The Role of Extra-cellular Matrix in Tumour Progression and Treatment Response

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Statement of Originality

This is to certify that, to the best of my knowledge, the thesis content is solely my work, except as referenced or acknowledged in the text. This has not been submitted for any other degree or to any other institution for any other purposes.

Maha Naeem Aman

30/12/2023

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Dr Naisana Seyedasli and I mentored and co-supervised the following students for their research project, oral and poster presentations, and thesis.

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List of publications and conference presentations

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Conference Presentations:

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  The Role of Extracellular Matrix in The Regulation of Tumour Cell Properties and Therapy Response (Poster)

- 2019 - Westmead Hub Symposium, Sydney, NSW, Australia

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  The Role of Extracellular Matrix in The Regulation of Tumour Cell Properties and Therapy Response (Poster)

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Abbreviations

ABC	ATP-binding cassette transporter
ACTA2	α-smooth muscle actin
AD-MSC	Adipose-derived mesenchymal stem cells
AOCS	Australian ovarian cancer study
ATCC	American-type culture collection
BM-MSC	Bone marrow-derived mesenchymal stem cells
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
COX-2	Cyclooxygenase-2
CTGF	Connective tissue growth factor
CSC	Cancer stem cell
CSPG4	Chondroitin sulphate proteoglycan 4
DAPI	4',6-diamidino-2-phenylindole
DBS	Double-stranded breaks
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DPBST	TWEEN 20 in DPBS
ECM	Extracellular matrix
EGF	Epithelial growth factor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular-signal-regulated kinase
FAP	Fibroblast activation protein α
FBS	Fetal bovine serum
FTE	Fallopian tube epithelium
GAG	glycosaminoglycans
Glut1	Glucose transporter 1
GusB	Glucuronidase beta
Gy	Gray
HA	Hyaluronic acid
HDF	Human dermal fibroblast
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HGSOC	High-grade serous ovarian cancer
HIF	Hypoxia-induced factor

Hh	Hedgehog
HNSCC	Head and neck squamous cell carcinoma
IHC	Immunohistochemistry
IL	Interleukin
IR	Ionising radiation
ITGA11	Integrin subunit α-11
ITH	Intra-tumoural heterogeneity
MCT4	Monocarboxylate transporter 4
MFB	Myofibroblast
mTOR	Mammalian target of rapamycin
NK	Natural killer cells
NTS	Native Tissue Scaffold
OSCC	Oral squamous cell carcinoma
OC	Ovarian cancer
PBST	Phosphate buffered saline tween
PGE2	Prostaglandin E2
PI3K	Ptumouratidylinositol-3-kinase
qPCR	Quantitative polymerase chain reaction
Rho-ROCK	Rho-associated kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SPH	Spheroids (tumour model)
TAM	Tumour-associated macrophages
TCGA	The Cancer Genome Atlas
TEAD	Transcriptional enhancer associate domain
TGF-β	Transforming growth factor-beta
TME	Tumour microenvironment
TNC	Tenascin C
Treg	T cell (regulatory)
TS	Tumour stroma
VEGF	Vascular endothelial growth factor
YAP 1	Yes-associated protein 1
γH2.AX	Histone H2A phosphorylated at Serine 139
µM	Micro-Mole per Liter

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Thesis Abstract

Despite having different anatomical origins, cancers are the second leading cause of mortality worldwide. Due to inherent or acquired therapeutic resistance, most cancer subtypes have a high recurrence rate. Oral carcinoma and ovarian cancer (OC), the two research models examined in this thesis, are both considered to have a poor five-year survival rate with a high prevalence rate. OC develops from the malignant transformation of ovarian or fallopian tube epithelial cells lining the respective anatomy. High-grade serous ovarian carcinoma (HGSOC), the most prevalent and lethal subtype of OC, is distinguished by an aggressive clinical course and a high mortality rate. Although the usual conventional treatment for HGSOC is surgical cytoreduction, most patients are treated employing a platinum-based drug, carboplatin, which kills ovarian tumour cells by producing deoxyribonucleic acid adducts. Despite these therapies, 80% of patients will still develop recurrences because of chemotherapy resistance. On the other hand, oral squamous cell carcinoma (OSCC) is the most common cancer subtype, 90%, among those originating from the head and neck region. OSCC is named after their origin, squamous epithelium, and is driven by epigenetics and dietary factors such as alcohol and tobacco usage. The gold standard of treatment is primary surgical resection, with or without postoperative adjuvant therapy such as ionized radiation or chemotherapy. This complex multifactorial tumour targets key regulatory pathways such as EGFR (epidermal growth factor receptor), CDKN2A (cyclin-dependent kinase inhibitor 2a), and STAT3 (signal transducer and activator of transcription 3). This aggressive tumour has exhibited limited improvement in its prognosis in the last few decades.

It has long been known that a tumour's microenvironment (TME) plays a role in the tumour's survival and resistance to treatment. It comprises a specialized cellular component and a non-cellular component, the latter of which is called the extra-cellular matrix (ECM). Cancer cells and their ECM constantly remodel while interacting dynamically, impacting drug transport, and signalling pathways and leading to tumour cell survival and resistance. The diverse makeup of the cancer cell in conjunction with ECM dynamics makes it difficult to combat therapy resistance. In this study, we established a more reliable way to investigate the effect of ECM on therapy resistance, in both types of cancer cells, by generating cost-effective and reliable *in-vitro* study models.

An indispensable first step in the development of anticancer treatment interventions is the identification of biological alterations present in ECM that may be used to combat these important molecular players. To understand the nexus between tumour cells and their vital link to the TME, and ECM, this thesis focused on the following aims.

- Investigating the effect of ECM on therapy response, particularly in oral and OC cells.
- Establishment of a novel 3D model targeting the study of cancers in the head and neck region, specifically oral cancer, while distinguishing the model from other pre-existing research models through analytical data.
- Establishment of an *in-vitro* model for CAF generation and extracting their secreted ECM for further study of the TME.
- Analyzing OC cells' key response to differently sourced ECMs that can potentially lead to advanced targeted therapeutics.

- Utilizing the data achieved to forecast the model's probable implications for better patient prognosis.

This thesis has been developed in a hybrid format to address the above-mentioned aims, with Chapters 1, 3, 5, and 6 being conventional chapters and Chapters 2 and 4 being manuscripts that are submitted to the journal.

Chapter 1, Introduction, is a traditional thesis chapter that briefly talks about the common cancers affecting a vast population and the cancers that are the primary focus of this PhD. It states the characteristics of each cancer type that link it to therapy resistance, relapse, and metastatic potential.

Chapter 2 is a review article, submitted to BioEssays highlighting the significance of the gel-like matrix, ECM, in cancer homeostasis and its crucial role in modulating tumour behaviour. It further entails the involvement of transformed fibroblasts, known as cancer-associated fibroblasts (CAF), that engage in altered therapeutic response and about the various *in-vitro* cancer study models that have been established so far.

Chapter 3 is a research manuscript.. This article utilizes an established protocol, mentioned in the chapter, and generates a specialized *in-vitro* 3D model that incorporates ECM as a scaffold derived from native tongue tissues of mice. It is further analyzed by comparing its post-radiotherapy efficacy, as a protective milieu for cancer cells, with other models, namely monolayer cells grown in culture and tumour spheroids. We assessed this model by incorporating OSCC cells. Detailed transcriptomic data highlights the essential criteria our *in-vitro* model matches to reproduce an *in-vivo* tumour tissue.

Chapter 4 is a traditional research chapter discussing the critical role of CAF in OC progression. It also elaborates on the research focusing on in-vitro production of CAFs from fibroblasts and extracting their ECM to study their modulatory effects on the OC cell response to chemotherapy.

Lastly, *chapter 5*, cohesively summarizes and interprets all the data accumulated throughout this study confirming the existence of the ECM in altering cancer cell therapeutic response and supporting the hypothesis that analyzing the tumour response of the modulated tumour ECM, can provide insight towards the mechanism of action and tumour interaction leading to new drugs and developing more effective targeted therapies.

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Author's contribution statement

Chapter 1: The author compiled and wrote the chapter along with the preparation of the figures.

Chapter 2: The author compiled and drafted the manuscript along with the preparation of the figures.

Chapter 3: The author planned and performed experiments along with drafting the manuscript and creating figures with data analysis.

Ms Mara Cvejik assisted with imaging the samples through scanned electron microscopy at the Institute of Dental Research, Westmead Hospital.

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Mr Joey Lai assisted with RNA library preparation at the Westmead Institute of Medical Research.

Mr Grant Parnel assisted with the transcriptomic analysis and produced figures 4 C – H, 5 and 6 through our collaborative project at the Westmead Institute of Medical Research.

Mr Mitchel Lockwood assisted with figure 6 and revising the manuscript.

Chapter 4: The author planned and performed experiments along with writing the chapter and creating figures and data analysis.

Chapter 5: The author wrote the chapter.

Supervisor's declaration

As the supervisor of the PhD candidate, Maha Naeem Aman, I, Naisana Seyedasli can attest that the authorship attribution statements above are correct.

Supervisor's name: Dr Naisana Seyedasli

Date: 30/12/2023

Chapter 1: *Introduction*

1.1 Cancer

Cancer is one of the most prevalent illnesses in the world, and although it can affect people of any age and of any gender, its fatality rate is much greater in those aged 60 and older [1, 4, 5]. According to recent studies, the number of new cancers diagnosed in those aged 65 and 80 would double and triple respectively, globally in the next two decades, with significant regional and national variation [6]. If a patient manages to escape this deadly ailment, almost all cancer survivors have to deal with deteriorating neurocognitive after-math alongside emotional and financial repercussions [7]. Cancer differs from other infectious and environmental illnesses and disorders by developing within a living organism as opposed to through the introduction of a foreign body, and as a result, branching into multiple subtypes that can be distinguished by their sources (fig. 1.1) [8]. This unrestricted growth of tissues enabled by cancers is targeted at different cell-cycle phases and cellular components for a better prognosis. However, some of the resilient cancer cells survive anti-tumourigenic therapy resulting in treatment resistance and metastasis [9]. Every treatment mode is cancer-specific and can have one or more therapy forms involved.

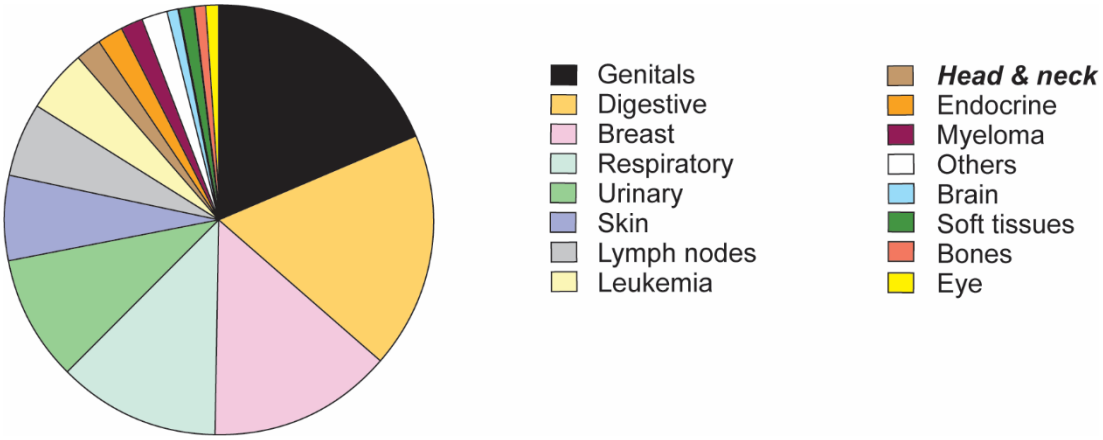

Figure 1.1 The prevalence of the major cancers seen worldwide [3, 7]

1.2 Cancer models discussed in this thesis

This thesis discusses two of the most common cancers with a very high mortality and recurrence rate, head, and neck squamous cell carcinoma (oral cancer) and ovarian cancer.

The cell lines used for experimental procedures are:

Oral cancer : SCC 25, derived from the tongue and collected by American Type Culture Collection (ATCC)

Ovarian cancer : AOCS 15 and AOCS 21 (ovarian cancer), derived from the ascites and collected by the Australian Ovarian Cancer Study (AOCS)

HDF cells used with the above-mentioned cell lines were purchased from Sigma-Aldrich (Merck).

More elaborated details about each cell line are given in Chapter 3 (methods).

1.3 Head and neck cancer

Cancers in the head and neck region are enlisted as one of the ten most widespread cancers worldwide with a mortality rate of more than 50% [10]. Head and neck cancer comprises carcinogenesis in a group of anatomical structures and the carcinomas linked to them hence the malignancies of this region are extremely diverse [11]. This prominent emergence of head and neck cancer encompasses major structures such as paranasal sinuses, nasal cavity, oral cavity (including lips, tongue, and salivary glands), larynx, pharynx, and the throat (fig. 1.2). Although there are many histological presentations for these carcinomas, one of the most frequent is seen originating from the squamous cells lining the mucosal surfaces, and hence named as squamous cell

carcinomas. Following the anatomical branches of the head and neck region, *oral squamous cell carcinomas* (OSCC) have one of the highest incidences and recurrence rates with less than a 5-year survival rate making it an extremely lethal cancer too [12-14].

Oral cancer is shown to be frequent in 3-25 % of all new cancer cases across countries in low to high-risk countries where tobacco and areca consumption is particularly popular, such as South Asia. Other contributing variables include genomic instability and excessive cigarette and alcohol intake [15, 16]. OSCC is particularly infamous for its deceptive onset and hence challenging diagnosis. The rapid progressive state of this cancer is typically accompanied by metastases and disfiguring treatments [17]. Therefore, OSCCs have been the primary focus of this project. Their therapy-resistant nature and treatment modalities are discussed in detail in Chapter 4.

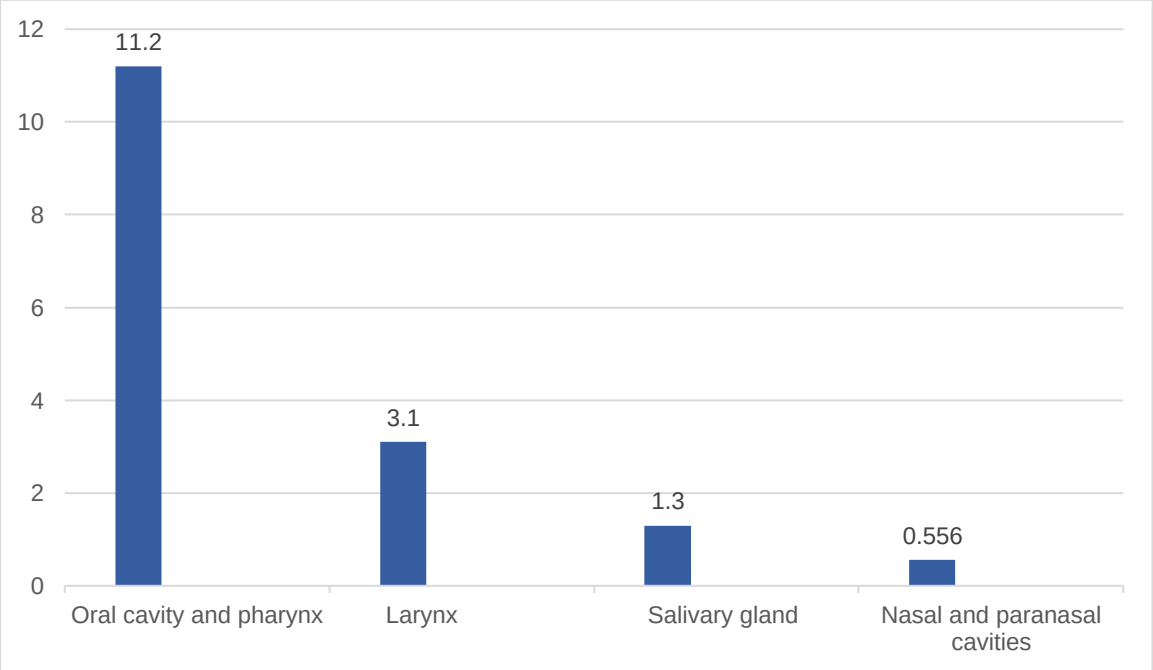

Figure 1.2 Incidence rate (/100,000) of head & neck cancers [1]

1.4 Ovarian Cancer

Ovarian cancer (OC), also sometimes referred to as the silent killer as it is frequently undiagnosed until a very late stage, is the most fatal cancer out of all female reproductive cancers with a 5-year survival rate of less than 45% [18, 19]. OC is the second most prevalent cancer type seen in women, particularly those over the age of 40 years [20]. Although morphologically classified, OC has several sub-types that are differentiated by origin and molecular biology, they all are treated as a single entity [21]. OC are broadly classified as epithelial and non-epithelial cancers which amount to 95% and 5% of diagnosed cases, respectively [22].

Epithelial OCs are further histologically divided into high-grade serous, low-grade serous, clear cell, endometrioid, and mucinous ovarian cancers with high-grade serous ovarian cancers (HGSOC) being the most common type and hence that is the core focus of this candidature (fig. 1.3). [22, 23]. Both cell lines used, AOCS 15 and AOCS 21, belong to the HGSOC family. HGSOC has been known to involve multi-gene mutations such as P53, BRAF, KRAS and PIK3CA and several pathway alterations including RB1 and NOTCH, according to the cancer genome atlas (TCGA) [24, 25]. Chapter 5 covers specifics on their therapeutic resistance and treatment options.

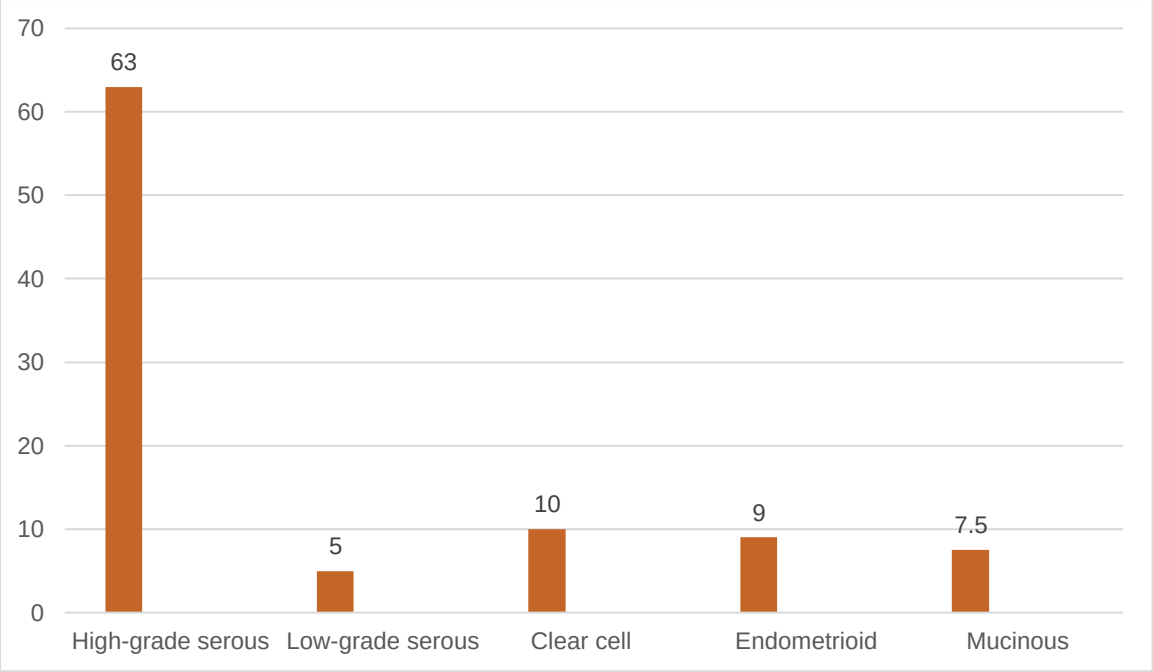

Figure 1.3 Prevalence (%) of histologically distinct epithelial ovarian cancers [26]

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1.5 Heterogeneity and commonality

Several factors demonstrate how tumours with various aetiologies each have distinctive traits, but there is also something fundamental to all cancer types that may serve as a common therapeutic target, both of which are discussed below.

1.5.1 Intra-tumoural heterogeneity

Metastasis, therapy, and relapse are widely based on some factors that are similar for most cancers and distinct for some, such as cancer-specific growth factors and mediators. Intra-tumoural heterogeneity (ITH) is one such common concept that allows cancer cells to escape the apoptotic effects of therapy [27-29]. ITH, defined as the existence of several clones of cancer cells within a single tumour mass, and intra-tumoural heterogeneity, defined as the presence of various genetic mutations in separate metastatic tumours from the same patient, are two types of heterogeneity that both lead to tumour evolution making them pass through the ‘survival of the fittest’

[9, 30]. Scientists have described ITH via two models - the cancer stem cell (CSC) model and the clonal evolution model, also called the stochastic model (fig. 1.4) [31, 32]. The first posits that tumour growth is driven by a small subset of cells with self-renewal capacity known as cancer stem cells (CSC) that are capable of tumour initiation, metastasis, and relapse and that the heterogeneity seen within a tumour, results from the differential positioning of these cells along the stemness hierarchy. The second theory argues that precancerous and cancerous cells accrue genetic and epigenetic modifications over time, owing to their inherent genomic instability. In a Darwinian-like evolutionary process, these alterations eventually confer selective benefits on the cells and resulting subpopulations can trigger tumourigenesis in their isolated space [33, 34]. As a result of this clonal evolution therefore, subsets of cell populations, each with their metabolic profile, phenotype, cellular level of plasticity, and unique response to endogenous and exogenous stimuli and drugs will form which will further complicate the treatment [35]. ITH has been repeatedly associated with tumour advancement and therapy resistance due to its link to highly complex biological mechanisms that encapsulate temporal occurrences idiosyncratic to each patient [36]. ITH has been seen in a variety of malignancies, including renal, prostate, ovarian, breast, and oral cancers and the findings indicate that variability exists not only at the genomic but also at the epigenomic and transcriptome levels [37-39].

Studies based on oral cancer's ITH demonstrated that malignant cells varied within and between tumours in their expression of programs related to cell cycle, hypoxia, stress, epithelial differentiation, and epithelial-to-mesenchymal transition (EMT) [40]. A hypoxic environment is a characteristic feature of oral cancer cells that initiates many transcriptional programs including those involving the key hypoxia-related

transcription factor, hypoxia-induced factor (HIF-1) [41]. The diminished supply of oxygen to cells leads to the stabilisation of this fundamental hetero-dimeric structure that regulates a gene pool responsible for various pathophysiological processes such as cell survival, proliferation, motility, biogenesis, extracellular matrix (ECM) function, inflammatory cell recruitment and angiogenesis by inducing the expression of their downstream target genes [42]. Hypoxia is a key biochemical factor in the tumour microenvironment (TME), regulating cell resilience and tumour heterogeneity [43-45]. The importance of ITH has been further highlighted by several studies which have shown delivery of small interfering RNA against TWIST1, a transcription factor embroiled in chemoresistance and EMT, sensitized ovarian tumours to platinum drugs and anti-tumour efficacy was further improved if nanoparticle carriers were coated in hyaluronic acid and therefore targeted to CD44+ cells [46-48]. KMT2C, KMT2D, and SMARCA4, which are the most often changed across all molecular subtypes, are some of the most typically reported epigenetic modifiers arising from ITH [49, 50]. It is critical to investigate the environment in which a cancer cell evolves to comprehend and assess the evolutionary pattern and the negative consequences connected with it, as both internal and external, i.e., genetic, and environmental variables impact heterogeneity and thus the cancer's fate.

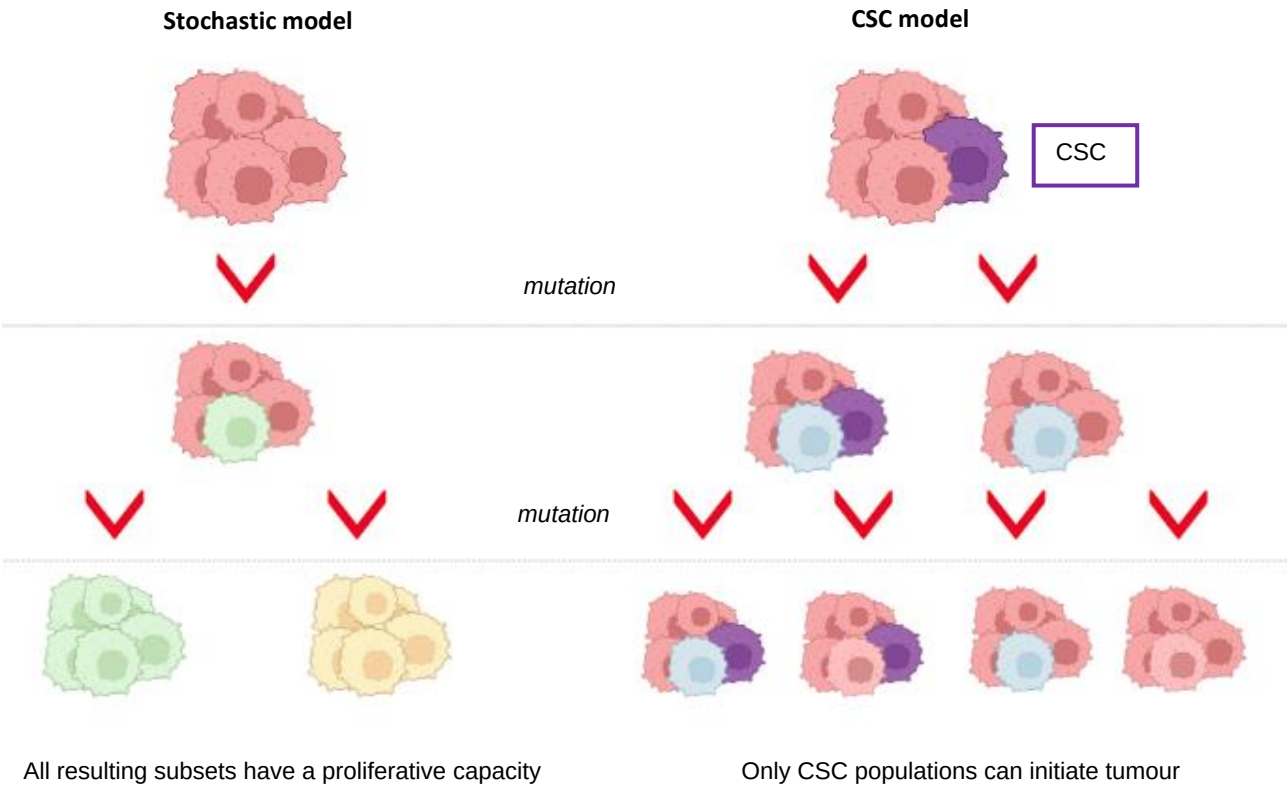

Figure 1.4 The difference between the two theories of ITH [31,32]. This figure is created with biorender.com

1.5.2 Tumour microenvironment

While the ITH is distinct for every clone and every cancer, the TME is the common factor found in all cancers regulating cancer growth and survival [51, 52]. A healthy tissue microenvironment provides cells and tissues with an optimal balance of molecules, proteins, and enzymes that govern cell behaviour in the absence of any aberrant cell physiology. Once tumour growth initiates, the cells struggle to locate adequate microenvironment components to support their excessive growth functions [53, 54]. Consequently, tumour cells remodel the microenvironment to overcome its function as a barrier, while trying to develop and disseminate. This entails changing the biochemistry and biophysics of the normal milieu to produce a tumour-specific microenvironment [55, 56]. The TME, which is principally the habitat of tumour cells,

is characterized by a complex matrix of specialized cells and its physiological state has been strongly affiliated with tumourigenesis [57]. The oncogenic transformation of TME triggers a characteristic phenotype of the cancer cells influencing their survival rate [58]. The extracellular matrix (ECM), fibroblasts, basement membrane, mesenchymal stromal cells, pericytes, adipocytes, blood, and lymphatic vascular channels, as well as T- and B-lymphocytes, NK cells, and tumour-associated macrophages (TAM), make up this structure. In the presence of a tumour, this intricate web of cellular networks and channels has been shown to orchestrate anomalous biological processes, assisting in clonal evolution, and facilitating hallmark cancer progression features [58, 59]. Biochemical cues in the TME(also called tumour stroma) can modulate cellular conduct, metastatic potential, and cancer stem cell features. It includes benign and malignant cells, as well as soluble mediators that drive tumour formation [60]. The tumour–stroma ratio (TSR) is the ratio of tumour cells relative to the surrounding stroma. A low TSR means a high stroma content, which contrary to popular belief, has been repeatedly linked a to higher invasion rate, lymph node metastasis and a more deleterious clinical stage of cancer (fig. 1.5) [61-64].

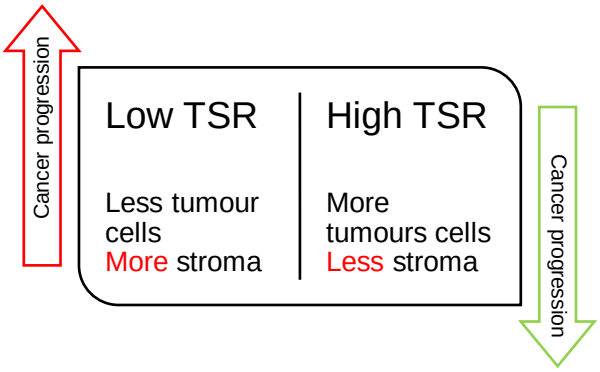

Figure 1.5 shows the balance between TSR and cancer progression [61-64].

As mentioned, epithelial-to-mesenchymal transition (EMT) plays a key role in the establishment of the tumour ITH. EMT is a dynamic process where the cells lose their phenotypical profile of epithelial cells to become mesenchymal [65]. This conversion involves loss of cellular polarity while gaining mobility allowing the cells to migrate from their primary site and spread further to a secondary site causing metastasis. The TME plays a huge role in the induction of this transitional process [66, 67]. TME promotes EMT via a variety of channels and pathways, including those activated by transformed fibroblasts, cancer-associated fibroblasts (CAF), such as transforming growth factor (TGF)-, hedgehog, Wnt, and Notch signalling, Akt pathway, and others. Through cellular proliferation and differentiation, all of these mechanisms promote tumour development and therapeutic resistance [33, 68]. The role of CAFs in OC's TME has been further elaborated in chapter 5. Several research studies have validated that promoters of ATP-binding cassette (ABC) transporters, which collaborate to perform inter-membranous transportation of molecules that are involved in a multitude of cellular processes, bind to EMT-transcription factors and these ABC transporters have been reportedly linked to multidrug resistance [69, 70] suggesting that TME consisting of its cells and ECM induces EMT which eventually results in invasion, metastasis and therapy resistance. There are multiple studies linking EMT, genetic and epigenetic modulations, and a myriad of signalling mechanisms emanating from the TME to tumour cells' plasticity [33, 71, 72].

ECM is a three-dimensional network consisting of a multitude of proteins like collagen, glycoprotein, elastin, fibronectin etc. The acellular structure, ECM, serves in mechanical signal transmission to the parenchyma and as a reservoir for chemokines, cytokines, growth factors, and other signalling molecules by managing their local

concentrations [73, 74]. The structural composition of the ECM permits it to provide stability to tissues while also allowing it to transmit mechanical stresses [75]. A further detailed description of ECM, its constituents and its role in tumour progression has been described in detail in chapter 2, ‘The roles of the extracellular matrix in tumour progression and treatment response’. Both oral and ovarian cancers have a common mode of metastasis – through the ECM [76, 77]. The ECM is required for the TME's survival and the onset of metastasis. The contents of this dynamic structure, as well as any communications between the ECM and the cellular content, are critical for the TME's functionality [78]. These cellular-acellular interactions play a direct role in weakening the host defence and initiation of ECM remodelling, both of which contribute to treatment failure. Furthermore, ECM draws immune cells into the TME, particularly TAMs, which have been shown to maximize CSC survival while limiting the recruitment of anti-tumourigenic immune cells [79, 80]. It can help CSC immune escape by activating pathways including PI3K/AKT, which has been identified as a pro-tumourigenic survival pathway. Furthermore, ECM has the potential to resist T cells from reproducing and activating, which are integral for the destruction of CSCs [81]. The current research focuses on one of the most fundamental components of the TME, the ECM.

OSCC cells, like any other cancer cell type, undergo biochemical and biological changes to sustain their invasive profile. These modifications are procured by the TME compartments that include both, TME cells and the ECM [82, 83]. The first stage of metastasis in solid epithelial cancer is the invasion of cancer cells into the underlying stromal compartments, which happens when they cross the basement membrane, a physiological barrier between the epithelium and other extra epithelial tissue

structures. The ability to invade and metastasize is a key aspect of cancer growth [84]. As previously stated, the ECM is made up of various components, each of which plays a pertinent purpose in allowing the tumour to meet a metastatic potential by incorporating radio- and chemotherapy resistance. ECM remodelling, which occurs as part of tumour-mediated TME remodelling, boosts glucose metabolism in cells and tumours, providing them with the excess fuel they need to proliferate exponentially [85, 86]. As a result, Glucose transporter 1 (GLUT 1) is overexpressed in tumours, resulting in radiation and chemotherapy resistance leading to poor prognosis [87]. Matrix metalloproteinases (MMPS) are multifunctional proteases or enzymes that have an integral role in cancer advancement by stimulating locomotion, influx, and angiogenesis by degrading the majority of healthy ECM proteins during cell differentiation, growth, and tissue turnover [88, 89]. In multiple studies, MMP expression has been reported to be considerably greater in OSCCs [90, 91]. MMP has also been acknowledged as inducing EMT as one of the prime features of the TME [92]. Periostin is another component that is notably overexpressed in the ECM of OSCC. This protein enhances tumour-initiated angiogenesis, cell motility and dissemination. It is also an EMT driver that accelerates MMP-9, MMP-10, and MMP-13 expression, which as highlighted earlier, breaks down ECM, further aiding in metastasis and invasion [93, 94].

Unlike other solid tumours, ovarian cancer (OC) sheds single cells or aggregates by extending into the peritoneum and eventually invades the ECM [76]. The modification of ECM in the TME of fallopian tubes has been associated with tumourigenesis in several studies. In TP53-mutated fallopian tube epithelium (FTE) cells, augmented laminin 1, an isoform of one of the heterotrimeric chains of the glycoprotein laminin, is

dramatically elevated compared to normal FTE cells and P53 mutations have been demonstrated to influence laminin expression in MCF-10A cells, resulting in protein expression in the acinus and alterations in cell polarity [95-97]. Therapy resistance, which is the focal point of this project, has been studied to be caused by excessive ECM remodelling that activates numerous signalling pathways such as integrin and cytokine-mediated events that are implicated in tumour growth and survival [98, 99]. According to further research on the matter, suppression of ECM-integrin signalling by focal adhesion kinase (FAK), inhibition reduced drug resistance and triggered death in OC cells, proving that ECM-cell communication is also pivotal in OC cells' response to platinum drugs. Proteomic analysis revealed that ECM proteins were enhanced in the samples of chemo-resistant OC patients [100, 101].

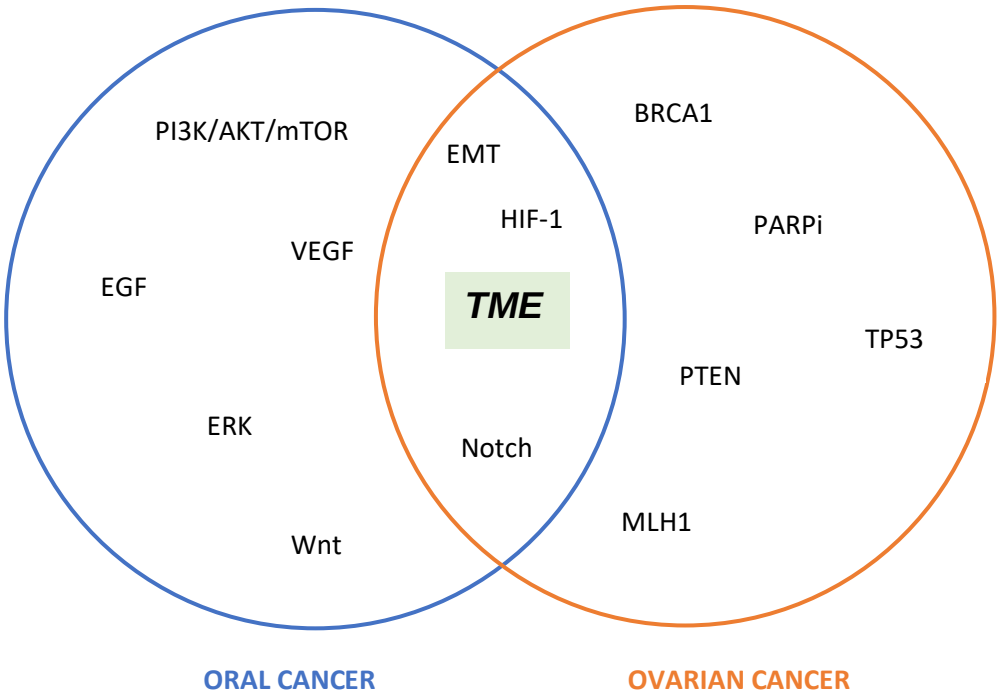

Figure 1.6 Common genes, pathways, and other factors between oral and ovarian cancer [24,25,41,50,33,68,81,121,122]

Chapter 2: [Manuscript] *The Roles of the extracellular matrix in tumour progression and treatment response*

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Abstract

Extra-cellular matrix (ECM) has been established to be a significant cell growth promoter and regulator by monitoring inter- and intracellular communications. In addition, it has also been under the recent spotlight as a target for cancer treatment. The tumour microenvironment (TME), which is the ecosystem within which the tumour expands and grows, influences the ECM surrounding healthy cells and structures to undergo mechanical changes enabling the malignant cells to thrive through several mechanisms including bypassing the immune surveillance. This process is aided by the recruitment of cancer-associated fibroblasts (CAF) further allowing metastasis. This review highlights the present state of knowledge on ECM's dual pro- and anti-tumourigenic roles discussing the challenges involved in ECM-targeting therapeutic approaches.

Introduction

The extracellular matrix (ECM) has been established to be a significant cell growth promoter and regulator by monitoring inter- and intracellular communications. As such, it often contributes to carcinogenesis as an interface of cell-cell and cell-matrix communication as well as an aid in growth and metastasis. Here, we have critically reviewed the literature on the basics of ECM composition, its alteration and remodelling during carcinogenesis and its position as a therapeutic target. In addition, we have looked at the ECM dynamics and interactions with the various stromal components of the tumour microenvironment (TME), and the current state of ECM-based in vitro models. Given the upcoming significance of ECM cancer progression and disease adaptation, a comprehensive overview of this key structural component will inform better and more effective therapeutic decisions.

Cell behaviour is regulated at the interface of cellular and non-cellular control centres

It is a well-known concept that homeostasis being the central tenet of integrative physiology for any living organism, is modulated by the co-existing cellular and non-cellular components in all tissues and organs [2, 3]. While the cellular elements including the cell's powerhouse, mitochondria, nuclei, and other functional structures have been extensively studied, the non-cellular module has received far less attention [4, 5].

The non-cellular compartment essentially called the extracellular matrix (ECM), is secreted by the cells to provide them with the milieu within the body and, also to initiate and regulate signalling mechanisms mandatory for differentiation and homeostasis [1,

6]. Based on analyses using optical and electron microscopy, ECMs have been ubiquitously detected across metazoa, underlying and surrounding the cells while possessing distinctive morphological patterns [7]. The dynamic morphological and compositional changes in the ECM often align with different characteristics and functions of each organ and tissue [8]. The three-dimensional macromolecular structure of the ECM is commonly composed of collagens, glycosaminoglycans, elastin, fibronectin, laminins, and other glycoproteins [9]. Moreover, ECM also binds to growth factors and other signalling molecules which further facilitates its biological and topographical properties as a local reservoir for these molecules [10]. In healthy tissue, these factors are continually reassessed for production and secretion to physiologically remodel ECM to maintain homeostasis [11]. The physical interaction of the ECM with the neighbouring cells and proteins is also a critical balancing factor in cellular regulation [10]. As expected, the ECM-cell interface is heavily regulated during pathogenesis including in carcinogenic events [12]. In fact, the architectural network of ECM has a prominent role in cancer cell homeostasis by fostering the malignant microenvironment [13]. Despite the common role of ECM as a regulatory milieu across different cancer types, distinctive roles have also been demonstrated for ECM in specific cancers [11].

ECM components and their roles in cancer progression and resistance

ECM has been described to be functionally involved in a variety of native and diseased tissue remodelling processes [14]. The structure has been described in two major forms: The interstitial matrix- which operates as a buffer against local and systemic stressors, and the basement membrane- which acts as a physical stand while

coordinating behavioural cues among cell clusters [15]. ECM (Fig. 1A) is a polymeric structure, comprised of a variety of macromolecules details of which will be covered below.

Collagen: Collagen is one of the most abundant components of the ECM, commonly found in the majority of tissues [16]. This key matrix protein is organised into fibrils giving the tissues the structural stability they require [17, 18]. The 28-typed protein has a triple-helical structure that interacts with a vast number of molecules that enable imperative biological processes to occur including providing tensile strength to the cell, molecular filtration, binding to cell adhesion receptors, and basement membrane anchorage [6, 19, 20]. During the processes of cellular metabolism, proliferation, differentiation, and death, collagen serves as an additional channel for communication between cancer cells. The major way collagen interacts with cancer cells is through physically attaching to their receptors [21]. During tumorigenesis, the ECM is extensively remodelled, and hence discrepancies in the collagen structure, composition and metabolism contribute to imbalances in these processes promoting cancer growth hence collagen turnover within the ECM has been acknowledged as an important tumourigenic biomarker [22, 23]. During carcinogenesis, the pre-existing ECM is remodelled to a tumour-specific ECM, which is frequently more linearized, more rigid, and significantly richer in collagen. The high stiffness of tumour tissue is correlated with increased collagen deposition and crosslinking of collagen fibres, which can also affect and drive many stages of tumour progression, including malignant transformation and increased metabolic adaptability as well as enhanced intravasation, facilitating metastasis through PI3K signalling and consequent EMT transition [24, 25]. Fig. 1B shows collagen (green) stained with immunocytochemistry in ECM extracted from fibroblasts, one of the most commonly expressed sources of

ECM. This is elaborated later in this review.

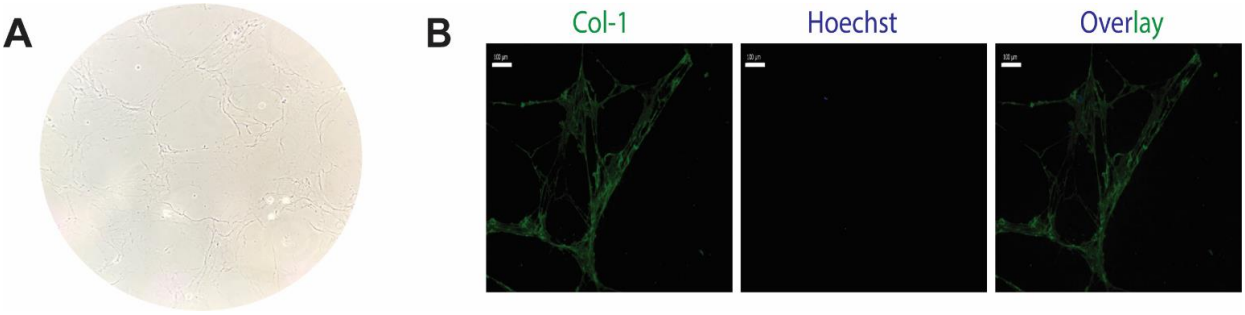

Figure 2 Fundamentals of ECM (A) Core aspects of the multi-dimensional ECM. Green arrows indicate how each segment assists in fighting tumours and the red arrows signify the damage the same ECM molecules might inflict in case of a tumourigenic imbalance **(B)** Key factors and pathways associated with tumour progression through ECM.

Elastin: Elastin is produced and secreted by numerous cell types, including endothelial cells and fibroblasts [26]. Elastin and collagen work in partnership to guarantee the mechanical strength of the ECM. Lysyl oxidase (LOX) catalyses the initial step in the covalent crosslinking of the ECM proteins, collagen and elastin, which subsidise the rigidity and mechanical characteristics of the ECM [27]. ECM macromolecules are proteolyzed to produce ECM-derived peptides which have novel biological functions and have the potential to be used as therapeutic targets. Elastin is one such excellent example of a proteolytically degradable ECM macromolecule [26]. Elastin has deleterious functions in facilitating the growth of tumours, cellular invasion, and adhesion. For instance, the Val-Gly-Val-Ala-Pro-Gly (VGVAPG) elastin is implicated in the growth of tumours due to the peptide's high affinity binding to a specific surface receptor and post-transcriptional control of enzymes such as matrix metalloproteinases (MMP), which are involved in the breakdown of ECM during tumorigenesis [28, 29].

Fibronectin: Fibronectin is a non-collagenous protein that also contributes to the fibrous network of the ECM [30]. This protein is released by fibroblasts and drives cell activity by acting as a fundamental factor facilitating the communication between the intra and extracellular environments by harbouring various binding sites for collagen and other extracellular molecules [31]. Existing in two, soluble and insoluble forms, fibronectin plays a crucial role in cell adhesion, migration, growth, and proliferation. Premature cellular senescence has also been associated with fibronectin overexpression. By acting as a mechanical interface between cells and the surrounding ECM, fibronectin fibrils can modulate mechano-transduction signals as they operate as a bioactive molecule reservoir. Growth factors, other small molecules, and a variety of ECM components that are necessary for and have an impact on subsequent ECM formation and remodelling are bound by these fibronectin fibrils [32-34]. This protein has also been identified as one of the key factors responsible for inducing therapy resistance [35].

Matrix metalloproteinases (MMP): These molecules are the proteases accountable for ECM degradation [36]. Although they are ECM proteins, some of their domains are found intracellularly, highlighting their importance in a broad range of biological processes [37]. They are structured by a variable-length linker peptide known as a hinge region and a 200-amino-acid hemopexin domain, which is essential for integration with other MMPs and their tissue inhibitors [37]. These enzymes are activated via proteolytic cleavage or oxidation [38]. Since not all members of the MMP family have the same substrate to break down, they play their role in both extremes of biological functions of physiological development and pathological conditions such as cancer [36]. Tissue inhibitors of metalloproteinases (TIMPs) limit the action of MMPs, acting as protagonists. As such, a homeostatic environment is dependent on the

equilibrium of these two molecules, which is disrupted in the event of a tumour [35]. Overexpression of MMP1, MMP9, MMP14 and MMP 26 has been co-related with invasive, chemo-resistant and metastatic phenotypes of cancer cells and hence their inhibitors have been identified as anti-cancer agents [39-42].

Proteoglycans (PG): PGs drive a multitude of homeostatic and pathophysiological mechanisms due to their structural complexity and heterogeneity. This idea is bolstered by the fact that they are expelled by all cells and commonly found in all ECMs, making it even easier to carry out their functions. Proteoglycans are comprised of a protein core and one or more covalently linked Glycosaminoglycan (GAG) chains that allow the protein cores to interact with a variety of other proteins [43]. The negatively charged GAGs allow PGs to retain water and cations, allowing them to expand in space and provide lubrication. PGs are widely documented to contribute to the angiogenesis, proliferation, invasion, and metastasis of cancer. In addition to controlling cell metabolism, acting as a sensor of the mechanical characteristics of the ECM, influencing immunological control, and contributing to therapeutic resistance to various kinds of therapy, PGs have a significant impact on wider aspects of the development and spread of cancer [44, 45]. Any alterations in the ratio of PGs, GAGs, or ECM components may be indicative of local TME abnormalities in a variety of malignancies, including breast, colon, glioma, liver, and pancreatic cancers [46-50]. High levels of hyaluronan, a sulphated GAG, have been linked to a detrimental effect in colorectal, breast, and prostate cancer patients [10].

Integrins: These molecules are transmembrane receptors for the ECM proteins that mediate signal transduction and cell adhesion while serving as a connection between different cells and the ECM. Integrin and ECM proteins function in a one-to-many ratio for both entities. Structurally, they are heterodimers with 2 sub-units, each having

particular functions that assist them in carrying out their cellular roles [51]. They topologically regulate cell adhesion by connecting intrinsic and extrinsic triggers. Integrin-mediated tumour stroma sensing, stiffening, and remodelling are crucial processes in the development of cancer stem cell characteristics as well as treatment resistance with the β1 integrin most commonly linked to tumour invasion and lymph node metastasis [52, 53]. The RAS-MAPK and PI3K-AKT signalling nodes are activated by altered integrin expression, which is frequently observed in tumours. Integrins facilitate oncogenic growth factor receptor (GFR) signalling and GFR-dependent cancer cell motility and invasion. Integrins further contribute to the metastatic cascade by enhancing the production of MMP genes, which are in charge of degrading the ECM [54, 55].

As mentioned above, all these components of ECM are known to shift the nature of their key roles in a healthy and tumorigenic environment (Fig. 2A).

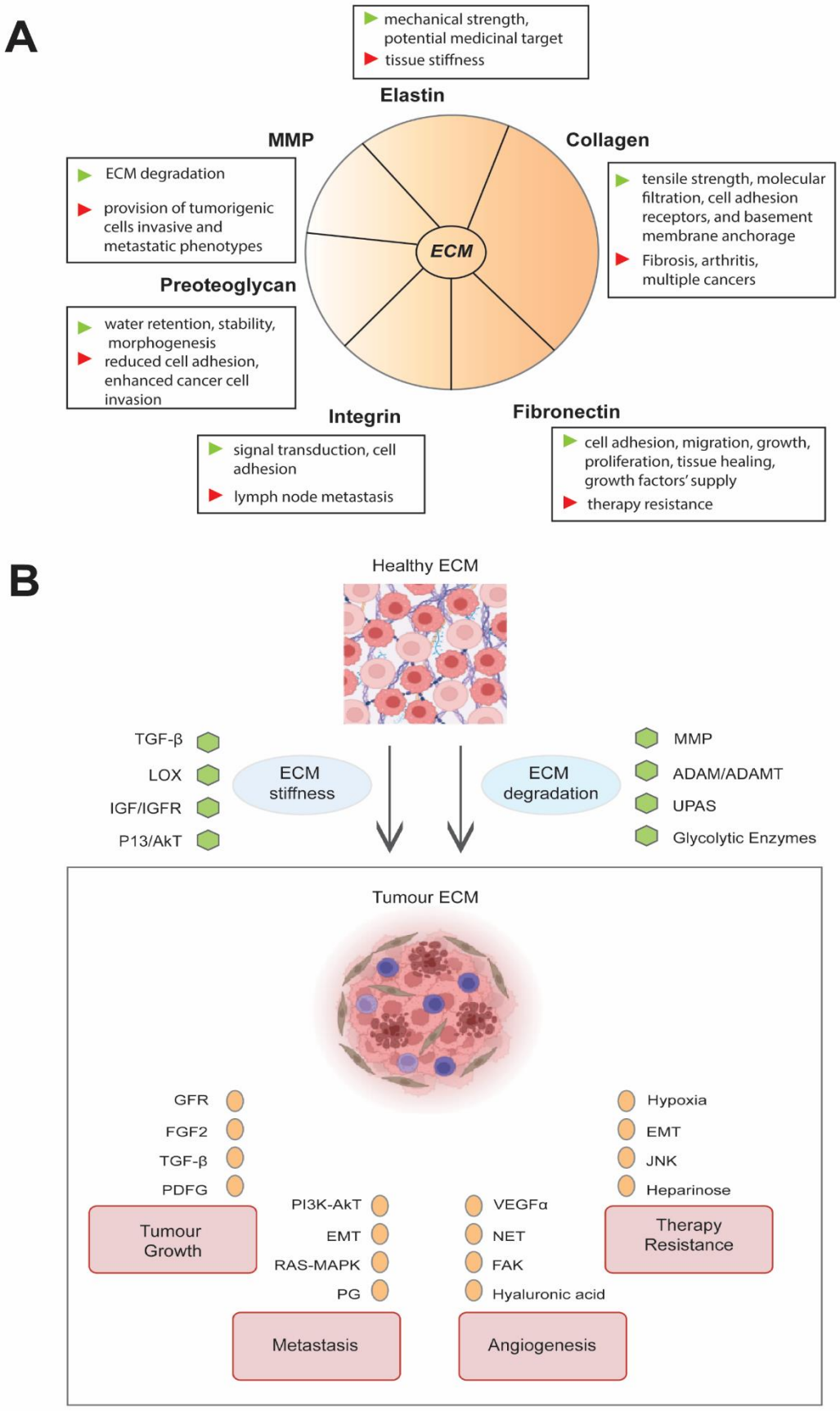

Figure 2 Fundamentals of ECM (A) Core aspects of the multi-dimensional ECM. Green arrows indicate how each segment assists in fighting tumours and the red arrows signify the damage the same ECM molecules might inflict in case of a tumourigenic imbalance **(B)** Key factors and pathways associated with tumour progression

ECM remodelling during tumorigenesis

Two major changes in the ECM are reported to be tumorigenic: Increased stiffness and degradation (Fig. 2B), both of which collectively lead to the growth of cancer cells, migration, invasion, disruption of morphogenesis and angiogenesis [38]. Solid tumour micro-environments are often desmoplastic – fibrotic and dense – lending to the description of them being an unhealed wound, with many similarities in presentation and signalling [56].

ECM Stiffness: The stiffness of the ECM plays a vital role in the tumourigenic process and it is often indicative of the severity of the disease [57]. In solid tumours, this change is extremely common, for instance in Liver cancer [58], breast cancer [57], pancreatic cancer [59], glioblastoma multiforme [60], and high-grade serous ovarian cancer [61]. The separation between malignant and benign tumours for diagnosis in breast cancer has been explored in depth using the elastography strain ratio, a measurement of ECM stiffness, and although it is promising there is yet to be a universally agreed upon value [57, 62]. Given this, unravelling the mechanisms of ECM stiffness has been the focus of many studies.

The mechanisms behind the stiffening of ECM are varied, but collagen has been identified in numerous studies as a key player in the regulation of matrix stiffness [63, 64]. For example, changing the dynamics of collagen interconnectivity through cross-linking via the Lysyl Oxidase (LOX) family of enzymes, including the LOX-Like (LOX-L) enzymes. LOX post-translationally modify telopeptide lysine (Lys) and hydroxylysine (Hyl) on collagen through oxidative deamination [24]. The resulting covalent bonding between collagens as well as other ECM proteins, such as elastin,

greatly increases the stiffness of the matrix. It is unclear if LOX-induced cross-linking occurs in all tumour types but it has been implicated in liver cancer [65], colorectal cancer [66], breast cancer [67], and pancreatic ductal adenocarcinoma [68]. It has also been recently noted that advanced glycation end products [69] and peroxidases [70] also lead to increased cross-linking of collagen in the TME. Collagen crosslinking is comprised of a variety of mechanisms and is strongly linked to cancer progression.

Besides physical crosslinking, another factor is the increase in the concentration of ECM proteins. Commonly up-regulated cancer pathways contribute strongly to ECM deposition, these include TGFβ [71], IGF/IGFR [72], and PI3/Akt [73]. The excessive accumulation of stiff ECM encapsulates the tumour cells can also form a physical barrier contributing to therapy resistance [35, 74]. Further, by encouraging integrin clustering, a stiff ECM can accelerate cell movement, invasion and chemoresistance [75, 76]. The stiff ECM has also been demonstrated to impair epithelial morphogenesis and tissue polarity, which encourages extracellular-signal-regulated kinase (ERK) activation, the central signalling mechanism for cell survival and motility. It also induces focal adhesion kinase (FAK), a cytoplasmic tyrosinase kinase involved in cell migration and angiogenesis [77, 78].

The stiffness of the ECM has been noted to play a role in therapy resistance. The excessive accumulation of ECM that encapsulates the tumour cells can obscure treatment access by forming a dense physical barrier and alter signalling within the tumour cells, for example in Ilk-mediated YAP activation in breast cancer, leading to drug resistance [35, 74, 79]. The desmoplastic ECM promotes a hypoxic tumour micro-environment leading to the upregulation of drug resistance signalling, increasing vascular permeability, and compromising the immunological micro-environment [76, 80]. Additionally, epithelial-to-mesenchymal transition (EMT) has been noted as a

critical contributor to drug resistance [81] and ECM stiffness has been demonstrated to play a guiding role in EMT [64]. In high-grade serous ovarian cancer, increased matrix stiffness through stiffness-dependant tumour-promoting collagen 6 (COL6) induced FAK and YAP-dependant resistance to cisplatin DNA damage [82]. In breast cancer cell lines it was demonstrated that ECM stiffness mediated Sorafenib, a Raf kinase inhibitor, resistance through JNK signalling [83]. However, in a more recent study, it was identified that JNK signalling in a stiff matrix sensitised breast cancer cells to ionising radiation and paclitaxel [84]. The story and exact mechanisms of ECM stiffness-mediated therapy response remain complex, with much still to be understood, but ECM stiffness is playing a vital role.

ECM Degradation: ECM degradation in cancer is undertaken through several main enzymatic groups, including the matrix-metalloproteases (MMPs), the adamalysin group (ADAMs and ADAMTs), urokinase plasminogen activation system (uPAS), glycolytic enzymes, and cathepsins.

MMPs play a mediator role in the cancer microenvironment and are considered a key player in the EMT axis [85]. They not only aid in remodelling, by cleaving and breaking down ECM for new deposition, but expose ‘cryptic’ binding sites for cell proliferation and survival that were previously obscured by the ECM [86]. Additionally, MMP-driven ECM breakdown leads to downstream pathways that aid tumour migration and dissemination. MMP-1,8,11,13, and 16 have been shown to boost the bioavailability of growth factors for tumour cells, augment osteoclastic activity and chemokine cleavage, affect cell proliferation, decrease cancer cell susceptibility to immune surveillance, and promote adhesion, among other tumourigenic functions [87].

ADAMs and ADAMTs are responsible for the degradation and turnover of several important ECM proteins, collagen, fibronectin and periostin [35]. In pancreatic cancer, it was demonstrated that ADAM8 expression correlated with reduced patient survival [88]. The ADAMTs can play tumour-suppressive (ADAMTs 8, 9, and 1) and tumorigenic roles [89]. ADAMTs 5 has been noted as being strongly expressed in endometrial cancer [90].

The UPAS is a series of proteolytic enzymes that are usually tightly controlled under normal physiological conditions. However, its expression is commonly up-regulated in cancer, for example in breast cancer [91], ovarian [92], and skin cancer [93]. It is often localised to the invasive 'front', breaking down the normal ECM for replacement and metastasis [94]. Inhibition of this system has also shown pro-tumorigenic effects. SERPINE1, an inhibitor of the uPAS, has been noted as inhibiting fibrinolysis [95], which in turn encourages the desmoplastic TME. The activation and inhibition of the uPAS both being pro-tumorigenic have been raised in the past and the mechanisms are still being investigated [96], but this apparent contradiction is beginning to be unwound.

Glycolytic enzymes include heparinase and hyaluronidases that cleave heparan sulphate chains on proteoglycans and Hyaluronan [97]. Heparan sulphate proteoglycans – the target of heparinase – are the most abundant of the proteoglycans and play a diverse role in ECM connectivity and cell signalling [98]. Recently, it was demonstrated that the presence of heparinase in triple-negative breast cancer cells increased their resistance to radiation therapy [99]. Hyaluronidases target hyaluronan, also referred to as hyaluronic acid, an essential proteoglycan in joint lubrication and tissue integrity [100]. The hyaluronan-CD44 axis is widely recognised as being an extremely strong driver in cancer stemness and tumorigenesis [101] and

hyaluronidase plays a critical role in the turnover of hyaluronan and its fragmentation for signalling. A recently published study investigated the role of HYAL1, a hyaluronidase, in pancreatic ductal adenocarcinoma and demonstrated that downregulation of HYAL1 decreased proliferation and increased apoptosis *in vivo* [102].

Cathepsins are mainly found as lysosomal proteases that are active at acidic pHs [103]. Their role in altering the ECM in cancer has been noted in breast cancer [104], pancreatic cancer [105], and brain cancers [106]. It was recently demonstrated that 1,2,3,4,6-Penta-O-galloyl-β-D-glucose (PGG) regulates cathepsin-B ECM degradation and could potentially be an anti-metastatic therapy in colorectal cancer by interrupting EMT [107].

ECM degradation plays a key role in the regulation of tumourigenesis and cancer progression.

Therapeutic Targeting of the ECM

The role of the ECM in cancer is apparent and this has led to the development of many therapies with the intent of disrupting ECM-driven carcinogenesis. These therapies can be broadly classified as direct and indirect in their targeting approach.

Indirect Targeting of the ECM

These therapies target the pathways that lead to the deposition and alteration of the ECM. Of the signalling pathways that target ECM deposition TGF-β is perhaps the best understood. The rationale behind targeting TGF-β is that TGF-β is a potent stimulator of collagen biosynthesis [108] and that decreasing the collagen content of

a tumour can increase the effectiveness of adjuvant therapies and decrease the effectiveness of collagen-mediated mechano-transduction in ECM signalling [109, 110]. There have been promising results in pre-clinical models of pancreatic cancer using the TGF-β antibody NIS793 [111], lung cancer using pirfenidone [112], and melanoma using halofuginone [113]. These results have been extended into clinical trials for pirfenidone and halofuginone. For halofuginone, while it has been investigated in stage 1 trials (NCT00027677), results were not posted and no papers were published, it can be surmised that the results were not favourable. Pirfenidone has shown good tolerability in non-cancer conditions [114] and is currently under investigation in non-small cell lung carcinoma (NCT04467723). It is worth noting that the TGF-β anti-body fresolimumab has also undergone clinical trials (NCT00356460) showing preliminary evidence of activity [115], but the lack of further clinical trials since completion suggests that it may not be as effective as hoped. Given the seemingly poor results of therapies targeting TGF-β, there has also been an exploration of small molecule inhibitors of the TGFβR receptor such as Ki26894 [116] and LY210976 [117]. These appear promising but have yet to make it to clinical trial. TGF-β is a difficult molecule to target as it plays far more roles in tumorigenesis than just collagen biosynthesis, and any results should be interpreted carefully as to whether the response was due to ECM-specific effects [118]. Perhaps even more indirectly, losartan affects the major regulatory pathways of TGF-β signalling and has been noted as reducing tumour collagen and hyaluronan [119, 120]. Using the same rationale as TGF-β there exists potential for other growth factor-driven pathways to be targeted such as VEGF and PDGF [121], but indirectly targeting the ECM through growth factor-driven pathways has proven difficult as the ECM is only one of many downstream effects of the blockade [121, 122].

Another approach to indirect targeting of the ECM is to target the cells responsible for the deposition of ECM. In a recent study, it was demonstrated that co-targeting cancer associated fibroblasts (CAFs) with nano drug emulsions of Doxorubicin and siRNA in mouse models of colorectal cancer not only eliminated a significant source of crosstalk between cancer cells and CAFs but also that collagen deposition was significantly disrupted, eliminating a physical barrier to therapy penetration and therefore drug resistance [123]. It has even been recently suggested that theranostic targeting of FAP using CAR-T cells could deplete CAFs within the TME and remove their capacity for ECM remodelling [124].

Direct Targeting of the ECM: The ECM is a dynamic and complex composition, and with that comes a wide variety of targets—these range from inhibitors to using the ECM against itself. Much like with indirect therapies, collagen is a prime target.

Collagenases, a subset of MMPs, have seen extensive development for cancer therapy [125]. Nanoparticles have proven to be the vehicle of choice for delivering collagenases to tumour sites in recent studies, avoiding unwanted degradation of normal tissue [126-129]. These have yet to progress to clinical trials but show great promise as combination therapies in pre-clinical models.

Hyaluronan (HA), also known as hyaluronic acid, a sulphated GAG has also been investigated as a direct target through the development of therapies such as Pegvorhyaluronidase alfa (PEGPH20) – which enzymatically degrades HA. PEGPH20 is currently undergoing clinical trials: HALO-109-101 (NCT00834704), HALO-109-102 (NCT01170897), and HALO-109-201 (NCT01453153). Early phase trials indicate it is acting as expected, depleting HA and decreasing water content in patient tumours

[130]. Unfortunately, PEGPH20 has shown little clinical activity in pancreatic ductal adenocarcinoma and gastric cancer when used as a combination therapy with Atezolizumab [131] and with nab-paclitaxel/gemcitabine [132]. Given its good tolerability, it is likely to undergo more clinical trials, perhaps when the mechanism of failure is better understood.

The LOX family of enzymes is another interesting target for combination therapies. As discussed earlier, LOX aids in the cross-linking of collagen and elastin and through this increases the stiffness of the matrix. Recent studies have demonstrated that combination therapies in *in-vivo* and *in-vitro* models that pan-LOX inhibitors, such as β-APN in breast cancer [133] and PXS-5505 in pancreatic ductal adenocarcinoma cancer [134], can be effective in potentiating front-line chemotherapies in difficult to treat cancers. PXS-5505 is currently undergoing phase II clinical trials (NCT04676529) after favourable phase I results [135]. The LOXL2 inhibitor Simtuzumab has also been tested in phase II trials of KRAS colorectal cancer (NCT01479465) but showed no improvement in clinical outcome [136].

Matrix metalloproteases present a likely target for therapy and several inhibitors have made their way to clinical trials [87]. There have been many inhibitors for MMPs developed but this review will focus on the action of MMP for an example of targeted therapies. MMP-9 has a well-documented role in cancer and clinical trials have explored it as a promising target for combination therapies, for example: andecaliximab. Andecaliximab initially performed well in phase I and II trials in advanced gastric or gastroesophageal junction adenocarcinoma in combination with nivolumab (NCT02864381) but when progressing to phase III (NCT02545504) it failed to replicate the success [137, 138]. MMP-9 has attracted but much attention and as it stands there have been no successful FDA-approved inhibitors [139]. Unfortunately,

given the structural similarities between many MMPs, it is difficult to design selective inhibitors and many of those tested showed non-specific binding [87].

The common theme of both direct and indirect therapies is their capacity to act as combination therapies. The rationale is that they reduce the barrier to treating the cancer cells and this has shown success in the clinic. Given its complexity, in function and signalling, the limiting of the ECM as a target only to be removed to improve other therapies could be a reflection of the models we use and our methods of analysis. As will be discussed in the modelling section of this review, there is much to be improved when it comes to isolating and studying the effects of ECM *in vitro*. However, the promise of the ECM as a source of therapeutic targeting has been demonstrated and the further development of therapies targeting this niche promises to enhance our understanding and treatment of cancer.

Stromal Components of the ECM

The cellular component TME is comprised of more than cancer cells and plays a significant role in the ECM. Fibroblasts are the most obvious and well-studied ECM modulators, but tumour vasculature, immune cells, and exosomes also play a significant role in the composition and function of the ECM.

Cancer Associated Fibroblasts (CAFs): When it comes to interactions between ECM and cancer, CAFs can be found at the forefront of research (Fig. 3). In normal tissue, fibroblasts are responsible for the majority of ECM deposition and modelling, although the fibroblasts themselves are often dormant until needed such as in wound healing [140-142]. Tumour development is associated with the activation of these

fibroblasts. The tumour cell/fibroblast crosstalk triggers a transition of these healthy fibroblasts to CAFs that in turn further restructures the ECM to create a prime environment for tumour proliferation [143]. Although a majority of CAFs are traced from fibroblasts, the heterogeneity of CAFs extends beyond their characteristics, phenotypic indicators, transcriptomics, and functionality to include their sources. These transformed fibroblasts can also originate from cancer stem cells or cells undergoing epithelial-to-mesenchymal transition. CAF induction could be mediated through, or factors secreted by the cancer cells, or another cell type in the TME including immunological, epithelial, and inflammatory cells [142, 144]. Both α-SMA and TGFβ, are shown to be associated with CAF proliferation, ECM stiffness and local invasion and hence they are well-known biomarkers for active fibroblasts [77, 145, 146]. The growth factors produced by cancer cells and infiltrating immune cells, such as Fibroblast growth factor 2 (FGF2) and Platelet-derived growth factor (PDFG), play a prominent part in stromal fibroblast recruitment to the tumour, hence providing an additional biomarker for the fibroblasts [77].

CAFs both synthesize and degrade the major components of the ECM. It was shown that CAFs are responsible for the deposition and organisation of elongated collagen fibres in head and neck cancer [147], this same study also suggested that LOX and LOX-like enzymes could have been playing a role in this process by cross-linking the secreted collagen. A potential mechanism for this LOX crosslinking was investigated, and it was demonstrated that cancer-cell/CAF/myofibroblast cross-talk leads to the inactivation of PRDX1 which in turn leads to an increase in LOX [148], and through this an increase in cancer-promoting microenvironment much like discussed earlier in this review. Collagen is not the sole focus of research in CAFs, it has also been shown that CAFs secrete fibronectin into the TME and through the combination of fibronectin

and contractility that CAFs exerted on the ECM they promoted invasion. The study demonstrated that in the absence of fibronectin, the contractility of CAFs did not induce the same level of invasion [149]. Interestingly, extracellular vesicle packaged TGF-β1 promoted fibroblast to CAF conversion, as has been widely investigated, but in head and neck cancer this was shown to be further potentiated by fibronectin [150].

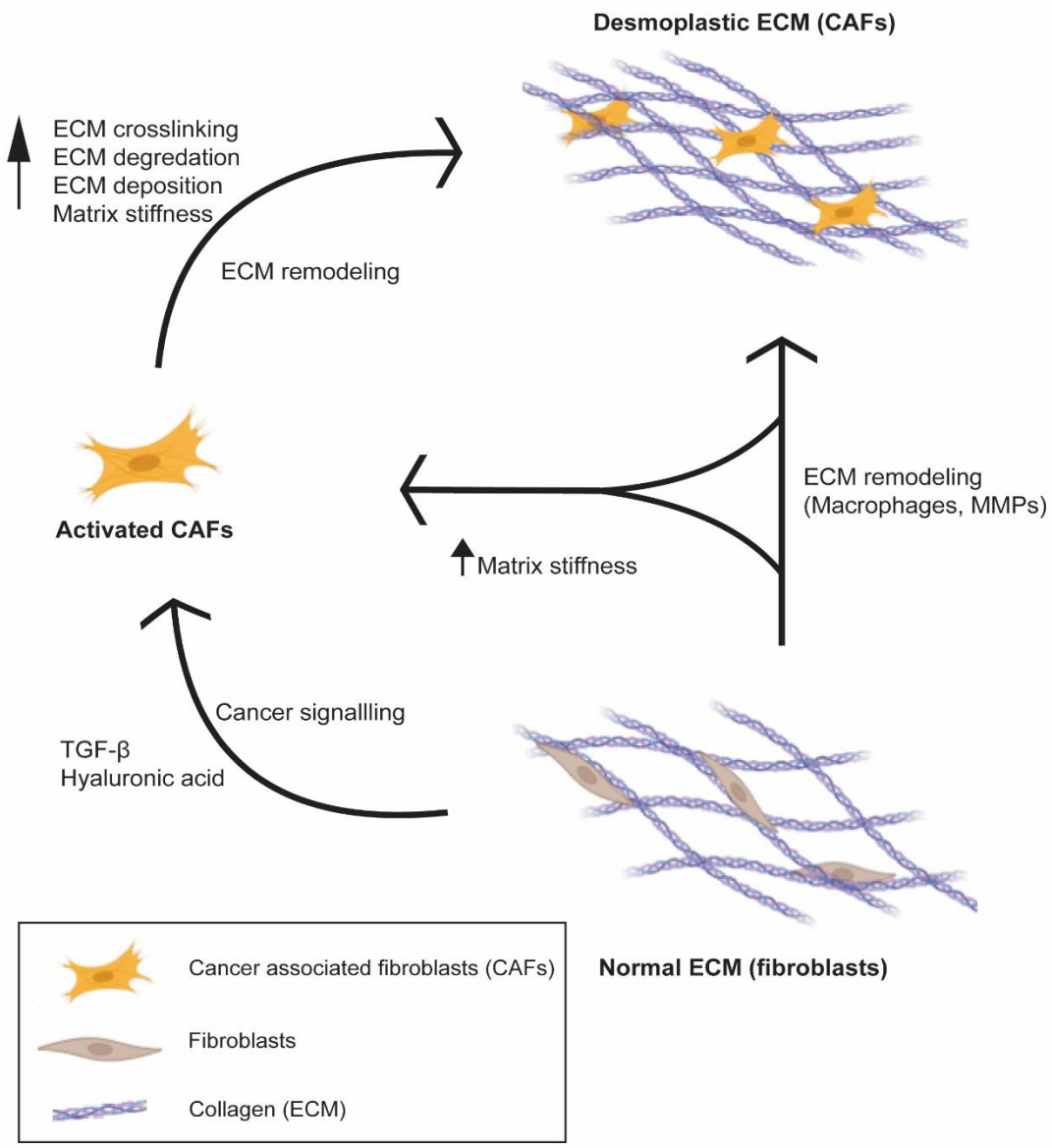

Figure 3 The interaction between CAF and ECM

The role of hyaluronan has been touched on multiple times in this review, and it is unsurprising to find CAFs playing a role in its deposition. Hyaluronan synthase 2 (HAS2), primarily responsible for the deposition of HA in CAFs, is significantly correlated with α-SMA myofibroblasts which indicate poor clinical outcomes in oral squamous cell carcinoma [151]. A recent study demonstrated that not only do CAFs secrete HA, but they are also responsible for the degree of crosslinking of HA and through this the resulting malignancy in breast cancer. The study revealed CAF downregulation of genes responsible for crosslinking HA increased malignancy and that restoration of this pathway could present a new target for future therapies [152]. The deposition of ECM components by CAFs plays a large role in driving the malignancy of tumours but they are also responsible for the breakdown of ECM. It has been known for some time that CAFs secrete MMPs to remodel the ECM [153]. It was demonstrated that the combination of CAF-excreted MMPs and CAF contractility creates tracks in the ECM for the invasion and migration of cancer cells [154].

Given their large role in the shaping of the ECM, it is unsurprising that there has been development of therapeutic approaches whose goal is to eliminate the action of CAFs [123, 124] – as was discussed in the targeting section of this review. The exact populations, markers, and their actions vary from cancer to cancer in their grouping although there have been studies that have attempted a pan-cancer classification system. In non-small cell lung cancer, it was recently reported that the CAF subpopulations could be separated into three separate populations that influenced clinical outcomes, these were defined by HGF, FGF7, and p-SMAD2 expression status [155]. In a study across several cancer types – melanoma, head and neck squamous cell carcinoma, and lung cancer – it was found that CAFs could be separated into six pan-cancer groups based on their molecular characteristics. Interestingly, in this study,

it was demonstrated that subtypes could be separated based on interaction in ECM remodelling or immune modulation (not necessarily mutually exclusive). Two subtypes were involved directly in ECM remodelling and it was suggested that due to increased *TWIST1* expression epithelial-to-mesenchymal transition may be necessary to activate these aggressive CAFs [156]. In another recent study in ovarian cancer, this separation between ECM remodelling CAFs and other functions of cancer has been noted. They separated them into four subtypes and identified one which played a significant role in ECM remodelling which demonstrated high expression of α-SMA, CD29, and FAP [157]. These high FAP aggressive CAFs have become the target of therapies as discussed earlier in this review [124].

The interaction of CAFs and ECM is not a one-way relationship, the ECM also plays a role in modulating CAFs. A recent study demonstrated that advanced glycation end product (AGE) accumulation in the ECM led to increased collagen crosslinking, they then demonstrated that this increased matrix stiffness and subsequently activated fibroblasts to CAFs [69]. The effects of stiffness in the ECM on activating CAFs were also seen in another recent study that explored the relationship between collagen matrix density and CAF activation. The difference is that in this study lower density matrix (less stiff) facilitated the activation of fibroblasts in cancer cell-conditioned media vs the higher density matrix [158]. It is important to note that in both these studies a collagen matrix was used as the base, and while this is somewhat accurate it does not provide the full picture of signalling in the ECM as will be discussed later in the review. It has also been demonstrated that while CAFs are depositing HA [151], HA is also responsible for the push towards fibroblast-to-CAF activation – potentially running a positive feedback loop. It was demonstrated in *in vitro* and *in vivo* models of lung cancer that HA deposition, in a p38/MAPK dependant manner, drives the early

activation of stromal fibroblasts to CAFs and is responsible for priming of the tumorigenic niche [159].

The role of CAFs and their complex interactions with ECM indicate an inextricably linked system that supports the growth and metastasis of cancer. The literature indicates that research is thoroughly underway in unravelling this relationship and its subsequent leveraging into therapeutic strategies.

Vascular endothelium: In normal and pathological tissue the ECM is a key driver in vascular endothelial morphogenesis [160] and the concentration of ECM has been demonstrated to play a role in the organisation of capillary-like networks [161]. The chaotic vascularisation of tumours was demonstrated to be regulated by the degree of collagen crosslinking in *in-vivo*, *in-vitro*, and *ex-ovo* models [162]. This crosslinking primarily increases the stiffness of the TME and stiffness has been linked with increased vascularisation in *in-vitro* experiments [163]. The stiffness of the matrix has also been shown to regulate the VEGF signalling in endothelial cells through integrin-mediated responses [164]. VEGF is a key mediator in the angiogenic process [165] and has been widely recognised as a driver of cancer progression [166-168]. Beyond the mechanical influence on VEGF signalling, It was demonstrated that MMP-13 expressing fibroblasts play a role in the release of VEGF from the ECM where it is bound [169]. The deposition of ECM by the vascular component of the TME is under-researched in comparison to fibroblasts in cancer. However, vascular endothelial cells do deposit ECM components such as fibronectin, VEGFR-1, and laminin [170-172] but the effects of this interplay in cancer have yet to be investigated in depth. There is much research on the effects of ECM on vascular endothelial cells in cancer but not so much on the reverse. There exists an opportunity for discovery within this niche

given the dynamic nature of the ECM and the ubiquity of vascularisation in solid tumours.

Immune cells: The immune makeup of the TME is complex and comprised of many different cell types, including Macrophages, T lymphocytes, B lymphocytes, natural killer cells, dendritic cells, and neutrophils [173] (Fig. 4).

Macrophages: Much of the focus of ECM remodelling is on the role of cancer associated fibroblasts (CAFs), however, this is a disservice to macrophages which play a pivotal role in the process. The role of macrophages can vary on a spectrum between the pro-tumorigenic 'M2' phenotype to the anti-tumorigenic 'M1' phenotype, although this is not a definitive rule, and there is strong evidence that the presentation of phenotype is influenced by the ECM [174, 175]. For instance, it was recently demonstrated in macrophage/monocyte cell lines that a hyaluronic acid/collagen-based hydrogel matrix will initiate a spontaneous polarising of monocyte cells into M2-like macrophages [176]. Adding more weight to hyaluronic acid as a mediator in this polarisation process, hyaluronic acid preconditioning in xenograft models further drove this pro-tumorigenic state with a specific focus on angiogenesis [177]. It was also recently demonstrated in mouse models of breast cancer that the stiffness of the matrix contributes to phenotype expression among macrophages. The study showed that as stiffness of the ECM in tumours increased so too did the expression of M2-like macrophages [178]. In these cases, the cancer ECM and its modelled components drove the macrophages/monocytes towards a pro-tumorigenic state. Interestingly, given the preoccupation with fibroblasts in ECM remodelling, it has been reported that macrophage recruitment precedes CAF recruitment in mouse models of breast cancer and that this initial recruitment correlates strongly to increased tumour collagen deposition [179]. This CAF-macrophage interaction has been noted in the past, with

evidence that fibroblast activation protein-α (FAP) driven collagen cleaving by CAFs causes co-localisation of CAFs and macrophages within the tumour stroma [180]. The crosstalk between these cell types once again highlights the interconnectedness of the TME and suggests that a full understanding of the cancer microenvironment relies on more than just one or two cell types. Another interesting avenue of research is the signalling through ECM proteins such as biglycan on macrophages. It was demonstrated in mouse models that biglycan signals through toll-like receptors 2 and 4 induce macrophage activity [181].

The ECM has a strong effect on macrophages and their presentation, but the reverse has also been thoroughly investigated. It has been demonstrated that macrophages excrete MMP-2 in co-culture breast cancer models, increasing the invasiveness of the breast cancer cells [182]. MMP-2, or gelatinase A, is an MMP targeting basement membrane, non-fibrillar collagen, fibronectin and elastin [87]. It was also recently reported that across 28 out of 33 patients of varying solid tumour types MMP-9 and macrophages strongly correlated to invasiveness of the tumour and poor prognostic outcomes [183]. MMP-9, or gelatinase B, is an MMP targeting basement membrane, non-fibrillar collagen, fibronectin and elastin [87]. This link between MMP-9 secreted by macrophages and invasiveness has also been noted in gastric cancer, Wilm's tumour, and hepatocellular carcinoma [184-186], for examples of recent studies. MMP-9 has attracted but much attention and as it stands there have been no successful FDA-approved inhibitors [139].

Macrophages also play a role in the turnover and deposition of ECM. M2 Macrophages were shown to play a role in not just the degradation of the ECM through MMPs but also the uptake and lysosomal digestion of collagen [187]. Further, it was demonstrated in colorectal cancer mouse models that tumour-associated

macrophages play a significant role in the deposition, linearisation, and cross-linking linking of collagen in the ECM [188]. This further supported the earlier discovery of collagen IV-producing macrophages [189]. This turnover and degradation of the ECM has consequences beyond just the mechanobiological ECM environment. It was recently demonstrated that the turnover of collagen in tumour-associated macrophages can lead to an increase in arginine levels leading to an increase in reactive nitrogen species, which then creates a profibrotic environment that increases collagen deposition [190]. These studies combine to give a picture of macrophages as playing a very significant role in the degradation, digestion, and deposition of ECM and through this the TME. This role in remodelling was also linked to joint activity with CAFs. The relationship between ECM and macrophages is a complex one but one that is also being given due attention in the research process.

T Lymphocytes: T lymphocytes play an extremely important role in the adaptive immune system as part of the systemic response to cancer. Given that T lymphocytes must migrate to their target, it is perhaps unsurprising that the ECM modulation can affect them. They can be broadly separated into CD8+ and CD4+, with CD8+ cells representing the cancer cell killer population whereas CD4+ represents the maintenance of the inflammatory environment [191]. In *in-vitro* 3D models of cancer, the stiffness of the ECM plays a definitive role in the activation and deactivation of T lymphocytes. It was found that in models with higher ECM density CD4+ T cells differentially survived vs CD8+ T cells after 24 hours in culture [192]. The effect of ECM on CD8+ competence in the TME has been demonstrated in real-time imaging on tissue slices in lung cancer and ovarian cancer samples. There were altered migratory pathways for the CD8+ cells based on ECM composition, especially the amount and organisation of collagen fibres [193]. It has also been demonstrated that

the invasive capacity of T-cells is dependent on the expression of ECM genes and that better responders to immunotherapy are partially based on the ECM loss in the tumour [194]. T lymphocytes can also influence the ECM. Somewhat counter to the role of CD4+ as less damaging to the ECM of the TME, it was shown that CD4+ T cells can stimulate cytotoxic T cells to secrete MMPs and through this increase their ability to invade the TME through the degradation of ECM [195]. In a recent study, it was suggested that ECM and CD4+ helper T cells be used as a combinatorial biomarker in triple-negative breast cancer based on the collagen content of tumour samples and the patient response [196]. The composition of the ECM plays a role in the modulation of T lymphocyte competence, but in addition to this T lymphocytes are also impacting the ECM.

B Lymphocytes: B Lymphocytes have been demonstrated to play a role in the regulation of the immune environment in solid tumours. This has been controversial as they appear to play both pro and anti-tumorigenic roles [173]. In the context of ECM interactions, it has been demonstrated in oral squamous cell carcinomas that B lymphocyte infiltration into solid tumours provides an indicator of positive outcomes [197], and this has been linked to stromal complexity and an inverse relationship between high levels of interstitial fibrosis (collagen driven) and CD19+ B cells on predicting outcomes [198]. In mouse models of hepatocarcinoma it was shown that the depletion of B lymphocytes reduced fibrinogenesis, the authors suggest that B lymphocytes drive fibrinogenesis through direct secretion of pro-fibrotic cytokines such as TNFα and IL-6 [199]. However, the mechanics of this interaction have yet to be fully uncovered. There exists a niche for discovery here. Discovering how the ECM affects B lymphocytes and their migration into the TME could potentially introduce new therapy modalities.

Natural Killer Cells: The infiltration of natural killer cells (NKs) into the stroma of tumours is believed to be regulated by the ECM [200]. The ECM is once again playing a regulatory role in the trafficking of immune cells into the TME. Evidence of this is through knockout studies of heparinase (a glycolytic enzyme targeting heparin, an ECM sulphated GAG) in mice where it was shown the expression of heparinase plays a significant role in the anti-tumour effects of natural killer cells, with increasing heparinase increasing effectiveness of NK cells. The authors state that they believe the action of heparinase to be directly related to its ability to degrade the ECM and through this regulate NK access to tumour cells [201]. Increased extra-cellular Galectin-3, a potent bridge between cell-cell and cell-ECM interaction [202], has been demonstrated to regulate NK cell infiltration in mice models, and through this NK cell-mediated anti-tumour effects through galectin-3 binding to NKp30 [203]. As a soluble, ECM binding molecule, tumour-released galectin-3 could be stored in the ECM. It was also demonstrated in colorectal cancer that NK cells with high expression of matrix-metalloprotease 9 remodelled the ECM to a pro-angiogenic state [204]. These studies combined demonstrate the back-and-forth role of ECM and NK cells.

Dendritic Cells: Dendritic cells play an important role in the anti-tumour immune ecosystem. They are antigen-presenting cells that bridge the gap between innate immunity and adaptive immunity through induction of the T lymphocyte immune response in cancer [205]. Versican, an ECM proteoglycan, has been identified as playing a role in the increased activation of BATF-3 dendritic cells in colorectal cancer. This is mediated through versikine, a proteolysis product of versican, which induced differentiation in conventional dendritic cells towards BATF-3 dendritic cells and through this an increased T lymphocyte activation [206]. This heightened immune response through ECM-derived signalling was also identified with low molecular

weight hyaluronan (HA), which was demonstrated to cause an increased immune response in dendritic cells [207]. This presents an interesting connection to CD44 which is a HA receptor [208]. In dendritic cells, it has been demonstrated that there are specific differences between migrating and non-migrating CD44 splice variants [209]. Interestingly, in dendritic cells, the literature appears to focus more on ECM-derived signalling as opposed to trafficking and action as a physical barrier. As for whether dendritic cells are depositing or affecting the ECM directly, there appears to be little to no literature. Although, this may be due to the highly connected nature of dendritic cells and the adaptive immune system. There is room for investigation here as it seems unlikely that dendritic cells would have no impact on the ECM surrounding them.

Neutrophils: Neutrophils are generally considered the first line of defence in the innate immune system but in cancer, their role is altered, potentially contributing to pro- and anti-tumorigenic pathways [210, 211]. Their role in the context of the ECM has been identified in the release of proteolytic enzymes, whose action is widely to degrade the ECM while also releasing the signalling molecules contained within [212]. It was demonstrated that tumour-infiltrating neutrophils are a major source of MMP-9, which through degradation of the ECM is responsible for the release of VEGFα and through this angiogenesis [213]. The role of VEGF signalling, and the ECM was discussed earlier in this review, demonstrating the interconnectedness of this stromal system and the many cell types at play. It was recently reported that tumour-activated neutrophils, and through them, MMP-9, correlated with poor clinical outcomes in breast cancer patients by supporting invasion and metastasis [214]. The role of neutrophil-mediated MMP-9 in invasion and metastasis has also been noted in an earlier study in lung cancer [215]. MMP-9 is not the only neutrophil-mediated protease

to be implicated in the metastatic process. In mouse models of lung cancer neutrophil elastase and cathepsin G degraded thrombospondin-1, a cell-ECM mediator protein, to promote metastasis [216]. A more recent study focused on the role of neutrophil cathepsin G in hepatocellular carcinoma, showing an increase in metastasis but in this case, it was through the association of cathepsin G and neutrophil extracellular traps (NETs) [217].

NETs, a heterogeneous collection of DNA and granule proteins extruded by neutrophils, have a clear interaction with the ECM – in particular ECM proteolysis [217, 218] – and have been demonstrated to interact widely with carcinogenesis through metastasis, angiogenesis, awakening dormant cancer cells, physically shielding cells, and cancer associated thrombosis [219]. It was demonstrated in mouse models of breast cancer that NETs awakened cancer cells through ECM remodelling, specifically through cathepsin G, neutrophil elastase, and MMP-9 and their action on the ECM protein laminin. Interestingly, this proteolysis was driven by NET preferential binding to laminin [218]. It has also been demonstrated that masking collagen discoidin domain receptor 2 (DDR2), a tyrosine-kinase receptor specific for collagen, can significantly interrupt neutrophil chemotaxis, suggesting yet another strong link for ECM and neutrophil activity [220]. The discoidin family of collagen receptors has recently been shown to drive poor outcomes in pancreatic cancer and breast cancer through interaction with NETs [221, 222]. The role of the ECM in neutrophil-driven cancer interactions is particularly interesting, especially regarding NETs. Interrupting this NET-driven activity in cancer has been recently suggested through the use of drugs such as thrombomodulin [223] but this direction of research is still in its infancy. The mechanics of NET-ECM interaction in cancer have yet to be fully elucidated and may provide valuable insight into the neutrophil-cancer ecosystem.

Exosomes: Exosomes are a subclass of extracellular vesicles, ranging between 40nm to 120nm, which were once thought to be nothing more than extracellular junk. Exosomes are released by many cell types but are especially abundant in tumour-derived cells [224]. Recent research has shown this to be untrue and that they are responsible for a wide variety of tumorigenic functions, of which remodelling the ECM is one [225].

The ECM's composition has been demonstrated to affect exosome signalling in recent literature. For example, matrix stiffness was demonstrated to play a YAP/TAZ-dependent role on exosome release in breast cancer cell lines, with stiffer matrices inducing greater release. The same study also showed that the exosomes secreted were overloaded with thrombospondin-1 and this was stimulating cancer invasion [226]. This stiffness-dependent increase in exosome signalling has also been noted in pancreatic ductal adenocarcinoma, where the authors suggest that YAP signalling may be playing a role in exosome release in this cancer as well [59]. Beyond increasing the release of exosomes, it was demonstrated that the proteomics profile of exosomes also shifted between soft and stiff matrix with increased expression of c-MYC, Jagged1, Sox9, and Hes1 in stiff matrix exosomes [227]. The stiffness of the matrix is intrinsically linked to its ECM components. Each of these studies used collagen-I to model the ECM and while this is undoubtedly a viable model, it may need modulating to mirror the true complexity of the ECM of which collagen-I is only a part. However, it has been shown in sufficient detail that the ECM is affecting exosome signalling.

The effect is not one way, and it has been recently demonstrated that ECM stiffness modulates the signalling of exosomes in prostate cancer which in turn modulates ECM composition. They did this through differential packaging of miRNAs in exosomes and

demonstrated that as ECM stiffness increased so too did miRNAs responsible for various functions of invasion, cell motility, interaction with nerve cells, and ECM remodelling [228]. MMP-13, a collagenase [229], has also been implicated in hypoxia-driven exosome signalling. They demonstrated that in hypoxic conditions in nasopharyngeal carcinoma, tumour-derived exosomes showed increased MMP-13 and this drove the cells towards EMT and invasion [230]. It was not directly covered in the paper, but it can be safely assumed that the action of MMP-13 on its target ECM substrates played a significant role. Beyond degradation, it has been recently demonstrated that exosomes play a role in the establishment of the ECM in human dermal fibroblasts. This is through the enrichment of exosomes with ECM proteins associated with the microsome, MMP-2 and 9, and the signalling molecules such as IL-6 [231]. It has also been demonstrated that exosomes in melanoma can activate fibroblasts to CAFs [232] and, as is explored in the previous section, this drives the remodelling of the ECM. The interplay between the ECM and exosomes is complex but this is being investigated in recent literature.

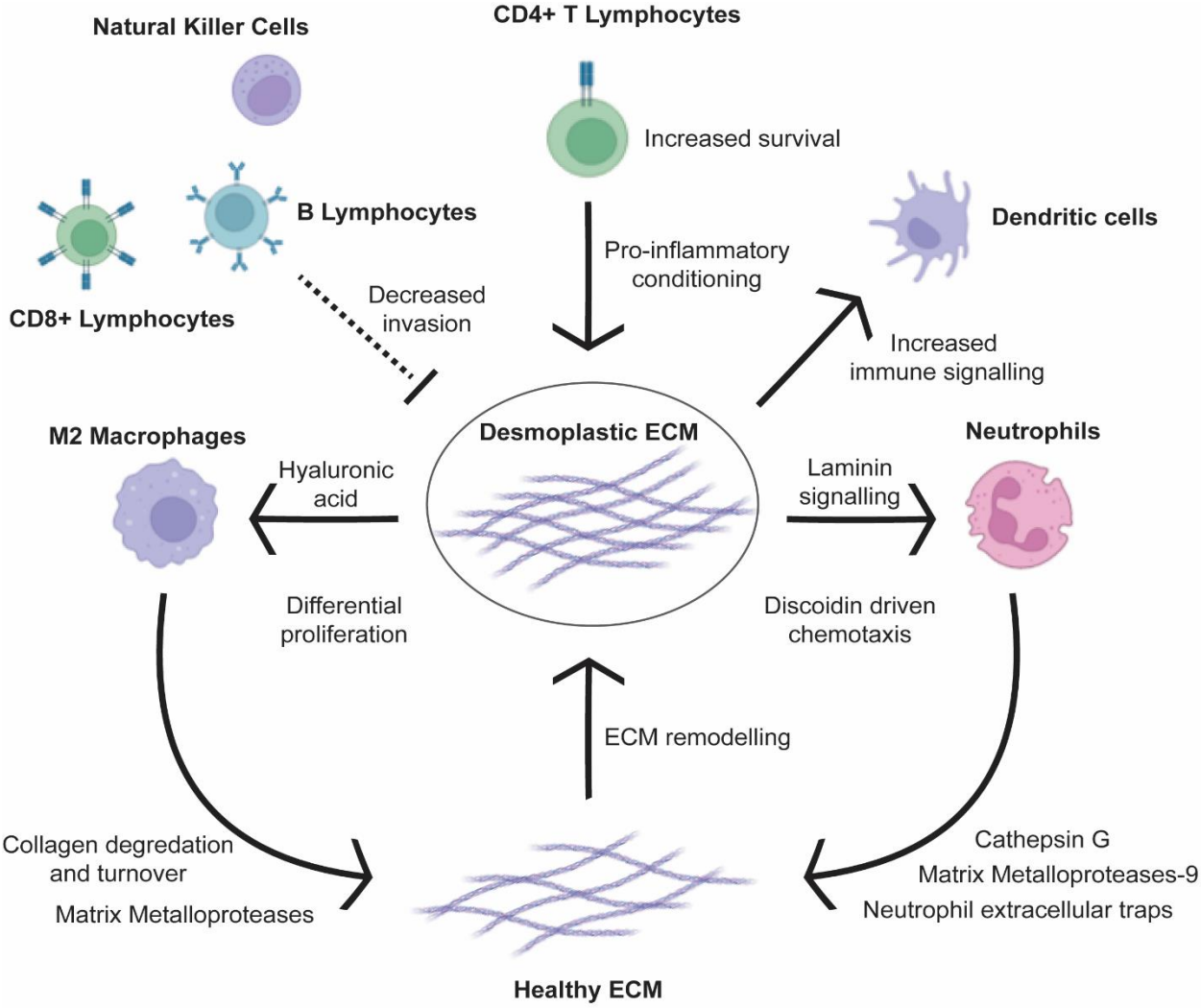

Figure 4 Immune signalling system activated through the ECM

Overall, the message of this section is that the relationship between the stromal components of the TME and ECM is not omnidirectional. In each case, except for dendritic cells, there is documented evidence of the stromal component affecting the ECM and the ECM affecting it. This leads to the question of modelling the ECM. If such complex interplay is present in deciding the final ECM composition of the TME then what model could accurately represent this?

In-vitro ECM models

As described, tumorigenesis has been demonstrated to result in considerable changes in the ECM, such as enhanced deposition leading to a stiffer ECM, crosslinking, orientation, and organization [233-235]. Furthermore, ECM stiffness has been connected to characteristics of cancer cell invasion and migration, and it is observed to be higher in malignant tissue than in normal tissue [236-238]. Tumours may develop resistance to treatment through gene mutations, gene amplifications, or changes in cellular interaction with various drug molecules.

Numerous cancer cell types have been shown to use their interactions with the envir onment to acquire resistance to treatment, either within the primary tumour or during the spread of metastases to other organs. Additionally, an increased matrix stiffness may serve to further promote resistance to specific medicines including chemotherapy [239-241].

Hence, ECM is an important prognostic therapeutic target for a variety of cancers, such as gastric, breast, oral and ovarian, and is best replicated in study approaches using three-dimensional (3D) *in-vitro* models [242, 243]. Cells grown on plastic in a monolayer setup also called 2D experimental models, are far from producing robust data compared to native tumours that cells grown in 3D models. Although the 2D models have been commonly used in many studies since it is cost- and time-effective and easy to manipulate, they are restricted in their potential to replicate physiological variables, resulting in sometimes inconsistent results between *in-vivo* and *in-vitro* studies since cells in tissues and organs are not isolated entities; rather, they are dependent on one another for the coordination of multiple cellular processes, all of which depend on cell-to-cell communication between various cell types and tissues

[242]. Similarly, to control cancer differentiation, proliferation, invasion, and metastasis, tumour cells interact with the matrix and tissue around them as well as the physical and biochemical characteristics of the TME. 2D models overlook spatial cell–ECM and cell–cell interactions and are unable to replicate the intricate and dynamic interactions of the TME [244, 245]. A comparison of tumour cells grown in 2D, and 3D cultures revealed clear differences in the morphological aspect of the cells as well as important biological properties like growth rate, invasive behaviour, and gene/protein expression. Significant differences in the sensitivity of 2D and 3D-cultured cells to either targeted or traditional chemotherapy drugs have also been discovered in various carcinomas such as head and neck squamous cell carcinoma (HNSCC), colorectal and glioblastoma [246-248]. Moreover, 2D attachment of cells results in unwanted morphological changes, such as flattening of the cell, modifications to the cytoskeleton, and changes in nuclear morphology, all of which affect how genes and proteins are expressed [249, 250].

Another commonly used study model is the tumour spheroids (SPH). SPH is in vitro-cultivated, 3D spherical cell aggregates. The characteristics of SPH include an internal quiescent zone encircling a necrotic core and an external proliferating region. This organisation mimics the cellular heterogeneity typical of solid tumours and is dependent on the gradient of nutrients and oxygen diffusion [251]. Therefore, the distinct architecture of spheroids replicates the morphology, proliferation, oxygenation, absorption of nutrients, excretion of waste, and drug uptake of *in-vivo* cells. It also permits the preservation of cell–ECM interactions and signalling, which in turn controls molecular processes and cellular phenotypes [252, 253]. They have been used as a potent tool in cancer research since they presented as a promising means of bridging the gap between *in-vivo* cultures and 2D cultures due to their similar

nutrient distribution and enhanced 3D cellular communication and intercellular crosstalk to true *in-vivo* tumour [254]. Numerous significant facets of the cellular transformation process, including angiogenesis, gene expression and cell-cell and cell-ECM interactions, can be thoroughly studied using these 3D *in-vitro* models [255]. Only compact, stable, spherically shaped cell aggregates that are pliable enough to be moved or manipulated without fragmenting down should be referred to as SPH. The term "spheroids" should not be used misleadingly to describe loose packages of cells that aggregate but do not form tight structures [245]. SPH can be generated through multiple channels and can be used as single or multicellular tumour models. However, all these methodologies are prone to some pros and cons. For instance, using microwell arrays [256] or a rotating flask method [257] to generate SPH is although cost-effective and easy to operate, it is difficult for the SPH production to be reproducible without any variation in the SPH size and shape leading to a variation in the results. Also, these protocols have been reported to result in the inability to stimulate cell-ECM interactions [258, 259]. Hanging drops [260] is one of the other globally utilized methods to generate SPH, and although it allows good control over the size of SPH and easier cultivation of multicellular SPH, handling and transferring of the SPH has been reported to be challenging affecting the success rate of the experiment [261]. Several other techniques such as microfluidic [262] provide a dynamic TME but require professional equipment and are expensive to be run at all research laboratories. Furthermore, microfluidic devices employ materials permeable to oxygen and growth factors affecting proliferation [262]. Another major limitation is the inability of various tumour cells such as breast cancer cell line SK-BR-3 to form SPH [245].

Therefore, designing an ECM prototype that mimics the macromolecular environment and any possible underlying mechanisms is essential. The technologies for the assembly of these micro tumour models are being comprehensively studied and optimized since they represent cell-matrix bio-mechanics and intricate interactions that lead to tumourigenesis [263]. Here we discuss the main ECM-centred 3D models and study approaches developed for diagnostic studies [264].

Microfluidics has received great attention for impersonating ECM. These models, also known as organ-on-a-chip (OOAC), reproduce the physiological and pathophysiological conditions in a way that traditional cultures cannot. These devices, which can potentially also serve as bioreactors, consist of tubular microchannels supporting laminar fluid flow through which, with the help of a syringe or pump, culture medium perfused with growth factors, nutrients and drugs are transported into the system. This allows for the cells to be triggered by recreating biomimetic stimuli such as rigidity and fluid flow, as well as chemical signals such as chemotaxis and oxygen gradients [265]. Furthermore, the microvalve technology aids in the investigation of cell migration and crosstalk in cell co-cultures, allowing for the imaging of cell adhesion and metastasis [266]. OOAC can be designed to accommodate a variation of cell culture methodologies such as a 2D setup allowing to study of cancer cell migration or viability by exposing the cells to a gradient of a solute [267] or using a 3D scaffold to create lumens or tumour compartments, which are usually used to model blood vessels in tumours [268]. They can also be employed as compartmentalised chips where a combination of 2D and 3D cultures are examined with the ability to manipulate the different matrices and cells used [269], or also as a membrane chip. This multi-layered tool can be used to study the interactions between cancer cells and other cell types [270]. The membranes employed are semipermeable thin films with pores that

are frequently smaller than cells. As stated above, they are involved in medium perfusion, cell communication, and the selective transport of drugs, metabolites, and nutrients. However, gas permeability can also be a disadvantage because it can distort the TME through the absorption and adsorption of small molecules. Cell adherence and growth are nonetheless affected by the composition and intrinsic stiffness of certain of the substitute membranes, which are substantially distinct from the native ECM. Moreover, certain materials such as polydimethylsiloxane may produce hydrophobicity, which prevents proteins from adhering to biomolecules and absorbing them [271, 272].

Hydrogels are also used as an ECM-based 3D *in-vitro* model that simulates ECM as a matrix providing a 3D scaffold for tumour cells to grow and invade. Its tumour ECM-mimetic biomaterials composition also shows exceptional potential as preclinical evaluation for the development of drugs [273, 274]. Hydrogels are used to examine cellular drug response, metabolic activity, invasion, and migratory activity employing biomaterials like Matrigel, which is a sophisticated hybrid of ECM proteins, collagen, laminin, or hyaluronic acid to recreate the distinct TME. To elicit the desired cellular response, the ECM source, particle size, cell types, and implantation concentration of these hydrogel composites may all be customised making a vast variety of experimental approaches possible [146, 275]. Furthermore, new findings indicate that hydrogels have been studied for their self-healing qualities—that is, their capacity to reestablish their original structure following damage. Through the reversibility of dynamic covalent crosslinks without the need for an external stimulus, such as low pH, an enzymatic environment, or temperatures, this phenomenon appears comparable to the healing process that occurs naturally in cells [276, 277]. The inadequacy of 2D cultures and SPH to reiterate tumour-ECM components during the early development

phase is overcome with this approach [278, 279]. Additionally, regarding matrix stiffness which has been extensively linked to tumour progression, invasion, and drug sensitivity, 2D cultures have a stiffness of 1–2 GPa compared to normal breast tissue (160 Pa), and breast cancer tissue (4 kPa) while Matrigel and collagen are around 200–400 Pa [280-283]. Unfortunately, many of the hydrogel synthesis and gelation methods used today are ineffective, labour-intensive, costly, and unsuitable for large-scale manufacturing. Furthermore, at the moment, *in-vitro* experiments and *in-vivo* subcutaneous ectopic cancer models are the primary methods used to validate the hydrogel's properties. These studies don't offer a realistic and accurate simulation of the *in-vivo* state and tumour-specific microenvironment, despite being convenient and easy to monitor [284].

In addition to the above settings, a promising strategy that has recently emerged is decellularizing native organs to form scaffolds to mirror the spatial distribution of the macromolecular TME and its major constituent, ECM. Decellularized tissues and organs can be repopulated with cancer cells to understand the underlying mechanisms of metastasis. This is accomplished by the use of a multitude of agents and modes of action, including chemical compounds such as surfactants, biological agents like enzymes, or physical means for instance using a freeze-thaw cycle or high hydrostatic pressure [285-287]. In a surgical scheme to preserve ECM’s physicochemical characteristics, the native scaffold was isolated via a retrograde-shunted flow method, whereby all other vessels are clamped, permitting the decellularization components to perfuse through to target tissues or organs [288]. Detailed visualisation enabled by these techniques can potentially provide new insights for regenerative medicine and as an ECM catalogue for drug screening and biomarker targeting for tumour progression [289]. A parallel approach resulted in the formation of tissue-engineered

constructs (TEC), which are organ scaffolds re-cellularized with cancer cells to be used as study models after the process of decellularizing to remove its natural cellular content. It eliminates any means of artificial passaging of cells into the decellularized ECM scaffold allowing the cancer cells to naturally proliferate and grow. This technique maintains the structural integrity of the organ-derived-ECM models along with remarkable biocompatibility with little to no mechanical difference between the models and the native biological ECM. Research utilizing 3D models has resulted in discovering the significant difference in drug sensitivity between 2D and 3D models in colorectal, Ewing sarcoma and HNSCC [246, 248, 290]. However, several studies have raised concerns to determine whether there are any potentially toxic compounds left in the scaffold and more appropriate detection techniques ought to be created. The resting physiological motifs and bioactive receptors have been shown to cause the immunomodulatory effects of scaffolds and their breakdown products; however, the precise mechanism remains unclear and requires additional research. Surface modification, such as mechanical modification and loading with factors or other bioactive compounds, can be applied to scaffolds to offer biological cues that support tissue regeneration. Decellularized tissues frequently have markedly different mechanical characteristics from their native equivalents. This could therefore impair the scaffold's in vitro shape retention ability, change the behaviour of the cells, or lower its load-bearing capability [291-296].

As a solid application for ECMs in the de novo generation of 3D models, 3D bioprinting has come into the spotlight in several fields and disciplines. It enables not just mimicking biomimetic and complex tissue structures but also OOAC and tissue-engineered constructs to serve as research models with high resolution and accuracy and hence makes it a suitable technology for various applications, such as drug

screening and high-throughput assays, transplantation and clinical application, tissue engineering and regenerative medicine and cancer research [297-299]. This is carried out using a variety of bio-inks to match the interface between different tissues [300, 301]. The goal of creating biological tissues or organs that mimic their natural equivalents in form and function has led to the widespread usage of bio-inks that include isolated ECM components. These materials need to be highly degradable, excellently mechanical and have outstanding printability and biocompatibility [302]. Currently, the materials utilised for 3D bioprinting are broadly categorised into two groups: synthetic, including polycaprolactone, and alginate, and naturally derived biomaterials, such as collagen and hyaluronic acid. These materials each have their advantages and drawbacks. The naturally occurring biomaterials are superiorly biocompatible yet have poor mechanical characteristics [303, 304]. Since each tissue and organ's distinct ECM enables cells to execute a variety of activities, making it more vital to assess cancer prognosis, scientists are now employing decellularized ECM as bio-inks to bioprint biological structures [305]. These models have been further employed to analyse drug screening and metastasis [306]. Many researchers have incorporated bioprinted 3D models to study the interaction between breast cancer and bone stromal cells [301], OSCC stage progression [307] and breast cancer invasion into adipose tissue [308], all achieving extensively detailed results. Further comparison between results from 2D and bioprinted 3D cervical tumour models revealed a higher proliferation rate, higher protein expression, and chemoresistance similar in nature to *in-vivo* tumours in the latter [309]. The are several challenges faced when using 3D bioprinting. This includes the requirement to vascularize at the single-cell level, the intricate patterning of multicellular tissues, the identification of biodegradable and biomimetic printable materials that facilitate rapid

cell attachment and proliferation, and the maintenance of cell viability and long-term functionality post-printing until remodelling and regeneration have been completed [301, 310-313].

ECM replication	Advantages	Source
Spheroids	- Individual components and anti-tumorigneic drug can be assessed in depth - favours cell-cell interactions rather than cell-material interactions - In both animal and human subjects, gene expression profiles are more closely related to malignancies.	[146]
OOAC	- All variable and parameters including drug cocentration gradients, drug-organ concentrations, cell patterning can be controlled and monitored - Multiple organs can be tested at the same time	[263]
Hydrogels	- Enhanced cell attachment and differentiation is observed due to usage of materials that are characterized by preserved ECM molecules - Easy manipulation	[273]
TEC	-Architectural meshwork maintained -Biocompatibility preserved -Very close relation to true cancer	[275]
Bioprinting	- Increased ECM stiffness can be mimicked abling to closely replicate true tumor - Cellular differentiation and viabilty can be manipulated for experiments - Increased metabolic drug conversion	[289]

Table 1 Overview of the different in-vitro ECM models with a summary of potential applications

Conclusion:

ECM has been shown to play a role in cancer progression and prevention in multiple studies. ECM's building blocks and cell-ECM interactions, all play a critical role in improving patient prognosis and hence deserve further attention. As established, the macromolecular network, ECM, governs cell destiny in a multimodal manner and dysregulation of ECM can lead to tumourigenesis by allowing tumour cells to thrive [45]. ECM facilitates tumour progression throughout its timeline in multiple biological processes such as initiation, proliferation and metastasis [314]. The predominance of each component of ECM depends on the type and stage of healthy tissue development, or the type and differentiation level of the tumour tissue as each component acts as a tumour suppressive or resistant factor [10, 315]. In invasive breast cancer, increased deposition of collagen along with a plethora of activated macrophages and strong tissue growth factor (TGF) activity characterise the modified tumour ECM [316]. MMPs have been proven to have a low activity rate in healthy cells but are significantly raised in pathogenic cells and hence in individuals with oral and throat malignancies, elevated MMP-2 expression is a prospective indication of tumour invasiveness and poor prognosis [35, 317]. Following It is also understood that integrins are important cell surface receptors that allow cells to perceive the ECM, activating signalling cascades that control cell fate and development towards a malignant phenotype and treatment resistance. Recent investigations have emphasised the importance of integrins in influencing cell-ECM communications required for tumour growth and therapy resistance [51, 314]. High levels of hyaluronan, an ECM polysaccharide, have been linked to a detrimental effect in colorectal, breast, and prostate cancer patients [10]. Since *in-vitro* models are inexpensive simulations that are developed in a controlled physical environment, researchers have commonly

expressed their preference for them. This has led to faster development of novel therapies through several pharmacological studies at once with substantially less animal use. Developing an advanced *in-vitro* ECM study model that enables the study of cancer cells in a controlled environment as close as possible to the native tumour microenvironment has been a crucial milestone. Although many models have been devised and adopted thus far, the bulk of them have a set of inadequacies that do not fully mimic the TME and thus, would not fully reflect the native behaviour of the cancer cells. It may be possible to combine these techniques to supplement the limitations of each method and maximize the advantages. This review highlights the necessity of exploring and targeting ECM with a wider application in devised 3D models and screening platforms.

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Chapter 3: [Manuscript] *Establishment and validation of a novel 3D decellularised native tissue scaffold for human oral squamous carcinoma*

Abstract

The non-cellular component of tissues, known as the extracellular matrix (ECM), plays a crucial role in essential cell functions such as cell proliferation and homeostasis. The same macromolecular network, ECM, is also responsible for tumour heterogeneity and can contribute to failure to respond to treatment modalities and therapy resistance. To be able to investigate the changes in cell behaviour in the presence of ECM, it is vital to construct a 3D cancer model that can recapitulate the cancer cell-ECM interface. In this study, we have generated native ECM from decellularised tongue tissues for the culture and propagation of cells derived from oral squamous cell carcinoma. The cellular and molecular behaviour of the cells grown on these matrices has been further compared to established 3D spheroid models and cells grown in conventional 2D plastic dishes, highlighting key changes and advantages in the proposed native tissue scaffolds in terms of maintenance of baseline tumour cell signatures and behavioural dynamics in response to radiation.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the top ten most prevalent malignancies worldwide, commonly diagnosed over 60 years of age although occasionally also seen in age groups well below 60 [1-3]. The symptoms and treatments' side effects can have a significant impact on a patient's quality of life with a late clinical diagnosis, little to no chance of healthy recovery, a lack of clear biomarkers for the disease, and expensive therapy options [2, 4]. Even with a very poor current prognosis report of 5-year survival rates, survival rates can be expected

to rise if it is diagnosed in its early stages [1, 5, 6]. All tumours exhibit tumour heterogeneity, which is characterized by several factors including histological abnormalities, cellular behavioural changes, and molecular imbalances shown by various biological markers and genetic profiles [7-10]. These genetic and phenotypic features promote tumour growth and metastasis [11-13], It is difficult for clinicians to ensure a favourable therapeutic response since tumour heterogeneity also precedes treatment resistance, prompting studies into this heterogeneity to better clinical results [14, 15].

It is well established that cancer cell behaviour is heavily affected by its surrounding environment known as the tumour microenvironment (TME). Immunotherapies' efficacy and cancer prognosis are widely determined by this microenvironment [28-31]. TME includes vasculature, fibroblasts, immune cells, bone marrow-derived inflammatory cells and extracellular matrix (ECM). It is the transformed stroma that was formerly responsible for healthy cell regulation and growth but now facilitates the same for the tumour [32, 33]. In the occurrence of cancer, modulation of TME is governed by the intimate crosstalk, within and across the cellular structures, that occurs through the ECM. By producing extracellular signals, the tumour can interact with the microenvironment, enhance tumour angiogenesis, and induce peripheral immune tolerance [34-36]. The resulting hypoxia, oxidative stress, and acidity in the TME prompt the extracellular matrix to adapt [37-39]. Furthermore, radiation causes an overabundance of immunoinflammatory cells in the TME, which results in the production of growth factors and cytokines that promote genetic unrest and angiogenesis [40-42].

ECM is an essential component of the TME as it has been proven responsible for substantially influence tumour growth and therapy response [43-46]. ECM is a macromolecular network with extremely versatile functionality. Present in all tissue, it surrounds the cells providing them with growth factors and cytokines to maintain homeostasis along with providing mechanical strength and establishing and developing differentiated cells and tissues [47, 48]. This acellular architecture is densely packed with fibrous proteins such as laminin, collagen, proteoglycans (PG) encored with glycosaminoglycans (GAG), proteases and transmembrane receptors such as integrins [49]. All these elements work cohesively to provide the cells and tissues with structural scaffolding and biochemical support [50, 51]. Healthy cells' adhesion and motility are controlled by biochemical and biophysical signals elicited by the ECM, which is constantly and actively remodelling. As ligands for numerous cell surface receptors that support metabolism, development, and differentiation, ECM components affect intricate cellular signalling networks [52-54]. This remodelling process is hampered by cancer, which causes aberrant ECM deposition and stiffness, eventually promoting tumour growth [55-58].

In-vitro 3D models are amongst the greatest diagnostic tools for simulating and deciphering cancer behaviour and providing essential data for *in-vitro* drug screens [59, 60]. These models have been used to analyse every facet of tumour formation, including cellular proliferation, migration, invasion, and treatment response [61]. Researchers have aimed to create 3D models for several years and use a variety of experimental approaches. The multicellular tumour spheroids (SPH) are a common example of tumour cells grown in 3D into spherical structures [62-64]. These feature a necrotic core and an exponentially expanding cell layer around the periphery. They can mimic the 3D architecture of solid tumours, as well as physiological processes,

signalling molecule secretion, gene expression, and treatment-acquired resistance [65, 66]. The cells also deposit the characteristic elements of ECM such as collagen and PG [67]. Among many other methods, low adhesion plates, the hanging drop method, cell-seeded matrices, scaffolds, micropatterning, and agitation-based technologies are currently available for spheroid formation [68]. Since these models can replicate several key aspects of the true tumour, the SPH has been investigated and proven beneficial for the tumour's physiological and malignant properties [69, 70]. Although these solid tumours growing in 3D spatial conformation conserved the biological characteristics better than the traditional monolayer cultures [71], this prototype still had many constraints due to imprecisely representing the *in-vivo* tumour tissues. The static environment created by SPH fails to correctly reflect all the elements that impact cancer cell growth and uncertainty in pharmacokinetics and toxicological predictions has led to the creation of other models [72].

ECM characteristics including composition, stiffness, topography, and microarchitecture influence cell activity that aids in the development of tumours [73] and henceforth it is crucial to generate an ECM model that is not only capable of producing accurate cell-TME interactions but also largely available for extensive research. Bioprinting, an adaption of additive (3D) printing that uses the deposition of biomaterials and bioactive compounds, such as cells, growth factors, etc. which is employed as another synthetic model to operate as the ECM, has grown in popularity as a viable approach for creating intricate tissue scaffolds, drug screening, and genetic editing [74, 75]. The primary biomaterial used in 3D printing is called bioink, consisting of cells that need to be deposited. These cells are frequently encased in a carrier matrix that mimics the physical and biochemical environment of natural tissues to enable cell adhesion, proliferation, and differentiation. To offer structural and

biochemical support akin to the natural ECM, bioink's carrier material serves this goal [76]. However, the expensive equipment, extensive printing periods, and undesirable 3D build structures brought on by repeated laser exposure, along with temperature and shear forces used to create bioink impacting cell survival, compounds its limitations [77]. A model must accurately recreate complex tissue operations, including metabolite exchange, nutrition transit, and contraction, in addition to its histological and biochemical properties. One of the difficulties in replicating natural tissue is the intricacy of the tissue. No matter the biomaterial sources that are accessible, it is still difficult to accurately reproduce the biophysical and biochemical characteristics of the native ECM in a synthetic bio-microenvironment [78, 79]. Furthermore, optimal bioinks that are free from structural instability and insolubility in vivo and in culture are not yet available [80, 81]. ECM is unique to the tissue it surrounds, therefore finding biodegradable and biomimetic printed materials that promote rapid cell attachment and proliferation is a significant challenge [82]. The organ-on-a-chip (OOAC) device, another ECM simulation technique that labours perfused cells in the model, has comprehensive applications in precision medicine [83, 84]. However, because cells are particularly fragile under these conditions, the design, which is based on the principles of microfluidics, is subject to the creation of bubbles within the apparatus [85, 86]. Additionally, the research on cell interactome has been severely restricted by the fact that the longest-perfused OOAC models so far have only survived for 4 weeks [87, 88]. Another innovation involved using bioreactors to inject cells into different-sized porous sponges that served as scaffold templates. By providing tumour cells with nutrients and biomimetic stimuli in a controlled manner, these bioreactors were able to affect tumour growth and tissue development [89, 90]. But even so, when scaling up with expensive bioreactors, constant perfusion may not be sufficient for

tissue viability, limiting the eventual thickness of the construct [91]. Hence it is indispensable to reproduce this cellular habitat to examine cell-cell and cell-matrix interactions The key was to develop the closest depiction of *in-vitro* TME which is a dynamic cellular milieu where the tumour exists.

Despite offering valuable data on cellular behaviour, the majority of the above approaches introduce artificial settings for the study of cancer cell behaviour. To address the essential elements, present in a native tumour, in vitro models were developed where native tissues were used as growth scaffolds to home and propagate cancer cells [92, 93]. In fact, numerous studies have transformed tumour spheroid models into tissue-engineered constructs, which are primarily biological substitutes that can mimic tissues for diagnostic and therapeutic purposes [61, 94, 95]. These models are derived from native tissue scaffolds (NTS) and hence are recognised as advanced 3D models with the closest imitation of native carcinoma tumours [96]. Various organs and tissues have been decellularized to obtain organic ECM [97] using a diverse range of methods that involve chemical breakdown using acids, detergents, and solvents [98-100], or enzymatic treatments utilizing trypsin or nuclease [101, 102], or mechanical disintegration including freeze-thaw cycles, sonication and pressurization [103-105]**.** In this study, we introduce a novel NTS-based model for the study of oral squamous cell carcinoma cancer cells and further characterise the cellular and molecular state of OSCC cells when grown on NTS, as spheroids (SPH) and in conventional 2D plastic cultures both during normal growth and in response to ionising radiation (IR) [106, 107].

Materials and Methods:

General tissue culture and cell line information

SCC 25, OSCC, purchased from American Type Culture Collection (ATCC), were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture with GlutaMAX-I (1x, Gibco (Life Technologies)) supplemented with 10% Foetal Bovine Serum (FBS) (Gibco (Life Technologies)) and 1% GlutaMAX-I (100x, Gibco (Life Technologies) (RFHG) at 37 °C in a humidified incubator with 5% CO2. They were pre-tagged with fluorescent mCherry (red) for effective visualisation.

Specimen collection, decellularization protocol and validation

Native mouse tongues were collected from C57BL6 male wild-type mice, under 1 year of age. After being euthanized by CO2, tongues were surgically excised in sterile conditions and then decellularized using the previously described methods [108]. To verify a successful decellularization (DCL) process, the DNA content was measured for native and decellularized tongues, size 4 mm, using a DNA extraction kit (Qiagen, Cat. # 69504). Histological and immunohistology stained sections were also obtained for the same for further confirmation (Fig. 3).

Seeding of SCC25 cells on decellularized matrices

Before seeding the SCC 25 cells on the scaffolds, they were immersed in DMEM w/FBS for 5 days to enhance SCC 25 homing. The scaffolds were submerged in FBS for 3 hours on ice in a sterile hood on the day of transplantation. SCC 25 cells were extracted and resuspended in DMEM w/FBS when their culture reached around 85% confluency. 0.02 mL of the cell-suspended media was placed on top of the scaffold after aspirating FBS. Post-90 minutes incubation, re-cellularized models now known as the NTS, were replenished with fresh DMEM media. NTSs were transferred to fresh

wells after 24 hours and incubated for further 7 and 14 days to allow proper settlement of the cells.

Exposure to ionising radiation

NTSs were exposed to 2 Gy radiation, X-RAD 320 (Precision X-Ray), at a dose rate of 2 Gy/min. The treated NTSs and controls (non-radiated) were processed 24 hours post-irradiation for transcriptomic analysis or histology and immunostaining analyses.

Histology, immunostaining, scanning electron microscopy and image analysis

All samples, in triplicates, were fixed in 4% paraformaldehyde (PFA)/PBS for 24 hours at room temperature and after 3 washes of 70% v/v ethanol, the samples were embedded in paraffin using a 4-hour cycle. A detailed protocol has been mentioned in Chapter 3, Methods, under section 3.1.5. A microtome (Leica Biosystems RM2245) was employed to section the samples. Following this, each slide with sections was dewaxed by incubating them at 60 C for 2 hours and rehydrated with 3 consecutive washes of Xylene for 2 minutes each, and with 3 washes of 100% v/v ethanol washes each, 1 wash of 70% v/v ethanol for 2 minutes ending with a wash in running tap water. A (NexGn) decloaking chamber was used for antigen retrieval by the manufacturer's manual using a citrate buffer of pH 6.0 at 95 C for 20 minutes. Following wax removal, slides were washed with running water and dried on a hot plate. Sections were then stained with hematoxylin solution (Mayer's modified (Abcam, Cat. # 220365)) for 1 minute followed by a rinse in running water again. A counter Eosin stain (Mayer's modified (Abcam, Cat. # 246824)) was applied for 1 minute and then they were passed through a change of water and 75% alcohol before being them to dry again on the hot plate. Lastly, sealed with a glass coverslip using a mounting agent. Monolayer cells

were grown on round coverslips (ThermoFisher, Cat. # 174950; 13mm) in 6-well plates and later stained for immunohistochemistry (IHC). Tumour SPH, produced using the protocol mentioned in [109], were gently rinsed in PBS for 5 minutes, fixed in 4% PFA/PBS for 10 minutes at room temperature, and then washed once more in PBS for 5 minutes for IHC staining preparation. The fixed SPH were treated with 25% sucrose for 30 minutes and then transferred to cryomolds containing O.C.T compound (Emgrid, Cat. # 4583), where they were frozen on dry ice before being moved to -80°C. On a cryostat (Leica Biosystems), frozen blocks containing the SPH were cut into 6-8 micron slices and processed for downstream staining. NTS sections were treated with an hour of blocking and permeabilization with 0.1% Triton X-100, 3% donkey serum (Sigma, Cat. # D9663) and Bovine Serum Albumin– Reagent Grade pH7 (BSA) (1%, Moregate Biotech) in DPBS for IHC. Samples were incubated with primary antibodies including mCherry (Abcam, Cat. # ab183628), ki-67, Col-1 (ThermoFisher, Cat. # PA1-26204), SMA (Cy3™ antibody, Mouse monoclonal Cat. # C6198), anti-γH2AX (Abcam, Cat. # 11174), at 4 °C overnight. The next day, samples were washed with 0.1% Tween-20 in PBS before incubating with either Alexa Fluor 555-, or Alexa Fluor 48-conjugated donkey anti-rabbit (Abcam, Cat. # 150073 and Cat. # 175649), secondary antibodies for 1 hour at room temperature. Afterwards, the samples were washed, and the cell nuclei were stained with Hoechst 33342 (Invitrogen, Cat. # H3570) and sealed with a coverslip using covered ProLong Gold mounting solution (ThermoFisher, Cat. # P36930).

The tongues and oral cancer cells were imaged using a Scanning Electron Microscope (SEM). A healthy mouse tongue was used as a negative control, and scc25 cells and an NTS were fixed in 4% PFA. All samples were then washed with PBS thrice. Followed by dehydration in a series of ethanol concentrations (50%, 70%, and 90%,

for 10 minutes each. Final rinses with 100 % alcohol thrice, for 10 minutes each cycle. The samples were then critically dried using a critical point drying apparatus (Balzers Union 010) to remove all alcohol residue and replace it with carbon dioxide. This was achieved by heating up to a temperature of 45 degrees Celsius and 90 mbar pressure. All samples were gold coated with Balzers Union Au coater and then viewed and photographed at various magnifications using a scanning electron microscope, JEOL SEM 840.

Fluorescent images were acquired using an Olympus U-RFL-T fluorescent microscope and Leica Application Suite and quantitatively analysed with ImageJ software. At least five fields in each of the three biological replicates were quantified for each quantification, and averages were derived. Samples imaged using Scanning Electron Microscope (SEM), JEOL SEM 840, critical point drying apparatus (Balzers Union 010) and gold coated with (Balzers Union Au coater), were fixed in 4% PFA, washed with PBS and dehydrated with ethanol concentrations (30%,50%, 70%, 90%, 10 minutes each followed by 30 minutes in 100%). NTSs were first cleared using a standard protocol [110] which enabled superior visualisation of the cells and then pictured using the Zeiss Light sheet Z.1 system, according to the manufacturer's guidelines, analysed with Imaris software.

Statistical analysis

Standard deviation (SD) was calculated for all (at least three) biological replicates and error bars were generated using ANOVA (GraphPad Prism). (P-value < 0.05: *; P-value < 0.01: **; P-value < 0.001: ***). All P-values above 0.05 were regarded as non-significant (NS).

Total DNA measurement, RNA-sequencing and data analysis

DNA content was extracted to evaluate the decellularization process using the DNeasy Blood & Tissue Kit (Qiagen, Cat. # 69504). Using Isolate II RNA Mini Kit (Bioline, Cat. # BIO-52073), total RNA was isolated from irradiated 2D cultures, SPH and NTS (on the 14th successful day of oral cancer cells seeded) 24 hours after radiotherapy. The whole messenger RNA content of each sample was converted to cDNA using TruSeq RNA Library Prep Kit (Illumina, Cat. # 20020595) to prepare the cDNA libraries, which were then sequenced on an Illumina Hiseq2500 System on 100 bp single-end mode. Reads from the sequencing were aligned to the hg38 genome with STAR v2.7.8a (32), discarding multi-mapping reads. Aligned reads were then summarized to gene counts using the encode v26 gene annotation. Gene counts were normalized using TMM (33) to account for library size differences. Tests for differential expression between groups were performed using voom (34) with robust estimation of the prior variances. Adjusted p-values that account for multiple comparisons were calculated using a Bonferroni correction with a threshold of 0.05 used to call statistical significance. Pathway analysis was performed using the goSeq v 1.44.0 package (https://pubmed.ncbi.nlm.nih.gov/20132535/) and MSigDB gene sets, as accessed on December 14, 2022 (WEHI Bioinformatics - mouse and human versions of the MSigDB in R format)

Results:

Generation of the decellularized ECM and the NTS

To generate the 3D native ECM-based scaffold, native tongue tissues from C57BL6 male wild-type mice were decellularized using sodium dodecyl sulphate (SDS) for 14

days and after treatment with their culture medium and FBS, the scaffolds were seeded with SCC 25 and processed for downstream analyses (Fig. 1A). The mice tongues were imaged consecutively throughout the 14-days of the optimized DCL process to certify this methodology, and a stark difference was observed in colour from red to pale dictating the loss of all the cellular content while preserving the acellular matter, ECM, as well as the tissue morphology (Fig. 1A). The use of SDS in this protocol allows the breakage of the cellular membrane allowing all contents to be flushed out while avoiding any integral damages that are caused by enzymatic or temperature-based protocols.

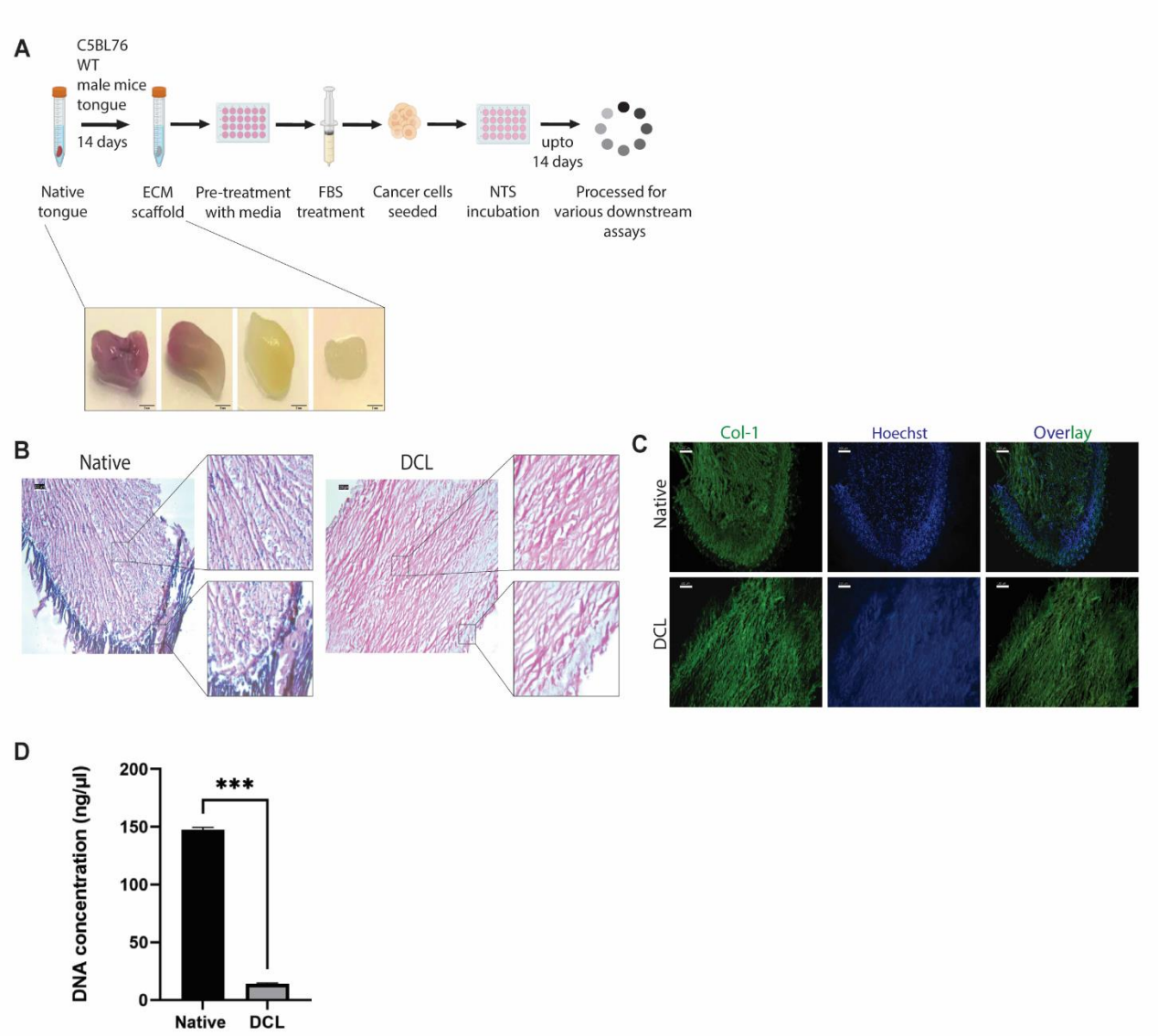


Figure 1 Establishment and characterization of the decellularized native tongue tissues (A) (Top) Graphical abstract

demonstrating the protocol followed to generate the decellularized ECM model, NTS (This figure is created using Biorender) and (bottom) images of the tongue through the 14-day decellularization process (Scale bar: 2mm). This figure was created using Biorender. **(B)** Hematoxylin and Eosin staining of native (left) and decellularized (right) tongue sections (Scale bar: 100 µm), **(C)** Immunostaining of native (top) and decellularized (bottom) tongue sections with Col-1 and Hoechst (Scale bar: 100 µm), **(D)** Statistical analysis of cellular DNA content of both samples; unpaired t-test, n=3 replicates for each, SD error bars, P-value < 0.001: ***.

Histological staining of the native and DCL tongue (Fig. 1B) confirmed the same results with the loss of cells (purple nuclei) while the structural integrity of the tongue remained intact. To further certify a successful procedure, IHC staining of Collagen, ECM's predominant and vital ingredient, using Col-1 antibody (green) and nuclei stained by Hoechst (blue), supported the same conclusion that the ECM was maintained but the majority of the cells were removed during the treatment as evidenced by the loss of majority nuclei (Fig. 1C). Quantitative analysis of DNA content in samples (Fig. 1D) revealed a significant drop after the decellularization further validating the protocol of a successful establishment of the ECM scaffold without any residual tongue cells to interfere with the experimental analysis.

OSCC cells successfully home and propagate on the decellularized matrices

Once the ECM scaffolds were obtained through decellularizing mouse tongues, mCherry-tagged SCC 25 cells were transplanted on them to allow a natural homing and integration into the matrices. Sections of NTS were then imaged to evaluate the protocol's efficacy using brightfield and fluorescent microscopy (Fig. 2A). SCC 25 can be visualised as distinguished circular black figures, under brightfield, equally dispersed through the grey stroma, representing the ECM while immunostaining of parallel sections using an antibody against mCherry (red) and the nuclear stain Hoechst (blue) are seen representing the healthy growth of these cells across the NTS

(Fig. 2A). After 14 days of seeding, the migration and emergence of mCherry SCC 25 into the scaffold were recorded and examined using light sheet microscopy showcasing the 3D integration and migration of the SCC25 cells in the NTS (Fig. 2B). (Supplementary Fig. 3S shows the embedded cells in the NTS at day 13 through live sheet microscopy.) Further analysis of the model using scanning electron microscopy (SEM) displayed the circular SCC 25 cells, that when seeded on the native tongue, flattened its pointed papillae, and completely adhered to the scaffold forming a blunt surfaced NTS (Fig. 2C). Further histological analysis (Fig. 2D) confirmed the effective homing of SCC 25 cells into the NTS. The cells were seen to be more densely populated in the tongue's outer parenchyma as compared to the rest of the structure.

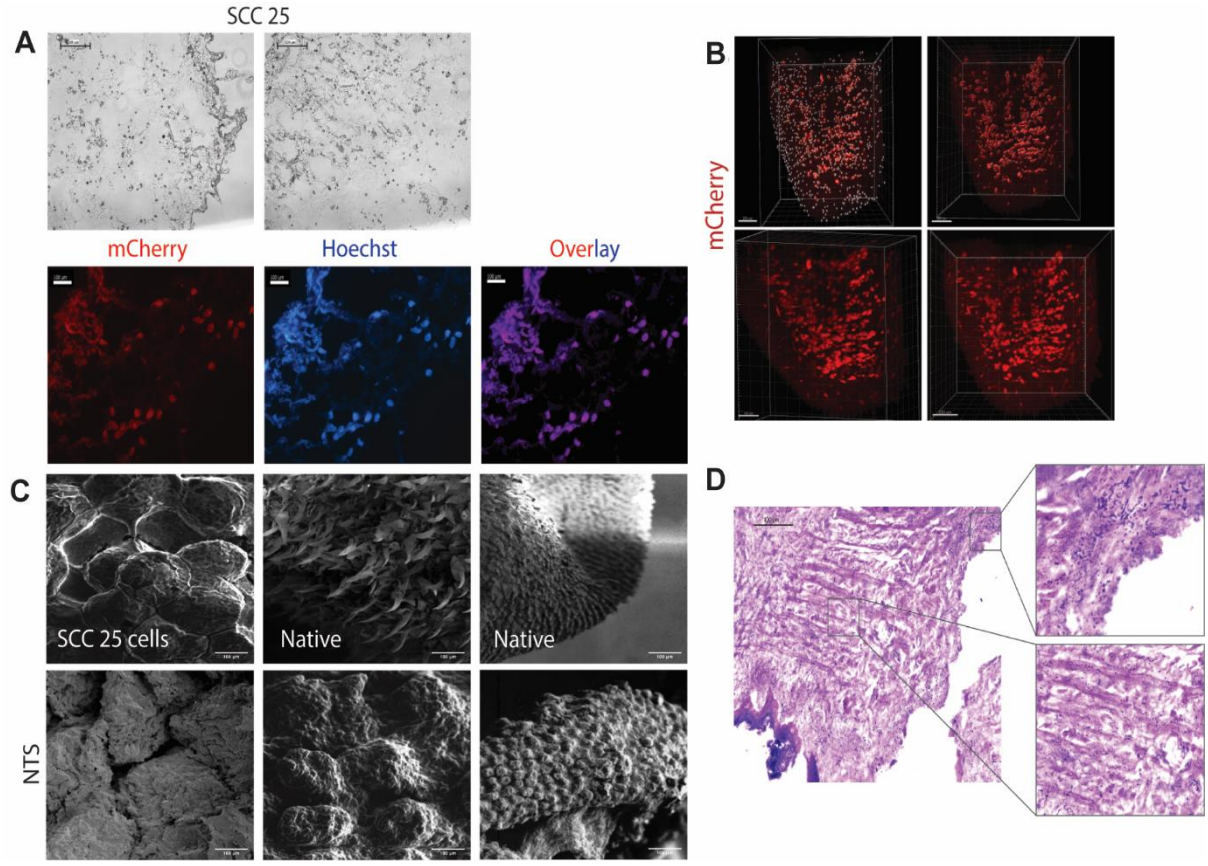


Figure 2 Characterization of the NTS through different methods (A) Brightfield (Scale bar: 100 µm)(top) and IHC (bottom) of the NTS sections using an antibody against mCherry (Red) and nuclear counterstain (Hoechst) (Scale bar: 100 µm), **(B)** Light sheet microscopy of mCherry-tagged SCC25 cells in NTS (Scale bar: 200 µm), **(C)** From top left; SEM images of SCC25 cells

1000x magnification, native mice tongue 150x and 50x magnification. From bottom left, NTS with SCC 25 cells seeded on the tongue scaffold 1000x, 1000x and 150x magnification (Scale bar: 100 μm), **(D)** H&E staining of the NTS' sections (Scale bar: 100 μm).

Tumour growth models differentially modulate the levels of cell proliferation and IR-induced double-strand breaks

The proliferative capability of each model was analysed quantitatively by comparing its expression of ki67, and the accumulation of IR-induced double-strand breaks (DSB), indicating its therapy resistance, was determined by comparing the expression of phosphorylated histone γH2AX (Fig. 3). This was accomplished by cultivating 2D cells until confluent, NTS cells for 14 days, and SPH cells for 2 days. To get an equal number of SCC 25 cells growing in each of the three models, the "n" number of days was optimised through vigorous trials. The models were then subjected to 2 Gy ionising radiation after successful cell implantation and underwent IHC staining. Ki-67 is a prominent cancer indicator that is strongly expressed during mitosis and the G2 phase of proliferating cells and is undetected in quiescent cells. Thus, it has become the de facto method for determining cancer's growth rate [111, 112]. The signalling and repair components needed to mount a suitable response to DNA damage are both recruited and retained by the protein γH2AX that accumulates at the sites of double-strand breaks. The average concentration of γH2AX is maintained across the entire nuclear volume. The quantitative signals of γH2AX increase on average 5-fold within the first 15 min after exposure to IR, by a general increase in the amount of γH2AX immediately following exposure to IR [113].

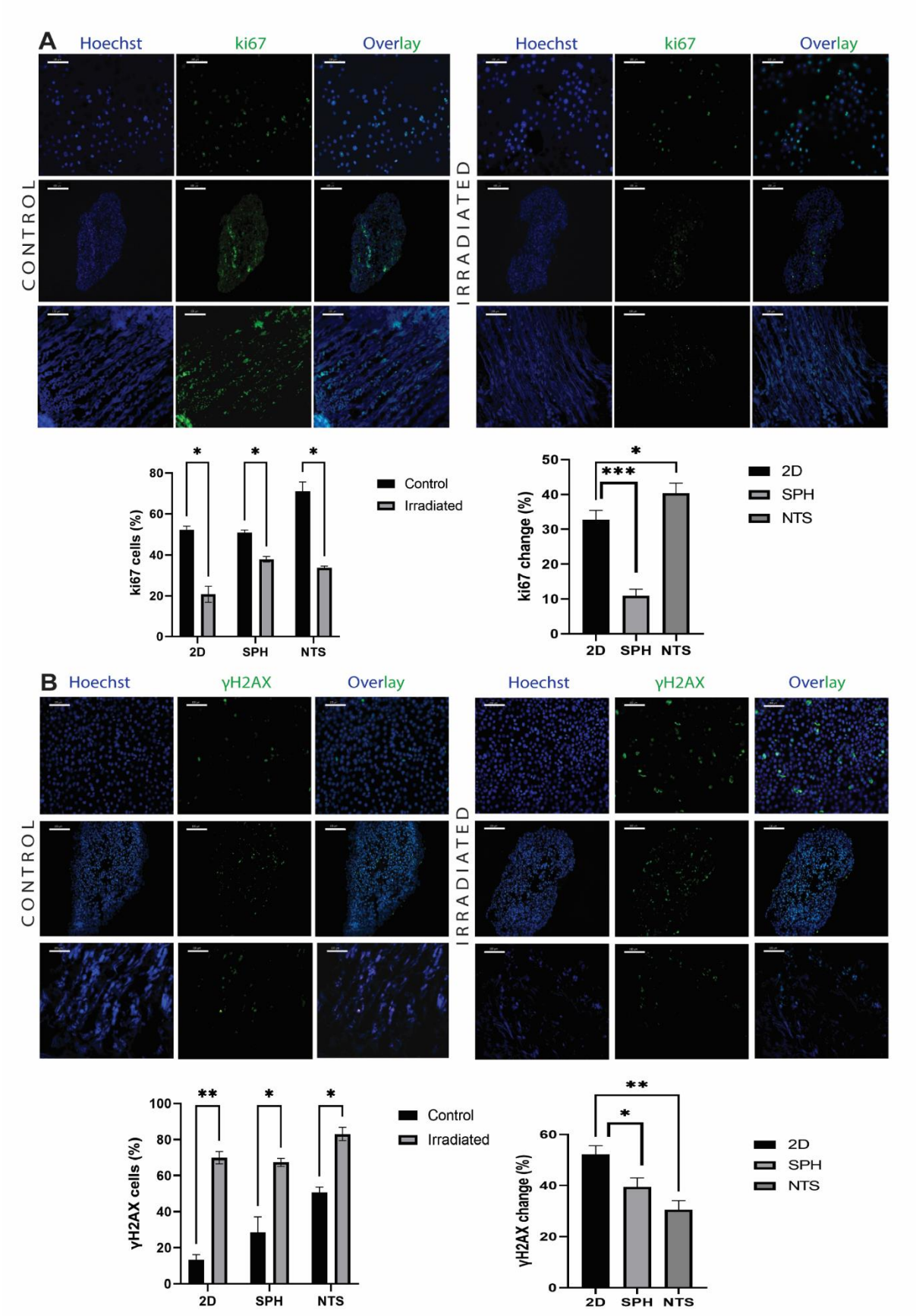


Figure 3 Demonstration of the models' proliferative capacity, through ki67 signaling, and accumulation of IR-induced DNA damage by γH2AX expression (A) Change in activated ki67 signals pre- and post-radiotherapy across all 3 study models through IHC (Scale bar: 100 μm). The graphs quantify the % ki67 change for the same. **(B)** Change in activated γH2AX signals pre- and post-radiotherapy across all 3 study models through IHC (Scale bar: 100 μm). The graphs quantify the % γH2AX change

for the same. (n=3; n=independent experiments; data shown as mean ± SD).

The three study models exhibited varied levels of ki67 expression both in control and irradiated states with NTS showing the highest downfall of ki67 index of 40.4% after radiotherapy and SPH the least significant change of 10.9%. 2D exhibited a decrease of 32.7%. This indicates the SCC 25 cells entered a quiescent stage after irradiation and hence are not seen exhibiting a higher ki67 index, which is usually seen post-radiation due to inhibited mitosis [114]. However, it's important to note that the effects of radiotherapy can also depend on various factors, including the type of cancer, the stage of the disease, and the overall health of the patient. In some instances, the reduction in Ki-67 activity may not be immediate and could take time to manifest as the damaged cells undergo cell cycle arrest or death [115]. Even though the NTS displayed the most drastic decrease in the ki67 index post-irradiation, it still had a proliferative capacity of greater than 15% which has been linked to metastasis and recurrence [116]. Furthermore, the untreated NTS displayed the highest ki67 signals towards a better replication of a true carcinoma, in comparison to the 2D and SPH models with higher proliferative rates of the SCC 25 cells.

An identical experimental set-up was utilized to quantitively assess the levels of IR-induced DSB in different SCC25 cells grown in different models (Fig. 3B). As hypothesized, NTS showed the least change in the expression of γH2AX of 30.5%, suggesting a radio-protective effect in the presence of the decellularized matrix while the 2D model showed the highest level of IR-induced DNA damage with an increase of γH2AX levels by 52.1%. SPH displayed an increase of 39.4%.

The three models differentially regulate the tumour cell signatures in control and irradiated samples

To further elaborate on the impact of different models on the cell biology and molecular profiles of the tumour cells, the total RNA from control and irradiated samples was exposed to RNA sequencing and signature analysis in each model (Fig.4A). We first compared the over and under-represented genes across all 3 study models. Fig. 4B shows the total number of genes differently regulated among each study model and between control and irradiated samples, with NTS representing the greatest amount of transcriptional changes. Furthermore, we found that while genes coding for cell transport, membrane structure, migration, cellular communication, and stimulus-response were downregulated in 2D v/s SPH (Fig. 4S), those coding for DNA structure, cell cycle and division were downregulated in NTS v/s 2D. NTS v/s SPH showed a similar range of genes down-regulated as in 2D v/s SPH. When analysing the genes upregulated in 2D v/s SPH, we saw genes for cell cycle, DNA repair and cell division (Fig. 4S), and ECM organisation, cell development and migration and cell morphogenesis were upregulated in NTS v/s 2D (Fig. 4C). NTS v/s SPH showed a similar range of up-regulated genes (Fig. 4C) as in 2D v/s SPH. It is interesting to note while 2D v/s SPH and NTS v/s SPH showed a similar range of up and downregulated genes via the heatmaps, the pattern and clustering are still very different. Moreover, only the NTS v/s 2D graph showed genes related to the ECM upregulated indicating that NTS is a superior representation of the *in-vivo* setting by mimicking the characteristic niche required for cancer cell survival.

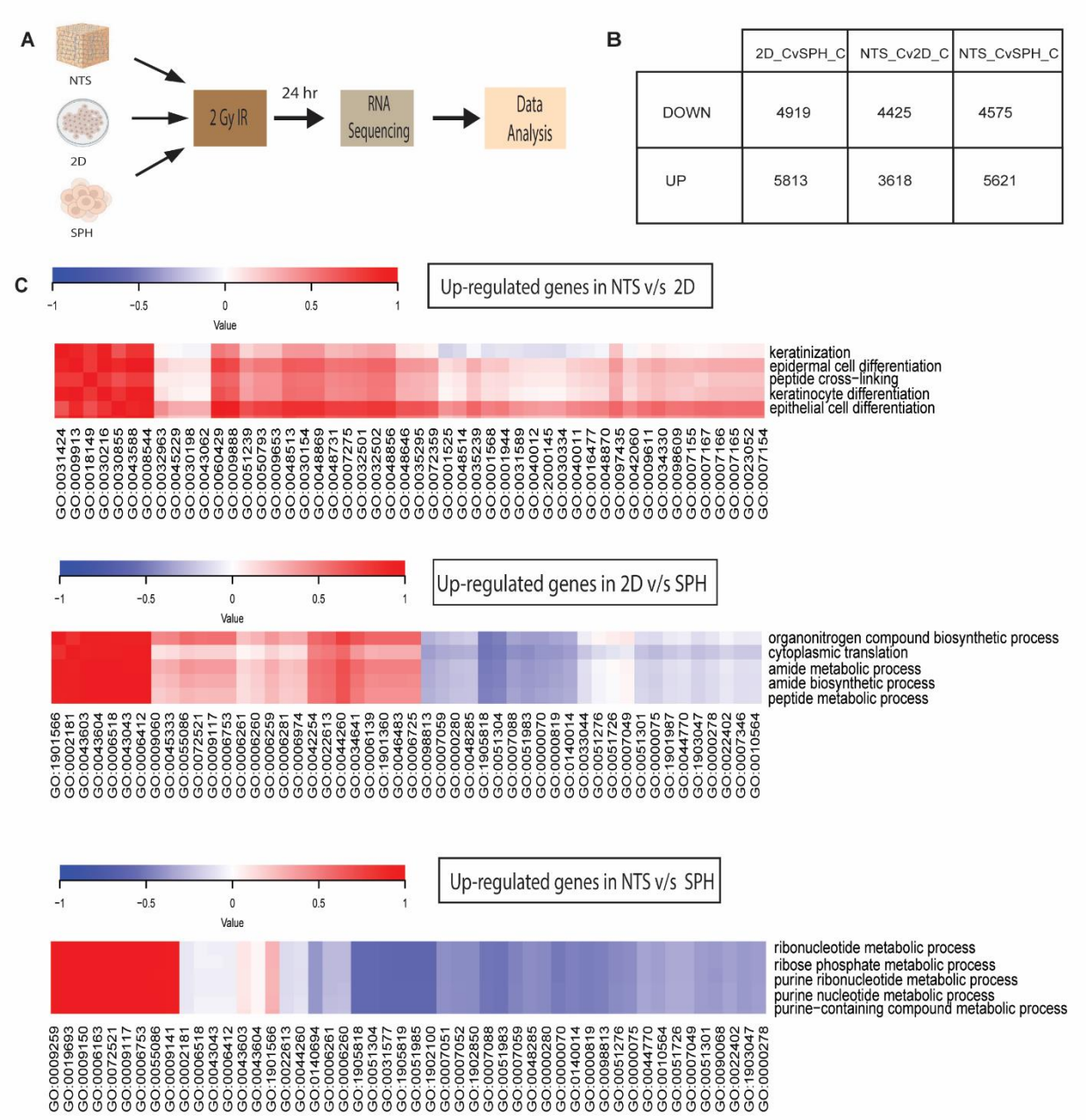
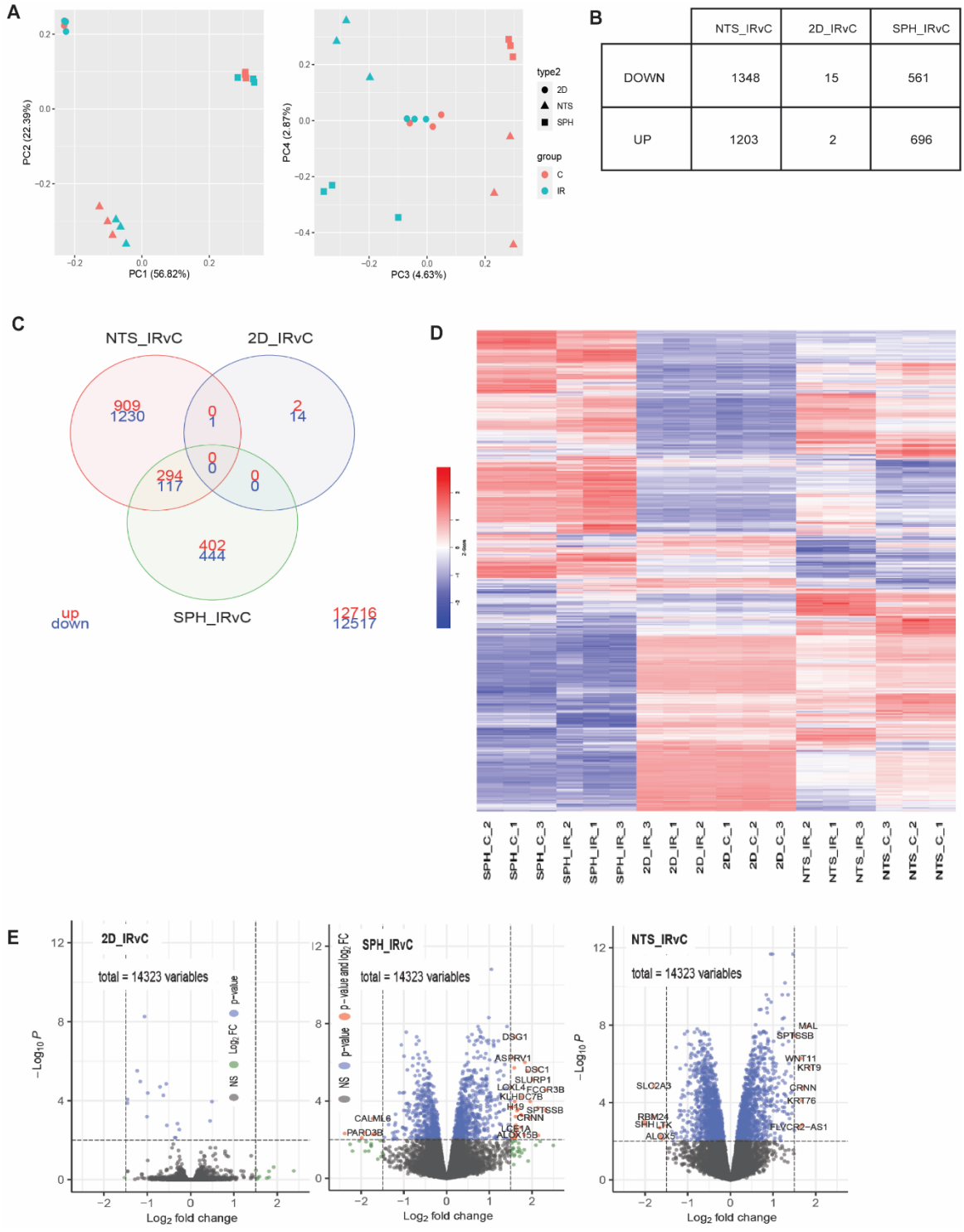


Figure 4 Study's schematic representation and heatmaps demonstrating baseline controls of upregulated genes of all three models (A) Experimental design for quantitative and qualitative RNA analysis **(B)** Number of dysregulated genes across all untreated study models **(C)** Dysregulated genes in NTS v/s 2D, 2D v/s SPH and NTS v/s SPH. All data was normalized against the Z-score.

The overall analysis of signatures through principal component (PC) analysis showed significant clustering of each model away from the others (Fig. 5A), through clear segregation. The differential clustering further continued into PC3 and PC4, while at this stage, samples were segregated based on their states of control or irradiation. NTS and SPH show distinct segregation between the control and IR samples and

hence suggest that they are better a reflection of the IR effect in the study model, compared to 2D. Fig. 5B,C shows the number of genes up and downregulated in the study models pre- and post-irradiation with NTS representing the largest change in all aspects indicating the best reflection of gene signature in IR exposure. Despite the overall segregation of the signatures among different models, more detailed analysis confirmed distinct transcripts co-regulated between the three different models, while a significant number of transcripts were also differentially dysregulated between different models (Fig. 5D). This is also represented in the volcano plots (Fig. 5E) where we can visualize how significantly large the differences are between the models.

Further, heat map analysis confirmed the distinct patterns of gene regulation in control and irradiated states between different models. SPH and 2D models have a mirrored pattern of gene regulation, however, NTS shows a multitude of gene clusters that show a shift in regulation hence indicating that NTS is more reflective of transcription dynamics in response to IR.

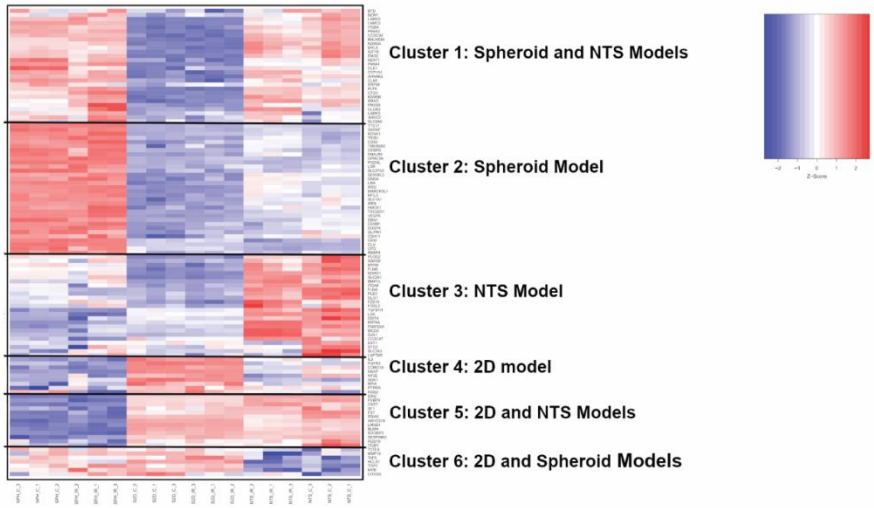


	NTS_IRvC	2D_IRvC	SPH_IRvC
DOWN	1348	15	561
UP	1203	2	696

Figure 5 Transcriptional profiling of different experimental models (A) PC Analysis based on similarities in different time-point-based irradiated samples **(B)** Number of genes down and upregulated in control models **(C)** Venn diagram showing commonly up and downregulated genes for the study models **(D)** Heatmap illustrating the classification of study models normalized to Z-score **(E)** Volcano plots showing gene regulations in all 3 models in control and IR samples.

To further assess the study models in reflecting specific radiation-response-related transcriptional dynamics, we first compiled a comprehensive list of genes that were implied in the cellular and molecular response to radiation based on published literature. The transcriptional profiles of control and irradiated samples were then assessed against this specific gene list. Heatmap analysis of upregulated radiation response genes (Fig. 6A) confirmed three distinct gene clusters each overrepresented in a specific model: cluster 1 was majorly present in the 2D model, cluster 2 in the NTS and cluster 3 in the SPH model. Likewise, a similar pattern was evident in the downregulated gene category (Fig. 6B), with three clusters (1-3) respectively downregulated in 2D, NTS and SPH models.

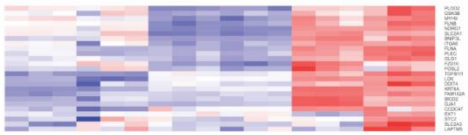
A



B

Cluster 2: Spheroid model
Hypoxia, Cell Stress, and Apotposis

TTC17, SARAF, KCNK1, TRIB1, CD55, TMEM260, CEBPG, DNAJB9, GPRC5A, PODXL, LSR, SLC27A3, SERINC3, GNG4, LBH, IRS2, MARCKSL1, NFIL3, SLC1A1, IRF6, HMOX1, TSC22D1, VEGFA, DBN1, CEMIP, DUSP4, GLIPR1, CDH11, GEM, CLU, CPD, RBBP4



Cluster 3: NTS Model
Epithelial to Mesenchymal Transition

PLOD2, GSK3B, MYH9, FLNB, NDRG1,SLC2A1, BNIP3L, ITGA6, FLNA, PLEC, GLG1, FZD10, FOSL2, TGFB1I1, LOX, DDIT4, KRT6A, FAM162A, BICD2, GJA1, CCDC47, EXT1, STC2, SLC2A3, LAPTM5

Cluster 4: 2D Model
Proliferation

IL6, FGFR3, CORO1A, NASP, NT5E, SGK1, RPIA, PTPRR, RGS2

C

Pearson's Chi-Squared with Yates' Continuity Correction

	Differntially Expressed Genes	X-Squared	Degrees of Freedom	P-Value
NTS	54	30.088	1	4.13E-08
SPH	24	7.936	1	0.004846
2D	0	2.74E-30	1	1

Native Tissue Scaffold Differentially Expressed Genes

Common Mehanism of Action	Genes
EMT	LAMA3, LAMC2, P4HA2, CLK1, CLCN3, KDM5B, FLNA, DDIT4, TGFB1I1, LAPTM5, EXT1, SLC2A3, PLOD2, GJA1, KRT6A, FZD10, BICD2, TNFRSF10B, ACTN1, ANXA2
Apoptosis	CD55, PODXL, MARCKSL1, ANXA5, PPM1D, CTSD, CDKN1A, PHLDA3, HTRA1
Proilferation	ITGB1, LMNB1, NASP, CORO1A, NT5E
DNA repair/Genomic Stability	LBH, DDB2, SERPINE1, POLD3, CDC6, EXO1
Cell maintenance	ARHGDIB, CAST, FST
ER stress	PDIA5, TRIB1
Stemness	CITED2, MDM2, EMP2
Hypoxia	DUSP4, IRS2, SLC2A3
Inconclusive	CYP1A1, KCNK1, NINJ1, DBN1

Spheroid Differentially Expressed Genes

Common Mehanism of Action	Genes
EMT	NEAT1, CLK1, CTSV, BHLHE40, KLF6, CLCN3, RRAD, ST14, CCND1, TNFRS10B, LOX, FLNA, FAM162A, PRSS8
Apoptosis	ANXA5, CD55
Proilferation	RGS2, SGK1, SQSTM1
Cell maintenance	FKBP9
Stemness	MDM2, DAG1
Cellular Stress	PL3K
Inconclusive	DBN1

Figure 6 Bespoke Upregulated Gene Expression Profile and Radiation Induced Differential Gene Expression. Expression. (A) Heatmap normalized to Z-Score, predicted upregulated gene expression profiles separated into clusters. **(B)** Clusters representing a single model significantly upregulated compared to other models and the common mechanism

of action of the genes within the cluster. **(C)** Radiation induced differentially expressed genes as identified through Pearson's Chi-Squared with Yates' Continuity Correction and organized based on cluster analysis of common mechanisms of action.

Using the bespoke set of genes predicted to be upregulated in response to IR, we saw significant differences in regulation based on model type regardless of IR status. The NTS model demonstrated comparative upregulation in Clusters 1, 3, and 5; the Spheroid model in Clusters 1, 2, and 6; and the 2D model in Clusters 4, 5, and 6 (Fig. 6A). While the models shared the comparative upregulation status in Clusters 1, 5, and 6 with another model, each model was singularly upregulated in comparison to the other models in Cluster 2, 3, and 4 (Fig. 6B). The genes in Cluster 2, representing the Spheroid model, shared common mechanisms of action in hypoxia, cell stress, and apoptosis. Cluster 3 genes, representing the NTS model, was identified as sharing Epithelial to Mesenchymal Transition (EMT) as their mechanism of action. Cluster 4 genes represented the 2D model and was linked through proliferation as their common mechanism of action (Fig. 6B). There was commonality observed between the models in Clusters 1, 5, and 6 but none that encompassed all three models. It can be stated that the models are regulated differently at the transcript level and that, while there is some crossover between them, overall, they represent different transcriptional profiles.

The bespoke gene expression profile was analysed through a Pearson's Chi-Squared with Yates' Continuity Correction test to identify those genes that changed their expression under IR exposure. The NTS model demonstrated greater responsiveness to IR with 54 differentially regulated genes, followed by the Spheroid model with 24, and the 2D model with 0 (Fig. 6C). There was an over-representation of EMT related genes in both the NTS and Spheroid models. For both, EMT related genes represented the bulk of IR responsive genes: 25/54 genes in the NTS model and 18/24

genes in the Spheroid model. This commonality was extended to apoptosis and proliferation associated genes. The NTS model is the most responsive model at the transcriptional level when exposed to IR and EMT appears to play a key role in this responsiveness.

Discussion

The role of the ECM in the presence and absence of a tumour has been well established [117, 118], however, detailed knowledge of the ECM composition and structure in both healthy and diseased tissue, as well as its impact on the surrounding cells during normal lifetime or in response to therapy is yet to be fully defined. In our study, we have established a 3D model based on decellularized native tongue tissue that fully allows the SCC 25 cells to home in and effectively proliferate since decellularized matrices' biochemical compositions and the native extracellular matrices' microstructures provide tissue-specific microenvironments for anchoring cells [119, 120].

Further comparative analysis of the NTS model with previously established 3D SPH or conventional 2D cultures, demonstrated that studying complex phenomena like the spread of cancer reveals inconsistent and varied responses from the mentioned models as transitioning cells into a 3D *in-vitro* or *in-vivo* environment causes significant changes in cellular activity [121, 122]. Even though many IR-mediated stromal alterations are advantageous, such as the polarisation towards immunity that suppresses tumours, unfavourable side effects may promote the propagation of cancer and treatment resistance [123-125]. One effect of IR on the tumour stroma is chronic inflammation, which is characterised by an increase in ECM elements such as MMPs and collagen, all of which are crucial for tumour development, resistance, and

metastasis which explains the radio-protective feature of the NTS demonstrated through data [126, 127]. Additionally, the ECM regulates radiation response by affecting oxygen availability and moderating the stability and bioavailability of growth factors and cytokines, which further promotes treatment resistance [128-131]. Transcriptional analysis of the molecular signatures between the three models also in the presence of radiation, showed distinct clustering among samples belonging to individual models despite the signature changes in response to radiation. The presence of ECM can promote integrin clustering, which can activate downstream signalling pathways such as mitogen-activated protein/ extracellular-signal-regulated kinase activation and focal adhesion kinase activation that encourages tumour cell survival, proliferation, metastasis, and invasion. Integrin activation has been linked to activities including angiogenesis and treatment resistance and is crucial for controlling the phenotypes of tumours [123, 129, 132]. Hence, the epithelial and ECM-associated pathways that have been elevated support the NTS's strong relationship to the dynamic TME.

Difference at the transcript level was expected to be seen between the models as each represented a particular element of in vitro modelling; beginning at the 2D model, moving the 3D of the Spheroid model and then to the more complex 3D ECM environment of the NTS model. In terms of responsiveness, this may explain the hierarchy of IR response seen in Figure 6C where the NTS model demonstrated greater differential transcriptional changes. Perhaps as the 3D microenvironment increases in complexity, so too does gene expression dynamics when treated with IR.

The over-representation of EMT related genes in the 3D models is interesting, especially given the comparison between the NTS and Spheroid model and their

differences. Cluster 1 in Figure 6A, where both the Spheroid and NTS models are represented as being upregulated, was identified as representing ECM related EMT interactions. This is most evident in the LAMA3, LAMC2, P4HA2, P4HA1, and ITGB4 genes which play a direct role in ECM biogenesis and signaling (Supplementary figure 6), potentially indicating ECM remodeling – a known action of EMT [133].

Interestingly, Cluster 3 genes, upregulated in the NTS model and not the Spheroid model, was identified as EMT related cytoskeletal change through regulation FLNA, FLNB, MYH9, PLEC, and KRT6A (Supplementary Figure 6). Given EMTs strong role in invasiveness and dissemination of cancer cells [134], and the active re-arrangement of cytoskeletal components in EMT driven metastasis [135], the NTS model could potentially represent a more responsive and accurate model of metastasizing cancer cells in oral squamous cell carcinoma in comparison to the 2D and Spheroid models.

Conclusion

Globally, the quantity of wasted organs from diverse research is of great concern. Therefore, the goal of this work is to create a study framework utilizing the native tongue tissue of lab mice that would otherwise be discarded after experimental use offering a tremendous potential to harvest biomaterials for medical research use and to create increasingly practical cell behaviour analysis techniques. Significant implications for tissue engineering, cancer treatment, and developmental biology result from our growing understanding of the ECM's function in regulating cell destiny. However credible *in-vitro* simulations that incorporate tissue-specific microenvironments are missing. Only with a precise study model is a greater

comprehension of the ECM's composition, architecture, and dynamics feasible. Hence using a revised protocol, we generated an *in-vitro* ECM scaffold, the NTS, to establish an effective analysis medium for tumour propagation that has been demonstrated to have a substantial aggregation of vital ECM components. We also have successfully mapped the effective proliferation of re-cellularized cancer cells into the model that is a time and cost-efficient prototype and can be easily adapted for multiple studies. We then contrast the outcomes of radiating oral cancer, SCC 25 cells, using both the revised 3D ECM model and the pre-existing models. Our statistics reveal that NTS surpasses other models' proliferative capacity while acquiring therapy resistance. The phenomenon behind the significant downfall in NTS of the ki67 index needs to be explored.

NTS has demonstrated the ability to secure the cancer cells in a treatment-resistant environment and also provides the required medium to metastasize with genomic data showing upregulation of ECM-associated pathways that can aid in recapitulating aspects of the complex 3D micro-tissue environment.

Henceforth, this study has great potential for structural studies based on ECM remodelling addressing targeted therapeutic approaches as well as regenerative research.

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Supplementary Figures:

https://vimeo.com/manage/videos/898688642

Fig. 3S Live sheet microscopy Embedded SCC 25 cells in the NTS at day 13

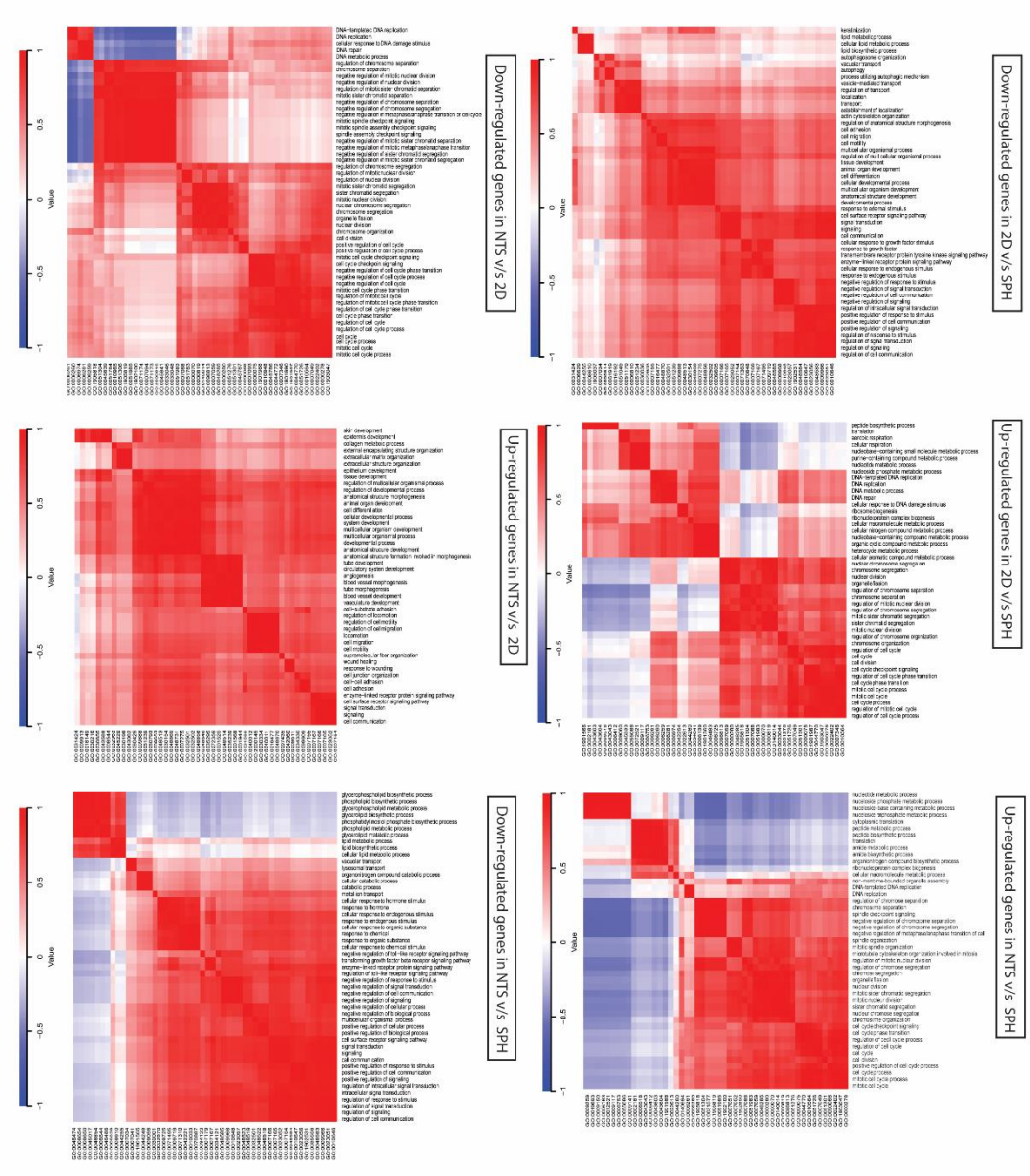

Fig. 4S Down and up-regulated genes in NTS v/s 2D, 2D v/s SPH and NTS v/s SPH. All data was normalized against the Z-score.

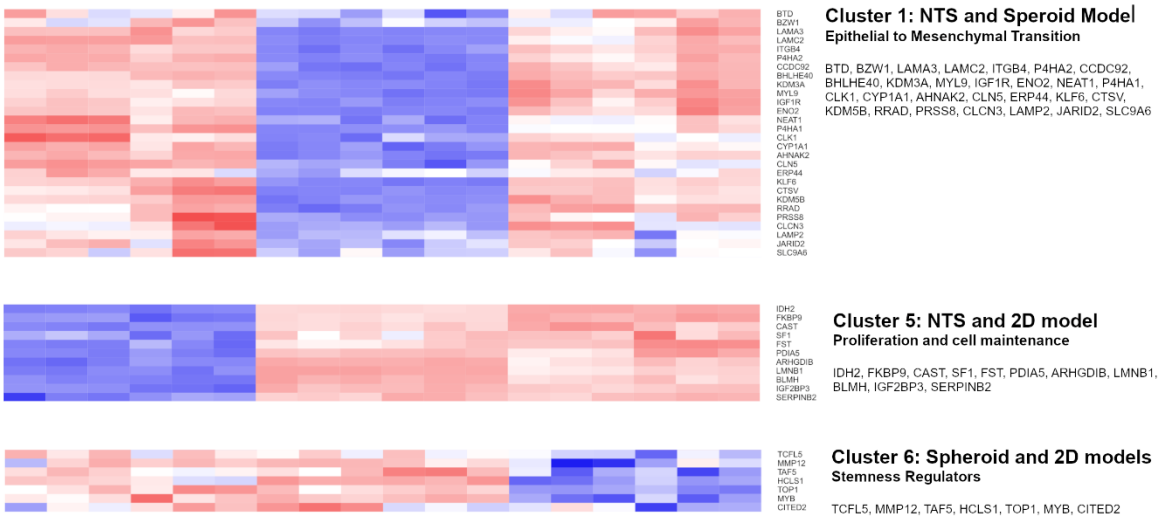

Fig. 6S Heatmap normalized to Z-Score, predicted upregulated gene expression profiles separated into clusters.

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Chapter 5: *The Effects of stromal ECM on platinum response to ovarian cancer cells*

5.1 Abstract

The tumour microenvironment's (TME) main role is to provide for the tumour's growth, and every component of the TME contributes to this goal. The cancer-associated fibroblast (CAF) is one such component. They are a pervasive cell group with a wide range of sources, all working together to craft the tumour immune-resistant and spreadable. Very limited research has been done into how CAFs could promote the development of cancer cells at the molecular level. So far, a huge variety of CAF subsets has been discovered that are distinct to each cancer type. For instance, four CAF subgroups, CAF-S1 through CAF-S4, have been established in the context of breast and ovarian cancer. It was shown that the transcriptional patterns of CAF subsets change from an assumed immunoregulatory programme to wound healing and antigen-presentation programmes throughout the evolution of breast, head and neck, and lung tumours. This suggests that CAF functions vary during oncogenesis. CAFs modify a variety of physiological processes to promote cancer growth, including inflammation, angiogenesis, and metabolism. They also cause ECM modification, which disturbs immune surveillance and hence affects treatment outcomes. CAFs have also been established to emit soluble factors that facilitate them to modulate tumour-promoting signalling systems while also activating them. This chapter meticulously discusses CAFs in depth. From their heterogeneous origin, what induces these origins, how are CAFs identified and how do they help amplify the biological process of tumour cells to proliferate and produce resistance clones to metastasize and recur. We utilized this understanding to further evaluate CAFs in relation to the ECM to observe how they alter one another's roles in cancer cells' survival to resist therapeutic effects post-platinum chemotherapy and to better comprehend their involvement in treatment sensitivity. This part of the study also aids in the assessment

of the cellular differences between CAFs and HDFs, which will help us better appreciate how CAFs evolve and improvise targeted therapy.

5.2 Introduction

Cancer-associated fibroblasts (CAF) have recently caught a great deal of scientists' attention because of their critical involvement in cancer development and progression. They have been established as the most abundant and vital stromal cells in the TME, and their dominance allows them to control the cellular biology of healthy and malignant cells [1-3]. The heterogeneity in origin (Fig. 1), phenotype, and function of these spindle-shaped cells allow them to modulate cancer propagation and this combined with significantly high plasticity results in a vast variation amongst these cells [4, 5]. CAFs are recognised for being extremely dynamic and resilient, allowing them to desensitise to treatment and generate resistant clones, however, there is still a lot of ambiguity around their impact on cancer cells [6]. Researchers continue to identify their multiple sources, prognostic biomarkers, cellular population subsets and most importantly the fundamentals of their cellular biology amplifying cancer progression and metastasis. CAFs co-evolve with cancer cells and develop a pro-tumour phenotype, allowing them to survive in the complex TME, fill it, and contribute to tumour development [7, 8]. They do this via a combination of techniques such as intercellular interactions, the release of various regulatory factors, and the construction and remodelling of the ECM, along with impacting angiogenesis, tumour biomechanics, medication permeability, and therapeutic responses. [9, 10]. As a result, CAFs have been proposed as a promising target for anticancer agents [11-14]. Since they occupy a major part of the TME and dictate treatment response and

eventually patient prognosis, this project brings light to CAF and its association with tumour progression.

It has recently been shown that there are several subsets of CAFs, some of which exhibit pro-tumourigenic features while others exhibit stronger anti-tumourigenic traits [15, 16]. Through the use of Gene Expression Profiling Interactive Analysis (GEPIA), it was demonstrated, for instance, that greater amounts of cytokines produced by CAF are associated with impressive cumulative colorectal cancer patient survival, hence better prognosis [17]. Furthermore, it has been demonstrated that the CAF-derived slit homolog 2 protein (SLIT2) prevents breast cancer cell invasion by blocking the phosphoinositide 3-kinase (PI3K) and -catenin signalling [18]. While it is believed that CAFs possess anti-tumourigenic traits, they are also documented as tumour initiators [3, 19]. CAF populations that impede tumour development and progression by activating stroma-specific Hedgehog (Hh), have been reported in a myriad of murine tumour models, including bladder, colon, and pancreatic tumours [2]. They are also observed to be an essential factor in encouraging tumour growth in prostate cancer by upregulating angiogenic factors and activating the Hh signal [20]. Several studies indicate enhanced tumour development and aggressiveness following the eradication of ACTA2-expressing CAFs and/or targeting of the desmoplastic response generated by the Hh signalling pathway [20, 21]. Many solid malignancies, like HNSCC, where late-stage HNSCC, for instance, usually comprises up to 80% of CAFs, make up the bulk of cell populations. Advanced desmoplasia, or the formation of fibrous or connective tissue, is another condition in which CAFs are known to play a significant role [22, 23].

5.3 Origination, identification and tumorigenic features of CAFs:

5.3.1. Origin

(a) Fibroblasts

Scientists have observed that the fibroblasts, which are principally responsible for producing ECM, are abundant in the tumour stroma, sometimes outnumbering the cancer cells [24]. Fibroblasts are summoned upon in the event of tissue damage or injury, as well as to maintain homoeostasis, a process that is initiated and perpetuated by several cytokines. Typically dormant fibroblasts are transdifferentiated into myofibroblasts (MFB) as a result of the recruited fibroblasts depositing ECM proteins, which increases mechanical stress in the wound and is accompanied by a notable over-expression of smooth muscle actin (ACTA2), an actin isoform that contributes to cell-generated mechanical tension of the ECM. [25, 26]. On the contrary, in healthy tissue, wound healing drives these MFBs to undergo apoptosis to avoid ECM modification or become dormant, where they are no longer active to cause cellular apoptosis [27].

When cancer progresses, the cytokine TGF-β, which is necessary for both the activation of the cancer stroma and the production of a fibrotic response, acts as a tumour promoter. One of TGF-β's early functions is to increase the expression and production of the impactful ECM proteins collagen and fibronectin, which stiffens the ECM. Additionally, it regulates the production of MMP inhibitors like tissue inhibitors of metalloproteinases and cell adhesion protein receptors such as integrins (TIMP) [28]. In Chapter 2, it was discussed how these dysregulated proteins affect the prognosis of tumours. Furthermore, TGF-β is in charge of stimulating fibroblasts' differentiation into CAFs. It triggers several inherent molecular alterations in CAFs, potentially making them candidates for pro-carcinogenesis [29]. Thus, producing

CAFs in the lab has been the subject of several research that have employed and validated this hypothesis. This study uses the same technique, which is described later. Notably, TGF-β acts as a tumour suppressor in the early stages of tumour development, in contrast to its role in tumourigenesis, as shown by the fact that blocking TGF-β signalling impairs normal homeostatic activities and promotes carcinogenesis [9, 30]. As a corollary, considering how rapidly TGF-β transforms the outcomes from cancer cell failure to survival, it is pivotal to understand CAF conversion to target TGF activity for therapeutic benefits.

Despite the fact that CAFs are a mutant version of fibroblasts, they vary from both normal and malignant fibroblasts in several ways. Resting mesenchymal cells anchored in ECM are designated as fibroblasts in healthy tissues that can be triggered to aid in wound healing, tissue inflammation, and fibrosis repair and regeneration. Quiescent, non-proliferative, and low metabolic state and insignificant transcriptomic activity are fibroblasts' key properties [31, 32]. Activated CAFs, in comparison to their non-activated counterparts, have increased proliferative and migratory abilities. They are often bigger than inactive fibroblasts, with indented nuclei and more cytoplasm branching visible under optical microscopy [33]. Malignant fibroblasts and CAFs are both defined by the loss of certain fibroblast markers FAP and the accumulation of ACTA2, as well as augmented ECM production and restructuring and a contractile morphology [34]. Tumourigenic fibroblasts vary from CAFs in their ability to undergo apoptosis and/or dedifferentiate to a dormant state following tissue repair. Unlike normal fibroblasts, CAFs stimulate tumour cell proliferation and migration [35].

(b) Adipocytes and other cellular origins

CAFs have been postulated to come from a variety of sources other than fibroblasts, including adipose-derived mesenchymal stem cells (AD-MSCs), bone marrow-derived mesenchymal stem cells (BM-MSCs), endothelial cells, and epithelial cells [36]. Despite the fact that cancer-associated adipocytes play a significant tumourigenic role in fatty acid metabolism in TME adipocytes by, for instance, directly increasing ovarian cancer (OC) cell migration and invasion, adipocytes have not been recognised to play a purpose in augmenting CAF's disruptive conduct against non-malignant cells [37]. Whilst some studies have reported adipocyte conversion to CAFs in a variety of tumour types, they are not commonly acknowledged as a notable source of CAFs. Because activated fibroblasts interfere with adipocyte formation, adipocytes may be decreased or non-existent in pathological tissue. Even if they are not transformed into CAFs, adipocytes can interact with cancer cells and provide metabolic support [38, 39].

(c) ECM alteration

Physical modulation of ECM also triggers CAF activation. *In-vitro* studies have demonstrated that fibroblast stretching, which may occur as a result of the hyperproliferation of altered epithelial cancer cells, can activate serum response factor (SRF) - driven and Yes-associated protein 1 (YAP1) – transcriptional enhancer associate domain (TEAD) - driven transcription [40, 41]. Both YAP1 and TEAD, are a part of the Hippo pathway which is a key regulator of cell-contact inhibition and a signalling network that regulates healthy organ growth hence an overproduction of these proteins leads to uncontrollable cell division [42]. Moreover, these transcription factors collaborate to control the expression of several CAF-related genes, including connective tissue growth factor (CTGF; also known as CCN2). Additionally,

matricellular molecules like CTGF and CYR61 collaborate with the contractile cytoskeleton to increase tissue stiffness, which, as previously discussed in previous chapters, contributes to the progression of cancer. They also encourage SRF- and YAP1-dependent transcriptional programmes, involving CAFs in a self-sustaining advantageous circuit [43]. Apart from physiological, and genetic stressors can also incur fibroblast alteration. For instance, double-stranded DNA breaks (DSB), resulting from radiotherapy, can boost IL-6 and the TGF-β family ligand activin-A production that results in the progeny of CAFs [44, 45].

(d) Epithelial-to-mesenchymal transition

Induction of the epithelial-to-mesenchymal transition (EMT) is one of the most recently recognised mechanisms underpinning CAF-mediated regulation of oncogenesis and therapeutic resistance. [46]. It occurs when solitary tumour cells lose cell-to-cell adhesion in a gradual manner characterized by several cellular states expressing different levels of epithelial and mesenchymal markers and exhibiting intermediate morphological, transcriptional, and epigenetic features, between epithelial and mesenchymal cells [47, 48]. EMT is a well-known mechanism for cancer metastatic promotion. It appears to be the driving force behind metastasis start and inducing this phenotypic state in tumour cells necessitates cross-talking with stromal cells, particularly CAFs [49, 50]. A cell with a mesenchymal phenotype is more likely to develop drug resistance and invade. Varied EMT transition stages have different roles, with hybrid EMT having the most metastatic potential. Tumour initiation, spread and stemness, intravasation into the bloodstream, and therapeutic resistance have all been linked to EMT [51, 52]. The acquisition of an EMT improves cancer cell invasiveness and the ECM disintegration necessary for cell migration. TGF-β secreted

by CAFs also promotes EMT-mediated invasion [53]. CAFs' propensity to promote EMT via overexpression of TGF- β, which affects cellular cytoskeleton architecture, and cyclin-dependent kinases, and reduces the effectiveness of immune surveillance, might explain their histological abnormalities and variations. As a result, tumour cell migration and invasion are enabled, as well as the formation of pluripotent tumour cells [54].

(e) Hepatocytes

In the chronically injured liver, CAF is hypothesized to evolve from a myriad of cell types including portal fibroblasts, MFBs, fibrocytes, hepatocytes, hepatic progenitor cells, and mesothelial cells. During the hepatocellular progression, liver MFBs are believed to transform into CAF and further aid in their tumourigenic properties [55]. CAF have ACTA2 and fibroblast activation protein α (FAP) expression similar to activated hepatic stellate cells (HSC) [56]. In conjunction with secreting these growth factors, CAFs also mediate additional functioning cell surface proteins including PDGFR and the insulin-like IGFRII in addition to FAP. The TME's principal source of CAF is activated HSCs. Tumour cell predominance, motility, recurrence, and poorer prognosis are all linked to the interaction between activated HSC/CAF and tumour cells [57].

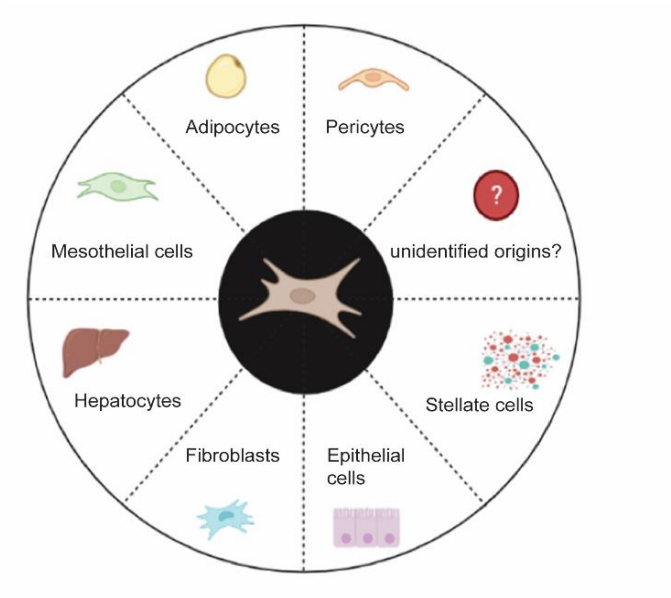


Figure 2 Cellular origins of CAFs

5.3.2. CAF identification markers

As established, fibroblasts mutate to form CAFs [24]. The molecular transformation from fibroblasts to CAFs has been studied extensively. Many studies have revealed how the fibroblastic component of carcinomas alters as they progress from hyperplasia to aggressive carcinoma in fibroblastic stroma patients [58]. The appearance of fibroblast proliferation is noted as a signature marker that often precedes the onset of cancer. Since they are structurally very similar to fibroblasts, they are identified by certain proteins that are highly expressed by CAFs and hence are termed ‘CAF markers’. The most often cited indicators that have a substantial expression include α-smooth muscle actin (ACTA2), fibroblast activation protein-α (FAPα), ACTA2, Integrin subunit α-11 (ITGA11), Chondroitin sulphate proteoglycan 4 (CSPG4) and Tenascin C (TNC) [59-61]. ACTA2 and PDGFR are some of the markers that are also highly expressed in CAFs and have been used to distinguish these populations in the past. However, several of these markers have limitations, such as low specificity, and

it's been questioned whether they can detect all CAFs or only a portion of them among the wider CAF population. These indicators, however, can also be difficult to distinguish between normal tissue-resident fibroblasts and CAFs [6]. Three proteins, including collagen 11-1, microfibrillar-associated protein 5, and asporin, are also primarily expressed in CAFs in addition to these indicators [62]. The cyclical nature of the changes seen has led to the conclusion that the majority of stromal fibroblasts are formed from tissue malfunctioning local fibroblasts [63, 64]. Another distinguishing and categorising feature of CAFs is their high capacity for ECM synthesis and remodelling during the collective response of various stromal cells to an initial tissue injury, which typically results in dense fibrosis or scar tissue in malignant neoplasms, also known as the desmoplastic reaction [65].

5.3.3. HDF conversion to CAF

A lot has been talked about where the CAFs are sourced from, in the previous trials established through studies but it did not provide a strategy for transforming them [66]. Multiple mechanisms and proteins are studied to be a part of this act and notch signalling is one such pathway. Though Notch signalling has been observed as a pathway for CAF conversion through contact between breast cancer cells and fibroblasts, a depletion of the same signalling mechanism is seen to enhance CAF phenotypes in squamous cell cancer [67, 68]. As a result, Notch signalling cannot be documented as a universal protocol. Various cytokines and chemokines are also involved in the conversion of normal fibroblasts into CAFs, and some of these form a feedback loop between cancer cells and CAFs. By stimulating TGF-β associated signalling pathways, lysophosphatidic acid from ovarian cancer and exosomes encourages the differentiation of MSCs originating from adipose into CAFs, which are

identified by the expression of ACTA2, FAP, and PDGFR [69]. Several inflammatory modulators can enhance CAF activation, with interleukin-1 (IL-1) working through NF-B and interleukin-6 (IL-6) acting largely on the transcription factor signal transducer and activator of transcription (STAT). CAF activation is further aided by crosstalk and positive feedback involving Janus kinase (JAK)–STAT signalling, the contractile cytoskeleton, and changes in histone acetylation through IL-1 induction, that promotes the generation of inflammatory CAFs [70].

5.4 CAF derived tumour promoting biological processes and factors

CAFs have been shown to promote tumour growth by controlling a variety of activities such as cancer cell proliferation, tumour cell invasion, inflammation, metabolism, angiogenesis, and ECM remodelling by immunomodulatory pathways, including soluble factor secretion. For instance, they generate important proteins such as Periostin and TNC, which aid tumour support and metastasis [71]. CAFs also secrete a wide range of growth factors, including hepatocyte growth factor (HGF), Epidermal growth factor (EGF), connective tissue growth factor (CTGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), leukemia inhibitory factor (LIF), prostaglandin E2 (PGE2) and TGF as well as cytokines like CC-chemokine 2 (CCL2), CCL5, colony-stimulating factor 1 (CSF1), CXC-chemokine 5 (CXCL5), CXCL9, CXCL10, CXCL12 (also known as stromal cell-derived factor 1 (SDF1)) and IL1, IL-4, IL-6, IL-8, IL-10, metabolites, ECM components, such as collagens, fibronectin, and TNC, and ECM remodelling enzymes, such as MMPs, LOXs, when activated [72, 73]. CAFs' secreted factors can affect tumour proliferation and antitumour immunology, both directly and indirectly, in a jargon fashion, since CAFs dynamically change across malignancies [74]. Other

CAF-secreted factors, including LIF, IGF1, HGF, IL-6, WNT5, and bone morphogenic protein 4 (BMP4), have been revealed to boost tumour development and progression through bidirectional communicative connections between CAFs and cancer cells [75, 76].

5.4.1 Angiogenesis

Tumours require to quick creation of a new circulatory network to sustain the fast proliferation of cancer cells. The TME can be profoundly affected by tumour blood vascular dysfunction, which can result in hypoxia, reduced immune cell infiltration and activity, and higher chances of metastatic spread. The aberrant amounts of growth factors released by tumour and stromal cells contribute to the inappropriate development of tumour blood vessels, which is called angiogenesis [77, 78]. In a tumourigenic state, CAFs undergo epigenetic alterations that result in the production of secreted factors, exosomes, and metabolites that boost tumour angiogenesis [79]. CAF-produced cytokines and chemokines, as well as the CAF-remodelled ECM (discussed later in this chapter), regulate immune responses and angiogenesis in tumours, resulting in an immunosuppressive TME that promotes therapy resistance through a range of mechanisms, such as regulation of interstitial fluid pressure, cell adhesion, and cancer cell survival [80, 81]. PGE2, a hormone that promotes tumour formation, angiogenesis, and resistance to traditional cancer therapies, is found to be produced mostly by CAFs. Moreover, PGE2 inhibits the immune system by altering the balance from anti-tumour to immunosuppressive, as well as lowering T-cell cytotoxicity and enhancing regulatory T-cell (Treg) activity [82, 83]. Furthermore, CAF is high in COX2, the enzyme that catalyses the creation of PGE2, leading to enhanced Treg recruitment in malignancies [84]. CAFs also enhance tumour progression via

VEGF which consequently promotes angiogenesis [85]. Additionally, CAFs also have an impact on tumour-associated macrophage (TAM) differentiation that aids pro-inflammatory signalling, angiogenesis, metastasis, and treatment resistance, among other tumour-promoting effects [86].

5.4.2 Metabolism

Glycolysis, or the conversion of glucose to pyruvate, is a crucial metabolic mechanism for healthy cells to create energy in the form of ATP under normal and healthy circumstances. Conversely, cancer cells prefer to create energy by converting glucose to lactic acid, even under aerobic settings, to generate ATP faster. This process is known as the Warburg effect [87, 88]. Recent discoveries, on the other hand, have shifted the way scientists believe about TME, particularly the crosstalk between CAFs and tumour cells and how this crosstalk hinders metabolism. The Warburg effect, which was previously thought to be exclusive to cancer cells, has now been found in the fibroblasts that surround cancer cells [89]. The *'reverse Warburg effect'* was coined to separate this CAF-related phenomenon from its cancer cell-related analogue [90]. According to this theory, cancer cells first encourage normal stromal cells to become CAFs, creating a tumour-friendly milieu in which tumour development may take place. Following the conversion of lactate to pyruvate, lactate from CAFs is directly supplied to cancer cells as fuel for aerobic metabolism. As a result, glycolytic enzyme expression levels, such as monocarboxylate transporter 4 (MCT4), are increased in the CAFs of breast and lung malignancies [91, 92]. In prostate, pancreatic, and breast cancer, high expression levels of genes implicated in the lactate shuttle system, and even more so high expression levels of MCT4, are tied to a poor prognosis. As a result, mounting data shows that MCT4 transporters might

be attractive cancer therapeutic targets [93].

5.4.3 Remodelling ECM

As outlined in the early chapters and as the core principle of this project states, ECM vastly affects cancer state and treatment response by undergoing stiffening or degradation. One of the many ways ECM is constantly being remodelled to negatively influence tumour prognosis through the aforementioned processes is via CAFs. CAFs, implicated in metastatic niche development, have been discovered to play critical roles in ECM modifications aimed at encouraging cancer cell adherence and proliferation [94]. The metastatic potential of cancer cells is affected by biophysical changes such as matrix stiffness, pore size, the degree and density of crosslinked proteins, and the configuration of the fibre network [95]. CAFs degrade this matrix and assist in invasion by building tracks in the ECM, allowing cancer cells to escape the place of origin and henceforth build a secondary or metastatic site. The development of these tracks necessitates both force-mediated and protease-mediated matrix remodelling [40, 96]. The sophisticated and dynamic interplay between cancer cells, CAFs, and immune cells also causes ECM strain. MMPs are also released as a result of interactions between cancer cells and cells in the tumour stroma, including CAFs [97]. As discussed in the previous chapters, MMPs destroy all ECM proteins, while degrading them, which is necessary for oncogenesis to develop. MMPs degrade ECM, allowing cancer cells to change their structure and acquire proliferative, invasive, and migratory characteristics [98]. Fibronectin synthesis is stimulated by the infiltration of other cells into the TME, which results in a stiffer ECM, however, CAFs have a more substantial impact in this respect. [99]. These converted fibroblasts stiffen the ECM in response to YAP1 signals, which, as mentioned earlier, is one of the leading components in the

Hippo pathway that encourages immunosuppression and drug resistance in cancer cells. When combined with other oncogenic factors like COX-2, this pathway upstream amplifies collagen production in tumour cells linking and, along with TGF-β [100], it attracts more fibroblasts in the same process and hence is thought to be a driving force in the reduction of cancer metastatic time [101, 102]. The excessive deposition of structural ECM proteins (such as collagen I-III, V, and IX), increased production of protein crosslinking enzymes and local topographical realignment of the ECM fibres brought on by contraction force are all ways that CAF can further stiffen ECM. CAF enzymes, such as LOX, operate on newly deposited ECM proteins to stiffen the ECM more; stored growth factors are then released to enhance the circuits between the tumour cells and their ECM. Ultimately, this causes metastases and drug intervention tolerance in the ECM [71].

As tumours grow, CAFs have been found to modify the ECM quantitatively, making it more amenable to tumour invasion into the surrounding tissue. TGF-β, certain interleukins and metalloproteases are all significantly secreted ECM remodelling components [103, 104]. MFBs and CAFs, which are prevalent in the stroma of carcinomas and in particular around the edge of the tumour that is invasive, yield ECM proteins and growth factors, increasing the amount of ECM deposited, which in turn stimulates the propagation of cancer cells and the de-differentiation and migration of cancer stem cells too [105, 106].

5.5 Treatment-resistant and therapeutic targets

CAFs have been shown to be a good predictor of tumour recurrence in studies. Interestingly, they've also been linked to a tumour-suppressive effect via the I kappa

B kinase/nuclear factor kappa-light-chain-enhancer of activated B cells (NFB) pathway, which is known to reduce tumour size and slowing metastasis by lowering HGF synthesis, a growth factor that promotes tumourigenesis and sustenance, intensifies tumour aggressiveness, and provokes metastasis [107, 108]. By secreting TGF to encourage a radioresistant cancer stem cell phenotype, CAFs can also aid in the radio-resistance of tumours. CAFs are radioresistant and can endure doses of up to 50 Gy in cell culture. Stress-induced cellular senescence and permanent DNA alterations are brought on by doses greater than 10 Gy [109-111]. Along with factors including growth factors, proteases, inflammatory mediators, and extracellular matrix protein that enhance radiotherapeutic resistivity, CAFs also produce exosomes that interact with tumour cells via retinoic acid-inducible gene-I (RIG-I) and increase their radio-resistance. The DNA damage-induced senescent-messaging secretome from irradiated CAFs is thought to have several effects, including enhancing integrin expression and encouraging tumour cell survival through EMT. However, high radiation doses may also have a direct therapeutic impact in part because the tumour's CAF levels have decreased [112-114].

Nevertheless, there are significant roadblocks in the way of current efforts to alter CAFs for therapeutic purposes. The paucity of understanding about CAF origins and variance in CAF function are two of these difficulties with several other challenges to overcome in this sector, not least the fact that CAFs can have both pro-tumourigenic and anti-tumourigenic properties. Hence, this project dives deep into CAF's therapy response in association with the ECM starting with establishing a protocol to produce CAFs in the lab that can be employed for future cancer treatment studies too.

5.6 Ovarian cancer and the ECM

OC is one of the most prevalent causes of death in women, as was previously mentioned in Chapter 1, Introduction. The epithelial lining of the ovary or the fallopian tube undergoes a malignant change to develop OC. HGSOC, the predominant subtype, accounts for more than half of all cases of OC, as was previously established, and is characterised by various histopathological profiles or molecular markers. The disease's enigmatic symptoms, which are commonly misinterpreted, contribute to the high fatality rate along with a high recurrence rate owing to the intrinsic therapy-resistant mechanisms triggered within the cancer cells and TME. Mature OC has a higher metastatic rate, making treatment more challenging.

Research has proven surgery and/or chemotherapy to be the most effective treatment form for OC. The surgical approach involves partial or complete hysterectomy while adjuvant chemotherapy relies on platinum-based drugs, namely, carboplatin and cisplatin [115]. Due to lower side effects, carboplatin has emerged as the more successful therapeutic alternative to cisplatin [116]. Carboplatin forms covalent connections with DNA, RNA, and a multitude of proteins, targeting the gap2 (G2)/mitotic phase of the ovarian cell cycle and suppressing the S phase. Consequently, carboplatin limits apoptotic activity in cells by disrupting the nuclei adversely, which has a negative impact on the tumour’s metastatic potential [117, 118]. The aptitude of OC cells to identify and restore DNA damage defines their responsiveness to DNA-damaging agents [119].

As an adjunct to surgery, platinum-based chemotherapy is the most used treatment strategy chosen for OC patients, which includes two primary drugs - Carboplatin and Cisplatin. Although cisplatin and carboplatin (1,1-cyclobutane dicarboxylate) are both equally effective platinum-based medicines for treating OC, carboplatin is currently the most preferred drug attributable to its reduced risk of adverse effects. It limits the growth of OC by altering its DNA. Early chemotherapy frequently fails to completely eradicate the tumour cells, which causes the cancer to return. Despite the substantial response to the initial platinum therapy, more than 65% of patients recur within 5 years.

One of the major reasons for such a high recurrence rate and poor prognosis to chemotherapy is drug resistance [120]. OC cells gain chemotherapeutic resistance through a multitude of channels that include DNA-repair activation, imbalance of pathways, autophagy, drug efflux, presence of dynamic cancer stem cell subpopulations, mutations caused by the TME etc. Some of the ubiquitous genes and proteins that are dysregulated and have been highlighted by various research for being associated with therapy resistance include BRCA1, PTEN, TP53, MLH1, ErbB3, E-cadherin and Vimentin [121, 122]. This project brings the TME aspect to light and how its associated alterations induce therapy resistance to OC cells. Moreover, whole genome sequencing has revealed that a quantifiable amount of non-silent coding single nucleotide variants is amplified inconsistent with the frequency of exposure to platinum-based therapies, suggesting that the primary tumour evolves during treatment [123, 124]. Alongside modification of the genetic make-up, generation of the cancer-associated fibroblasts (CAFs) have also been studied to play an impactful role by secreting CCL2 and CCL5, which in turn promotes IL-6 production in ovarian, gastrointestinal and several other cancer cells and subsequent chemoresistance [125-

130].

Mechanisms of carboplatin resistance are diverse [117, 129]. The intracellular characteristics of cancer cells are the main focus of conventional research on carboplatin resistance. This includes diminished blood flow, decreased intake of carboplatin, elimination of carboplatin, faster DNA repair, improved DNA damage tolerance, downregulation of proapoptotic proteins, elevated levels of apoptosis inhibitors, and heightened levels of carboplatin efflux which is also the most frequent mechanism and a major contributor to chemoresistance [130, 131]. A possible therapeutic target for the treatment of OC is CD44, a non-kinase glycoprotein transmembrane receptor that has been associated with cancer metastatic progression, cancer stem cell survival, and the emergence of chemoresistance OC subpopulation. The majority of epithelial OC tumours express CD44, and larger levels of CD44 are associated with later stages of the illness as it is closely correlated with the development of metastases and disease recurrence. This proves that CD44 concentrations are a useful marker for clinical diagnosis and prognosis [132, 133]. The expression of stem cell markers by CD44-positive (CD44+) ovarian tumour cell subpopulations has been documented, and these cells are capable of initiating carcinogenesis and encouraging disease recurrence by reiterating the initial tumour. The only cells that survive chemotherapy and can subsequently regenerate the tumour are those that have CD44 expressed on their surface. They are reputedly markedly resistant to carboplatin, a component of platinum treatment. The Type I chemo-resistant and Type II chemo-sensitive EOC cell fractions have been referred to as CD44+ and CD44-, respectively [134-136]. Collagen, laminin, fibronectin, and many growth factors all have binding sites in the CD44 extracellular domain, but hyaluronic

acid (HA) is the most common and specific CD44 ligand [137, 138]. HA, a subtype of GAG present in the ECM, is responsible for regulating cell adhesion, differentiation, and motility. Since serum, HA levels were greater in chemo-resistant patients at relapse than at diagnosis, but not in chemo-sensitive patients, indicating that HA may help enhance platinum resistance in the formation of malignancies, HA is essential in controlling tumour matrix stiffness together with collagen [139-141]. Chemoresistance is promoted by HA by activating downstream survival and antiapoptotic pathways. By binding to HA-CD44, the most often genetically altered pathway in OC, PI3K, is induced, promoting cell growth. HA also facilitates CD44 complex formation triggering the MAPK pathway through Ras signalling in SK-OV-3 cells, another aggressive OC cell line. In addition to activating NF-kB, coupling with CD44-HA may also elicit size reduction in the SK-OV-3 cells treated with carboplatin and paclitaxel [142, 143]. The development of OC can interfere with the signalling pathways for PI3K, MAPK, and NF-kB, further increasing resistance to platinum therapy [144]. However, due to the limited study, the extracellular functions are still poorly known. Henceforth, we evaluate the effect of ECM on carboplatin therapy response through this project.

Our theory, that CAFs and the effects of the ECM drive the growth of tumours, as a strong relation between CAF and OC prognosis has already been established [145, 146], was tested using 2 HGSOC cell lines, AOCS15 and AOCS21. Since OC treatment plans often include chemotherapy and surgery, followed by radiotherapy, if necessary, this portion of the research concentrates on the chemotherapeutic element rather than ionising radiation.

5.7 Methods:

5.7.1 Generation of CAFs

a) Optimising fibroblasts' conversion

As previously discussed, TGF-β induced fibroblasts are one of the pathways through which CAF develop. To create an *in-vitro* ECM study model, HDFs were plated in 6-well plates and subjected to TGF-β to start the fibroblasts' conversion to CAFs. Each step of this process was heavily scrutinized and optimised to produce the most amount of healthy CAFs efficiently. This method was used to examine the chemotherapeutic resistance component of ECM. Every contingent parameter, including the amount of HDF seeded, the HDF incubation duration, and the TGF- concentration, has been adjusted to produce a repeatable sum of CAF.

Healthy HDFs were cultivated in 6-well plates for 7 days at a low (50,000 cells/well) or high confluency (270,000 cells/well). A control with no TGF-β1 treatment was incorporated for each time point. The wells were then treated with 2 µl of Recombinant Human Transforming Growth Factor beta 1 (TGF-β1) (10 ng/ml, PeproTech) in either DMEM, RPMI or AOCS15- or AOCS21-conditioned RPMI medium. AOCS15- or AOCS21-conditioned medium was used to account for any effect from extracellular vesicles and other secreted signalling molecules derived from the OC cells and to further assess the ideal condition for activation of normal fibroblasts. The medium was changed every 2 days. Here, the term "conditioned media" refers to the RPMI medium extracted after the AOCS cells were incubated in them for a minimum of 5 days. Following 5 and 7 days of TGF-β exposure, cells were washed with PBS, harvested by incubating with TryplE Express for 5 minutes at 37°C and resuspended in the

growth medium. the expression of important CAF markers mentioned prior, namely ACTA 2, FAP, ITGA11, CSPG4 and TNC, that have been established through literature, were evaluated by running a Stratagene Mx3000P qPCR after extracting RNA for all samples, in triplicates, utilising the cDNA samples and the SensiFAST SYBR Lo-ROX Kit (Bioline). The housekeeping gene glucuronidase beta (GusB) (NC_000007.14) was run for data normalisation. Primers (table 1) were verified using Universal Human Reference RNA (Agilent Technologies) by ensuring there was only one melt curve peak for each. Primer3 Input (Whitehead Institute for Biomedical Research), Primer Blast (National Centre for Biotechnology Information) and OligoAnalyzer (Integrated DNA Technologies) were used to design primers, ensure primer specificity and check for secondary structure formation respectively. The primers (10μM) spanned exon-exon junctions to target CAF markers in order of validity: smooth muscle actin α 2 (ACTA2 also known as α-SMA) (NC_000010), fibroblast activation protein α (FAP) (NC_000002), tenascin C (TNC) (NC_000009), chondroitin sulphate proteoglycan 4 (CSPG4) (NC_000015) and integrin subunit α 11 (ITGA11) (NC_000015) human genes.

Human Target Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
Smooth muscle actin α 2 (ACTA2 also known as α-SMA)	GCGTGGCTATTCCTTCGTTACT	GGCCATCTCATTTTCAAAGTCC
Fibroblast activation protein α (FAP	ACAGCAAGTTTCAGCGACTACG	GTCCATCATGAAGGGTGGAAAT
Tenascin C (TNC)	CCACAACCAAAACCACACTCAC	GCTGGATTGCTCTCCTTGTCTT

Chondroitin sulphate proteoglycan 4 (CSPG4)	AGAAGGAGGACGGACCTCAAG	GTCATGCACGTAGCGGATCA
Integrin subunit alpha 11 (ITGA11)	CTTGGCAACGCTGTGATTCT	ATGTTGATCTTGGATGGCTCAA

Table 1 Forward and reverse series of primers used to authenticate CAF generation.

b) Statistical Analysis

Data were analysed by unpaired t-tests under the assumption of the Holm-Sidak method. Data shows the fold change in mRNA expression for the control with no TGF-β treatment, normal medium, and AOCS15 and AOCS21 conditioned medium normalised to the same control for HDFs cultured to low confluency and treated with TGF-β for 5 days (±SD, n=3 for all subsets except all controls where n=2; $p^{*}<0.05$) or low confluency and treated with TGF-β for 7 days (±SD, n=3; $p^{****}<0.0001$) or high confluency and treated with TGF-β for 5 days (±SD, n=3 for all subsets except ACTA2 and ITGA11 controls where n=4 and FAP, TNC and CSPG4 controls where n=2; $p^{*}<0.05$; $p^{**}<0.01$) or high confluency and treated with TGF-β1 for 7 days (±SD, n=3 for all subsets except ACTA2, FAP, CSPG4 and ITGA11 controls where n=2; $p^{*}<0.05$; $p^{***}<0.001$).

c) Validation of CAF establishment

The successful generation of CAFs was essential for their study of intrinsic therapeutic resistance. Hence, the morphological transition of CAFs from HDF after TGF-β exposure was routinely imaged under the microscope. Immunohistochemistry (IHC) was further carried out to ensure the conversion of HDF to CAF via staining for ACTA2 as that is one of the most well-established hyperactive proteins in CAFs with

significantly low activity in HDFs. Thus, the activity of ACTA2 was further evaluated in both sets of cells. HDFs and CAFs were cultivated on coverslips and then stained for the ACTA2 antibody with Hoechst, to identify the nuclei. A similar experimental setup was employed to analyse the collagen distribution, through the Col-1 antibody, which is known to be one of the most predominant proteins found in these cells. The nuclei were identified using the Hoechst stain. Further detailed IHC methodology can be read under section 3.2.2 of Chapter 3, Methods.

5.7.2 Treatment of Ovarian cancers with varied ECMs and carboplatin

a) Establishment of ECM extraction protocol

ECM was secreted from both, HDF and CAF cells. Each cell line was cultured and grown in 6-well plates for 7 days until reached a minimum confluency of 270,000 cells/well with growth media change every 2 days. All cells were then removed to allow the ECM to remain intact in the well plate. This was done by aspirating the media and washing the wells gently with PBS by rocking and tilting the well plate and using a glass pipette to collect the medium against the walls rather than from the floor which can puncture the ECM. The wells were decellularized of HDF cells by using Ammonium Hydroxide (20 mM, Sigma-Aldrich (Merck)) and Triton X-100 (0.5%, Sigma-Aldrich (Merck)) in PBS without Ca2+/Mg2 for 1.5 minutes at room temperature, to form a thin, transparent, and shiny ECM layer on the bottom of each well of the 6-Well plates. During the 1.5-minute incubation period, gently agitate the dish every minute to ensure the lysis of all the cells. The remaining ECM was gently but thoroughly washed with PBS twice to ensure the complete removal of all lysing

reagents. The wells were then ready for the next step, the implantation of OC cells on this ECM. The same protocol was used to extract ECM from CAF, as explained below. The same process was used to establish a counter-comparison as a positive control of no ECM plate. This is further illustrated in Figure 2 below.

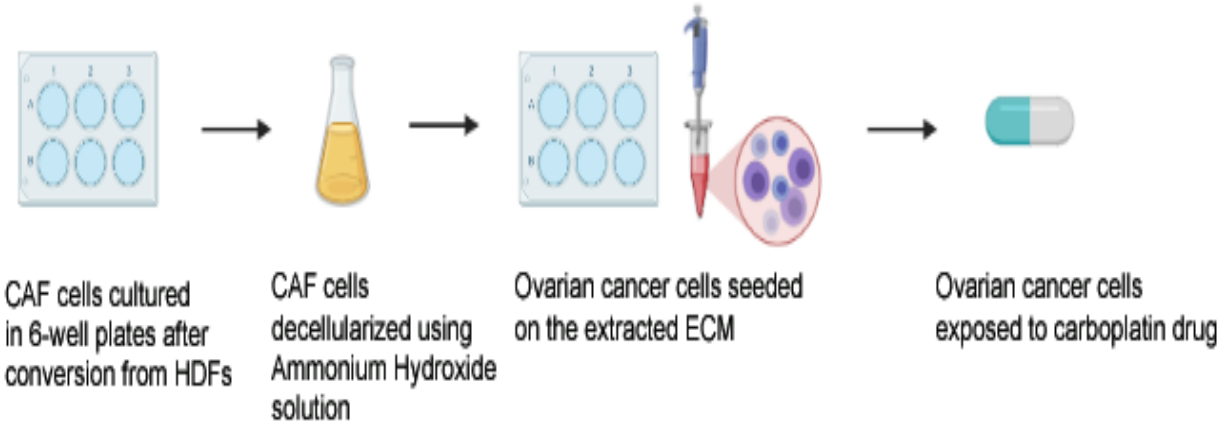


Figure 2 Schematic representation of the procedure for testing ECM's efficacy against platinum therapy

b) Decellularizing OC cells to extract their ECM

We further looked at how OC cells responded to chemotherapy under the influence of their ECM as it is believed that different ECMs generated from different cells may potentially have distinct components that could end up resulting in changes in cancer prognosis. The identical procedure used for HDF and CAF was adopted to extract AOCS15 and AOCS21's ECM.

c) Validating ECM extraction

The ECM extract was imaged under the microscope and also stained for IHC using collagen antibody and Hoechst stain for nuclei. The key was to remove all remnant cells without wiping out the thing ECM. Different concentrations of the ammonium hydroxide mix were employed to achieve the most amount of healthy ECM.

d) Evaluating ECM response under chemotherapeutic approach for OC cells

After being seeded on the various ECMs (CAF, HDF, and without an ECM as a control), the cells from both OC cell lines, AOCS15 and AOCS21, were incubated for 24 hours at 37°C and 5% CO2 to facilitate adhesion to the ECM. The use of the same number of live cells (40,000/well) for each sample served as internal quality control. After 24 hours, carboplatin (1, 10 and 100 µl; Sigma-Aldrich (Merck)) was added to each well. A negative control involving no carboplatin was incorporated for both ECM and no ECM samples for normalisation. Samples were incubated at 37°C and 5% CO2. After 3 days of carboplatin treatment, live cells from all samples, in triplicates, were harvested with TrypleE, stained and diluted 9:1 with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Using CytExpert (Beckman Coulter Life Sciences) and a CytoFLEX Flow Cytometer (Beckman Coulter Life Sciences, the number of live cells after chemotherapy was counted for comparative results against a template of cell count with the medium.

The number of live cells for each sample at 1, 10, and 100 µM were normalized to the average number of live cells for the no carboplatin control on each and was expressed as a percentage using the formula below.

$$\textit{Live cell \% normalised to control} = (N^{\textit{LIVE CELLS}} / \textit{Mean}^{\textit{CARBOPLATIN CONTROL}}) \textit{ X } 100$$

d) Statistical Analysis

Carboplatin response for both OC cell lines was assessed after 3 days as live cell counts using CytoFlex and analysed by two-way analysis of variance (ANOVA) under the assumption of Holm Sidak's multiple comparisons tests. Data show the mean number of live cells normalised to the negative control for AOCS21 (±SD, n=3 for 10 and 100 μM of carboplatin with ECM subsets, n=8 for 1 μM of carboplatin with and without ECM subsets, n=9 for 10 and 100 μM of carboplatin without ECM subsets) and AOCS15 cells with and without HDF and CAF induced ECM (±SD, n=6 for 1 and 10 μM of carboplatin without ECM subsets, n=7 for 100 μM of carboplatin without ECM subset, n=8 for 1 μM of carboplatin without ECM subset, n=9 for 10 and 100 μM of carboplatin without ECM subset; $p^*<0.01$).

e) Cellular analysis of CD44 marker post-chemotherapy

Due to the strong correlation between chemo-resistivity and CD44, as mentioned earlier, we examined its association with the OC cell lines functioning under the influence of differently sourced ECMs. Henceforth, a replicate batch of the aforementioned experimental setup for HDF- and CAF-inducted ECM and non-ECM treated AOCS15 and AOCS21 cells were stained with APC conjugated CD44 antibodies to be processed for flow cytometry enabling us to visualise their CD44 activity profile post-chemotherapy. All variable concentrations with controls were run for sorting after the triplicates from each were combined and centrifuged at 4C, 250 RCF for 5 minutes. The cell pellets were resuspended with 2% FCS/PBS solution and equally divided into Eppendorf tubes for control v/s stained antibody sorting for CD44 (BD antibody, Cat. # 559250). All tubes containing cells and the FCS buffer were kept

ice-cold for 30 minutes. They were then centrifuged at the same setting as mentioned above and washed with the buffer twice before moving them to the cell sorting facility. On a FACS Aria III (BD), cells were separated to isolate those that expressed CD44 at various levels, and data was collected and analysed using FACS Diva software (BD)

e) Statistical Analysis

FlowJo was used to evaluate quantitative data, and two-way ANOVA was implemented for statistical significance using GraphPad prism under the premise of Tukey's multiple comparison test (AOCS15 and AOCS21, n=3; n=independent experiments; data displayed as mean +-SD; p*<0.01).

The complete flow of experiments from HDF culture to data extraction has been shown below in Fig. 3.

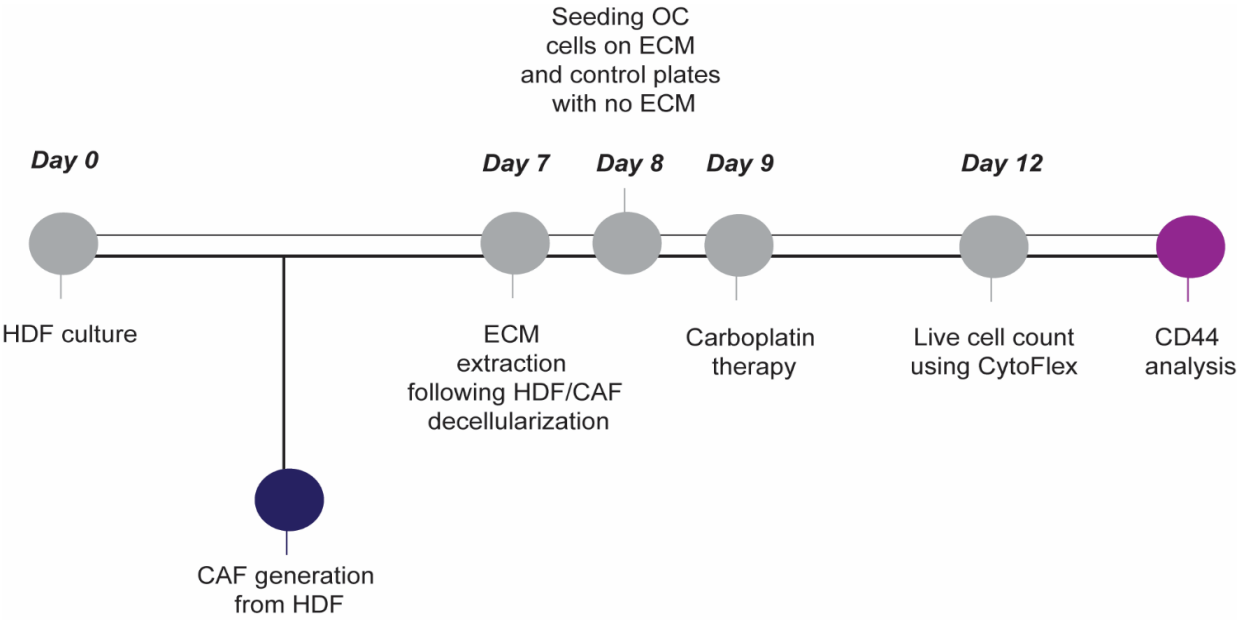


Figure 3 Timeline demonstrating the project outline for CAF establishment and analysis of chemotherapy on the ECM.

5.8 Results:

5.8.1 Production of CAFs

The samples collected to be analysed for HDF to CAF conversion under the influence of TGF-β were accounted for in their RNA statement. The mRNA expression results from qPCR demonstrated a significant increase of ACTA2 and ITGA11 markers, however, highly confluent samples in normal media that were treated with TGF-β for 5 days (Fig. 4C) revealed an upregulation of ACTA2, FAP, TNC, CSPG4 and ITGA11 markers when compared to the control without TGF-β treatment. Conversely, samples grown in AOCS15 or AOCS21 conditioned media showed a reduction in the expression of certain markers, whereas samples for the other conditions did not raise the expression of any CAF markers. Although highly confluent samples in normal media treated with TGF-β for 7 days showed a substantial rise in the expression of the most reliable marker ACTA2 and the ITGA11 marker, the increase was larger for highly confluent samples in a normal medium treated with TGF-β for 5 days. In the end, this implies that utilising a normal medium in cells grown at the high confluence with 5 days of TGF-β therapy is the ideal setting for producing CAFs. The controls denote no TGF- β exposure.

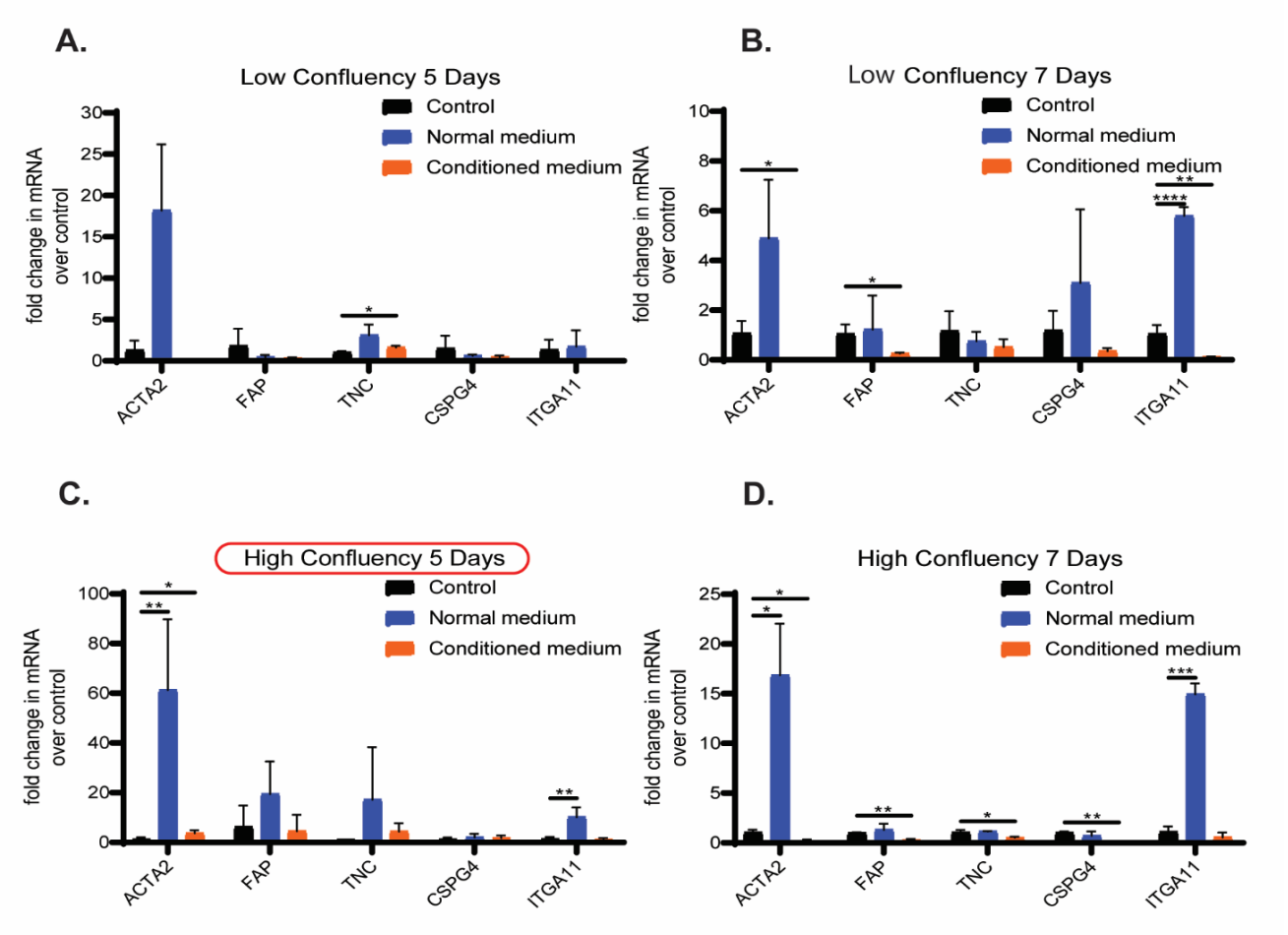


Figure 4 qPCR analyses for optimised CAF conversion. Results displaying CAF conversion by heightened CAF markers in different culture mediums, confluency rate and number of days for incubation (A-D). TGF-β treatment, normal medium, and AOCS15 and AOCS21 conditioned medium normalised to the same control for HDFs cultured to (A) low confluency and treated with TGF-β for 5 days (±SD, n=3 for all subsets except all controls where n=2; p*<0.05) or (B) low confluency and treated with TGF-β for 7 days (±SD, n=3; p****<0.0001) or (C) high confluency and treated with TGF-β for 5 days (±SD, n=3 for all subsets except ACTA2 and ITGA11 controls where n=4 and FAP, TNC and CSPG4 controls where n=2; p*<0.05; p**<0.01) or (D) high confluency and treated with TGF-β1 for 7 days (±SD, n=3 for all subsets except ACTA2, FAP, CSPG4 and ITGA11 controls where n=2; p*<0.05; p***<0.001).

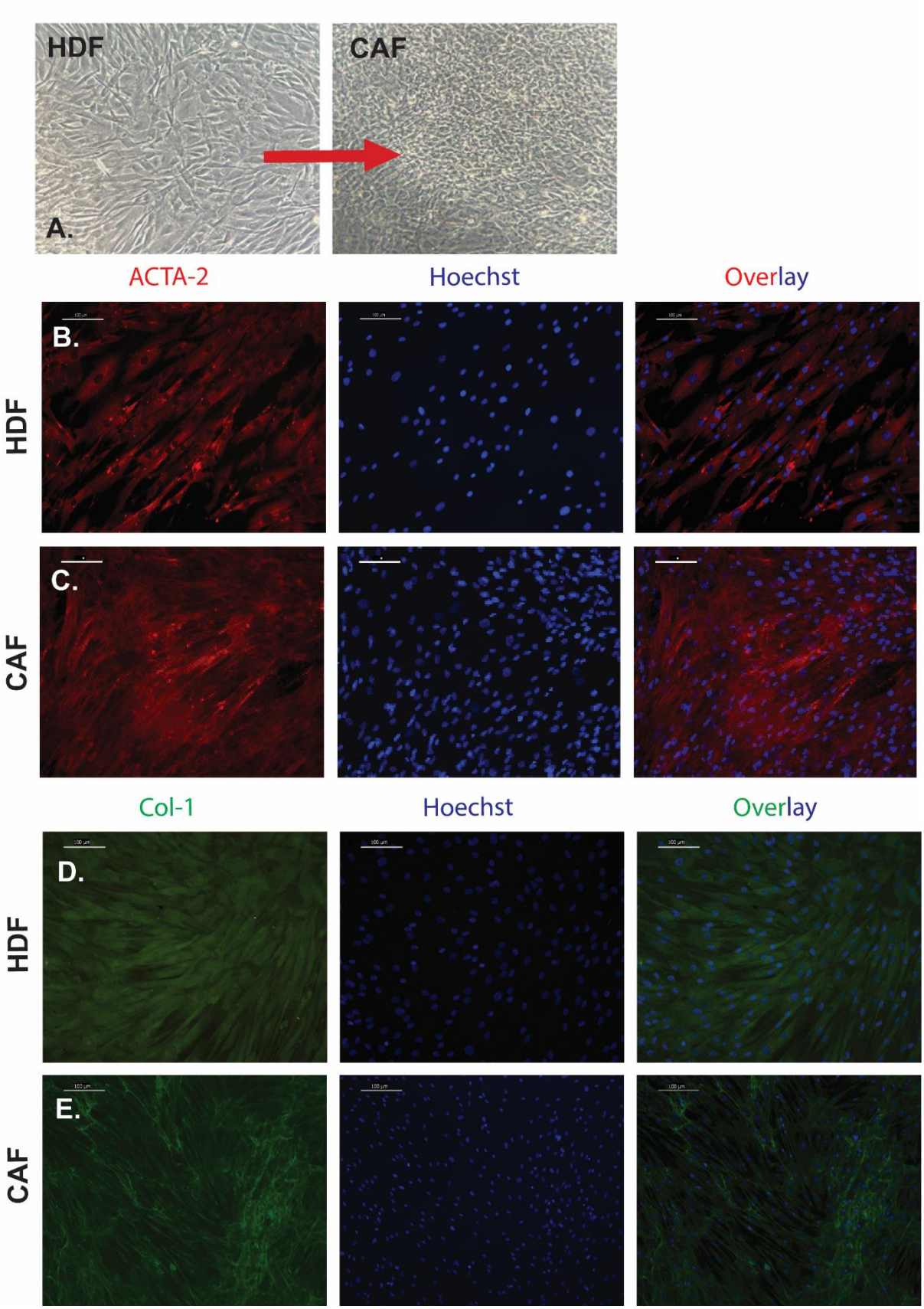


Figure 5 Comparative characterization of HDF and CAF (A) Transitioning of CAFs from HDFs under a microscope, (B) Presence of ACTA2 activity in cells of HDF (top) and (C) CAF (bottom), (D) Collagen dominance in HDF (top) and (E) CAF (bottom).

The CAF conversion was supported by microscopic images as seen as spindle-shaped cells [149], heavenly dense during the metamorphosis and haphazardly arranged themselves unlike their organised predecessor, HDF (Fig. 5A). The cells were subjected to IHC staining to obtain the level of ACTA2 and Col-1 activity in both samples. The over-expression of ACTA2 (red) seen in Fig. 5C coincides with the characteristic signature CAF activity of an over-stimulated activity of ACTA2 hence proving a successful fibroblast transformation. Fig. 5D and E display the same transition, from HDF to CAF, with an overabundance of collagen (green) stained in CAF, supporting the successful validation of the CAF conversion protocol used. The nuclei in Fig. 5B-E can be identified with blue Hoechst staining.

5.8.2 Establishment of *in-vitro* ECM model

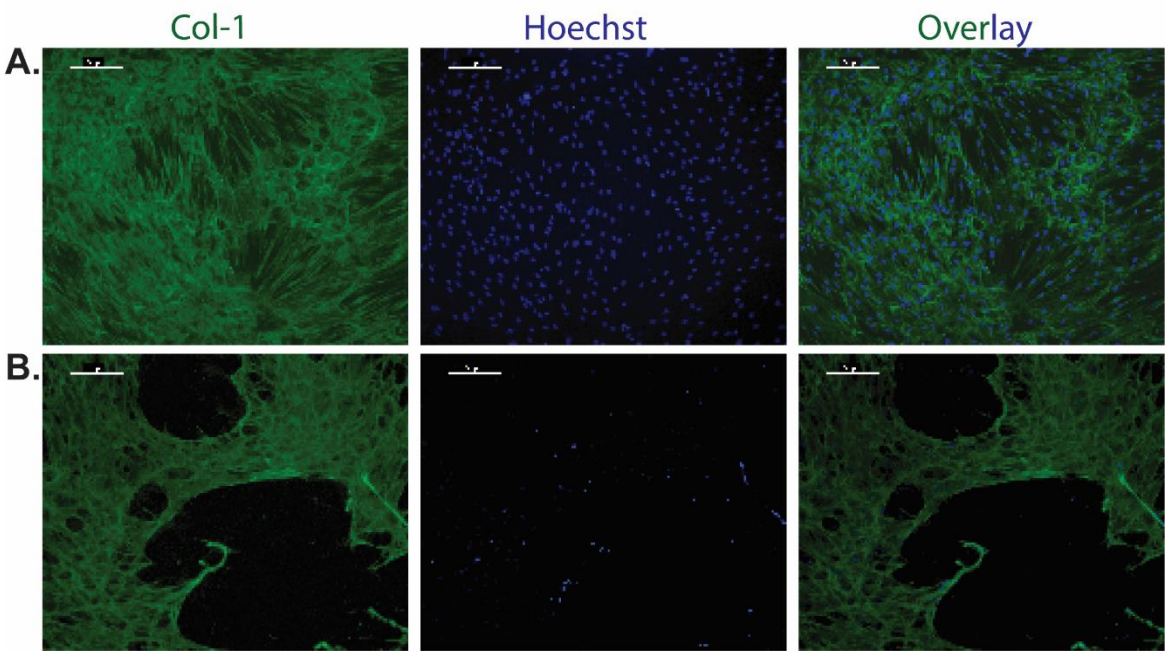


Figure 6 Characterization of CAFs (A) CAF cells observed with collagen and Hoechst staining through IHC before decellularization **(B)** IHC images after removal of CAF cells leaving the ECM behind

Images from a highly confluent plate of CAF cells stained with IHC for collagen (green) using Col-1 antibody and nuclei (blue) using Hoechst are shown in Fig. 6 A and B, before and after the decellularization procedure, respectively. The undisturbed HDF cells (A) display an intensive and significant quantity of collagen in contrast to the images representing the CAF cells (B) after the vast majority of them have been meticulously eliminated, leaving behind the collagen that constitutes the bulk of the ECM hence showing a successful ECM extraction for the tests to be conducted on. Fig. 6C exhibits the essence of this chapter through the microscopic alteration of HDF cells to CAF while both cell lines are then utilised to extract their ECM for further chemotherapeutic studies, as discussed below.

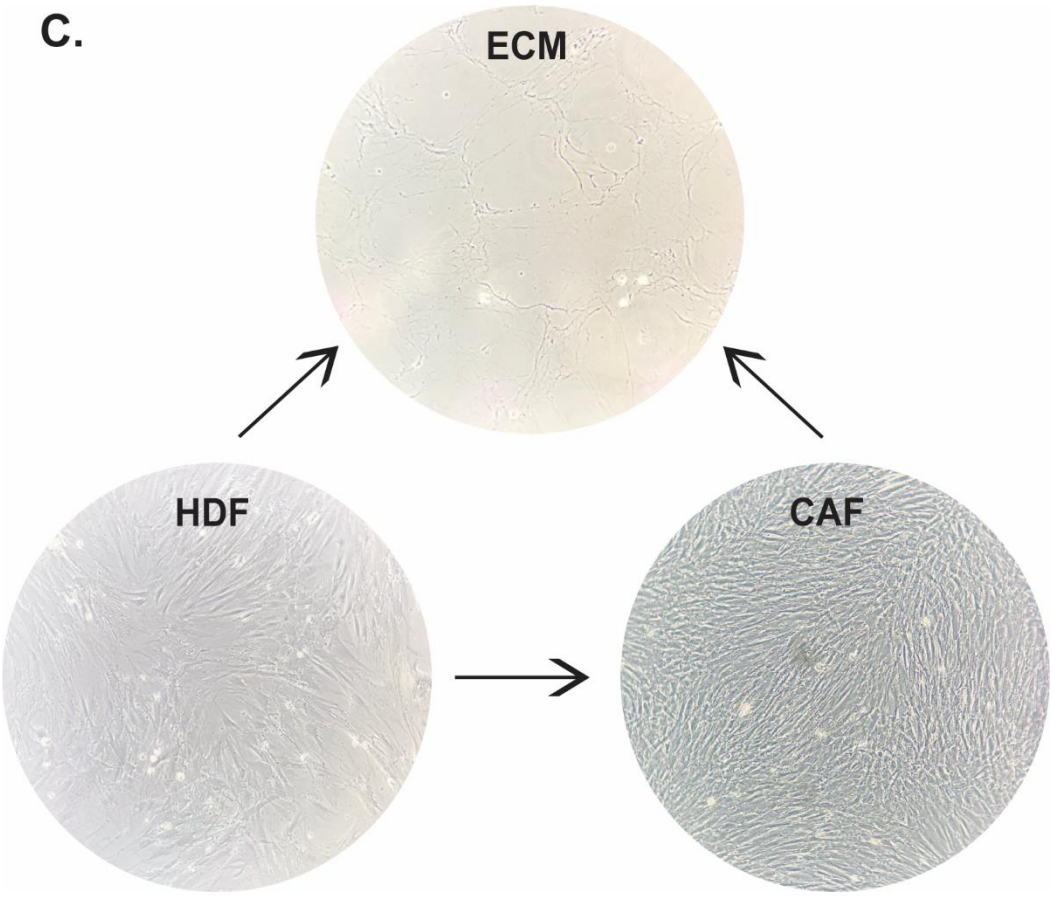
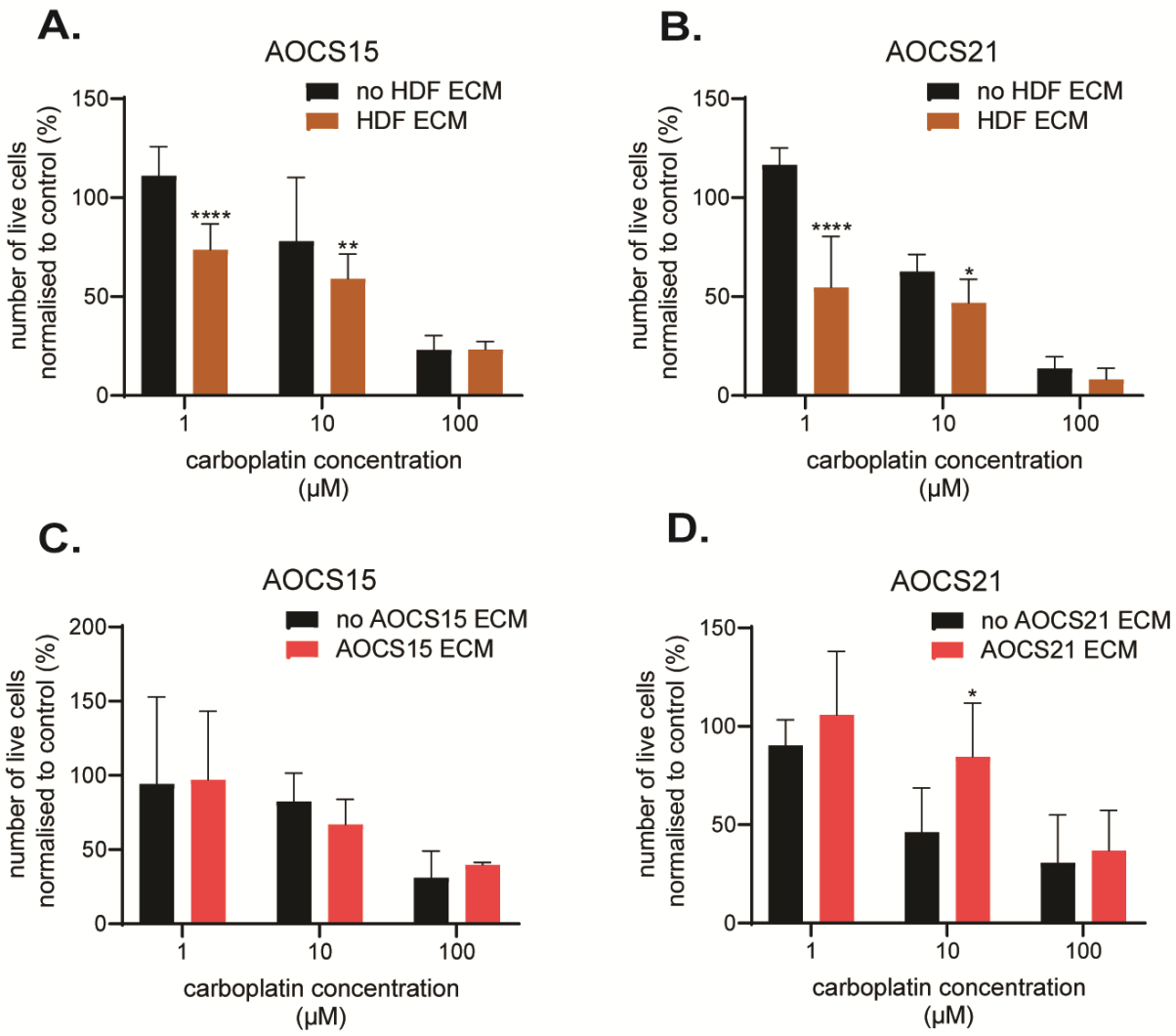


Figure 6 (C) Microscopic images of HDF cells, extracted ECM and CAF.

5.8.3 Chemotherapeutic response of HDF and CAF-derived ECM

(a) Carboplatin assay



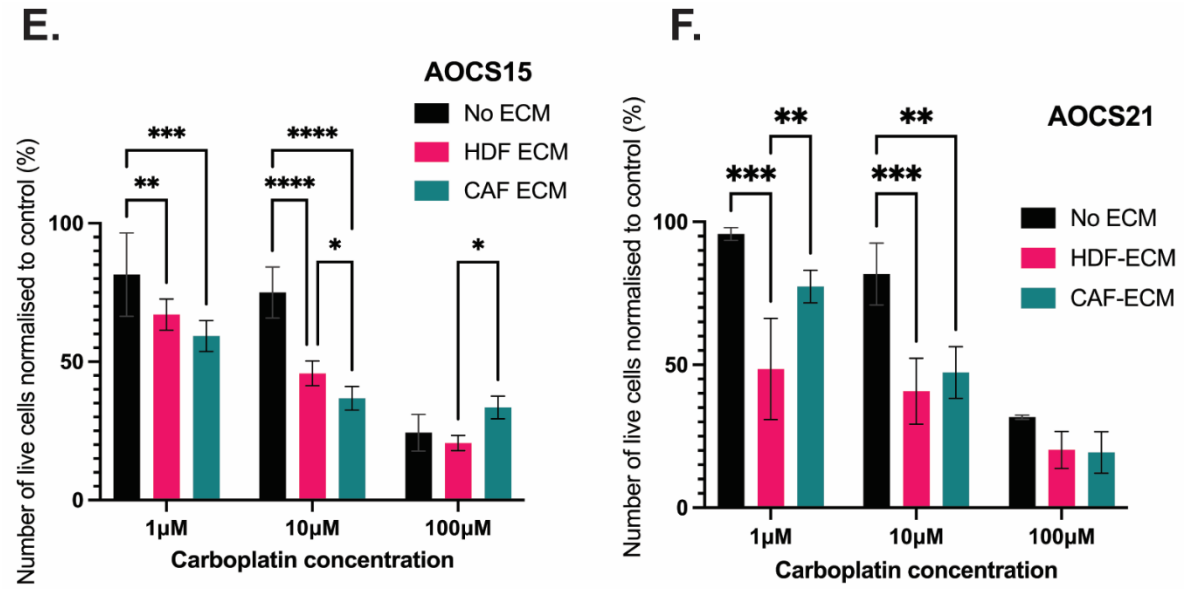


Figure 7 Chemotherapeutic response of OC cell lines. The effect of different ECMs on carboplatin therapy is seen in AOCS15 and AOCS21. (A) AOCS15 with HDF ECM (B) AOCS 21 with HDF ECM (C) AOCS15 with AOCS15 ECM (D) AOCS21 with AOCS 21 ECM (E) AOCS15 with CAF ECM in comparison (F) AOCS21 with CAF ECM in comparison. (n=3; n=independent experiments; data shown as mean ± SD)

All data obtained from both cell lines were normalised against their experimental controls. It is interesting to note that the abundance of ECM, regardless of its source, is responsible for response behavioural change which is very cell-specific. In comparison to cells that were chemo-treated without the presence of any ECM, AOCS15 cells treated with HDF's ECM indicated sensitivity to carboplatin at concentrations lower than 100μM with a reduced proportion of cells surviving. However, their sensitivity was decreased as the carboplatin's concentration was increased with an almost insignificant difference between cells treated with and without HDF ECM at 100μM. The HDF ECM produced a similar reaction in AOCS21 as well.

However, when AOCS15 cells were treated with their own, AOCS15 ECM, it produced insignificant results at all carboplatin concentrations, whereas AOCS21 exhibited chemotherapy resistance with a higher percentage of cells surviving when compared to cells not treated with their own AOCS 21 ECM. AOCS21 cells treated with 100μM

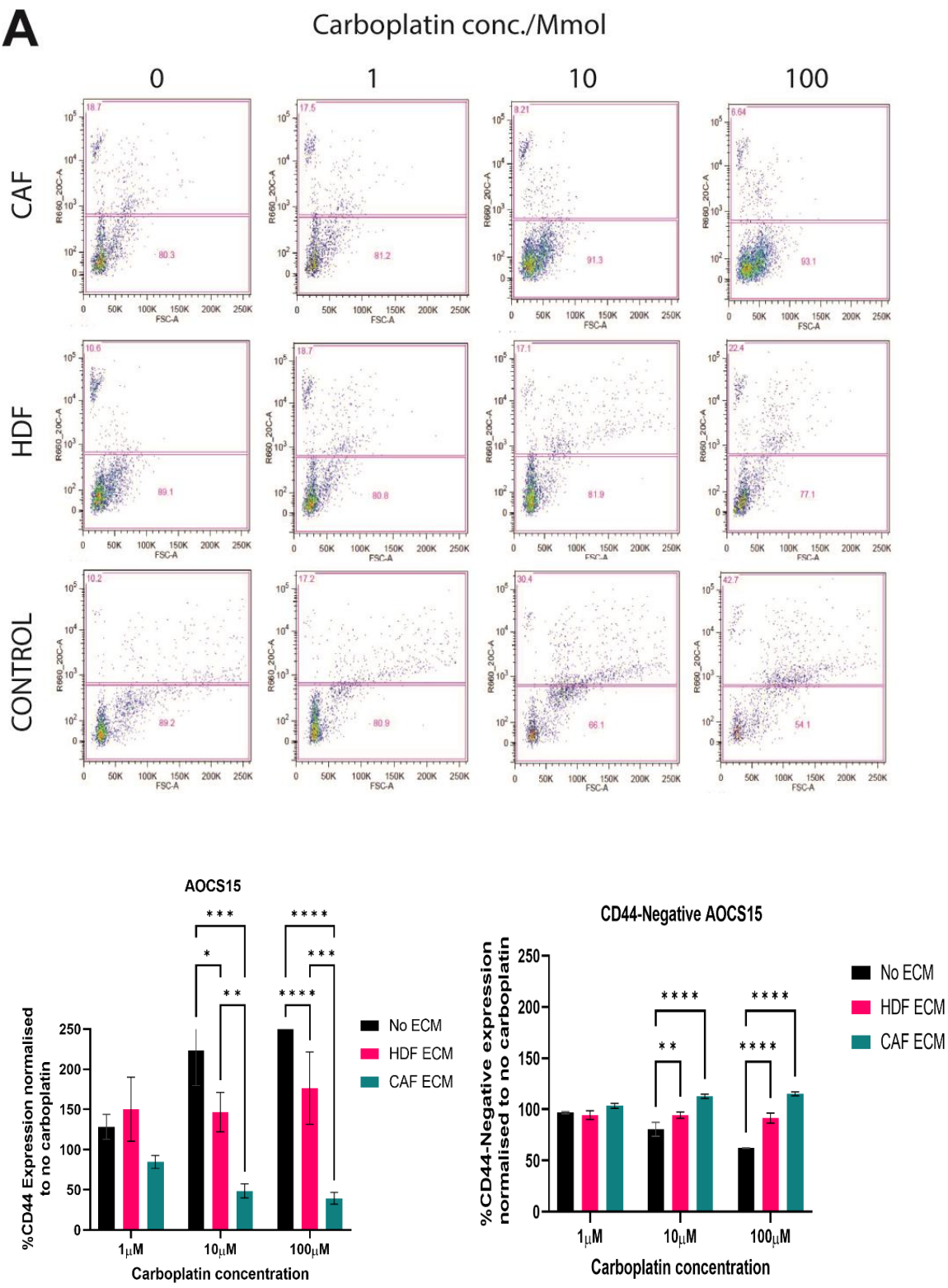
of carboplatin nevertheless demonstrated sensitivity, though this was still insignificant. The shift in therapeutic sensitivity or resistivity trend at the maximum carboplatin concentration of 100μM was a common component seen in both kinds of ECM. This explains a potential ECM alteration at higher chemotherapeutic dosages that, depending on the cell line, may operate as a protective or unprotected layer for OC cells. This has a significant impact on how treatment regimens could potentially be changed for a more favourable prognosis.

Interestingly, both OC lines exhibited varying trends in their survival race against therapy when overlapped with CAF ECM (Fig. 7E, F). AOCS15 demonstrated an increasing amount of therapy sensitivity as the ECM became more complicated and specialised. That is, the ratio of live cells after 72-hour treatment with Carboplatin was significantly lower in samples with CAF ECM as compared to HDF ECM or without any ECM. However, this trend did make a shift when the carboplatin concentration was raised to 100μM where the highest percentage of live cells was seen with CAF ECM displaying resistivity to the same treatment and the least resistance with HDF ECM and no ECM even though, the difference was marginal. Similar to how AOCS21 preserved the characteristic response of therapeutic sensitivity of cells to ECM in comparison to the no ECM control, OC cells were more sensitive to HDF ECM as opposed to CAF ECM. This was seen for both lower concentrations of carboplatin, 1 and 10 μM. However, at 100μM of carboplatin, AOCS21 displayed the same level of sensitivity to therapy to CAF ECM and HDF ECM by a lesser percentage of cells surviving by the end of the experiment, in comparison to the cells treated without any ECM. This effectively advocates that varying ECM bring about different therapy responses in OC cells. The result that there is a considerable difference in the chemotherapeutic response with CAF- and HDF-ECM at higher carboplatin

dosages is very valuable for targeted therapeutic strategies. It opens up the door to producing a treatment approach of an adjunct of lower concentration of carboplatin with HDF/CAF ECM, depending on the cancer type, for improved patient prognosis.

(c) CD44 analysis

As discussed earlier, OC cells' high CD44 expression was linked to chemoresistance, poor differentiation, high resistance, and recurrence rate for OC cells and thereby a negative CD44 expression is correlated to enhanced treatment sensitivity [150]. Analyzing the CD44 marker activity in AOCS15 revealed a varied response for all three differently sourced ECMs. AOCS15 cells grown on CAF ECM showed a drop in CD44 expression, displaying an increase in treatment sensitivity. This is correlated with a considerable increase in CD44 negative expression in AOCS15 cells as the carboplatin concentration was raised (Fig. 8A). This confirms the findings of our earlier studies showing that carboplatin therapy increased the sensitivity of AOCS15 cells when they were exposed to CAF ECM. Assessing the expression of CD44 across the AOCS21 cell population revealed that it significantly diminished as carboplatin concentration rose for both CAF and HDF-derived ECM, whereas AOCS21 cells seeded on no ECM maintained a similar abundance of CD44 expression irrespective of carboplatin concentration (Fig. 8B). The greatest increase in CD44 negative expression was observed in AOCS21 cells cultured on HDF ECM. This is aligned with the carboplatin assay of AOCS21's chemotherapeutic sensitivity pattern for the same ECM.


A
Carboplatin conc./Mmol
0
1
10
100
CAF
HDF
CONTROL
AOCS15
%CD44 Expression normalised to no carboplatin
Carboplatin concentration
1μM
10μM
100μM
No ECM
HDF ECM
CAF ECM
CD44-Negative AOCS15
%CD44-Negative expression normalised to no carboplatin

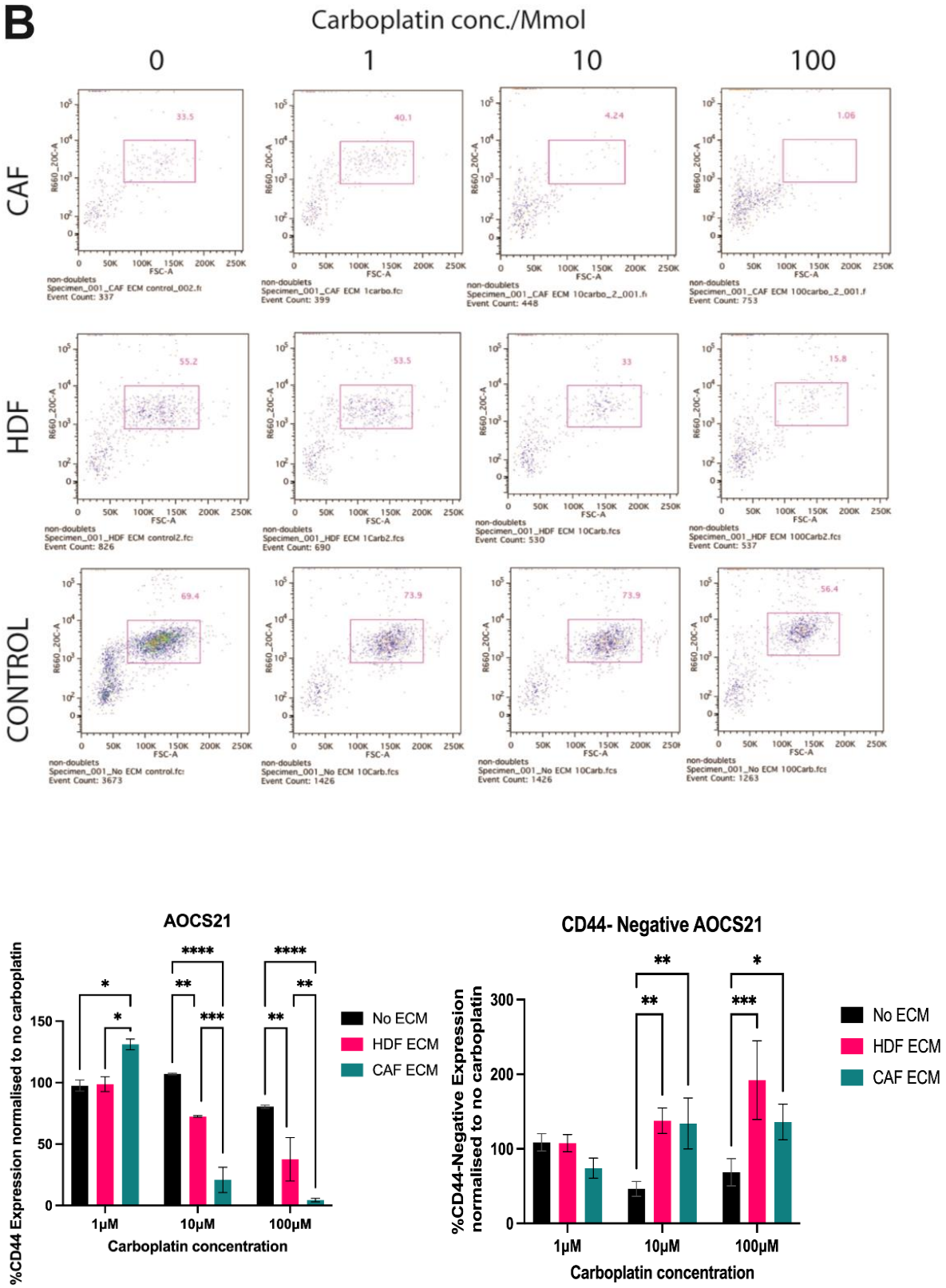


Figure 8 CD44 responsiveness of the OC cell lines (A) Results from flowcytometry displaying CD44 expression and subpopulation in AOCS15 **(B)** Results from flowcytometry displaying CD44 expression and subpopulation in AOCS2. (n=3; n=independent experiments; data shown as mean ± SD)

5.9 Conclusion

CAFs are key cellular structures that drive tumour progression from an early stage in the disease onset and have been allocated as the main source of ECM in tumours [151-153]. They are produced through a variety of biological mechanisms, such as EMT and endothelium-to-mesenchymal transition (EndMT) in epithelial and endothelial cells, respectively, bone marrow-derived and from the trans-differentiation of cells like adipocytes but, most commonly, TGF-β activation of the resident fibroblasts. Multiple pathways induced activation of ACTA2 in some cancer stromal cells, such as stellate cells, also causing them to trans-differentiate into CAFs. Furthermore, mesenchymal stem cells can be activated by a specific receptor or ligand, or TGF-β stimulation by osteopontin, to develop into CAFs [154-156]. With more than one way to originate, they often outnumber the cancer cells themselves, further aiding them to comply with a metastatic niche. Nearly every cell in the TME interacts with CAFs, which allows them to modify TME elements for tumourigenic goals with ECM as a primary target. Cross-talks between CAFs, cancer cells, and macrophage type 2 (M2) cells are crucial for ECM rigidity and disintegration. Hypoxia in the TME is a further effect of CAFs, and it plays a significant role in ECM rigidity and dissolution [157]. Both, hypoxia and ECM through heightened stiffness and degradation are being extensively studied in research labs due to their potential impact on chemotherapeutic response. CAFs have been repeatedly associated with chemoresistance through various pathways but specifically under hypoxic conditions [158-161]. Uncertainty exists about the function of the ECM produced by healthy fibroblasts, OC cells, and CAFs in connection to OC. CAFs' role in tumour initiation and progression has been elusive; hence, research was required to better understand

the same. A decellularized *in-vitro* model of the ECM was developed to test this involving planting OC cells on regular HDF-, OC cell-, and CAF-derived ECM and analysing the impact of these ECM after exposure to a range of carboplatin doses. A quality check that used collagen and staining for double-stranded DNA found in cellular nuclei was successful in establishing that the ECM was adequately preserved following decellularization and that the cells had been adequately removed. In particular, normal HDFs grown to high confluency in DMEM media and treated with TGF-β for 5 days showed the greatest upregulation of CAF markers, as opposed to other confluencies of HDF, conditioned media or longer exposure to TGF-β. This circumstance was consequently effective in producing CAFs to afterwards extract their ECM. OC cells planted on various ECMs showed intriguingly specific responses once *in-vitro* CAF production was optimised. While both OC cell lines showed a substantial increase in platinum treatment sensitivity when exposed to ECM as compared to the control of no ECM, both AOCS cell lines responded differently to ECM produced from CAF and HDF. At lower doses of 1 and 10 μM, AOCS15 showed increasing carboplatin sensitivity to CAF compared to HDF ECM and no ECM, but a flip in response was seen at 100 μM when cells showed treatment resistance with CAF ECM and increased sensitivity to no ECM and HDF ECM. On the other hand, AOCS21 exhibited the highest treatment tolerance at all doses of carboplatin when no ECM was provided, they were specifically responsive to treatment when ECM synthesized from HDF and CAF was present, with a switch in responsiveness occurring once more at 100 μM. While AOCS15 were more sensitive to treatment with CAF ECM, AOCS21 portrayed that response for HDF ECM. Interestingly, both cell lines showed insignificant variation in survival rate post-chemotherapy when exposed to a conditioned medium. This is further supported by the observation that colon cancer

cells' ability to migrate was not improved in conditioned media created using CAFs derived from tumours [34]. Furthermore. tests with CD44, a metastatic biomarker, revealed the dose-dependent variation in CD44 expression and alteration in CD44 negative cell population for cell lines seeded in ECM reflect the cell response. Data from CD44 negative expression coincided with the chemotherapeutic results for both, AOCS15 and AOCS21, suggesting that an interaction with HA or other ECM proteins is needed for cancer cells to have a meaningful response affecting the eventual outcome of chemotherapeutic treatment.

As previously indicated, CAFs affect cancer cell migration and invasion in various ways, including manipulating the ECM, influencing EMT in cancer cells, secreting growth factors that aid cancer cells, and controlling treatment responses [162]. This process is enabled through the interface between CAFs, cancer cells, and the ECM, which can eventuate via direct contact, cytokine secretion, or extracellular vesicles. Understanding the processes that contribute to metastatic spread is conducive to improving prognosis and establishing better treatment strategies [34, 163]. Further research can be carried out to comprehend the bias in these cell lines' susceptibility to carboplatin during chemotherapy. Whether or not this response is cell cycle stage-dependent or factors in any other potential variable and if the same trends are observed in other cancer cells, will be a crucial key to the more elaborated understanding of CAFs and their role in ECM.

Chapter 6: *Discussion & Conclusion*

6.1 Summary

This thesis has presented research on ECM's influence on oral and ovarian cancer cells in tumour progression against radio- and chemotherapy, respectively. As understood, ECM constitutes a major part of the tumour microenvironment (TME), which is described as the complex and diverse, multicellular, and acellular environment in which a tumour grows. Cancer hallmarks such as tumour proliferation, metabolism, angiogenesis, invasion, and metastasis, as well as therapeutic resistance, are directed by cellular components of the TME, and therefore cancer formation is strongly aligned to the TME's physiological state [1-3] which is about this study primarily based on.

The role of ECM was analyzed by summarizing the key research findings established by evaluating pre-existing ECM-based *in-vitro* research models, two-dimensional (2D) cell culture and three-dimensional (3D) tumour spheroids (SPH) and generating a modified and novel 3D *in-vitro* research model. This model has been utilized to study the tumourigenesis of cancers in the head and neck region, specifically oral cancer, SCC 25.

We also branched this thesis to investigate the role of cancer-associated fibroblasts (CAF) in the TME and their influence on the progression and survival of High Grade Serous Ovarian Carcinoma (HGSOC) cell lines (AOCS 15 and AOCS 21). As discussed in the preceding chapter, CAF accelerates cancer growth and even encourages cancer cells to proliferate at a different anatomic site. Changes in the rigidity and degradation of the ECM impact the development and progression of tumours, with CAFs being the primary cause [4, 5]. Thus, it is widely acknowledged that they have a role in treatment resistance and hence have been considered a target

for cancer therapy addressing the TME [6]. This contributory effect has been examined by generating an *in-vitro* CAF model that utilized their secreted ECM to analyse the ovarian cancer cells' behaviour against therapy. A major marker of various malignancies, CD44 overexpression is closely associated with acquired resistance to first-line OC therapies. Cancer cells with CD44 suppression, on the other hand, showed decreased proliferation and a shift in chemoresistance characteristics [7, 8]. We henceforth labelled the AOCS cells with an APC-conjugated CD44 antibody and used flow cytometry to analyze the expression of this marker to see how various ECM affected CD44 expression following carboplatin exposure.

This chapter will also review the limitations of this study and propose opportunities for future research.

6.2 Evaluation of the pre-existing and this study-generated *in-vitro* models for cancer research.

One widely used technique to study carcinogenesis was the monolayer cultivation of cells on a plastic culture dish. This method allowed for the development of an affordable, repeatable methodology. However, this only allowed the cells to expand in a 2D model; it was unable to provide the cancer cells with a complex meshwork of a highly macromolecular niche, the ECM, which typically exists and is known to play directive roles in native tumours [9, 10]. The SPH, on the other hand, is a structure made up of an external layer of proliferating cells, an intermediate layer of quiescent cells, and an internal necrotic core. cells aggregate because of the surface tension and gravity plays a key role in the formation of a spheroid. However, not all cells could form spheroids and adhere to the distinctive architecture, which presented a significant

obstacle to understanding the cell-TME interactome [11-13]. In this study, SCC 25 cells were grown in culture plates as 2D models and SPH were formed using the hanging drop protocol as enlisted in [14]. To generate a novel 3D model that overcomes the disadvantages of both these models, we established the Native Tissue Scaffold (NTS). The model was built on a native mouse tongue tissue to serve as the ECM scaffold on which the oral cancer cells were plated to allow a natural migration and proliferation as opposed to any artificial injection of the cancer cell, as done in previous studies [15]. The NTS was generated and optimized in partnership with our collaborators and by using a shared protocol [16]. The modification is discussed in depth in Chapter 3, Methods. Briefly, after sterilising the native mice tongues, we incorporated the additional exposure of the native tongue tissues to foetal bovine serum (FBS), which has been proven to enhance the quantitative attachment of cells on the scaffold [17, 18]. This modification enables studies utilizing a smaller tissue size or organ to successfully generate an *in-vitro* cancer study model without any potential disruption caused by injecting the cells into the scaffold. The scaffolds were then seeded with SCC 25, forming the NTS.

This methodology was rigorously optimized and verified to ensure its effectiveness in using it as an approach to mimic ECM. The scaffolds were analyzed before and after decellularization with histological staining that showed complete removal of the cellular content. This was further examined by immunostaining collagen for all samples since collagen is one of the most abundant components of ECM [19]. As seen in the existing literature, thorough decellularization of scaffolds results in the complete removal of the native cells but should preserve a considerable amount of collagen for the scaffold to be used as an ECM substitute [20] which was shown in our results. DNA extracted from the decellularized, and the control sample showed a significant drop in the DNA

content after the decellularization process, indicating a successful procedure.

All study models analyzed in this project, 2D, SPH and NTS (used as an ECM prototype), were exposed to 2 Gy of ionizing radiation after 2 weeks of allowing SCC25 cells to grow, in a fractionated fashion to mimic the irradiation regimens administered to oral SCC cancer patients as a common mode of therapy in adjunct with surgery [21]. The same circumstances were used to seed a non-radiated control in each model using SCC 25. Throughout this project, we aim to compare the efficacy of all three different models, 2D, SPH and NTS, in replicating an *in-vivo* carcinoma.

The models were stained for various proteins and markers that included ki67 and γh2ax. Ki67 is a clinical proliferation marker that has been established as a predictor of therapeutic responses. The proportion of tumour cells that have been positively stained out of all the malignant cells analysed is known as the Ki67 percentage score [22, 23]. A low percentage is one of less than 10% whereas 20 per cent or more is regarded as high [24, 25]. The NTS model with SCC 25 showed a drastic downfall in the proliferative capacity after radiotherapy, indicating a quiescent stage which is usually observed after IR similar to the results produced in small cell lung cancer [26] and meningiomas [27]. Cancer cells can react differently to radiation treatment. Radiotherapy can sometimes result in cell cycle arrest or even death by preventing mitosis, which lowers the tumour's ability to proliferate [28]. As a result of fewer cells actively dividing, the ki67 index may drop [29]. This illustrates the excellent effectiveness of our design in creating the desirable milieu for cancer cells to thrive in, which may be therapeutically translated into insightful, constructive findings.

Nevertheless, it's essential to remember that many variables, such as the patient's general health and the type of cancer, can also affect how radiation treats the disease.

γH2AX is a readily detectable molecular marker of DNA damage and repair because it is sensitive to radiation exposure. The processes leading to chromatin de-condensation following DNA double-strand breaks (DSB) require H2AX, namely the phosphorylated form of H2AX [30, 31]. The project's outcome displays a percentage of the post-irradiation sample displaying treatment resistance acquired by the NTS with the least degree of DSB damage revealed with the weakest γH2AX signals in contrast to 2D and spheroid models emphasizing a greater accuracy of NTS to an *in-vivo* tissue tumour as compared to other *in-vitro* models.

RNA-sequencing analysis revealed NTS has the greatest number of differentially expressed genes while 2D had the lowest. A clear segregation of gene clusters between the control and radio-treated NTS and SPH samples indicate a better representation of an *in-situ* carcinoma. Furthermore, the main purpose of this research project was to identify and enable an enhanced ECM-based prototype for in-vitro studies and NTS's upregulation of ECM structures and organization associated genes indicate its superiority against the other study models.

6.2.1 Challenges

Due to time and financial constraints, we worked on unicellular models subjected to a single dose of IR. This heavily limits the results as a true carcinoma is subjected to multiple IR treatments in the presence of immune modulatory cells, which is discussed below.

This thesis generated results from single culture 2D models and unicellular SPH and NTS models. However, SPH and bioprinted hydrogels as ECM models have been

optimized in other research to function as multicellular (double co-culture and triple co-culture) prototypes [32, 33]. A similar approach can be optimised with NTS that could diversify the scope of this project and produce results with greater molecular data. However, when incorporating co-cultured techniques, one issue with 3D *in-vitro* models is the absence of a medium appropriate for every type of cell. This problem can be solved by distributing the required cytokines, growth factors, and MMPs into the tumour or stromal compartments in a way that promotes cell development at each compartment [34] but would require intensive optimisation, technique training and time.

Furthermore, the cell seeding protocol and radiotherapeutic effect on the cancer cells can be further evaluated by comparing an additional sample of NTS with incorporated macrophages and regulatory T cells, which present a true carcinoma and regulate the reaction of immune cells, to identify any characteristic change in the behaviour of the SCC 25 cells or pathways.

Adding a multiple treatment regime with repeated radiation dosages, which is a treatment regime for radiotherapy patients, could further expand the current study; however, since some SPH are susceptible to disintegration at doses greater than 7 Gy, it might not be possible to interpret data from higher dosages [35].

6.2.2 Prospects

Mass spectrometry has been used to analyse differences in protein expression between tumours and healthy tissue, to identify critical components of ECM that trigger particular cell behaviours [36, 37]. With this, customised treatment plans can be created, including the anticipation of potential side effects and the determination of

how a patient will react to particular medications. It can also be used to forecast the likelihood of a disease recurrence.

Decellularized scaffolds have been used to generate various *in-vitro* organ models such as cardiac [38], lung [39], kidney [40], hepatic [41], bone [42], skin [43], as well as for drug screening, cell therapy, or disease modelling. However, a multiple model approach incorporating the NTS model along with other competent *in-vitro* models such as self-repair hydrogel for wound healing [44] would enable significantly enhanced results and better understanding of a true carcinoma leading to improved treatment plans, and hence prognosis.

Future innovation of the novel NTS model can be carried out by incorporating different viral infections that occur in the oral cavity such as human papillomavirus. This will allow us to visualise the reflective characteristic response of cancer cells to the virus via such a study model.

Moreover, similar experimental setup of detailed RNA sequencing analysis can be carried out for other study models to achieve a hybrid of the best performing models that can be put together for a better prognosis and improved therapeutic results.

6.3 In-vitro generation of CAF

As highlighted in this study, a pivotal cellular component of the TME is cancer-associated fibroblasts (CAF). CAFs can be sourced from a wide assortment of cells and pathways helping them to aid cancer survival against treatment and metastasis. The majority of them are resident fibroblasts that get activated in response to environmental signals and growth factors such as transforming growth factor-β (TGF-β), simulating the quiescent fibroblast activation process in a wounded tissue just

before healing [45]. Hence, we created an *in-vitro* protocol to generate CAF from Human Dermal Fibroblasts (HDF).

HDF cells were cultivated in 6-well plates for an optimized time necessary to secrete ECM—7 days—while being critically examined for contamination and having the medium changed every two days. This procedure has been refined after several revisions to the variables, including the number of HDFs seeded, the incubation period, and the concentration of the ECM extraction reagents. The most superior procedure was approved, enabling the prompt and healthy development of HDFs. Chapter 3, Methods, offers a detailed explanation of every detail about this approach. The validation of ECM-secretion and HDF to CAF conversion using the TGF-β protocol has been widely discussed in the previous chapter.

In summary, overexpression of collagen through Col-1 antibody and smooth muscle actin (SMA) through α-SMA antibody was detected in CAFs after conversion from HDF reflecting the acquired aggressive phenotype of these cells [46, 47].

The generation of ECM on a plastic plate helped us to test for its viability against platinum treatment, namely Carboplatin, a very commonly used drug for the treatment of ovarian cancer, assisting us in analyzing the chemotherapeutic response of various specialized ECM [48]. Additionally, this research also offers a cost and time-effective protocol to produce CAFs and extract ECM from any ECM-producing cells.

6.3.1 Effects of cell-ECM Interactome on Therapy Response

We investigated and compared the effects of HDF-derived ECM and CAF-derived ECM towards the carboplatin response of OC cells and further analysed the changes in CD44 expression of the same OC cells. Carboplatin has been known to sensitize

the OC cells to therapy [49, 50]. It was hypothesized that the presence of ECM will enhance chemo-resistant response towards carboplatin and ECM derived from Fibroblast, and CAFs will differentially affect the response of the OC cells towards carboplatin exposure. Moreover, we expected CD44 to be highly expressed in chemo-resistant samples. The data summarised reveals that while both cell lines investigated were sensitive to ECM against carboplatin therapy at the highest given dosage of 100µm, AOCS 21 was most sensitive to CAF ECM while AOCS 15 was more sensitive to chemotherapy with HDF ECM and no ECM control. The results were supported by the CD44 analysis for both OC cell lines. This could indicate lowering therapeutic dosages of Carboplatin while targeting the ECM can lead to improved survival rates of patients diagnosed with OC.

6.3.2 Challenges

OC is treated through multiple regimes including surgery and polypharmacy. Due to the time constraint, we were able to deduce data from one commonly used anti-cancer drug. However, using multiple drugs such as cisplatin and paclitaxel, two others widely known OC therapeutic drugs [51, 52], can replicate a more similar response in patients giving us a better response to the chemo-resistant and/or sensitive response of the OC cells.

The rationale behind the variations in the OC cells' responses to various forms of ECM has not been sufficiently determined by this study. Mass spectrometry can be used to compare the composition of the ECM before and after exposure to carboplatin, giving additional insight into the possible variations in ECM protein deposition. The expression of CD44 in the AOCS cells was monitored in our study; however, investigating the effects of chemotherapy on surface receptors, including integrins,

growth factor receptors, and their binding counterparts (collagen, fibronectin, laminin, etc.), may also shed light on the mechanisms underlying the alterations in chemoresistance and cell survival. The ECM stiffness changes could also be confirmed or quantified, and the effects observed in this study's mechanism could be clarified by using cell signalling assays or Pamgene kits to look for additional downstream kinase signalling pathways.

6.3.3 Prospects

Remarkably, CAFs interact with other cell types in the TME not only through paracrine signalling but also through direct contact. For instance, in a 3D invasion model, SCC cell migration necessitated contact between CAFs and SCC cells [53]. As seen through our data too, CAF and its secreted ECM modulate cancer progression differently in each cancer cell type. This shows that targeted therapy for CAFs cannot be generalised for all cancer subtypes and requires further investigation to establish a treatment strategy based on the type of cancer in every individual.

In previous research, CAFs were co-cultured with tumour cells in an OOAC and with colorectal cancer cells in an SPH model supporting the theory that the presence of CAFs promotes drug resistance [54, 55]. A hybrid model incorporating CAFs into the NTS models would further cohesively enable both parts of this study to be used to determine clinically relevant drug combinations and drug resistance patterns.

6.4 Conclusion

3D cancer models have long been used for diagnosis, drug screening, genome editing, biobanking, and molecular biology against therapy resistance [56, 57]. This study

aimed to investigate the role of ECM in tumour progression against different modes of therapy via *in-vitro* 3D ECM study models as any imbalance during a tumourigenic state triggers ECM modification, which causes cancer to advance, disseminate, and finally develop resistance to treatment [58, 59]. The results indicate that our established 3D model, NTS, is more radioresistant in comparison to the pre-existing study models while showing a quiescent or a less proliferative aspect within 24 hours of IR. Further findings show the susceptibility of OC cells to CAF-specific ECM.

Validation of these models and results through carefully planned prospective, multi-centre clinical trials is also crucial for clinical translation. Therefore, further study using these models can potentially contribute to the development of a precision medicine strategy that provides tailored treatment for cancer patients.

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Chapter 1

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Chapter 4

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Chapter 5

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