation due to rapid cell proliferation and production of dense ECM. This phenomenon is the result of malfunctioning of the vasculature system resulting in regions of insufficient O_2 delivery (hypoxia) (Gurusamy et al., 2017).

2.4 Targeting the Tumour Microenvironment for Cancer Therapy

Chemotherapy combined with surgery and radiotherapy have been the standard cancer treatment strategies worldwide. Despite the recent discoveries of different anti-cancer drugs and their significant effects initially, the treatments many times results in drug resistance and tumour relapse. After many years of research, it has been gradually realized that tumour treatment has focused on the cancer cells exclusively and has neglected the various crucial parts of the TME in tumour progression (Roma-Rodrigues et al., 2019; Tsai et al., 2014). Understanding the composition of the TME and major events occurring in the TME of each specific cancer allows a better prognosis and efficient therapy. TME-targeted therapy or normalization of TME combined with current common treatment strategies such as chemotherapy will increase the possibility to attack a tumour at various fronts to achieve enhanced therapeutic efficacy.

The current existing strategies that primarily target TME for cancer therapy includes targeting hypoxia, the cancer cell-ECM communication, tumour vascularization, the immune system, and CAFs (Roma-Rodrigues et al., 2019). The highly disorganized, stiff, and heterogeneous collagen-rich ECM in late-stage tumours can be targeted in cancer treatments (Roma-Rodrigues et al., 2019). Several studies have shown that inhibition of the TGF-β signalling pathway can reduce collagen secretion by CAFs, inhibit tumour progression, and improve the efficacy of anti-cancer effects in solid tumours (Coulson et al., 2017; Diop-Frimpong et al., 2011). As CAFs are the most abundant stromal cell types in the TME, they have also been recognized as a promising target for cancer therapy. Several strategies have been adopted to influence CAFs such as targeting their signals and downstream effectors such as interleukin 6 (IL-6), use treatments to normalize or inactivate them, and target CAF specific markers such as fibroblast activation protein (FAP) and α-SMA which are in general not expressed in normal fibroblasts (Chen and Song, 2019; Sounni and Noel, 2013). Although various anti-CAF compounds have been under clinical and/or pre-clinical evaluation, the heterogeneity of CAFs and their diverse cellular origins makes anti-CAF drug development a challenging task.

As mentioned before, the hypoxic TME triggers a series of cellular responses such as cell proliferation, angiogenesis, and metastasis mainly due to increased HIF-1α gene expression. Several HIF-1α inhibitors have been designed that are currently in clinical trials to suppress tumour progression (Paolicchi et al., 2016; Yu et al., 2017). Moreover, hypoxia is able to induce angiogenesis in the TME with release of high concentration of vascular endothelial growth factors (VEGF) by tumour cells (De Palma et al., 2017). Therefore, several anti-VEGF drugs were developed to target anti-angiogenesis showing improved patient survival in clinical trials (Fukumura and Jain, 2007; Sounni and Noel, 2013) and are used in the clinic today (Garcia et al., 2020).

Rapid advances in the knowledge of the TME have contributed immensely to the development of numerous novel therapeutic agents that can target various components of the TME. Many of them are currently under clinical and/or preclinical evaluation. Together, co-targeting the TME with conventional cancer therapy and immunotherapy will probably enhance the therapeutic outcomes of cancer treatments.

3 Current Pre-Clinical Tumour Models

The development of different tumour models and approaches is essential for understanding cancer biology and for the discovery of new and better anti-cancer drugs. Different experimental models of human tumours are being utilized during the pipeline of pre-clinical drug discovery. Based on the purpose of the study, appropriate pre-clinical simple models need to be selected initially to first discover anti-cancer cell activity and then to identify the mechanism of action of the drug candidate. However, using more complex models should also be considered in order to validate the therapeutic efficacy before clinical drug development (Ireson et al., 2019). Although all these models have contributed to the advancement of cancer therapy in the past several decades, each model have intrinsic limitations and drawbacks. Therefore, a great number of studies in the pre-clinical stage, particularly in cancer research, result in misleading outcomes. As a consequence, despite successful pre-clinical testing, a majority of anti-cancer drug discoveries fail in early clinical stages with the success rate of less than 8 % (Cavo et al., 2016; Langhans, 2018). To overcome this problem, there is a desperate need to design novel pre-clinical models that incorporate the tumour physiological microenvironment which can improve the efficiency in drug screening and reduce the high attrition rate (Dhandapani and Goldman, 2017).

3.1 Two-Dimensional Cell Cultures

A cell culture system is basically about growing and keeping the cells alive outside their natural environment for a long time under a controlled *in vitro* condition. Most cells cultured *in vitro* require an artificial substrate for attachment, proliferation, and maintenance in an optimal condition with sufficient nutrient supply. Culturing of cells on flat plastic dishes commonly known as adherent or monolayer culture is considered two-dimensional (2D) cell culturing. This model is a standard technique used since more than half a century in a wide range of applications in life science studies such as basic research, stem cell and cancer biology. 2D *in vitro* techniques are widely used by many cancer biologists to study various cellular and molecular mechanisms in different types of cancer.

Pre-clinical drug screening also has been extensively carried out, largely due to the use of established cell lines, in flat 2D cell culture vessels made of polystyrene or glass. 2D monolayer cultures are simple to use, easy to maintain, replicable, and cost-effective and can be used in various high-throughput screening (HTS) cellular assays (Imparato et al., 2015). However, they do not allow optimal study of mixed cell populations and the multi-dimensional cell-cell communication through three-dimensional (3D) physical contact and paracrine signalling (Lu and Wang, 2007). Classically, 2D studies have been favoured by researchers for a long time but due to the inherent flaws of traditional 2D cell cultures, alternative methods need to be developed and utilized.

3.2 Limitations of 2D Cell Cultures

Although 2D culture approaches have significantly contributed to our understanding of biological and disease processes, it does not accurately model many of the important physiological events *in vivo* (Duval et al., 2017). One of the most dramatic differences between the native tissue microenvironment and that of the 2D culturing models is that 2D systems are unable to mimic the complex 3D heterogeneous microenvironment cells experience in tissue (Costa et al., 2016; Lv et al., 2017). 2D methods lack the ECM, supporting stromal cells, and subsequent interconnections between cells and ECM that surrounds them in a complex 3D fashion (Burdett, 2010; Langhans, 2018). There is a substantial body of work that reports the effect of ECM component such as growth factors and proteins on cell proliferation, progression, differentiation, migration, survival, and therapeutic response (Jensen and Teng, 2020; Quail and Joyce, 2013; Langhans, 2018). Therefore, 2D cultures where cells are grown under non-physiological conditions can hardly represent the essential physiology of the TME present *in vivo*.

The unique biochemical composition of the ECM defines physical and mechanical properties of each tissue such as stiffness of the extracellular environment (Langhans, 2018). In normal tissue, both chemical and physical features of the ECM is crucial for tissue homeostasis and therefore, ECM imbalance leads to disease development and progression (Handorf et al., 2015). Breast tissue has a comparatively lower degree of stiffness (800 Pa) compared to the surface stiffness of 2D culture plastic plates made of polystyrene (1-2 GPa) (Butcher et al., 2009). It has been reported that the mechanical properties of flat substrates regulate various key cellular behaviours such as cell adhesion, growth, survival, differentiation, and motility in a different way compared to *in vivo* (Duval et al., 2017; Paszek et al., 2005; Yeung et al., 2005).

Tumours are usually stiffer than surrounding healthy tissue due to high abundance of ECM deposition and remodelling (Frantz et al., 2010). Ulrich et al. (2009) observed that ECM rigidity can alter cancer cell morphology and proliferation. An extensive number of studies have shown that tissue stiffness can induce

angiogenesis, metastasis and drug resistance (Frantz et al., 2010; Langhans, 2018). When cells are grown on the flat and rigid surface of tissue culture plastics, it usually leads to significant morphological changes compared to the morphology in the native 3D microenvironment (Fig. 7). 2D cultures do not provide control of cell shape and induces forced apical-basal polarity which can change cellular functions (Baker and Chen, 2012; Duval et al., 2017). Under traditional 2D monolayer culture conditions, metabolic factors (such as growth factors and cytokines), nutrients, and gases (such as oxygen and nitric oxide) are mainly equally distributed in the cell culture medium, often containing fetal bovine serum (FBS). Nutrients and oxygen are exposed uniformly and freely to the cells from the side not attached to the bottom of the plate and therefore all cells are rather well-nourished and oxygenated (Baker and Chen, 2012; Rubashkin et al., 2014). Contrary to the limited metabolic gradients in 2D systems, the nutrient and oxygen availability is highly variable in the TME. The presence of these gradients in the TME is dictated by surrounding cells, the structure and porosity of the EMC, and the natural 3D architecture which creates a highly heterogenous disorganized structure (Fang and Eglen, 2017; Leng Lee et al., 2017). Biomolecular gradients are essential for many biological processes such as growth, migration, and differentiation of cells. It has been shown that gradients of metabolites such as specific proteins play a crucial role in cancer metastasis and angiogenesis (Keenan and Folch, 2008). Taken together, due to the shortcomings of 2D models (Fig. 7) associated with the lack of dimensionality and microenvironmental complexities, there is a great need for the development of more *in vivo*-like models that can physiologically imitate the structural and physiological functions of a tumour (Cavo et al., 2016).

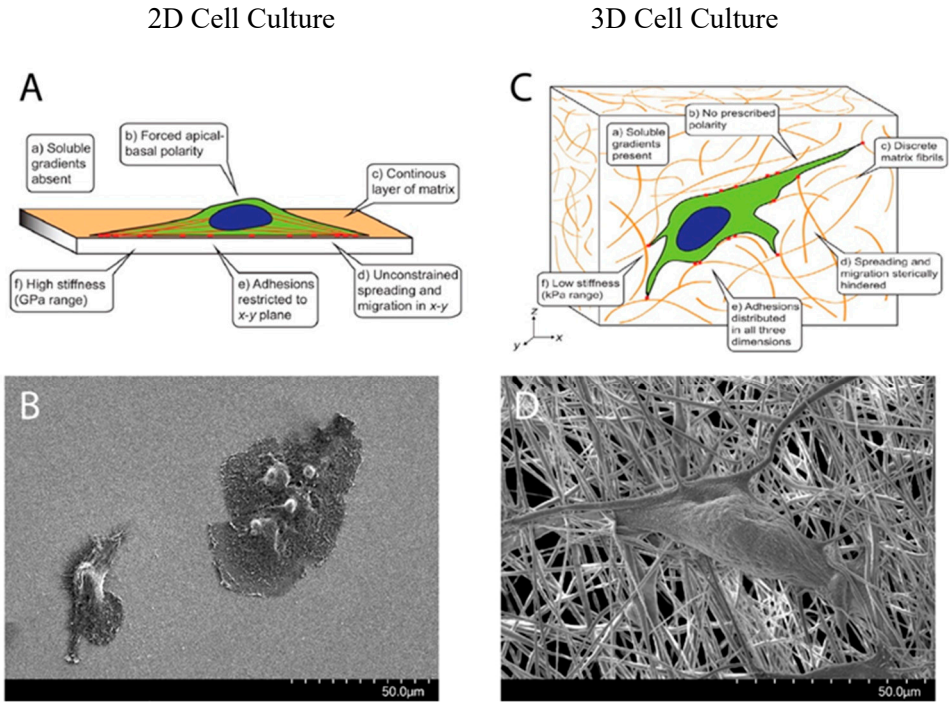

Figure 7. Comparison of the morphology of cancer cells grown in flat 2D culture *versus* 3D culture. Schematic illustrations highlight some of the main features of cellular behaviour and limitations of the 2D monolayer cell culture (A) compared to the cells grown in a 3D culture (C) (adapted from Baker and Chen, 2012). 3D models are defined by the presence of oxygen and nutrient gradient and optimal physical properties like lower stiffness compared to 2D monolayer cultures (Langhans, 2018). Scanning electron microscope images of 2D-cultured JIMT-1 breast cancer cells (B) and 3D-cultured JIMT-1 cells (D) in electrospun PCL fibre network. These images show how the morphology of the cells differs in 2D compared to 3D cell culture. Scale bars indicate 50 µM.

3.3 Animal Models

In vivo studies using animal models follows after *in vitro* pre-clinical efficiency assessment of potential therapeutic compounds in order to validate the results in a whole organism (Barre-Sinoussi and Montagutelli, 2015; Breslin and O'Driscoll, 2013). Historically, carcinogen-induced skin tumours in rabbits initiated the development and use of animal models in cancer research (Jung, 2014). Before, the development of current advanced pre-clinical models, mouse cancer models were used in drug screening for selection of effective cytotoxic drugs during the 1960s-70s (Day et al., 2015). Using animals to model human anatomy and physiology for biomedical research increased dramatically at the beginning of the twentieth century and resulted in remarkable disease-related discoveries and cues (Barre-Sinoussi and

Montagutelli, 2015; Ericsson et al., 2013). The anatomical and physiological commonalities between humans and other mammals motivated researchers to use different type of animals, particularly rodents, for investigating scientific questions. Another reason is due to the fact that human and other animal species share similar diseases such as type I diabetes, allergies, and cancer (Barre-Sinoussi and Montagutelli, 2015). In cancer research, two common ways to induce tumours growth in rodents is either by implanting the cancer cells in immunocompromised animals or by modification of gene expression which result in spontaneous tumour develop. A variety of approaches of mouse cancer models for *in vivo* tumour experiments currently exists including ectopic and orthotopic xenograft cancer models, patient-derived xenograft models, chemically-induced cancer models, virally-induced cancer in mice, and genetically-engineered mouse models (Cekanova and Rathore, 2014). However, due to the enormous attrition rate of anti-cancer compounds in pre-clinical stage, the results obtained in animal models are not necessarily confirmed in further human studies. Therefore, it is important to be aware of strengths and limitations of different types of animal models and consider serious assessment of these models for further development to achieve evidence-based reliability.

3.4 Limitations of Animal Models

There is no such thing as a perfect tumour model. Most *in vivo* models are also imperfect in the extrapolation to human cancer and there are certain problems connected with animal experiments that are still remains. The maintenance and use of sufficient number of animals for each research project is extremely labour-intensive and costly. It has been reported that the crucial genetic, molecular, immunological, and cellular differences between rodents and humans prevent the reliable predictions in human clinical trials (Mak et al., 2014). Different variables such as an animal's age, sex, weight, and species, can also lead to substantial variation in the results of studies. Moreover, differences in unrealistic drug doses used, and exposure durations, timetable of drug delivery, testing on small experimental groups, and nuances in laboratory techniques affect the outcome (Bracken, 2009). Highly unnatural experimental environments such as transportation of animals, insufficient time for adaption, in sufficient handling, and unnatural group randomization (too many male/females in one cage) can cause stress in animals (Hartung, 2008; King and Rowan 2005). The anxiety effect subsequently has large impact on physiological parameters and can cause immunosuppression, reduced growth rate, higher blood pressure, activation of the sympathetic nervous system, and reduced longevity (King and Rowan 2005).

A commonly used means to study cancer biology in animal models is to induce tumours in immunocompromised host through injection of human cancer cells. This will affect the animal's well-being and has raised many concerns regarding ethical issues. It has been estimated that approximately 154 million vertebrate laboratory

animals were used worldwide for scientific purposes in 2005 (Taylor et al., 2008). Therefore, as *in vivo* studies are costly, unpredictable, and require ethical approval, it is important to develop and validate non-animal-based-models that are based on human cells and put the Three R (3Rs: replacement, reduction and refinement) principles in practice (Anderegg et al., 2006). Alternative methods are crucial to continuously reduce non-humane and unnecessary suffering of animals and increase the quality of robust research results.

3.5 Three-Dimensional Cell Cultures

Over recent decades, a new approach known as "*in vitro* 3D tissue models" has gradually emerged that bridge the gap between 2D cell cultures and *in vivo* experiments in cancer research (Griffith and Swartz, 2006). The first use of the words "three-dimensional cell culture" started with the use of laminin-rich hydrogels developed by Barcellos-Hoff et al. (1989) and Petersen et al. (1992) in which cultured normal human breast epithelial cells and a subset of human breast carcinoma cells were used. Since then, many papers based on simple to more complicated 3D cell culture applications in medical studies have been published exponentially (Fig. 8) and marked advances have been made in this field. With emphasis on the importance of dimensionality by scientists, a broad range of different 3D platforms have been developed for cell biological research including cancer research, pre-clinical drug screening, cancer stem cells research, and many other aspects.

Novel 3D culture models have proven to allow cells to grow in a microenvironment closer to the complex *in vivo* conditions (Ravi et al., 2015). The key advantage of *in vitro* 3D models is that they allow dynamic homo- and hetero-typical cell-cell and cell-ECM interactions similar to what is found in naive tissue that is lacking in 2D cultures (Chaicharoenaudomrung et al., 2019). They have the potential to reproduce the dimensionality, complexity, and heterogeneity of the matrix structure to some degrees by embedding the cells in natural or synthetic ECM (Hickman et al., 2014). According to Cukierman et al. (2002), 3D cell cultures allow the 3D arrangements of cells and optimal spatial organization of cellular aggregations in a tissue-mimicking manner, that promotes ECM deposition allowing cells to maintain their natural 3D shape contrary to monolayer cell culturing in flat flasks/Petri dishes. On the other hand, 3D scaffolding of cell cultures provides external mechanical and chemical cues that contribute to cell adhesion and regulate intracellular signalling with establishing a tissue-scale solute concentration gradient for growth factors, cytokines, enzymes, and other diffusible molecules (Griffith and Swartz, 2006). This phenomenon ultimately will contribute to *in vivo*-like cellular response to drugs and ensure accurate and more reliable cytotoxicity results. Furthermore, since cells reach confluency in a short time in monolayer cell cultures due to contact or density inhibition of proliferation, it is preferable to replace them with 3D models for longer cellular lifespan and more stable growth condition. In this regard, 3D models are an

appropriate platform to perform long-term experiments and evaluate long-term toxicity of drugs on cultured cells (Chaicharoenaudomrung et al., 2019).

The optimally-designed scaffold for 3D tissue models should provide the associated features of natural ECM (Chan and Leong, 2008) that is summarized by Raeisdasteh Hokmabad et al. (2017) as follow:

- Architecture: Scaffolds should provide volume with adequate interconnected pores to favour cellular infiltration.
- Biocompatibility: Scaffolds should be made of biocompatible materials with surface bioactivity that facilitates cellular adhesion, proliferation, and differentiation.
- Mechanical property: Scaffold should stimulate the mechanical properties of natural tissue in order to provide suitable composition and biological environment for the cells.

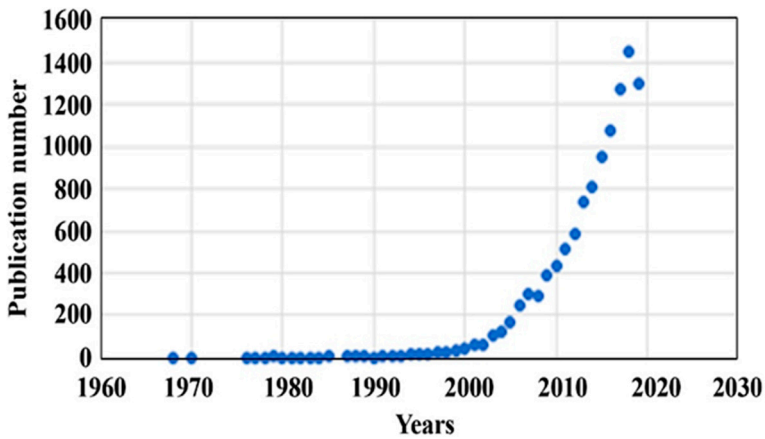

Figure 8. Number of publications per year (1968–2020) that use 3D cell cultures. The number of publications per year for PubMed searches using the phrase "3D cell culture" is shown in blue circles (Jensen and Teng, 2020).

3.6 Overview of 3D Cell Culture Techniques

A wide range of different culturing techniques have been constructed and developed to be chosen based on the purpose of the scientific investigation (Fig. 9). The 3D techniques can be broadly divided into two groups as they have been defined as scaffold-free or scaffold-based cultures. The field is evolving rapidly and with advances in technologies many different 3D techniques have been developed. These techniques include using spheroids, hydrogels, porous scaffolds, bio-reactors, magnetic-levitation, bio-printing, microfluids and many more. Each of these 3D culturing techniques are different in both principles and protocols and have their own distinct strengths and limitations. Therefore, it is important to consider the most appropriate 3D model for scientific implementation depending on the desired research applications. This section aims to briefly describe the unique set of advantages of some of the most widely used technique including multi-cellular spheroids, hydrogels, and scaffold-based technologies.

3.6.1 Multicellular Spheroids

Multicellular spheroids, the simplest and most widely used methods of 3D culturing are anchorage-independent aggregations of cells that can be formed by preventing cell adhesion to the surface. Cell aggregation can be obtained through different methods such as using liquid overlay culture, low-attachment hydrophobic substrates, and hanging drop plates (Fig. 9). Liquid overlay culture is the simplest and a highly reproducible 3D method for formation of cellular aggregation and spheroids by using an artificial matrix such as agar, Matrigel™, or agarose and on non-adhesive surfaces. Ultra-low attachment plates coated with a layer of hydrophobic polymer can be utilized for production of spheroids (Chaicharoenaudomrung et al., 2019). Hanging drop plates is another technique for scaffold-free generation of self-assembled multicellular spheroid.

Spheroids are able to partly reflect the *in vivo* microenvironment because they contain various stages of cells physiology including highly proliferative, non-proliferative, and apoptotic cells, and also due to nutrients and oxygen gradient maintain the hypoxic and necrotic regions of a tumour mass (Fig. 6) (Lv et al., 2017; McKee and Chaudhry, 2017). Griffith and Swartz (2006) suggested that an ideal 3D model should have tissue-specific stiffness, oxygen, nutrient, and metabolic waste gradients, and a complex cell-cell/ cell-ECM interaction. In the field of cancer research, spheroids are considered a useful 3D model with a hypoxia gradient (Knight and Przyborski, 2015). However, regular use of spheroid models to study drug sensitivity on cancer cells can be misleading due to the lack of uniform spheroid production and morphology (Cho et al., 2020).

The techniques used to obtain 3D spheroids are highly variable in reproducibility and are difficult to control as they tend to result in spheroids of various sizes and morphologies (Bresciani et al., 2019; Cho et al., 2020). An undeniable drawback of

the hanging drop method to form spheroids is the low-throughput, labour intensity, and difficulty in changing culture medium during long-term cultivation (Chaicharoenaudomrung et al., 2019).

3.6.2 Hydrogels-Based Cultures

Scaffold-based 3D cultures are formed through mainly two approaches: hydrogels and preformed fibrous scaffolds. Hydrogels are networks of hydrophilic cross-linked natural or synthetic polymeric material that contain a high-water content. Single ECM components such as collagen, fibrin, and hyaluronic acid can be used to encapsulate cells in naturally-based 3D networks (Fig. 9) (Lv et al., 2017). Since, they are natural products, 3D hydrogels are biocompatible and can promote many cellular functions, however in an uncontrollable manner (Tibbitt and Anseth, 2009). The well-known commercial product Matrigel™ is one of the most commonly used ECM rich embedding hydrogels and it is obtained from the Engelbreth–Holm Swarm mouse sarcoma tumour cell basement membrane (Hughes et al., 2010). As the Matrigel™ protein composition is not clearly identified, it suffers from high batch-to-batch variability (Knight and Przyborski, 2015). Also, it is a highly questionable product from an animal ethical point of view. Thousands of mice are transplanted with the tumour so that researchers can obtain Matrigel™. Despite these negative aspects of Matrigel™ it has been used successfully. It has been shown that it is possible to distinguish normal breast cells from invasive carcinoma cells in 3D Matrigel™ models through the formation of organized acinar structures by benign breast cells (Pal and Kleer, 2014). However, it should be a goal in research to replace Matrigel™ because of the severe animal ethical concern.

One of the best characteristics of hydrogels is that they provide a soft tissue-like stiffness similar to the natural microenvironment. However, they lack mechanical strength necessary for cell culture handling (Chaicharoenaudomrung et al., 2019). Also, they do not allow the optimum diffusion of nutrients and oxygen and therefore can be used only for short periods of cell culturing (Knight and Przyborski, 2015). Although natural products are biodegradable, their biodegradability can be problematic in cell culturing since it can introduce uncontrollable effects on cell functions (Knight and Przyborski, 2015). In addition, products derived from animals still rise the questions of ethical issue as well as of the difference between humans and other animals.

3.6.3 Scaffold-Based Cultures

A wide-range of synthetic materials have been used for the fabrication of solid 3D fibrous or porous scaffolds (Fig. 10). The have different mechanical characteristics that can mimic the *in vivo* ECM structure of the tissues. 3D porous scaffolds provide mechanical, physical, and biochemical cues for cell proliferation and morphology in which cells can grow in a similar way to their natural environment in the human body (Chaicharoenaudomrung et al., 2019). Some of the most common Food and

Drug Administration (FDA)-approved synthetic polymers, such as polylactic acid (PLA), and polycaprolactone (PCL), and polyglycolic acid (PGA) have been used for this purpose (Fig. 10). They are free of animal-based components, biocompatible, and biodegradable, however, at a very low rate very slow degradation rate (2–4 years depending on the starting molecular weight) and their surface bioactivity and mechanical properties can be adjusted (Knight and Przyborski, 2015).

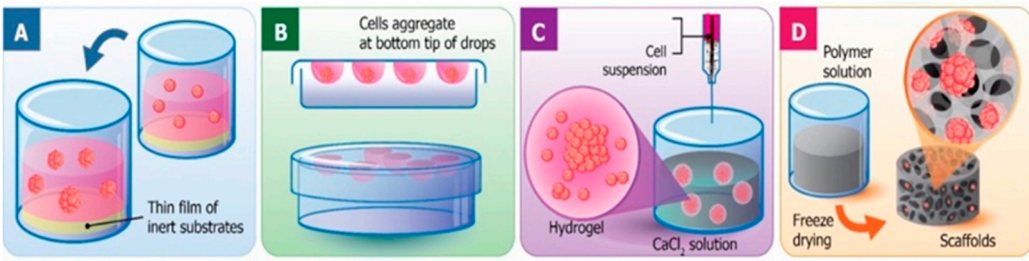

Figure 9. Currently available techniques for three-dimensional cancer modelling. A schematic diagram depicting the principles of these 3D methods including: Liquid overlay (A), hanging drop (B), hydrogel (C), and porous scaffold (D) (Chaicharoenaudomrung et al., 2019).

One of the advantages of these well-defined scaffolds is that they can be fabricated with high quality under controlled procedures in order to minimize batch variation and thus provide reproducibility and consistency (Knight and Przyborski, 2015). PCL is a linear and hydrophobic, semi-crystalline polymer with a glass transition temperature of -60 °C and a melting point of 55-60 °C (Nair et al., 2007). The stiffness of single PCL nanofibers is around 3.7 GPa, however, a scaffold of PCL fibres network has a Young´s modulus of 3.8 MPa which is softer than that of 2D cell culture Petri dishes with 2-4 GPa stiffness (Croisier et al., 2012; Elamparithi et al., 2016; Lv et al., 2017). Moreover, the stiffness of most synthetic biomaterials can be adjusted further based on the polymer components, concentration used, degree of crosslinking and the solvent used for fabrication (Lv et al., 2017).

It is possible to fabricate scaffold-based 3D cultures using various techniques among which electrospinning is one of the most widely used techniques. Randomly-aligned electrospun fibre meshes are produced by applying an electric field in which a polymeric solution will pass through to be collected on a grounded surface (Cipitria et al., 2011). It is a simple, low-cost, and highly reproducible method. According to Cipitria et al. (2011), electrospun fibrous scaffolds have attracted increasing attention among researchers due to several reasons. Firstly, nanoscale fibre diameters are similar to fibrous ECM proteins, in particular collagen, and the fibres can mimic the architecture and organization of fibrous structures found in the tissue.

Secondly, electrospun meshes are characterized by large surface area to volume or mass ratio, which is favourable for cell adhesion and spreading. Furthermore, high porosity of fibrous scaffolds allows uniform cell infiltration and distribution and at the same time allows nutrient and waste exchange. Electrospun scaffolds with submicron fibre diameters can maintain their mechanical integrity and have tuneable features such as desirable pore size, elasticity, and fibre size.

It has been reposted that cancer cells cultured in porous/fibrous scaffolds are able to produce small tumour-like structures or spheroids similar to *in vivo* (Girard et al., 2013; Lv et al., 2017; Rijal and Li, 2016). Also, cancer cell growth, cell-cell interaction, gene/protein expression levels, and drug responses in 3D scaffold cultures are relatively representative of tumours *in vivo* (Chaicharoenaudomrung et al., 2019; Gurski et al., 2010). Development and function of the normal mammary glands as well as of tumours, involves the complex crosstalk between mammary epithelial cells with surrounding stroma. Type I collagen is one of the most abundant components of the ECM in normal breast tissue and these fibres surround the mammary epithelial cells and have profound impact on morphogenesis (Provenzano et al., 2006; Conklin and Keely, 2014). The uncompressed randomly-oriented organization of electrospun fibres in PCL scaffolds are a representation of the stromal collagen fibres. They are in close contact with mammary epithelial cells in multiple directions similar to the native stroma (Conklin and Keely, 2014).

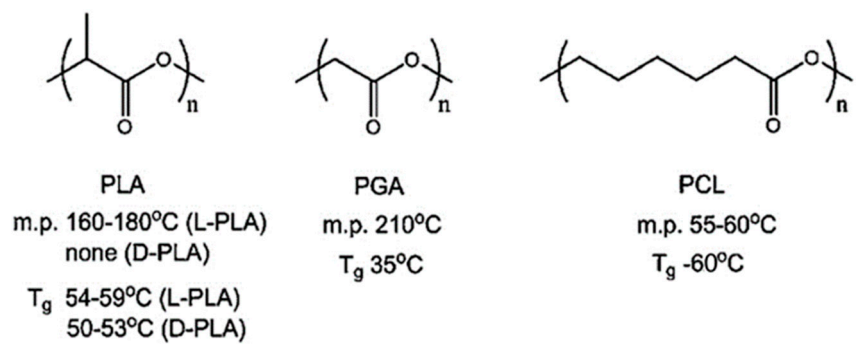

Figure 10. Chemical structures of common synthetic polymers used for fabrication of 3D fibrous scaffolds. Selected physical properties of these linear polymers are indicated as melting point (m.p.) and glass transition temperature (T_g) (Cipitria et al., 2011).

3.7 Limitations of 3D Cell Culture Models

In order to more closely recapitulate the *in vivo* cellular microenvironment in 3D cultures, there is a strive to increase the complexity of the system by addition of other matrix components to the system such as stromal cells, ECM proteins, growth factors, chemokines, and hormones etc. However, increasing the complexity of 3D cell cultures, rise several limitations and challenges that should be addressed. Higher complexity of 3D cultures usually results in higher volume of the system which makes it more difficult to incorporate the 3D models into high-throughput screening (HTS) platforms (Montanez-Sauri et al., 2015). HTS in drug design and discovery is a practical method to test cytotoxicity of a large number of compounds using microtiter plates and reduced assay volumes of 50-100 µl (Pereira1 and Williams, 2007).

A common laboratory technique for investigating the cells grown in 3D cell culture after drug treatment is staining and immunocytochemistry (ICC) assays. ICC allows the researcher to visualize the localization of cells and to a certain degree the amount of a specific protein in the cell as well as the ECM. However, imaging of 3D cultures is often interrupted by background signals and non-specific binding of primary or secondary antibodies (Montanez-Sauri et al., 2015). It is also possible to use other biochemical assays for further quantification of gene/protein expression in the cells such as Western blot, RNA sequencing, flow cytometry, and qPCR. However, it may be difficult to gently and rapidly extract all encapsulated cells from a tortuous multi-cellular 3D scaffold for post-culturing analysis (Comley, 2010).

One of the biggest challenges of working with 3D cell cultures which limit its applications is the visualization which requires advanced imaging equipment such as wide-field or confocal microscopes. Imaging of 3D models may become difficult due to several reasons such as optical light scattering, light absorption, and poor light penetration depending on the culture size, material transparency, and complexity of 3D geometrical structures (Antoni et al., 2015; Langhans, 2018). While, capturing only one single focal plane (*xy*) image of 2D cell cultures suffice for its visualization, a single confocal microscopy plane only shows a fraction of the possible imaging area at suboptimum plane in 3D cell cultures (Booij et al., 2019). Therefore, in order to capture images with sufficient data from the 3D cell cultures, a series of confocal focal plane images in the vertical direction should be obtained to give a 3D visualization of the culture (Booij et al., 2019). However, z stack imaging of 3D cell cultures is usually time-consuming and considerably increases data volumes and storage space. Furthermore, it is not optimal to use high magnification objectives such as 40× and 63× for imaging 3D cultures (Booij et al., 2019).

3.8 3D Cell Cultures in Drug Discovery and Screening

Drug development often starts with different disease modelling and understanding the disease mechanisms which is crucial for successful drug screening and discovery programs (Fang and Eglen, 2017). Cell-based 3D culture models with *in vivo* microenvironment characteristics are expected to yield more accurate and higher predictive values regarding drug efficacy and toxicity in early drug screening processes (Langhans, 2018). However, it has been suggested that the response to treatment with chemicals in 3D cell cultures can be very different e.g. resistance to increased sensitivity, cell type dependent, interaction with the stromal cells and ECM (Langhans, 2018; Stock et al., 2016). Therefore, the cytotoxic effect of a compound should preferably be evaluated in various types of 3D cell cultures using different states and conditions.

An ideal chemotherapeutic drug treatment induces a cytotoxic effect on cancer cells while normal cells are spared to minimize the side effects for the beneficent patient survival (Eastman, 2016). 3D tumour co-culture models then can provide an advantageous platform for assessing cell specific toxicity in heterotypic cultures of normal and abnormal cell populations cultured together and it also can be used to assess which cell type survives the specific treatment (Stulpinas et al., 2019). Also, combinational therapy with applying the optimal dose of each drug can be used to investigate drug resistance in cancer cells and lead to discovery of intolerable side effects (Bayat Mokhtari, 2017; Stulpinas et al., 2019). Therefore, 3D models might be valuable tools in the pre-clinical drug discovery pipeline to reduce the rate of failure of drug candidates and to obtain information on clinically relevant concentrations for cancer therapy (Horning et al., 2008; Lovitt et al., 2014). Still much work remains to be done to develop 3D culture models that truly represent *in vivo* conditions.

The Present Investigation

1 Background and Aims

The primary objective of the work presented in this thesis is to construct and establish a reproducible heterotypic and custom-made *in vitro* 3D tumour model outside the human body. There is a stark need for new simple and cost-effective animal free models for pre-clinical evaluation of chemicals as anti-cancer drugs. Various cellular behaviours such as cell proliferation, morphology, distribution, and infiltration in the physically defined 3D microenvironment was evaluated **(Paper I)**. Miniaturized 3D human tumour models may bridge the gap between the traditional 2D in vitro and *in vivo* experiments and can replace or be used as an adjunct to animal studies. 3D models provide an opportunity to use an alternative animal-free, human-based model in compliance with 3R principles recommended to refine, reduce, and replace animal experiments and develop alternative approaches in research. The ultimate goal of this project is to build a human tumour outside the body with minimum use of various animal-derived products such as Matrigel™ and collagen and to reduce both the numbers and types of animal models needed in medical research in the future. There is a long way to go before scientists can fully appreciate the potentials of 3D *in vitro* cultures. However, the field of 3D culturing is rapidly growing.

In order to further optimize a 3D cell culturing system with minimal use of animal-derived components, we replaced medium supplemented with animal-ethically questionable FBS with medium supplemented with DHHS. Collection of blood samples from donor horses are considered less stressful as the animals are trained to donate blood under very carefully controlled and calm conditions (Hashim et al. 2012). The use of DHHS eliminates the suffering of the preterm calves that are removed from the womb of the cow to permit blood extraction through heart puncture in order to obtain FBS (Jochems et al., 2020; van der Valk et al., 2004). Proliferation analysis of different breast cancer cells and other cell lines in DHHS-supplemented medium has been investigated in comparison with proliferation in FBS-supplemented medium in our lab (data not published). Moreover, it was previously found that a low concentration of DHHS (5 %) does support the culturing of cell lines in monolayer (Fedoroff and Hall, 1979).

The 3D tumour model has been used to recreate part of the complexities of a multi-cellular tumour by co-culturing malignant and normal cells in order to bio-mimic the behaviour of 3D cancer-stromal cell interactions. Furthermore, this 3D scaffold-

based tumour models can be used as a valuable therapeutic tool for further investigations of existing anti-cancer drugs and for discovery and development of novel anti-cancer compounds in a well-defined 3D microenvironment **(Paper II)**. To reach this goal, we have started investigations using two anti-cancer compounds: an experimental substance, a synthetic salinomycin derivative (SAEC) and a commonly used anti-cancer drug, paclitaxel. These compounds were used as single treatment in two different concentration in 3D mono- and co-cultured cells. 3D multi-culturing model can be used to evaluate the influence of TME including the stromal cells on the efficacy of therapeutic agents and microenvironment-mediated drug resistance in a *vivo-like* heterogenous microenvironment further.

As several studies had implicated the role of ECM as a major structural component of the TME in regulating tumour initiation, progression and cancer stemness (Nallanthighal et al., 2019; Walker et al., 2018), it seemed reasonable to investigate the deposition of ECM proteins in our 3D scaffolds (**Paper III**). Primarily, the objective was to assess the deposition of the three main fibrillar ECM proteins; fibronectin, collagen I, and laminin qualitatively and quantitively with immunostaining and confocal microscopy and investigate the influence of TGF-β1. TGF-β1 has an important role in TME modification and tumour growth and invasion is becoming increasingly evident (Massagué, 2008). We characterized the ECM deposition patterns of HDFs in mono-cultures and of JIMT-1/HDF co-cultures grown in a 3D PCL scaffold. In addition, we investigated the level of the cytokine IL-6 in the collected medium of the cultures and also the activity of MMPs following TGF-β1 stimulation. The study demonstrated that electrospun 3D scaffolds can be utilized as a 3D model to evaluate the macromolecular composition of tailor-designed human tumours outside the body.

Currently, the majority of cell-based HTS assays are performed using traditional 96- and 384-well plates for cells in monolayer cultures. As mentioned earlier, one of the major limitations of 3D tumour models is that they are not compatible for HTS cell-based assay drug screening and discovery. Some of the anchorage independent 3D cultures such as spheroid formation using hanging drop plates or low-attachment plates support medium- to low-throughput drug screening in microscale 3D cultures and have several limitations regarding the size and morphology consistency, robustness, labour intensity, and time consumption. Many other 3D systems use naturally-derived hydrogels of different types such as Matrigel™ which is not well-defined and also animal ethically questionable. However, scaffold-based cultures such as polymeric pre-fabricated 3D methods such as porous electrospun scaffolds used in this work are not scalable to 96- or 34-well nanofiber multi-well plates. We felt the need of developing a 3D HTS capable assay that can be produced with low cost and minimum level of expertise. To this end, we made a novel HTS-compatible drug screening set-up based on integrating well-defined PCL electrospun fibres in hydrophobic 96-well plates (**Paper IV**). This novel 3D HTS-based platform can be

used for rapid and feasible identification of potential drug candidates in drug discovery programs and other research fields.

In summary, the main aims of this thesis are outlined as follow:

- To evaluate biocompatibility of collagen-mimicking electrospun PCL scaffolds
- To establish *in vitro* 3D cultures of human cancer and normal cells in mono-cultures and co-cultures in PCL scaffolds
- To investigate the therapeutic efficacy of different anti-cancer compounds in 3D mono- and co-culture models
- To evaluate the ECM proteins produced by cells in the biocompatible but xenobiotic PCL fibre network and investigate cytokines secreted in the 3D culture medium
- To design and evaluate a 3D PCL-based and HTS-compatible multi-well plates assay to assess cytotoxic effects of anti-cancer compounds

2 Cell Lines Used in This Study

A number of different human normal and malignant cell lines were used throughout this research project. The epithelial human breast cancer cell lines used in the main work are JIMT-1 and MCF7 cells. However, we have also established mono-cultures of MiaPaCa-1 human pancreatic cells, co-cultures of MiaPaCa-1/HDFs as well as of SH-SY5Y human neuroblastoma cells (not published). The normal cells used are human breast epithelial MCF-10A cells and HDFs.

The human ductal carcinoma breast cancer line JIMT-1 was established from the pleural effusion of a 62-year-old woman with ductal breast cancer after postoperative radiation in 2003. The JIMT-1 cells are epithelial cells that can grow as an adherent monolayer. The cells overexpress the HER2 receptor mRNA and protein (Tanner et al., 2004). However, the JIMT-1 cells are insensitive to the HER2-inhibiting humanized monoclonal antibody trastuzumab, which is standard treatment for $HER2^+$ early and advanced breast cancer (Boekhout et al., 2011). The JIMT-1 population doubling time in monolayer in DHHS-supplemented medium is approximately 30 hours.

The human epithelial breast adenocarcinoma cell line MCF-7 was established from the pleural effusion of a 69-year-old woman with metastatic disease in 1973 (Soule et al., 1973). It is ER- and PR-positive and belongs to the luminal A molecular subtype. MCF-7 is a poorly-aggressive and non-invasive cell line, normally being considered to have low metastatic potential. It was demonstrated that the anti-

estrogen drug tamoxifen inhibited the growth of MCF-7 cells (Comşa et al., 2015). The population doubling time for MCF-7 cells in monolayer in DHHS-supplemented medium is about 34 hours.

The human breast epithelial cell line MCF-10A was established from benign proliferative breast tissue of a 36-year-old woman with fibrocystic disease and it immortalized spontaneously. The MCF-10A cells are not tumourigenic and do not express ERs. Their known molecular characteristics include the deletion of the chromosomal locus containing the p16INK4a and p14ARF tumour suppressor genes, both of which are critical in regulating senescence (Qu et al., 2015). They have shown to form duct-like structures and mammospheres when grown in 3D Matrigel and collagen cultures (Soule et al., 1990; Qu et al., 2015). The MCF-10A population doubling time in monolayer in DHHS-supplemented medium is around 20 hours.

HDFs are mesenchymal/stromal cells and are the main cell type present in skin connective tissue (dermis). HDFs are human dermal fibroblasts isolated from normal healthy adult skin. They produce ECM proteins to strengthen the dermal compartment and interact with epidermal cells (Böttcher-Haberzeth and Biedermann, 2019). The population doubling time of this cell line in DHHS-supplemented medium in monolayer is around 25 hours.

The L929 mouse fibroblasts cell line, commonly used in cytotoxicity assays, was established in 1948 from an initial strain derived in 1940 from normal subcutaneous areolar and adipose tissue of a 100-day-old male mouse (Sanford et al., 1948). This is a continuous and non-transformed cell line. The population doubling time of L929 cells in DHHS-supplemented medium in monolayer is around 25 hours.

All five cell lines described above were used to perform the initial studies of this project for development and establishment of 3D cultures in electrospun PCL scaffolds (**Paper I**). Considering our previous knowledge on the growth characteristics and sensitivity to treatments, the JIMT-1 cells were chosen as a malignant cell line in order to be used for co-culturing with a normal stromal cell line, HDFs for further investigations (**Paper II-IV**).

3 Summary of The Papers

The findings related to this work is presented here as four peer-reviewed published paper and manuscripts.

Paper I – Unique Animal Friendly 3D Culturing of Human Cancer and Normal Cells

Theoretically, in order to reconstruct an *in vivo*-like tumour model outside the human body, it needs to closely mimic the complexity and heterogeneity of the tumour tissues structure as much as possible. However, in practice the system needs to be simplified mimicking fundamental factors of the TME component to begin with. Collagen fibres are the foundation for shaping tissue structure and therefore mimicking this structure by artificial matrixes for *in vitro* biological studies is a good starting point. Thus, we have used uncompressed and highly porous 3D PCL scaffolds as a 3D matrix in an attempt to synthetically mimic collagen structures in our studies (Fig. 11). The 3D scaffolds with randomly-oriented fibres having specific parameters like fibre diameter and porosity were fabricated by a simple and rapid electrospinning technique with high reproducibility (Jacobsson et al., 2017). The scaffolds were purchased from Cellevate AB in Lund, Sweden. In **Paper I**, mono-cultures of human breast cancer cells (MCF-7 and JIMT-1) and human normal cells (MCF-10A and HDF) were established in the 3D biocompatible scaffolds. Confocal images of each cell line grown in 3D cell culture are presented in Figure 12.

Many factors should be considered in providing optimal conditions for cell attachment and growth for *in vitro* cell culturing. Cellular functions such as cell proliferation, adhesion, and migration are dependent on the nature of the matrix they are growing on. Above all, cells need a hydrophilic matrix to be able to attach and grow optimally. It is known that surface modifications of polymer scaffolds by plasma treatment can significantly increase their hydrophilicity and thereby enhance cellular performance (Martins et al., 2009). As the PCL polymer is naturally hydrophobic, we used O_2 plasma treatment to increase the hydrophilicity i.e. adding polar groups such as hydroxyl and carboxyl groups, to the fibres before cell seeding. Contact angle measurement using the sessile drop technique as a simple but sensitive standard technique (Drelich, 2013), was used for evaluation of the PCL fibres wettability before and after O_2 plasma treatment. The results show that untreated PCL scaffolds were hydrophobic while the hydrophilicity of O_2 plasma-treated PCL scaffolds increased significantly. The surface is defined as wettable and hydrophilic when the water contact angle (θ) is less than 90° (Xu and Siedlecki, 2007) and the measured contact angle of 43° ± 1, indicates good hydrophilicity of the fibre network. SEM analysis of the scaffolds furthermore shows that plasma treatment did not have any substantial effect on the scaffold topography.

We performed an initial study using the AlamarBlue™ assay to monitor the cellular growth pattern at different time points after seeding cells at low and high densities.

The AlamarBlue™ assay, which is an indirect indicator of cell number, showed that when seeding at a lower initial density there was a continuous growth of the cell in the PCL scaffolds for 1 week compared to seeding at the higher initial density for the cell lines investigated. Based on this finding, we decided to continue our experiments with the lower initial seeding density associated with each cell line. Our study in general showed excellent cell proliferation of human breast cancer and normal cells on electrospun PCL scaffolds. Confocal microscopy imaging revealed images of healthy cells in different layers of the 3D scaffolds.

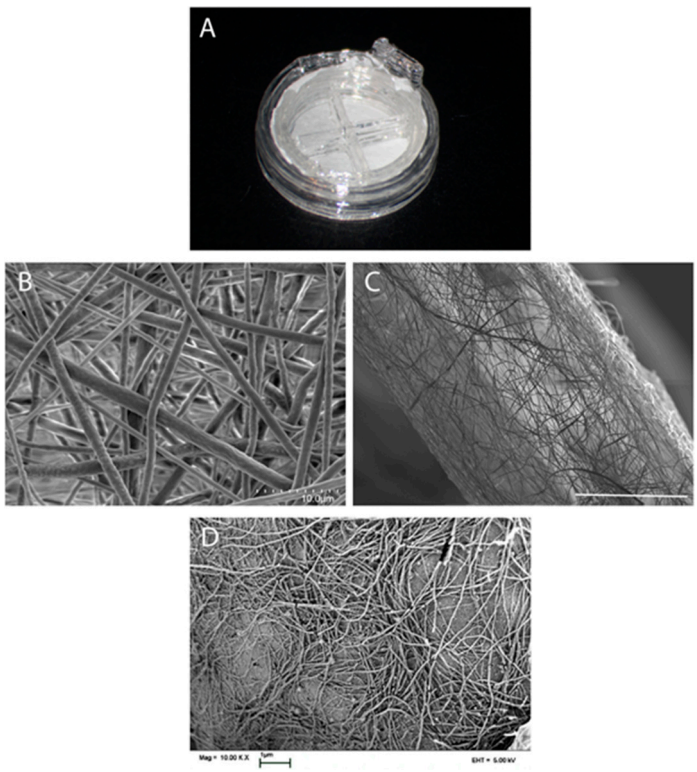

Figure 11. Structural characterization of the electrospun PCL scaffolds used for 3D culturing of cells in this study. Stereomicroscope image of (A) 3D scaffold consisting of the uncompressed PCL fibre mesh fabricated by electrospinning. The delicate PCL fibre mesh structure is supported with 3D-printed lock-rings and crossed-shape set-up using PLA polymer. The diameter of the set-up is 10 mm. SEM micrographs of (B) randomly-oriented PCL fibre network, (C) cross-sectional view of the PCL fibre mesh showing its porosity, and (D) collagen fibrillar structure immediately adjacent to the cell surface in the mammary gland *in vivo* (Provenzano et al., 2006). Scale bars indicate 10 µm (B), 100 µm (C), and 1 µm (D).

Although materials made of PCL for biomedical usage are approved by FDA, we were not sure if the process of scaffold fabrication, in this case electrospinning, handling before cell seeding, and leachables would affect cells cultured in the 3D

device. To address this issue, we performed a cytotoxicity assay. Extracts of different scaffold components (PCL granule, PLA granule, and PCL fibre mesh) were produced in deionized water according to ISO standard 10993-12. To study the potential toxicity of PCL/PLA nanofiber scaffolds, various concentrations of the extracts were tested for cytotoxicity using the MTT assay on L929 cells in compliance with ISO standard 10993-5. The cytotoxicity assay results clearly show that the collected extracts of the different components of 3D scaffolds had no adverse effect on the metabolic activity of the L929 cells.

3D cell culturing requires an optimum seeding concentration of cells to enhance cell proliferation, adhesion, and dispersion throughout highly porous scaffold. This is clearly a challenge in 3D systems since the area is less defined as compared to 2D systems. To address this, we decided to begin with seeding cells at two different densities to find a good seeding density that seemed to results in good proliferating of various cell lines in our 3D system.

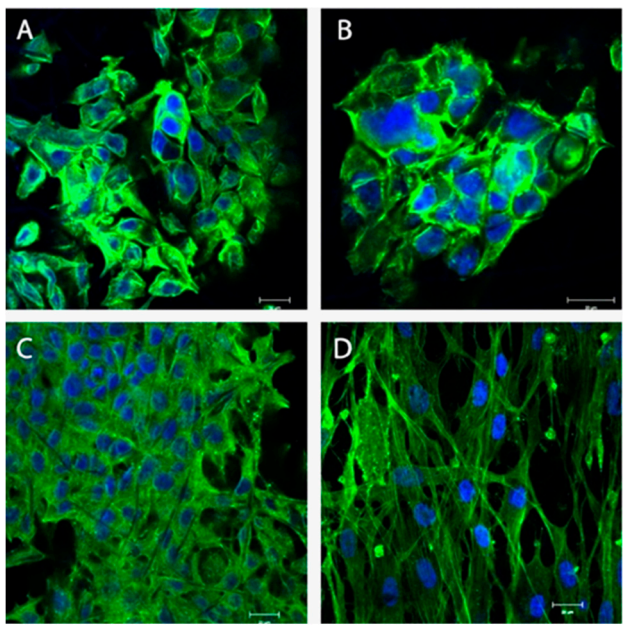

Figure 12. Morphology of cancer and normal cell lines cultured in 3D electrospun PCL fibre network. Confocal laser scanning microscopy images of (A) JIMT-1 cells, (B) MCF-7 cells (C) MCF-10A cells, and (D) HDFs grown in 3D PCL scaffolds for 1 week. The cells were fixed in 3.7 % paraformaldehyde and labelled with Alexa 488-phalloidin (green) to visualize the actin cytoskeleton and DAPI (blue) to visualize nuclei. Scale bars indicate 20 μm.

Cell-matrix interactions shape the cellular phenotype, attachment, and polarity of both normal cells and tumour cells. To understand the integration and morphology of human cancer and normal cells in the electrospun PCL scaffolds, both SEM and

confocal microscopy were used. MCF-7 and JIMT-1 breast cancer cells tend to form tight, irregular, tumour-like aggregates in the 3D PCL scaffolds similar to cancer cell clusters in *in vivo* tumours in the 3D PCL scaffolds (Fig. 12). This result is consistent with previous studies in which cancer cells were able to form micro-spheroids in electrospun polymer scaffolds (Feng et al., 2013; Girard et al., 2013). Both HDFs and normal-like breast MCF-10A cells were found to exhibit spread-out morphology and close interaction with both multiple fibres and other cells (Fig. 12). It is evident that the morphology of cells is highly dependent on the topography and geometry of their local environment.

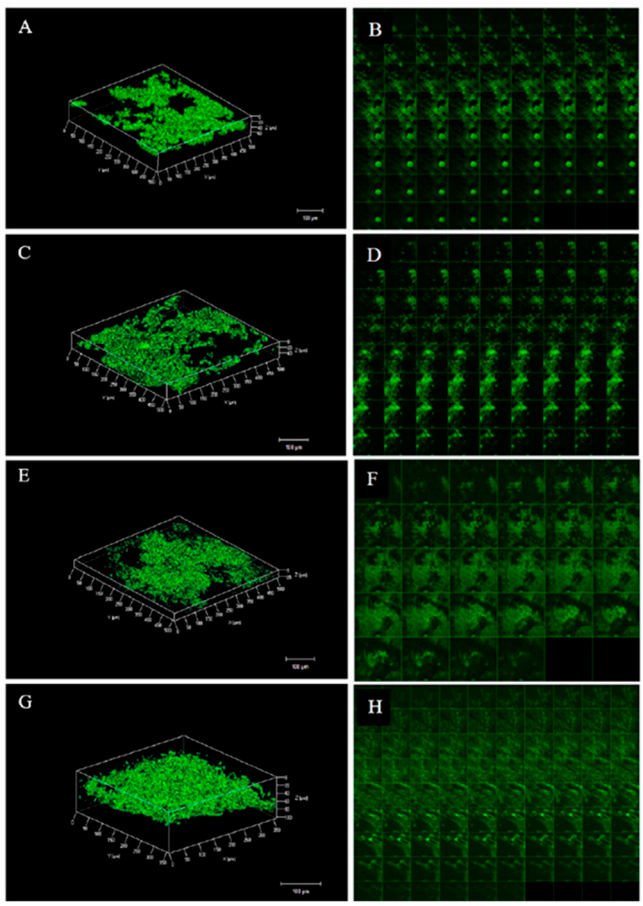

Figure 13. Infiltration of human breast cancer and normal cells in the 3D PCL scaffold after one week of incubation. Confocal microscopy Z-stack and montage images of (A and B) MCF-7 cells (70 µm), (C and D) JIMT-1 cells (56 µm), (E and F) MCF-10A cells (27 µm), and (G and H) HDFs (100 µm). The number in parenthesis shows cell infiltration depth. The distance between each plane is 1 µm. All the cell lines were stained with Alexa 488-phalloidin (green) visualizing actin cytoskeleton. Scale bars indicate 100 µm.

Considering the high porosity of the uncompressed PCL fibre network, we expected efficient infiltration and expansion of cells into the depth of the scaffolds. We used 3D laser confocal imaging to verify that the uncompressed structure of fibres allows distribution and infiltration of all the cell lines after one week of culturing (Fig. 13). We also used cryosectioning of stained samples to further confirm the 3D distribution of cells throughout the depth of the 3D scaffolds. The overall conclusion is that the cells are able to distribute/infiltrate throughout the entire scaffolds thickness.

Paper II – Three-Dimensional Co-Culture Model of Cancer Cells and Fibroblasts for Evaluation of Anti-Cancer Compounds

Initially, we confirmed that 3D mono-cultures of different human normal and cancer cells could be established in the 3D PCL scaffolds. In **Paper II**, we established an *in vitro* heterogeneous and miniaturized human tumour model outside the human body by co-culturing JIMT-1 cells and HDFs in the 3D PCL scaffolds. We additionally show that this 3D tumour model also can be used as a pre-clinical therapeutic tool for investigating the toxicity of different anti-cancer agents in which we can investigate how cancer cells and normal cells react to treatment. The co-culturing model then was then used to investigate the toxicity of the salinomycin analogue 20-ethyl carbonate-Na (SAEC) (10 or 50 nM concentrations) and the conventional anti-cancer drug paclitaxel (10 or 100 nM concentrations). We assessed proliferation of 3D mono-cultures and co-cultures treated with the compounds and incubated under normoxic (18 % O_2) and hypoxic (1 % O_2). conditions. The hypoxic culture condition was used to mimic the low oxygen level found in tumours *in vivo*. In these studies, the cells cultures were allowed to establish for one week, before applying the different compounds at two different concentrations for another 72 hours of incubation.

For initial toxicity assessment, the AlamarBlue™ method as an indirect means for approximation of the cell number was used. The AlamarBlue™ data from JIMT-1 mono-cultures showed that all compound treatments lowered the reduction of AlamarBlue™ in cultures incubated in both normoxia or hypoxia. However, only treatment with 50 nM SAEC had a significant effect on AlamarBlue™ reduction in HDF mono-cultures incubated in either normoxia or hypoxia. On the other hand, all treatments lowered the reduction of AlamarBlue™ compared to control in 3D JIMT-1/HDF co-cultures incubated either in normoxia or hypoxia.

To further analyse the differential sensitivity of JIMT-1 cells and HDFs in co-cultures to the treatments, the cells were manually counted in images taken randomly by confocal microscopy in the centre of the 3D co-cultures. The data show that SAEC treatment was more toxic to HDFs compared to the JIMT-1 cells in normoxia. In contrast, paclitaxel treatment did not result in any significant change in percentages of JIMT-1 cells and fibroblasts. However, when incubated in hypoxia, paclitaxel

treatment resulted in a significant decrease in the number of JIMT-1 cells implying a higher toxicity of paclitaxel on JIMT-1 cells compared to HDFs.

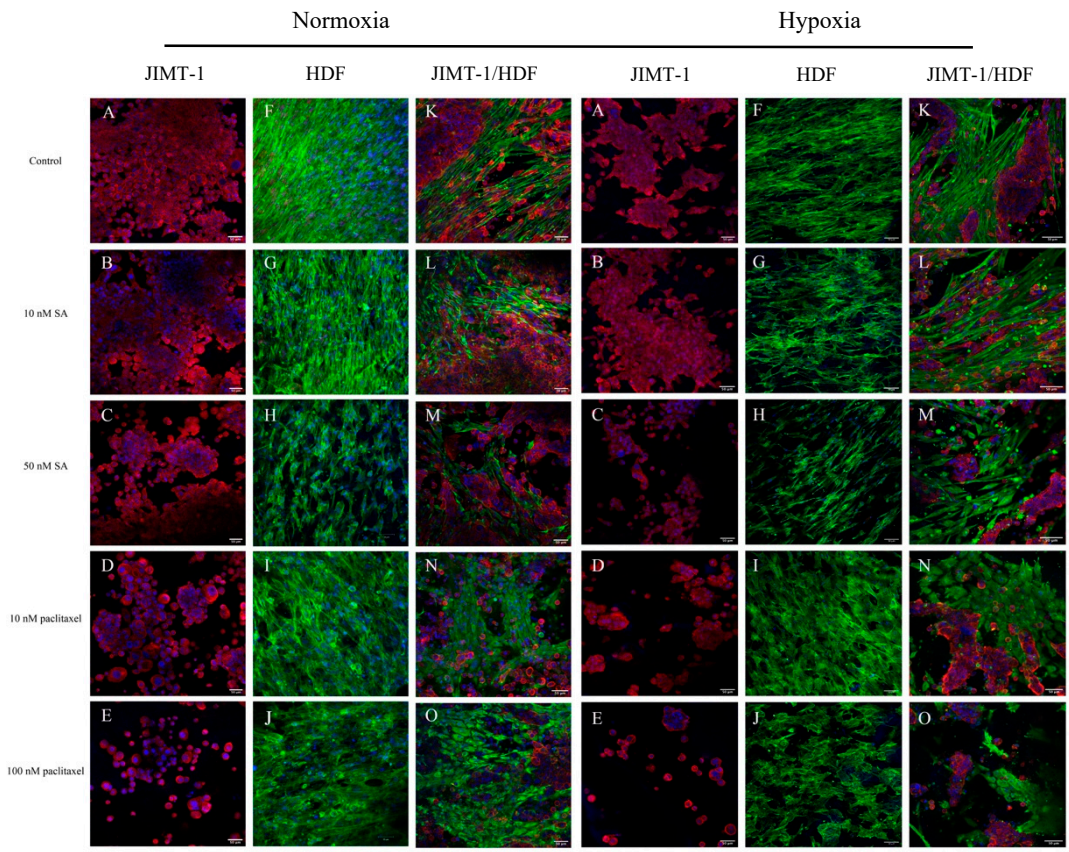

Fig. 14. Confocal microscopy single plane images of 3D mono-cultures of JIMT-1 cells (A-E) and HDFs (F-J), and co-cultures of both cell lines (K-O) treated with SAEC or paclitaxel in normoxia (18 % O_2) and hypoxia (1 % O_2). The images are taken in the centre of 3D cultures treated for 72 hours with the indicated compounds. The cultures were fixed in 3.7 % formaldehyde and labelled to visualize CD44 in JIMT-1 cells (red), actin filaments in HDFs (green), vimentin in HDFs in JIMT-1/HDF co-cultures (green), and cell nuclei (blue). The images are representative of 3-4 independent experiments. Scale bars are 50 µm.

In order to study the distribution and interaction of JIMT-1 cancer and HDFs in 3D cultures and also for morphological evaluation after treatment, cell-specific marker staining and then confocal laser scanning microscopy was used to identify the cells (Fig. 14). The identification of expression of different proteins in the JIMT-1 cells and the HDFs has enabled us to distinguish these two cell types in the co-culture system. The JIMT-1 cells express CD44 while the fibroblasts do not. The CD44 antigen is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion, and migration (Senbanjo and Chellaiah, 2017). On the other hand, the

expression of the intermediate cytoskeleton filament vimentin is much higher in the HDFs than in JIMT- 1 cells. The JIMT-1 cells and the HDFs vary in their growth pattern and organization. The cancer cells grow in tight clusters surrounded by the stretched-out HDFs in a manner which resembles breast tumour histology both in normoxia and hypoxia.

Overall, the data demonstrated that treatment with SAEC did not have an effect on the morphology of the JIMT-1 cells while treatment with paclitaxel decreased the number of JIMT-1 cancer cells in 3D mono-cultures incubated in normoxia. On the other hand, the morphology of HDFs was clearly affected by treatment with SAEC or paclitaxel. The HDFs were less elongated and stretched out compared to control, which was found for cultures incubated in normoxia and hypoxia. Treatment of co-cultured cells with SAEC affected the fibroblasts more than the JIMT-1 cells while the JIMT-1 cells were more sensitive to paclitaxel treatment in co-cultured cells incubated in normoxia and hypoxia. Furthermore, cryosectioning and focus stacking imaging of 3D cultures were used to give an impression of the 3D growth and distribution of the cells in cultures. Based on the data obtained, it was apparent that the cells in this study have infiltrated and distributed throughout the depth of scaffolds in all conditions. We have now established a 3D human tumour *in vitro* composed of cancer cells and fibroblasts that form a growth pattern similar to human tumours *in vivo*, with the cancer cells forming tight clusters surrounded by elongated fibroblasts.

Paper III – Identification of Extracellular Matrix Proteins Secreted by Human Dermal Fibroblasts Cultured in 3D Electrospun Scaffolds

In order to provide an accurate representation of the *in vivo* microenvironment, understanding the ECM molecules in an *in vitro* model is important. In **Paper III**, we investigated a set of ECM proteins deposited by HDFs in 3D mono-culture and in JIMT-1/HDF 3D co-cultures. The main reason for the investigation was to gain more insight on the level and distribution pattern of proteins secreted by the cells grown in the 3D artificial yet biocompatible PCL fibre network. We were particularly interested in filamentous ECM proteins with adhesion regulatory potentials, such as fibronectin, laminins and collagen I. Given the importance of TGF-β in regulating ECM gene expression, the cultures were treated with TGF-β1 in the hope to induce phenotypic differentiation of resident HDFs into myofibroblast-like CAFs. The TGF-β family thought to be a key regulator of fibroblast differentiation in most solid tumours during tumour progression (Massagué, 2008; Caja et al., 2018). We further were interested to elucidate and characterize the effect of TGFβ-1 exposure on the ECM protein deposition and on the level of the cytokine IL-6 in the medium as well as on the MMP activity in the medium.

In our study, we clearly demonstrated that fibronectin, collagen I, and laminin were deposited in a time-dependent manner in 3D mono-cultures of HDFs and in 3D co-

cultures of HDF/JIMT-1 cells and the proteins were found throughout the depth of the 3D structures. However, TGF-β1 exposure did not have any significant effect on the deposition of these ECM proteins. Also, we provide evidence that TGF-β1 treatment of HDFs mono-cultures and HDF/JIMT-1 co-culture mediates the enhanced alignment and linearization of the actin cytoskeleton in HDFs. Furthermore, tightly dense colonies of JIMT-1 cells in co-cultures seems to loosen up in the presence of TGF-β1 and tend to spread out along the streaks of elongated HDFs (Fig. 15). This result confirms previous studies in which CAFs could alter the architecture and physical properties of the ECM through force-mediated matrix remodelling that can ultimately promote directional cancer cells migration (Erdogan et al., 2017).

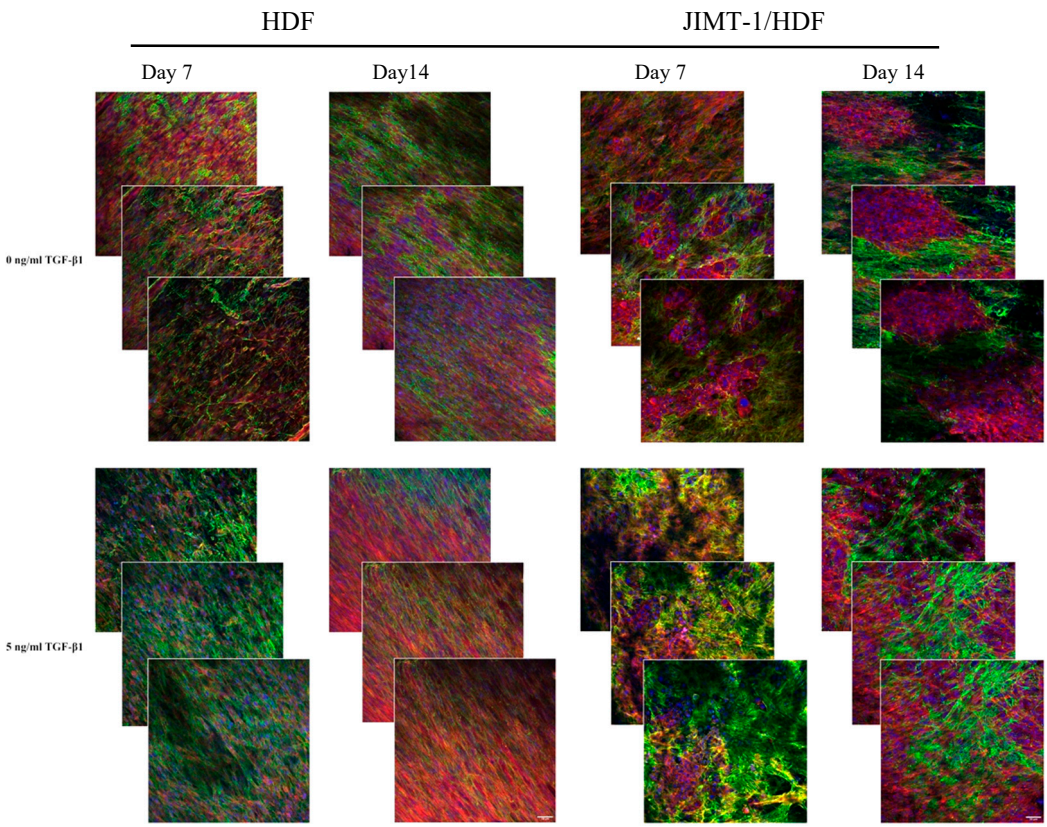

Figure. 15. Focus stacking images of 3D cultures of HDFs mono-cultures and JIMT-1/ HDFs co-cultures to visualize fibronectin deposition in 3D scaffolds. Three confocal plane images were obtained with approximately 10 µm distance from each other in the 3D cultures. Cultures were incubated in the absence (control) and presence of 5 ng/ml TGF-β1 for 7 and 14 days. Then, the cultures were fixed in 3.7 % formaldehyde and labelled to visualize F-actin (red), fibronectin (green), and cell nuclei (blue). All images are representative of 3 independent experiments. Scale bars are 50 µm.

In the next step, the level of the secreted IL-6 and MMPs activity were assessed in medium collected from JIMT-1 and HDF mono-cultures and JIMT-1/HDF co-cultures after 14 days of incubation. The ECM is constantly modulated by MMPs that are mainly secreted by fibroblasts (Lu et al., 2011). The MMP activity was lower in medium from JIMT-1 3D cultures compared to medium from HDF 3D cultures. The result show that TGF-β1 treatment significantly increased the MMP activity in the medium of HDF cultures which is an indication of MMPs ECM remodelling.

IL-6 is a pro-tumourigenic cytokine and plays various biological roles in the TME such as promoting proliferation of cancer cells (Chonov et al., 2019). We show that IL-6 is secreted by both JIMT-1 cells and HDFs in the culture medium and it was significantly modulated by TGF-β1 treatment in mono-cultures. However, HDFs mono-cultures and JIMT-1/HDF co-cultures generated significantly higher levels of IL-6 in the medium compared to JIMT-1 mono-cultures after 14 days of 3D cell culture.

Altogether we show that the secretion of ECM proteins and soluble factors changes over time in a 3D PCL scaffold-based tumour model to hopefully mimic the *in vivo* TME. Also, it provided a basis for further investigation of ECM molecules and cytokines found in different 3D systems as biological models.

Paper IV – A Novel 3D Polycaprolactone-Based High Throughput System for Evaluation of Toxicity in Normoxia and Hypoxia

The pre-clinical drug discovery process by cell-based HTS relies heavily on 2D microtiter 96- or 386-well plates. The study in Paper IV was performed in an attempt to design and validate a novel 96-well plates with 3D PCL fibre networks incorporated in the wells (Fig. 16A). Also, the evaluation of the toxicity of the two anti-cancer compounds used in Paper II *i.e.* SAEC and paclitaxel, was investigated using an MTT assay in 3D 96-well plates and compared with conventional 2D plates. This 3D assay allowed us to quickly assess drug cytotoxicity in an HTS-compatible and consistent manner.

In order to fabricate the PCL fibre network in the 96-well plates, aligned electrospun fibres were prepared by a regular electrospinning set up. Then, a solution of finely-cut PCL fibres in ethanol/water was prepared and added to the wells of round-bottomed hydrophobic 96-well plates. The PCL fibre pieces were thermally crosslinked and the plates allowed to dry at room temperature. SEM imaging was used to characterize the topography of the 3D fibre network and also to evaluate the pore size of the 3D network (Fig. 16B). The result shows different PCL fibre thickness and a mean pore are of $30 \pm 1\ \mu m^2$.

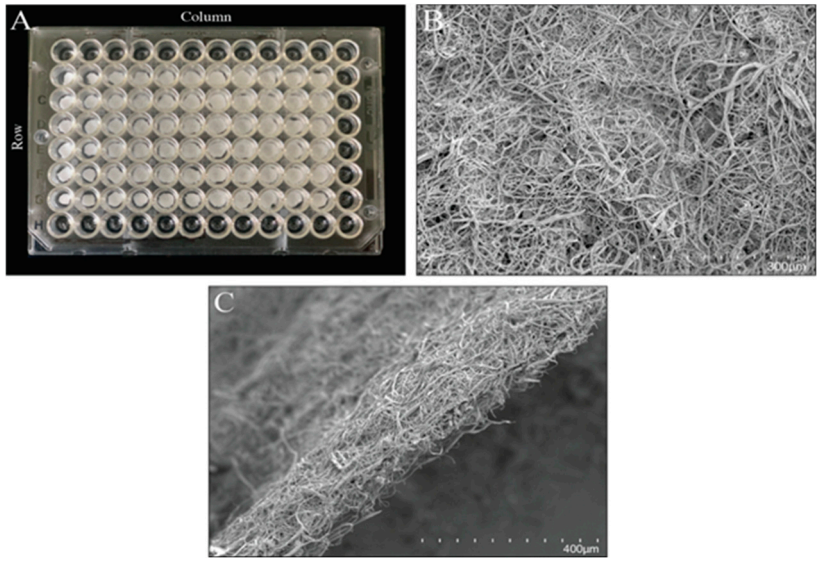

Figure 16. Image of the 96-well plate with 3D PCL-based fibre network (A), a SEM image of the top view (B) and cross-sectional view (C) of the fibre network extracted from a well. Scale bars are 300 µm (B) and 400 µm (C).

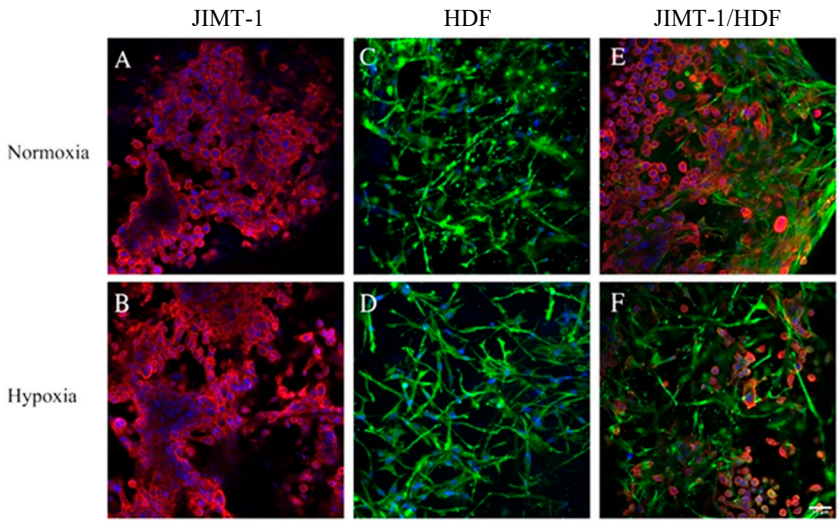

Figure 17. Fluorescent confocal microscopic images of cells grown in 3D HTS-based cell cultures incubated in normoxia or hypoxia. JIMT-1 cell (A and B) and HDF (C and D) mono-cultures and co-culture of both cells (E and F) were grown on 3D 96-well plates for 96 hours under normoxic (18 % O_2) and hypoxic (1 % O_2) conditions, respectively. The cultures were fixed in 3.7 % formaldehyde and labelled to visualize CD44 (red), vimentin (green), and cell nuclei (blue). All images are representative of 3-4 independent experiments. Scale bar indicates 50 µm.

Figure 16C shows a cross section SEM image of the 3D fibre network. Confocal microscopy and z-stack imaging shows there is adequate spacing and porosity for cell distribution and infiltration throughout the depth of the 3D fibre network. The mean infiltration depth of 112 ± 8.5 µm (n=10) was measured in the z-direction both in hypoxia and normoxia. As we have shown previously in our electrospun 3D model, the JIMT-1 cells are found growing in tight clusters of different sizes while the HDFs spread out along the PCL fibre network in both normoxia and hypoxia (Fig. 17). When co-cultured, the JIMT-1 cancer cells grow in irregular clusters surrounded by the fibroblasts in 3D fibre networks incubated in either normoxia or hypoxia. Generally, this HTS-compatible 3D fibre network replicates the conditions established in **Papers I-III** but it is more versatile since it can be used for drug screening in any possible way.

Table 1. IC_{50} values in nM concentrations for HDFs and JIMT-1 cells grown in 2D or 3D cultures treated with SAEC (A) and paclitaxel (B) incubated in normoxia (18 %O_2) or hypoxia (1 % O_2).

SAEC

A	HDF		JIMT-1	
	2D	**3D**	**2D**	**3D**
Normoxia	97.7 ± 11.8 [a]	150.3 ± 34.4	119.5 ± 13.2	271.3 ± 52.2
Hypoxia	239.3 ± 6.2	316.2 ± 35.1	80.4 ± 13.3	NA [b]

Paclitaxel

B	HDF		JIMT-1	
	2D	**3D**	**2D**	**3D**
Normoxia	61.7 ±13.8 [a]	NA	49.9 ± 14.8	332.9 ±95.6
Hypoxia	157.1 ± 34.0	NA	74.8 ± 18.6	NA [b]

a. Mean of three independent experiments ± SEM.
b. NA, not applicable within the dose range studied.

When the cellular response in 3D and 2D cultures were compared, based on the dose response curves and IC_{50} values (Table 1) obtained from the MTT colorimetric assay, the results show that the cytotoxicity in 3D cultures were lower compared to those of 2D cultures regarding both compounds. This indicates that the cells in 3D cultures were less sensitive to SAEC and paclitaxel treatment than 2D cultured cells under normoxic and hypoxic condition, regardless of the drug's mechanism of action. Our results are consistent with other studies showing that cells cultured in 3D such as multicellular spheroids are often more resistant to anti-cancer compounds compared to their 2D counterparts (Karlsson et al, 2012; Loessner et al., 2010). Also, the results show that the cells are much less sensitive to compound treatment in hypoxia compared to normoxia both in 2D and 3D culture set-up.

Previous studies suggest hypoxia may induce resistance against various anti-cancer agents such as paclitaxel in solid tumours through different mechanisms (Dong et al., 2012; Jing et al., 2019; Karakashev et al., 2015; Sullivan et al., 2008). Our findings in general show that using this HTS-based 96-well plate contributes to deducing more clinically-relevant results and can predicts the overestimation of drugs anti-cancer toxicity observed in conventional 2D monolayer cultures.

Conclusions and Future Perspectives

Most of the research findings described here may have a major role in cell and cancer biology. However, many novel heterogeneous 3D platforms are currently emerging that will address the shortcomings of 2D culturing and animal models. The main objective of the work carried out in this research project was an attempt to move beyond 2D and animal models and to emphasize the fact that context hugely matters and that dimensionality needs to be considered. Also, another objective was to contribute to reducing the number of animal experiments in cancer-research by recreating a complex humanized 3D tumour *in vitro*, which can be used to study cancer development and progression on cellular and molecular levels as well as for HTS of potential anti-cancer drugs. In order to make our model totally animal free, we have developed a fully defined animal product free medium (Manuscript). Figure 18 shows the images of 3D cultures of JIMT-1 cells and HDFs in 3D PCL scaffolds in this totally defined medium. Many different cell lines thrive in this medium.

3D cell cultures provide an exciting opportunity to study the cellular drug response in tissue-like models outside the body. A broad range of 3D models have been developed to partly address chemo-sensitivity of cancer cells to anti-cancer drugs under similar conditions to "*in vivo*". By utilizing 3D electrospun PCL scaffolds and HTS-based 3D 96-well plates, we showed that these scaffold-based models can be used for pre-clinical screening of anti-cancer drug toxicity. Our 3D system displays an *in vitro*-mimicking 3D human tumour cell culture model where the scaffold is free from animal-derived products. Thus, we have "proof of principle" that our tailor-designed human tumour outside the body indeed can be used for investigation of chemical toxicity. However, continued validation work comparing with established *in vivo* data of the outcome of treating with compounds is needed to further strengthen the use of this model. So far, we have co-cultured cancer cells with normal fibroblasts, however, in a tumour the fibroblasts have another phenotype than that of normal fibroblasts and are defined as CAFs. One of the cellular sources of CAFs are normal fibroblasts that are induced to undergo a phenotypic change under the influence of e.g. TGF-β1. We have investigated if TGF-β1 treatment can induce a phenotypic shift of the fibroblasts in our model. In addition, we characterized the proteins deposited by the cells to ascertain that the ECM in our tumour *in vitro* is similar to a human tumour specifically as we are using a synthetic scaffold. Furthermore, a deeper knowledge on cell-cell, cell-ECM interaction, ECM synthesis and many other cellular and molecular aspects of the

TME within a biologically complex microenvironment can be obtained. These data can elucidate the mechanisms of cross-talk between cancer and stromal cells and reveal the contribution of soluble factors in tumour progression and the response to therapeutic agents. In our study, the focus was on studying human breast cancer cells and normal fibroblasts, but this 3D system can provide an excellent *in vitro* model to investigate a wide variety of cell lines. Furthermore, for the future, this research group is interested in the possibility of extending the system further with other cells of the TME, *i.e.* immune cells and endothelial cells. Also, this complex co-culturing model can be used to investigate the presence of CAFs as the major component of the tumour stroma to further elucidate the potential role of the stromal cells in therapeutic resistance.

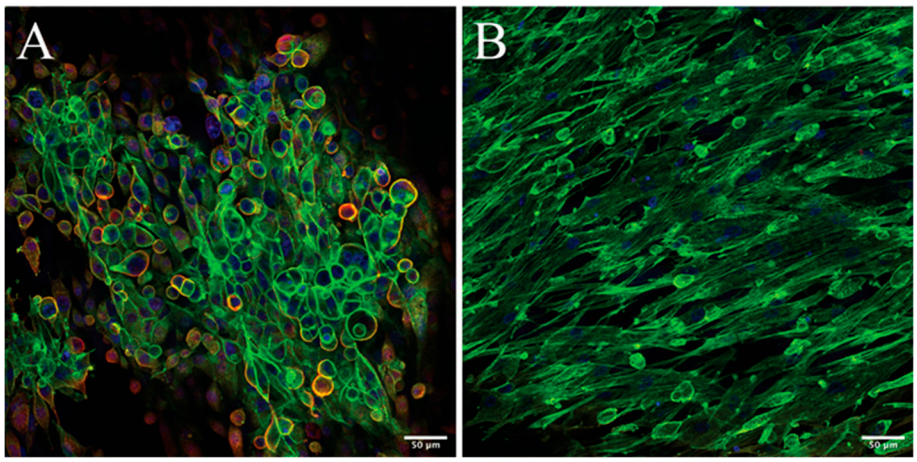

Figure 18. Confocal microscopic images of 3D mono-cultures incubated in totally defined animal product free medium. JIMT-1 cell (A) and HDF (B) 3D mono-cultures were grown for one week in totally defined medium (not yet published, in cooperation with Ólöf Birna Rafnsdottir, M.Sc.). The cultures were fixed in 3.7 % formaldehyde and labelled to visualize CD44 (red), actin (green), and cell nuclei (blue). Scale bar indicates 50 µm.

Although, immunotherapy is currently one of the promising modalities in cancer treatment, only a limited number of studies have investigated the application of immune cells in 3D culture systems. Understanding the specific role of the TME component and its effect on cancer and other normal stromal cells is crucial for improving immunotherapy strategies. Adding immune cells to our human 3D *in vitro* tumour model will bring it to the next level as these are important components of the tumour stromal compartment, contributing to cancer treatment and cancer progression. Therefore, the combination of incorporating immunotherapy within 3D tumour models will be likely to enhance the development of effective "*in vitro*" tool

for personalized cancer therapy. On the other hand, the synergistic combinational therapies targeting TME and immunotherapy may be the direction of future research. A further development in complexity of the 3D tumour *in vitro* is the incorporation of endothelial cells and cues to stimulate blood vessel formation.

Many scientists may still believe that animal research is the golden standard for obtaining a definitive result regarding the suitability of compounds to be used in the clinic and at which concentration they should be used. However, integrated testing strategies initially using 3D cultures followed by limited number of high-quality animal experiments maybe a promising tool at this stage for finding a treatment for various cancers. 3D models still are not fully developed and they come with their own obstacles and challenges like any other pre-clinical systems. However, simplicity of the 3D system can be an advantage at the beginning of its development, in order to reduce the threshold for researchers to start using it. The model presented here is easy to use, cost-effective, and can be used in early pre-clinical studies. We hope that the results from this work may persuade other scientists in academia and industry to switch from conventional tumour models over to 3D and pave the way for future 3D studies with minimal use of animal-derived products in the field of cancer research. The more research groups will be willing to come out of their comfort zone to use 3D culture models, the quicker the sophisticated 3D systems will be developed and will be widely accepted as a future 3D cell culture.

Obviously, it is still a long way to go and there is a lot more to do before any totally animal product free 3D model can be used as a common *in vitro* tumour model in academia and the pharmaceutical industry. The future will tell how the research scientists would travel this path.

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Athena
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