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MECHANOREGULATION OF ANGIOPOIETIN-LIKE 4 IN EPITHELIAL-MESENCHYMAL TRANSITION AND CANCER METASTASIS

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The cover illustration is designed by professional artist Manmo Zhao, based on the requirements of Zehuan Liao. In this illustration, the poised mantis ready to strike the cicada symbolizes cancer cells primed for metastasis within the patient. The siskin, poised to strike the mantis, embodies the medical researcher's drug/treatment as a potential cure for cancer. The vibrant hues on the left side of the illustration symbolize hope, while the subdued colors on the right side signify despair. The depiction of a timer in the artwork underscores the urgency and critical nature of our ongoing battle against the disease. (Please refer to the popular science summary of this thesis for more details regarding this cover illustration.)

Mechanoregulation of Angiopoietin-like 4 in epithelial-mesenchymal transition and cancer metastasis

Thesis for Doctoral Degree (Ph.D.)

By

Zehuan Liao

The thesis will be defended in public at Lecture Room 205, Karolinska Institutet, Solna, Sweden, on Thursday the 7th of December 2023 at 9.30 am.

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To my wife Zhang Yuying and my son Liao Chengjin, Alan,

Without whom this thesis might have been completed one year earlier.

For my parents Liao Zhenhua and Kong Qiong,

And my brothers Liao Zeyu and Liao Zeyao, Kevin,

This is probably the only page they will ever read in my thesis.

Popular science summary of the thesis

Inspired by the Chinese idiom "the mantis, the cicada, and the siskin" (螳螂捕蝉，黄雀在后) and the ongoing battle between medical researchers and cancer in saving patients' lives, we find a profound parallel between finding a cure for cancer and the predatory chain described in the idiom. Like the mantis swiftly moving and ready to strike the cicada, cancer cells possess a menacing ability to invade and migrate from one organ to another in patients via a process known as metastasis.

Cancer, an elusive adversary, often outsmarts conventional treatment strategies. The cells mutate, adapt, and spread with a stealth comparable to the mantis catching its prey, making it a challenge for medical researchers to intercept and treat effectively. Hence, despite significant advances in the early detection and diagnosis of cancer, new methods to treat cancer have not dramatically improved outcomes in many cancers over the last decade. Metastatic cancers are largely incurable because of their systemic nature and their frequent resistance to therapeutic agents, including immunotherapy. Hence, the efficacy of cancer treatment predominantly hinges on our capability to intercept and, potentially, reverse the metastatic process.

Our findings in medical research have brought new hope to the battlefield against cancer. We found that patients with high ANGPTL4-expressing cancers had a poorer prognosis and an overall shorter median survival time. Therefore, testing for ANGPTL4 expression as a prognostic marker in cancer patients helps to aid in early detection, treatment decision-making, and the development of more effective therapies, reminiscent of the siskin's ability to foresee and intervene in the predatory chain depicted in the idiom. Further findings from us also suggest that ANGPTL4 is a potential anti-metastatic target. Therefore, providing treatments targeting ANGPTL4 are analogous to the siskin at the top of the predatory chain, offering targeted approaches that aim to outmaneuver and defeat these metastatic cancer cells.

To conclude, the fight against cancer echoes the relationship between the mantis and the siskin, where medical researchers continually evolve their strategies to intercept, outmaneuver, and ultimately triumph over this formidable opponent. With each scientific breakthrough and innovative approach, we hope to edge closer to the ending of this age-old tale, aiming to save and improve the lives of cancer patients worldwide.

Abstract

The epithelial–mesenchymal transition (EMT) serves as a pivotal mechanism in the progression of metastatic cancer. However, current research, predominantly reliant on 2D monolayer cultures, inadequately replicates the intricate nature of a 3D tumor microenvironment. In the main project (Paper I), we investigated the transcriptomes of various cancer cell types undergoing EMT in both 2D and 3D cultures with different EMT inducers. We identified a 3D EMT gene signature that has broad implications across different types of human cancers. Angiopoietin-like 4 protein (ANGPTL4) was found to be a top ranked hub gene with clinical relevance and impact. Our study also revealed the mechanoregulation of ANGPTL4, which corroborated with its high expression in advanced tumors. Consistently, ANGPTL4 deficiency attenuated primary tumor growth and EMT of cancer cells. These findings suggest that targeting ANGPTL4 may be a promising approach to inhibit EMT and prevent cancer progression. In the collaborative project (Paper II), we studied the regulation of membrane microenvironment and signal transduction in natural killer (NK) cells, a group of innate immune cells involved in the tumor microenvironment (TME) and cancer immunotherapy. Here, we revealed the PIP2-regulated recruitment of DAP12 homodimer to lipid raft boundary of NK cells. In another collaborative project (Paper III), we introduce HTCA, a single-cell RNA-sequencing database with various user-friendly analysis tools. Collectively, our main findings reflect the intricate regulation of physical stiffness within the TME influencing EMT signaling in cancer cells, where ANGPTL4 emerges as a crucial player. Our comprehensive analyses strongly underscore the clinical significance of ANGPTL4, particularly in advanced stage cancer, aligning with our broader understanding of tumors in patients. In essence, our study vividly demonstrates how the TME's stiffness orchestrates the mechanoregulation of ANGPTL4, a hub gene within the 3D EMT gene signature.

List of scientific papers

I. **Zehuan Liao**, Joseph Jing Heng Lim, Jeannie Xue Ting Lee, Marcus Ivan Gerard Vos, Damien Chua, Yun Sheng Yip, Choon Boon Too, Huan Cao, Jun Kit Wang, Yufeng Shou, Andy Tay, Kaisa Lehti, Hong Sheng Cheng, Chor Yong Tay, Nguan Soon Tan. "Attenuating epithelial-to-mesenchymal transition in cancer through angiopoietin-like 4 inhibition in a 3D tumor microenvironment model." (Manuscript)

II. Ruijuan Dong, Yan Tan, Angran Fan, **Zehuan Liao**, Hangrui Liu, and Peng Wei. "Molecular dynamics of the recruitment of immunoreceptor signaling module DAP12 homodimer to lipid raft boundary regulated by PIP2." *The Journal of Physical Chemistry B* 124, no. 3 (2019): 504-510.

III. Lu Pan, Shaobo Shan, Roman Tremmel, Weiyuan Li, **Zehuan Liao**, Hangyu Shi, Qishuang Chen, Xiaolu Zhang, and Xuexin Li. "HTCA: a database with an in-depth characterization of the single-cell human transcriptome." *Nucleic Acids Research* 51, no. D1 (2023): D1019-D1028.

Scientific papers not included in this thesis

Review Papers

I. Youhuai Li, Mina Wang, Xueqiang Peng, Yingying Yang, Qishuang Chen, Jiaxing Liu, Qing She, Jichao Tan, Chuyuan Lou, **Zehuan Liao***, and Xuexin Li "mRNA vaccine in cancer therapy: Current advance and future outlook." *Clinical and Translational Medicine* 13, no. 8 (2023): e1384.

II. Yue Shi, Mina Wang, Liqun Wu, Xuexin Li, and **Zehuan Liao***. "COVID-19 associated liver injury: an updated review on the mechanisms and management of risk groups." *Liver Research* (2023).

III. Feiyu Xie, Mina Wang, Qishuang Chen, Tiange Chi, Shijie Zhu, Peng Wei, Yingying Yang, Le Zhang, Xuexin Li, and **Zehuan Liao***. "Endogenous stimuli-responsive nanoparticles for cancer therapy: From bench to bedside." *Pharmacological Research* (2022): 106522.

IV. Yue Shi, Li-Qun Wu, Peng Wei, and **Ze-Huan Liao***. "Children with type 1 diabetes in COVID-19 pandemic: Difficulties and solutions." *World Journal of Clinical Pediatrics* 11, no. 5 (2022): 408.

V. Jia-Ran Lin, Zi-Ting Wang, Jiao-Jiao Sun, Ying-Ying Yang, Xue-Xin Li, Xin-Ru Wang, Yue Shi, Yuan-Yuan Zhu,Rui-Ting Wang, Mi-Na Wang, Fei-Yu Xie, Peng Wei, and **Ze-Huan Liao***. "Gut microbiota and diabetic kidney diseases: Pathogenesis and therapeutic perspectives." *World Journal of Diabetes* 13, no. 4 (2022): 308.

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IX. Xuexin Li, Weiyuan Li, Mina Wang, and **Zehuan Liao***. "Magnetic nanoparticles for cancer theranostics: Advances and prospects." *Journal of Controlled Release* 335 (2021): 437-448.

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XIV. Mina Wang, and **Zehuan Liao***. "SARS-CoV-2 and COVID-19: How much do we know?." *Acta Virologica* 64, no. 3 (2020).

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Research Papers

I. Lu Pan, Paolo Parini, Roman Tremmel, Joseph Loscalzo, Volker Lauschke, Bradley Maron, Paola Paci, Ingemar Ernberg, Nguan Soon Tan, **Zehuan Liao**, Weiyao Yin, Sundararaman Rengarajan, and Xuexin Li. "The Single Cell Atlas." (Manuscript)

II. Lu Pan, Paolo Parini, Roman Tremmel, Joseph Loscalzo, Volker Lauschke, Bradley Maron, Paola Paci, Ingemar Ernberg, Nguan Soon Tan, Ákos Végvári, **Zehuan Liao**, Sundararaman Rengarajan, Roman Zubarev, and Xuexin Li. "Comprehensive analysis of multi-omics single-cell data using SingleCellAnalyst." (Manuscript)

III. Jiajia Zhang, Yingying Yang, Zahraa S. Al-Ahmady, Wenchong Du, Jinjin Duan, **Zehuan Liao**, Qinghua Sun, Zhiyun Wei, and Jing Hua. "Maternal exposure to PM2. 5 induces cognitive impairment in offspring via cerebellar neuroinflammation and oxidative stress." *Ecotoxicology and Environmental Safety* 249 (2023): 114425.

IV. Yingying Yang, Tingting Yang, Ji Zhou, Zhijuan Cao, **Zehuan Liao**, Yan Zhao, Xiujuan Su, Jia He, and Jing Hua. "Prenatal exposure to concentrated ambient PM2. 5 results in spatial memory defects regulated by DNA methylation in male mice offspring." *Environmental Science and Pollution Research* 30, no. 12 (2023): 35142-35152.

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VI. Yuanbo Fu, Mina Wang, Bingcong Zhao, Baoli Liu, Jie Sun, Yaohui Feng, Zhengfang Wang, Qian Li, Chunhong Shi, Yabo Xuan, Siqi Long, Huan Liu, Tiange Chi, **Zehuan Liao**, Bin Li, Qingquan Liu. "Psychological impact of COVID-19 cases on medical staff of Beijing Xiaotangshan

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Conference Paper

I. **Zehuan Liao**, Joseph Jing Heng Lim, Yun Sheng Yip, Marcus Ivan Gerard Vos, William Wei Ren Tan, Hong Sheng Cheng, Nguan Soon Tan. "Angiopoietin-like 4 is Potential Therapeutic Target for Non-Muscle Invasive Bladder Cancer." *Frontiers in Cancer Science 2022*. Singapore.

Contents

List of abbreviations

ACC	Adenoid cystic carcinoma
ANGPTL4	Angiopoietin-like 4
ASO	Antisense oligonucleotide
BM	Basement membrane
CAF	Cancer-associated fibroblast
cANGPTL4	C-terminal fibrinogen-like domain of ANGPTL4 protein
ChIP	Chromatin immunoprecipitation
DEGs	Differentially expressed genes
DHS	DNAse I hypersensitive sites
DMNC	Density of maximum neighborhood component
DMOG	Dimethyloxallyl glycine
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
ENCODE	Encyclopedia of DNA Elements
Erbb3	Receptor tyrosine kinase 3
FBS	Fetal bovine serum
FN1	Fibronectin 1
GelMA	Gelatin methacryloyl
GEO	Gene Expression Omnibus
GSEA	Gene set enrichment analysis
HIF	Hypoxia-inducible factor
ITAM	Immunoreceptor tyrosine-based activation motif
LAP	Lithium phenyl(2,4,6-trimethylbenzoyl)phosphinate
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP	Matricellular protein

MDSC	Myeloid-derived suppressor cell
MES DEGs	Mesenchymal DEGs
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
nANGPTL4	N-terminal coiled-coil fragment of ANGPTL4 protein
NK	Natural killer (cell)
PEGDA	Poly(ethylene glycol) diacrylate
PIP2	Phosphatidylinositol 4,5-bisphosphate
PMT	Proneural-mesenchymal transition
PPARs	Peroxisome proliferator-activated receptors
PPI	Protein–protein interaction
PPRE	Peroxisome proliferator response element
PRECOG	Prediction of Clinical Outcomes from Genomic Profiles
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SERPIN	Serine protease inhibitor
Snai1	Zinc finger protein SNAI1
TAM	Tumor-associated macrophage
TAZ	Transcriptional coactivator with PDZ-binding motif
TCGA	The Cancer Genome Atlas
TF	Transcription factor
TGF-β	Transforming growth factor beta
TME	Tumor microenvironment
Treg	Regulatory T cell
WHO	World Health Organization
YAP	Yes-associated protein
ZEB1	Zinc finger E-box binding homeobox 1

1 Introduction

Cancer remains a prevalent and critical global health concern, representing a significant cause of mortality. In 2020 alone, approximately 19.3 million new cancer cases were diagnosed, resulting in 10 million cancer-related deaths [1]. In the same year, the World Health Organization (WHO) reported the diagnosis of 20 million new cancer cases, and these numbers are expected to increase exponentially in years to come [2]. As per WHO estimates in 2019, cancer ranks as the second leading cause of death before the age of 70 in 112 of 183 countries [1]. Female breast cancer was the most commonly diagnosed cancer in 2020, accounting for around 2.3 million new cases (11.7%), followed by lung (11.4%), colorectal (10.0%), prostate (7.3%), and stomach (5.6%) cancers [1]. Furthermore, lung cancer remained the primary cause of death among cancer cases, resulting in approximately 1.8 million deaths (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers. Significant advances in the early detection and diagnosis of cancer enable interventions that often prevent death [3]. However, new methods to treat cancer only benefit patients with some cancer types and have not dramatically improved outcomes in others over the last decade. Well-confined, primary tumors are generally cured by surgical resection and adjuvant therapy. However, metastatic cancers are largely incurable because of their systemic nature and their frequent resistance to therapeutic agents, including immunotherapy [4, 5]. Indeed, the metastasis of cancer remains the primary cause of cancer-related deaths, accounting for over 90% of such deaths [6]. Hence, the efficacy of cancer treatment predominantly hinges on our capability to intercept and, potentially, reverse the metastatic process.

1.1 Overview of cancer

Tumorigenesis and tumor progression are complex multistep processes with different characteristics. The hallmarks of cancer provide a mechanistic framework for us to understand the multistep progression of a premalignant cell to a deadly metastatic cancer cell. These hallmarks and emerging characteristics of cancer are well described in two seminal reviews by Hanahan and Weinberg [7, 8].

The six hallmarks are (i) sustaining proliferative signaling, (ii) evading growth suppressors, (iii) activating invasion and metastasis, (iv) enabling replicative immortality, (v) inducing angiogenesis and (vi) resisting cell death. The four emerging characteristics are (i) genome instability and mutation, (ii) tumor-

promoting inflammation, (iii) avoidance of immune destruction, and (iv) deregulation of cellular energetics. In 2022, four new hallmarks were added: (i) unlocking phenotypic plasticity, (ii) nonmutational epigenetic reprogramming, (iii) polymorphic microbiomes, and (iv) senescent cells [9]. Appreciation of these hallmarks will help us to understand the response and resistance of various cancer therapies (**Figure 1**).

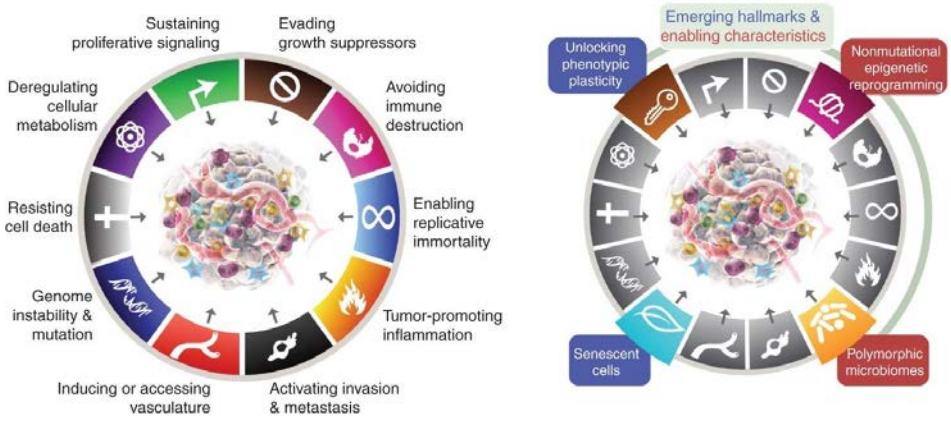

Figure 1: The 14 hallmarks of cancer adapted from Hanahan 2022 [9] under CC BY 4.0 license.

The ability of cancer cells to grow and metastasize has been one of the puzzles for researchers for many years. More details and discussions on the complex and multistep processes of epithelial–mesenchymal transition (EMT) and metastasis will be discussed in the later sections and chapters of this thesis [7, 8].

1.2 Epithelial–mesenchymal transition (EMT)

The process of metastasis is a complex multistep event that involves the spread of cancer cells from the primary site to distal organs. EMT is a key step in this process, which culminates in the loss of epithelial characteristics such as cell polarity and cell–cell adhesions and the de novo acquisition of mesenchymal phenotype features, including motility and metastatic potential (**Figure 2**) [10–14]. Upon aggressive tumor progression, EMT serves as a key step for cancer phenotypic plasticity and "stemness" properties similar to those of stem cells [15]. This transition is characterized by an increase in EMT transcription factors, such as Zinc finger protein SNAI1 (Snai1) and Zinc finger E-box binding homeobox 1 (ZEB1), and a decrease in epithelial markers, such as Receptor Tyrosine Kinase 3 (Erbb3). The adoption of a mesenchymal-like phenotype is a hallmark of increased

cancer aggressiveness. Although EMT is traditionally associated with the transition of epithelial cells, a parallel transformation is evident in cancers rooted in proneural cells, known as proneural–mesenchymal transition (PMT), as seen in glioblastoma multiforme. This shift towards a mesenchymal state seems to be a pivotal biological convergence, intensifying the metastatic capabilities of cancer cells. Whether originating from epithelial or proneural cell types, the transition to a mesenchymal phenotype imbues these cells with heightened invasiveness and aggressive behaviors.

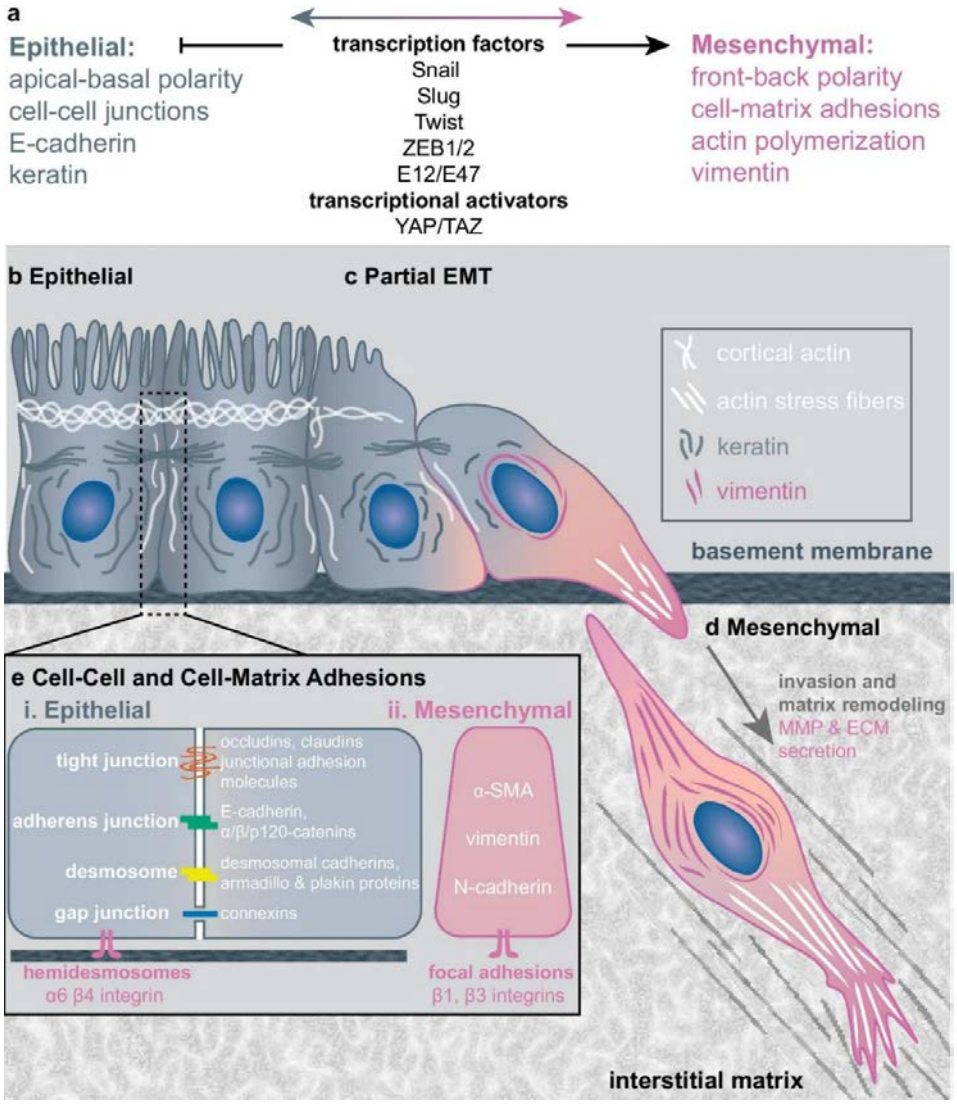

Figure 2: Epithelial–mesenchymal transition (EMT) adapted from Leggett et al. 2021 [14] under CC BY 4.0 license.

The highest clinical significance of the EMT process is linked to its crucial role in promoting tumor cell invasion, which is required for both the metastatic dissemination of carcinomas as circulating tumor cells and extravasation into distant organs [16]. There is also accumulating evidence that EMT-phenotype changes are associated with and might cause therapy resistance [17]. Therefore, the paramount clinical importance of EMT arises from its pivotal role in enhancing both the metastatic spread of carcinomas and resistance to traditional therapies. Developing effective strategies against mesenchymal transformation is crucial for successfully combating cancer.

Various signals received by cancer cells from the tumor microenvironment (TME) trigger EMT [18]. The activation of the EMT program can also be partial, and its success depends on the cancer cells and the TME, where the extracellular matrix (ECM) is a major component [13, 19-21]. The ECM composition and structure heavily influence the process of EMT and the eventual metastatic spread and therapy responses of cancer cells [22]. However, how exactly these factors affect EMT has yet to be determined.

1.3 The tumor microenvironment (TME)

Stephen Paget's "seed-and-soil" hypothesis postulates that tumor progression depends on the reciprocal relationship between tumor cells and their local environments [19, 23, 24]. Indeed, cancer progression is not an autonomous cell process, and it progresses in concert with the evolving cellular and acellular heterogeneity in the tumor stroma (**Figure 3**) [25-27]. In addition to a heterogeneous population of cancer cells, growing evidence suggests that various resident and infiltrating host cells, secreted factors, and ECM proteins coexist in the tumor mass [28]. The collection of these tumor-associated cellular components (fibroblasts, macrophages, regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), etc.) and acellular components (ECM, matricellular proteins (MCPs), etc.) forms the TME [29]. In addition, the TME can confer a proliferative advantage to tumor cells and reduce drug penetration during treatments [30]. Clinically, the TME also impacts drug response and resistance in treating cancer patients [31, 32]. Therefore, the interactions between cancer cells and their surrounding environment ultimately determine the fate of the tumor cells [33, 34].

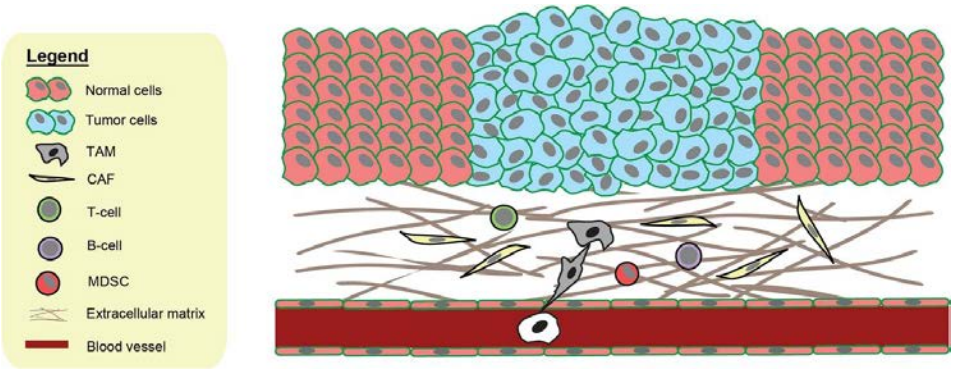

Figure 3: The tumor microenvironment (TME) adapted from Liao et al. 2019 [27] under CC BY 4.0 license.

While our ability to effectively treat cancer depends on our capacity to curb and perhaps even revert the metastatic process, it is realistically impossible to prognosticate when patients' tumors metastasize. Furthermore, anti-metastatic treatment options are severely limited due to the systemic nature of the disease and its associated characteristics, such as enhanced chemoresistance and invasiveness regulated by the complex crosstalk between cancer cells and the TME [5, 35, 36]. In order to effectively combat metastatic cancer, it is thus important to develop strategies that take into account the changes in the cellular and acellular components of the TME, curb the growth of the primary tumor, and eradicate cancer cells exhibiting a mesenchymal-like behavior [37, 38].

1.3.1 Cellular crosstalk in the TME

Cancer-associated fibroblasts (CAFs) play a central role in cellular crosstalk in the TME. CAFs are a major cellular component of many tumors and are known to influence cancer progression in many ways [25]. Thus, studies in CAFs may shed light on some of the most pressing clinical problems in cancer: metastasis, tumor relapse, and drug resistance [39-41]. Interactions between CAFs and tumor cells promote invasiveness and metastasis. For example, at the mechanobiological level, metastasis initiates when tumor cells invade and breach the basement membrane (BM), which typically provides mechanical support to epithelial tissues [42-44]. Central to this process, CAFs play a pivotal role in degrading the BM by upregulating matrix metalloproteinases (MMPs). Consequently, the formation of gaps in the BM compromises its integrity, making it more permissive to invasion and migration by tumor cells [45-47]. Moreover, the breach of the BM can be

facilitated by the mechanical interactions and signaling between CAFs and the BM [45-47].

Furthermore, CAFs notably contribute to cancer metastasis and invasion by creating paths conducive to tumor cell invasion. In a specific study involving CAFs isolated from two patients diagnosed with salivary gland adenoid cystic carcinoma (ACC), the conditioned medium collected from CAFs significantly augmented ACC cell migration and invasion [48]. During co-culture of CAFs with ACC cells in a microfluidic device, the ACC cells visibly followed a path established by CAFs positioned at the invasion front. The invasive track facilitated by CAFs within the ECM involves the activity of MMPs and the CXCL12/CXCR4 pathway [48].

Additionally, modulation of many areas of the immune system has been found to involve CAFs [49]. CAFs have been found to interact with tumor-associated immune cells to increase tumor cell dissemination [25, 50]. In essence, tumor-associated macrophages (TAMs), Tregs, and MDSCs can be recruited by CAFs to promote Th2 polarization of the TME [51]. TAMs and MSDCs are significant contributors to the production of Th2-inducing cytokines and various factors that actively inhibit the host's antitumor immune responses and foster tumor growth [52]. This alteration in the TME facilitates angiogenesis, lymphangiogenesis, and the inhibition of antitumor responses, effectively sustaining tumor growth and supporting metastasis.[53]. With the immunosuppressive role of CAFs in the TME as presented above, CAFs have major implications in cancer immunotherapy [49, 54].

Many studies have indicated that CAFs play a protumorigenic role via the secretion of various growth factors, cytokines, chemokines, and ECM components [25]. Tumor cells, as well as immune cells and stromal cells, can express chemokines in the TME. Chemokines attract infiltrating immune cells into the TME, enforcing communication between CAFs and immune cells and regulating tumor immune responses (**Figure 4**) [49, 55]. Chemokines can also directly target tumor cells to regulate cancer cell proliferation, plasticity, invasiveness, and metastasis [55]. While the roles of chemokines and growth factors in tumor progression are well established, much less is known about the roles of reactive oxygen species (ROS) in tumor pathology. Cancer cells and CAFs produce and respond differently to ROS [25, 50]. For example, an elevated level of intracellular ROS, stemming from

defects in either ROS production or detoxification processes, has the potential to convert a normal cell into a malignant one [56].

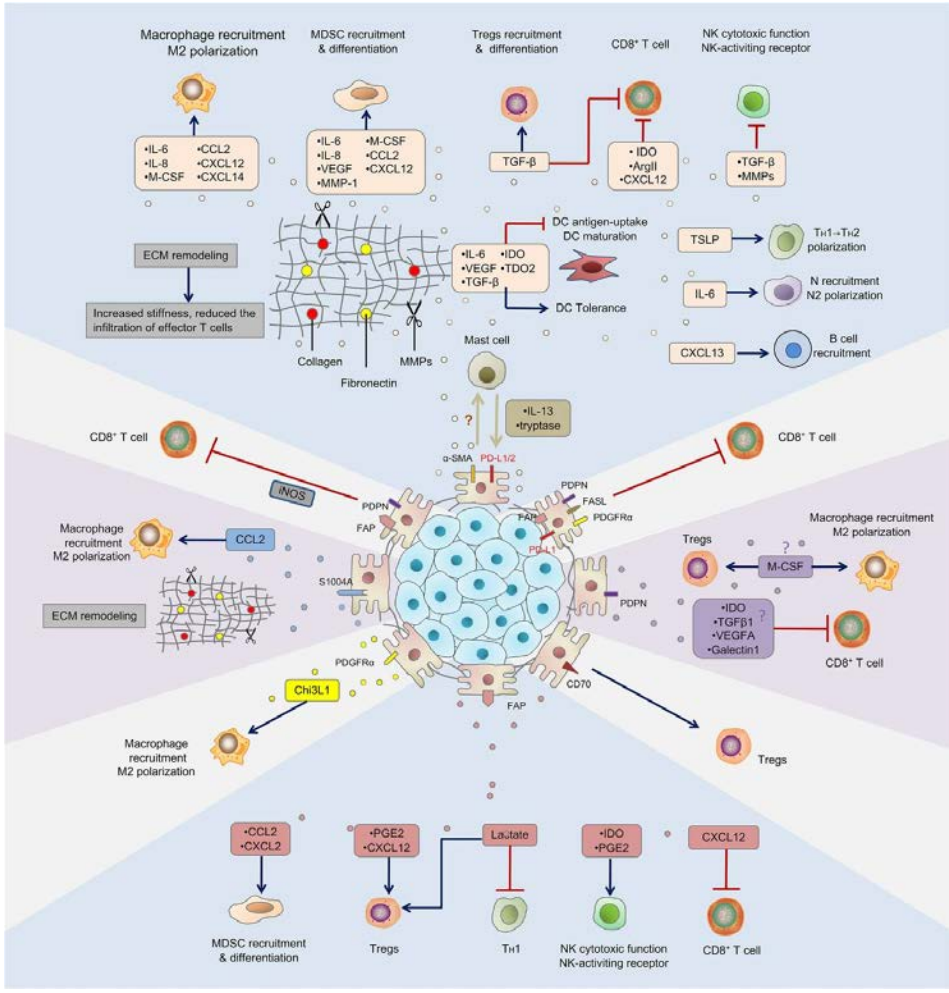

Figure 4: The immunosuppressive functions of different CAF subtypes in the TME adapted from Liu et al. 2019 [49] under CC BY 4.0 license.

While CAFs are highly abundant and play a major role in the TME, many CAF-targeting therapies hypothesized to be effective fail to achieve the expected clinical outcome [57]. This brings us to the noncellular components that increase the complexity of the study of the TME.

1.3.2 Extracellular matrix (ECM)

Cancer cells modify their microenvironment by secreting ECM components and ECM-modifying enzymes (**Figure 5**) [58, 59]. Similarly, CAFs alter the tumor stroma

by producing and depositing substantial amounts of ECM components [60, 61]. Under the influence of adjacent cancer cells, CAFs are differentiated from resident fibroblasts in the solid tumor mass [25]. Transforming growth factor-β (TGF-β) is a key regulator of fibroblast differentiation during wound healing and tumor progression [62]. The release and activation of TGF-β from the ECM promote the differentiation of fibroblasts into contractile CAFs, and increased tension promotes the further release of TGF-β [63].

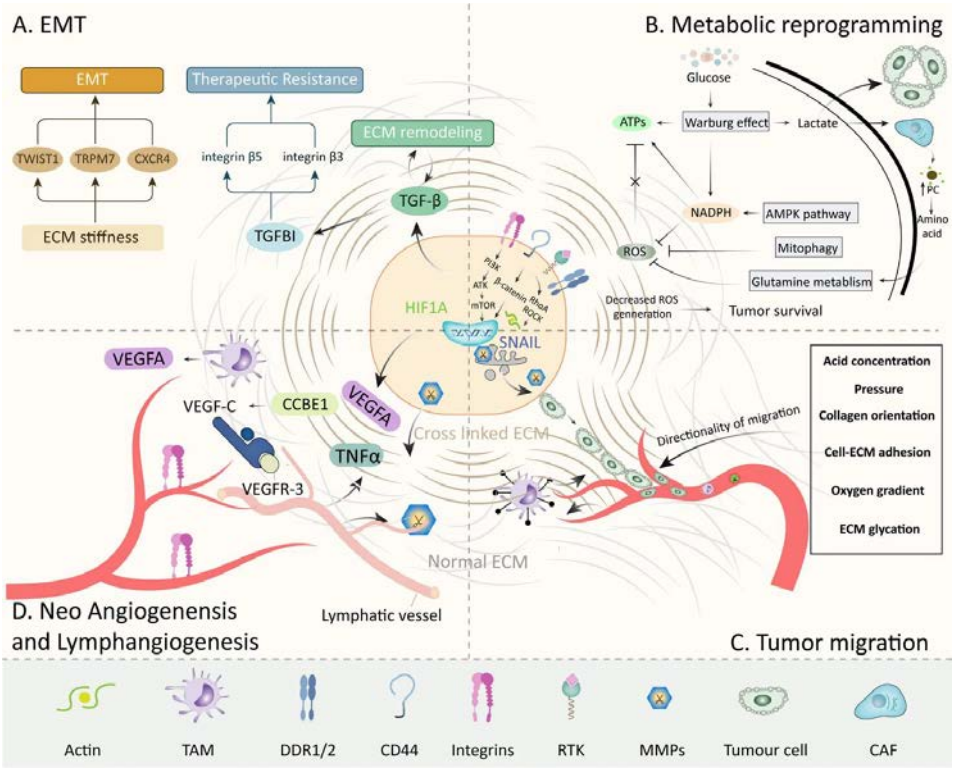

Figure 5: The regulatory network within the extracellular matrix (ECM) during tumor invasion and metastasis adapted from Yuan et al. 2023 [59] under CC BY 4.0 license.

A major space-filling structural component of the TME is the ECM, which consists of fibrous proteins, glycoproteins, proteoglycans, and polysaccharides. The cancer ECM is a highly dynamic structure undergoing constant remodeling, which provides a biochemical and physical niche for cancer cells. In cancer, abnormal ECM synthesis, secretion, and modification are achieved by the dysregulated expression of matrix modelling enzymes. Furthermore, the ECM changes drastically in its composition and relative abundance at the primary and

metastatic tumor site [5, 35, 36]. Abnormal ECM dynamics affect the overall biochemical, physical, and mechanical cues of cancer cells in the TME. These biochemical changes cause the ECM to alter its biophysical properties, such as stiffness, rigidity, and tension [64, 65]. Various common extracellular proteins, such as collagens, fibrins, elastins, fibronectins and laminins, contribute to these properties of the ECM [66].

Fibrin is a component of the ECM that plays a critical role in wound healing. It is produced by converting fibrinogen to fibrin via the action of thrombin, a clotting enzyme, during tissue repair [67]. Platelets release growth factors, drawing fibroblasts to replace the transient fibrin matrix with a collagenous one. Subsequently, resident fibroblasts undergo differentiation into myofibroblasts [68, 69]. Cancer resembles "a wound that does not heal" [70], suggesting that the cellular and biochemical processes associated with wound healing are similar to those of the tumor stroma. Local and systemic activation of blood coagulation appears to be a common and important host response to growing tumors. Tumor cells, TAMs, and tumor-associated endothelial cells contain proteins with potent procoagulant activities, and fibrin deposition has been observed histologically on the surface of both tumor cells and stromal elements within tumors in situ [71].

The involvement of fibrin in cancer biology has been documented for more than a century [72]. In 1878, Billroth observed the presence of fibrin around tumor cells. Fibrin play a crucial role in tumor cell growth and metastasis [73]. Fibrin facilitates tumor cell growth and migration. The fibrin matrix can also support the migration of other tumor-associated cells such as macrophages, fibroblasts, and endothelial cells in the TME [74]. The fibrillary proteins also possess adhesive ligands for cell attachment and crawling [75]. In addition, due to their chemotactic properties, fibrin fragments can promote the migration of endothelial cells and immune cells in the stroma [76]. Furthermore, fibrin also binds to and shields growth factors from degradation, playing a pivotal role in promoting angiogenesis. [77, 78]. However, the importance of the resultant fibrin deposition to tumor growth remains uncertain.

Tumor ECM is stiffer than normal ECM due to the overexpression of various ECM components, including collagen. Collagen is the most abundant type of fibrous protein and constitutes the scaffold of the TME. Collagen degradation and redeposition affect the TME and can promote tumor infiltration, angiogenesis, invasion, and migration. Collagen is traditionally thought to be a passive barrier to

resist tumor cells, as collagen must be degraded before tumor cell invasion [79]. However, current evidence highlights that collagen also plays an active role in driving tumor progression. Collagen alterations in the TME release biomechanical signals sensed by cancer and stromal cells, triggering a cascade of biological events. Integrin, when binding to collagen, regulates cancer cell behavior. This binding can activate signaling pathways involving AKT/PI3K, mitogen-activated protein kinase (MAPK), Rho family, and MEK/ERK, leading to the proliferation and invasion of cancer cells [80]. Furthermore, collagen-rich ECM often creates hypoxic conditions. Hypoxic signaling can activate, via various mechanisms, the promigratory and invasive phenotypes of tumor cells. For instance, by directly and indirectly regulating the transcription factors Snai1, Slug, Twist, and ZEB1, hypoxia-inducible factor (HIF) signaling can induce EMT [81]. In addition, HIF signaling can also enhance the upregulation of proteolytic enzymes, such as MMPs, cathepsins, lysyl oxidases, and prolyl-4-hydroxylases (P4H), to support further matrix remodeling [82, 83]. The collagen-rich ECM-induced hypoxic environment leads to the expression of chemokines and cytokines by tumor cells, recruiting macrophages and mesenchymal stem cells (MSCs) into the TME to further support invasion, migration, and metastasis [84, 85].

Apart from primary tumor sites, the ECM of distant organs/sites can be primed by soluble factors from the primary tumor and remodeled to prepare for the arrival of metastatic cancer cells [67]. These premetastatic niches may explain the organotropic preference to colonize certain sites by specific cancer cells during metastasis. It has been discussed heavily whether tumor cells actively target a specific tissue for metastasis or accidentally arrive at a suitable site for engraftment and growth. Both options are partially realized, as the primary tumor has been proven to influence and prepare distant sites for cancer cell arrival at the target sites [86, 87]. These primed secondary sites are known as premetastatic niches. The primary tumor releases chemokines, matrikines, and exosomes into the blood and lymph, distributing those contents to the targeted tissues [88-91]. Exosomes carrying integrins adhere to the ECM of targeted sites and merge with normal cells, releasing contents like proteins, translatable mRNA, and miRNA [90, 91]. These mechanisms induce transformations in distant cells, altering their metabolism and prompting the secretion of ECM proteins or enzymes that modify the ECM, such as LOXs [92]. LOX, in particular, has the potential to induce tissue stiffening, laying the foundation for the premetastatic niche and eventual metastasis. [93-97].

Over the past decade, cancer research has notably pivoted towards exploring the TME, particularly on the various cellular components and communication factors (e.g., cytokines, growth factors, and ROS) [98]. Current ECM research emphasizes biochemical mechanisms linked to tumor progression, particularly the intracellular pathways of signal transduction originating from the ECM and the cellular metabolic responses related to collagen remodeling [99, 100]. However, there is limited attention to the dynamic changes in ECM biomechanics, such as stiffness and elasticity, as crucial determinants of cancer progression [79].

1.3.3 Matricellular proteins (MCPs) in tumor progression

Apart from collagen and fibrin, MCPs are also an important part of the ECM (**Figure 6**) [101]. Tumor and neighboring stromal cells secrete MCPs, a class of ECM-associated and structurally diverse glycoproteins, in the TME [102]. These MCPs do not contribute significantly to the structure of the ECM but are involved in modulating cell-matrix and cell–cell interactions. Furthermore, these proteins facilitate cancer cells in the acquisition of various hallmarks of cancer such as metastasis, angiogenesis, cell proliferation and survival [103]. Various MCPs such as Angiopoietin-like 4 (ANGPTL4), tenasin C, osteopontin and SPARC are involved in invasion and metastasis. However, we would like to highlight the key roles that ANGPTL4 plays in metastasis. Metastasis-related ANGPTL4 is a secretory protein from the angiopoietin (ANG)-like family [104]. The expression of ANGPTL4 can be upregulated by hypoxia, TGF-β, and peroxisome proliferator-activated receptor, among others [104, 105]. Full-length ANGPTL4 is proteolytically cleaved by pro-protein convertases, giving rise to a functionally distinct N-terminal coiled-coil fragment (nANGPTL4) and C-terminal fibrinogen-like domain (cANGPTL4). ANGPTL4 regulates lipid and glucose metabolism, primarily as an inhibitor of lipoprotein lipase activity via nANGPTL4 [106, 107]. A premetastatic role for cANGPTL4, such as increased vascular permeability, anoikis resistance, cancer cell invasiveness and metabolic flexibility, has been described in many solid tumor types [104, 108-113]. Furthermore, ANGPTL4 secreted by stromal adipocytes also contributes to tumor growth [114].

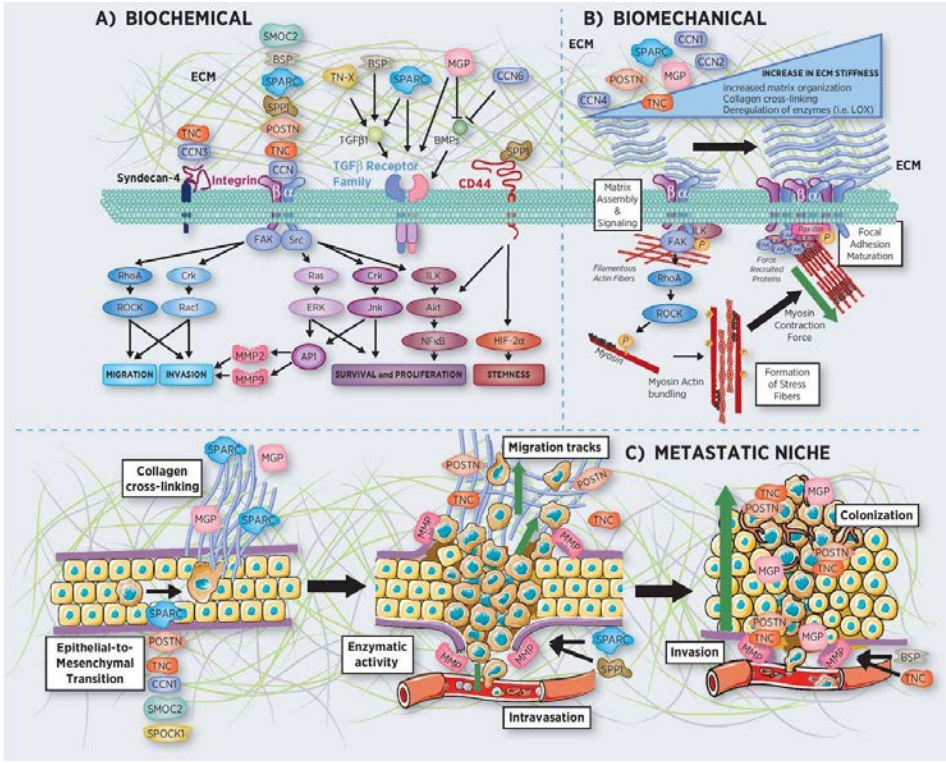

Figure 6: Activation of the biochemical, biomechanical, and metastatic effects by MCPs adapted from Gerarduzzi et al. 2020 [101] under CC BY 4.0 license.

1.4 Models used to study EMT

Many drug development studies have been performed on the primary tumor [115, 116]. There is also an increasing focus on targets that arrest cancer metastasis [117]. However, the effect of TME that propels cancer cells to acquire metastatic properties is often overlooked. TGF-β and hypoxia are well-established biochemical and microenvironmental cues that trigger EMT. There is a growing interest to investigate the impact of the biophysical properties of the ECM on EMT and its accompanying attributes, which reflect ECM remodeling during cancer progression [64]. Matrix stiffness influences exosome secretion and specific oncogene expressions, thereby promoting tumor growth [118, 119]. In a stiffer TME, cells activate EGFR/Erk, integrin-linked kinase and mechano-sensing signaling pathways to promote cell plasticity and EMT processes [120-123]. Certain cellular characteristics, like contractility and adhesiveness, allow metastatic cells to navigate with or against the stiffness gradient present in the TME [124]. Given these significant biological consequences, it is essential to unravel the ways matrix

biophysical attributes modulate cellular behaviors. In this context, a variety of 3D cell culture techniques – ranging from liquid- or scaffold-based 3D matrices to contemporary methods like microfluidics and bioprinting – have been employed to replicate the in vivo nuances of metastasis, TME, and the cancer cells' response to treatment [125, 126]. These 3D systems play a vital role during early preclinical drug development, creating more efficacious therapies and predicting therapeutic outcomes. Among these, scaffold-based 3D hydrogels, composed of natural biopolymers (like proteins, polysaccharides, and decellularized ECM) [127], synthetic materials (such as polyesters and self-assembling peptides), or their hybrids [128, 129], are particularly popular. Their widespread adoption can be attributed to their exceptional biocompatibility and tunability. These unique features of scaffold-based hydrogels allow the in vitro modelling of biophysical features of TME. While reliable 3D culture systems have been developed that recapitulate the growth of a primary tumor, the complex mechanism by which the metastatic cancer cells activate/utilize the multiple hallmark attributes/capabilities remain more challenging to model in vitro. Therefore, our improved understanding of the metastasis/EMT mechanisms in 3D TMEs is essential for our ability to treat the metastatic disease.

1.4.1 Cell culture models in 2D

There are various studies of EMT using 2D monolayer cultures [130-132]. The 2D monolayer culture model is a system whereby cells grow on flat dishes, usually made of plastic. The cultured cells adhered to the surface of the dishes and spread into a monolayer. 2D cell culture is still a popular method due to some of its advantages [133]. First, it is an inexpensive method to grow and observe cell growth and treatment [134, 135]. Therefore, it is an inexpensive method to conduct pilot cell experiments and replicate previous cell experiments. Second, the process has been well established since it was developed in the early 1900s and gained widespread acceptance in the mid-1900s [136, 137]. Hence, comparative studies are abundant for various cell studies in 2D cultures. It is easy to compare these new studies with previous studies. Finally, 2D cell culture is also a simple and easily understood cell culture method that does not require long lab training [134]. 2D cell cultures also make it easy to observe cell growth and analyze cell changes [138].

Although 2D monolayer cultures are popular for research, they have various limitations [139, 140]. Cells cultured on flat surfaces cannot adequately represent in vivo cell environments. In particular, growing cells on flat plastic surfaces are not

representative of the cancer cells' function, growth and adaptation in a tumor, where the cells are surrounded by other heterogeneous cells and are exposed to various signals and mechanical forces in three dimensions [141-143]. The stiffness of 2D culture (plastic culture) is also approximately 2.4 GPa, which is only similar to bone stiffness and is not tunable to suit other organ stiffness [144]. Furthermore, as 2D culture models are not representative of the in vivo environment, 2D cell drug screening is generally not accurately predictive [145-147]. This increases the costs and failure rate of drug discovery due to unnecessary further clinical trials and development [145-148]. Hence, other models that are more representative of the in vivo environment have been developed.

1.4.2 Cell culture models in 3D

In the early 1980s, Mina Bissell proposed the importance of studying the TME using 3D culturing techniques for cancer research [149, 150]. Her lab developed various 3D culturing techniques for cancer discovery and treatments [151, 152]. In the 21st century, research interest in 3D cell culture has grown enormously as researchers have realized the shortcomings of 2D culture models [153]. Recent research has indicated that various 3D ECM models are superior to 2D monolayer cultures, as 3D cell cultures can mimic the in vivo behavior of cancer cells within a tumor (**Figure 7**) [153, 154]. Although 2D cell culture techniques are still widely used in research, there is an upward trend in applying 3D cell culture techniques to cancer and stem cell research [135, 155, 156]. Indeed, cancer cells do not often grow in 2D surfaces in vivo, and it is necessary to model the actual growth environment of cells to understand cancer and develop more precise therapies against cancer.

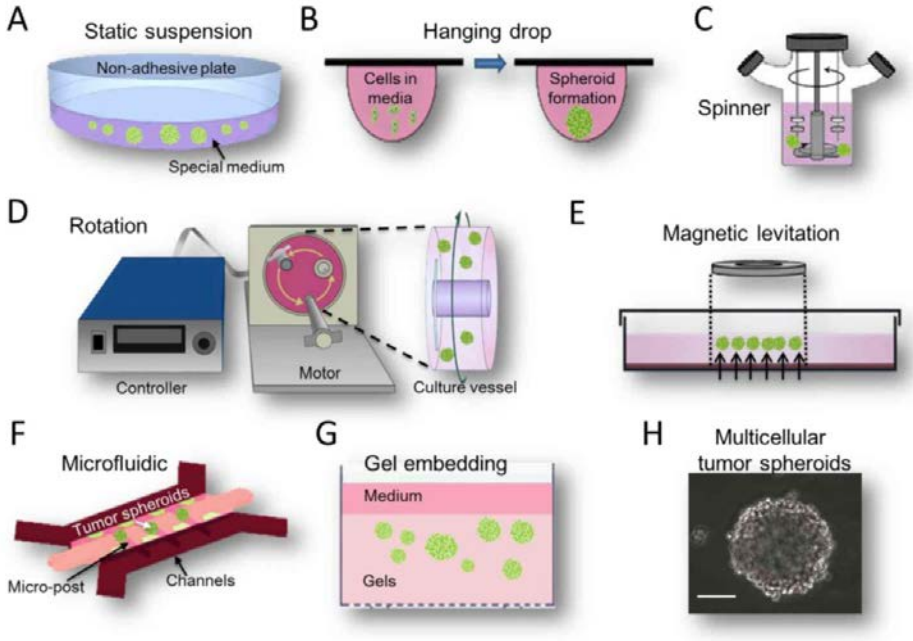

Figure 7: Some methods available for 3D cell culture adapted from Lv et al. 2017 [153] under CC BY 4.0 license.

There are various key advantages of 3D culture over 2D culture. First, biomimetic 3D cell cultures are much more physiologically relevant and predictive than 2D cell cultures [157-159]. The high degree of structural complexity in 3D cell cultures can mimic and maintain the cells' in vivo environment [157-160]. Such structural complexity is missing in 2D cell cultures. Hence, 3D culture systems are good in vivo simulators and are more realistic ways to grow and treat cancer cells, exhibiting similar growth and treatment patterns in vivo [125, 161, 162]. Second, different cell populations, such as CAFs and immune cells, can be cocultured with cancer cells in 3D spheroid structures, mimicking the cellular heterogeneity within in vivo tumors [163-165]. The interactions between these cells in vivo can be modeled and studied in 3D culturing systems [125]. In specific cases, 3D culturing systems can also act as barrier and help to understand the survival and function of cancer cells in 3D tissue microenvironments [166-168]. Finally, 3D cell cultures can more realistically simulate physical events in biological systems such as mechanical stress and fluid flow [139]. For example, blood flow is essential for the function of various tissues. The 3D models can be useful in studying how cells respond and adapt to changes in fluid flow (with changes in nutrients, etc.) [160, 169]. Additionally, changes in mechanical stress, physiologically or pathologically,

can be replicated by mechanically tunable 3D cell culture systems [170, 171]. For example, collagen-alginate 3D culture has been found to be tunable to mimic the stiffness of the breast, giving researchers the opportunity to study the organ tropism of cancer and associated cells under this stiffness [172]. Hence, 3D cell culture is a much better in vitro method than 2D cell culture in representing in vivo conditions. **Table 1** summarizes the various advantages of both 2D and 3D cell cultures. In addition, 3D cell culture is much cheaper and less cumbersome than animal work [136]. In some conditions, 3D cell cultures can be even more predictive and reproducible than those in vivo, and there is currently no universal in vivo model to study EMT and the TME [173]. In essence, as discussed above, 3D cell culture differs greatly in the biomechanical environment and type of substrates for cancer cells compared to those of 2D cell culture. Therefore, 3D cell culture is regarded as superior to 2D cell culture in mimicking actual tumor environments and progression.

2D cell culture	3D cell culture
Cheaper	More representative of in vivo condition
Very well established	Very versatile to study different physical conditions
Easily understood	Very versatile to study cell co-cultures
Easily analyzed	Tunable to represent different biological conditions

Table 1: Comparison of the advantages of 2D and 3D cell culture techniques.

1.5 Cellular signaling in the 3D model

Despite the advantages of the 3D model to recapitulate the in vivo TME and thus many phenotypes of cancer cells, such as increased resistance to drugs, the molecular mechanism by which the mechanical properties of the ECM affect cancer cell behavior remains unclear. Yes-associated protein (YAP), a transcription factor together with the transcriptional coactivator with PDZ-binding motif (TAZ), plays a crucial role in mechanotransduction [174]. Mechanical signals from the ECM are conveyed by YAP/TAZ to various intracellular signals [175]. High stiffness activates YAP, causing it to translocate to the nucleus from the cytoplasm [176]. Malignant cancer functions such as cellular proliferation and metastasis can be a result of enhanced activation of YAP.

Recently, studies have shown that YAP/TAZ-mediated ANGPTL4 expression is involved in human trophoblast cell invasion, ferroptosis and chemoresistance (**Figure 8**) [177, 178]. However, the exact roles of YAP/TAZ mediated ANGPTL4 expression in metastasis are still largely unknown. There is a paucity of information regarding EMT in 3D and the acquisition of associated characteristics of metastasizing cancer cells. Thus, investigation into 3D cell-matrix communication through comparative transcriptomic analyses and proof-of-concept in vitro studies will enhance the general applicability of targets from drug screening as new adjunctive or therapeutic treatments.

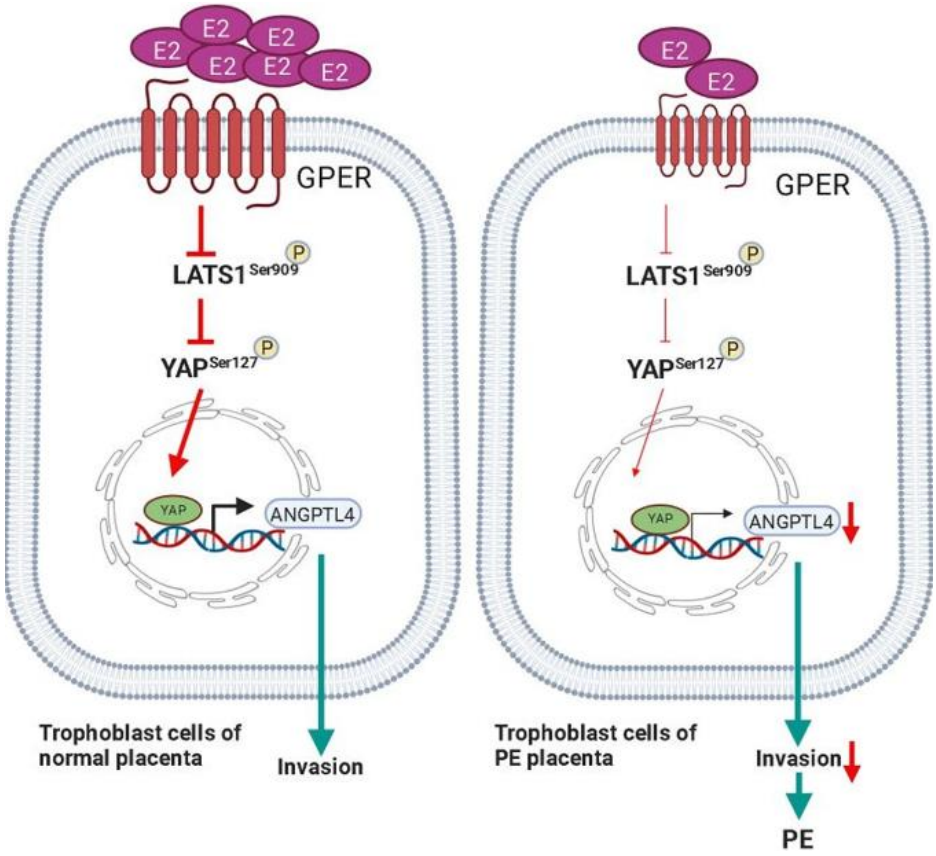

Figure 8: YAP/TAZ-mediated ANGPTL4 expression involved in human trophoblast cell invasion adapted from Cheng et al. 2021 [177] under CC BY 4.0 license.

2 Research aims

Our central hypothesis is that 3D culture activates a distinct transcriptome in cancer cells to confer metastasis-associated characteristics. We aim to prove this through the specific methods as stated below:

Aim 1: Identify common 3D EMT transcriptomes regardless of EMT inducers via meta-analysis of 2D vs 3D cultures of various cancer cell types.

Aim 2: Identify key hub genes involved in 3D EMT.

Aim 3: Validate the role of a key hub gene (ANGPTL4) as antimetastatic target using in vitro 3D culture models and in vivo animal models.

We leveraged interdisciplinary research among Nanyang Technological University (NTU), National University of Singapore (NUS) and Karolinska Institutet (KI) to provide clinically relevant insights into the cancer cell transcriptomes during EMT induced in 2D and 3D environments. The findings from our studies will provide new insights into the interaction between cancer cells and ECM in 3D cultures.

3 Materials and methods

The main materials and methods used for our studies are outlined in this section. For specific details, please refer to the individual papers.

3.1 Data retrieval and bioinformatics analyses

Datasets consisting of cancer transcriptomes from 2D and 3D cancer cell cultures with and without mesenchymal inducers were retrieved from the Gene Expression Omnibus (GEO) repository. An in-house generated dataset using gastric adenocarcinoma (MKN74) treated with either dimethyloxallyl glycine (DMOG) or TGF-β1 to induce mesenchymal transition was included. Bioinformatics analyses of these data and RNA-seq data of MKN74 cells grown in 3D collagen-alginate culture were performed and analyzed as previously described [179].

The protein-protein interaction network of 3D MES DEGs was constructed using Cytoscape [180]. Topological analysis of the network was performed using CytoHubba which computed the density of maximal neighborhood component (DMNC) scores of each node to reflect their interaction and importance [181]. Cohort data were retrieved from PREdiction of Clinical Outcomes from Genomic Profiles (PRECOG) [182] and The Cancer Genome Atlas (TCGA) Datasets.

3.2 Cell cultures

3.2.1 Cell lines

The polarized human gastric adenocarcinoma cell line MKN74 and human urinary bladder transitional carcinoma cell lines, T24 and UMUC-3, were used in our studies.

3.2.2 2D cell culture

MKN74 cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS). T24 and UMUC-3 bladder cancer cells were cultured in DMEM and EMEM, supplemented with 10% FBS, respectively. The cell lines were routinely passaged and maintained at 37°C in 5% CO_2.

3.2.3 3D cell culture

Micropatterned agarose hydrogel was used for the formation of 3D cell spheroids.

For 3D collagen-alginate cell culture, a mixture of collagen, sodium alginate, and medium solution were used. The stiffness of these 3D cell cultures was adjusted

using varying concentrations of calcium chloride. Rheology testing was performed on the hydrogels using a rheometer (Anton Paar).

For 3D PEGDA-GelMA cell culture, a mixture of Poly(ethylene glycol) diacrylate (PEGDA) and Gelatin methacryloyl (GelMA) in PBS with Lithium phenyl(2,4,6-trimethylbenzoyl)phosphinate (LAP) was induced to crosslink using a 405 nm wavelength light source. The stiffness of these 3D cell cultures was adjusted using varying concentrations of PEGDA. Rheology testing was performed on the hydrogels using a rheometer (Anton Paar).

3.2.4 Culture treatments

For EMT induction, each culture medium was replaced with the corresponding serum-free medium containing TGF-β1 or DMOG. Recombinant cANGPTL4 protein was produced and purified as previously described [111, 183].

For antibody treatments, indicated concentrations of 11F6C4mAb (antibody against cANGPTL4) were added. For negative control, IgG was used. ANGPTL4 silencing was also performed using ON-TARGETplus SMARTpool siRNA (Horizon Discovery) targeting ANGPTL4 as previously described [184].

3.3 Microscopy

The microstructures of the hydrogels were observed by scanning electron microscopy. 3D cell cultures were monitored using JuLi Stage: Real-Time Cell history Recorder (NanoEnTek, Singapore) or Inverted Fluorescence Live Cell Microscope AO7. Image processing and qualification were performed using ZEN software (Carl Zeiss) and ImageJ.

3.4 Real-time PCR and immunoblots

Total RNA was extracted using TRIzol® Reagent (Thermo Fisher Scientific, USA) followed by Pure NA. Fastspin). Total RNA was quantified based on the A260/280 absorbance using Nanodrop ND1000 (Thermo Fisher Scientific, USA). Total RNA was reverse transcribed using iScript cDNA SuperMix (Quanta Biosciences, USA). Quantitative PCR was performed as previously described [185]. Immunoblots were performed as previously described [186].

3.5 Chromatin immunoprecipitation (ChIP)

Human DNase-seq data across cancer cell lines were retrieved from Encyclopedia of DNA Elements (ENCODE). Active regulatory regions of ANGPTL4 gene were

identified. Chromatin immunoprecipitation (ChIP) experiments were carried out as previously described [187, 188]. Sonicated chromatin complexes were immunoprecipitated using an antibody against YAP.

3.6 Hydroxyproline assay

To examine the stability collagen-alginate hydrogel, 100 µL of culture media were collected from 0 h and 48 h wells and transferred to a 96-well plate. RPMI samples with known concentrations of collagen were included as standards. Chloramine T buffer was added to each sample and standard well, and incubated at room temperature. After that, Ehrlich's Reagent was added to each sample and standard well, and were incubated. Absorbance values at 560 nm were measured. Values from the standard wells were used to plot a standard curve. The relative amount of hydroxyproline present in the samples was determined from the standard curve to calculate the percentage of collagen degradation.

3.7 Animal experiments and ethics

Orthotropic xenograft for nonmuscle invasive bladder cancer in NSG mice was performed using UMUC3 cells as previously described [189].

To study the effects of stiffness in vivo, MKN74 cells were mixed with PEGDA-GelMA matrix (with LAP) and injected subcutaneously into NSG male mice. Crosslinking was triggered using 405 nm wavelength light source for 120 s.

All animal experiments in our studies are approved by the relevant local ethical committees. All animal handlers involved in our studies are licensed to carry out experiments in the university animal facilities.

3.8 Statistical analysis

Appropriate statistical analyses were used based on the sample size, number of groups and whether the groups were paired. Statistical tests were performed using GraphPad Prism software (GraphPad Software Inc., USA). A p-value of < 0.05 was considered significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s., not significant).

4 Results

4.1 Paper I – Attenuating epithelial-to-mesenchymal transition in cancer through angiopoietin-like 4 inhibition in a 3D tumor microenvironment model

4.1.1 Mechanosensitive gene signature of cancer cells undergoing mesenchymal transition in 3D culture

In a differential gene expression analysis using RNA-seq datasets from different cell lines and GEO projects, we examined two main parameters, namely, mesenchymal induction (control or MES) and culture condition (2D or 3D). Comparing 3D to 2D cultures, we found 848 differential expressed genes (DEGs), of which more than 70% (610 DEGs) were significantly upregulated. The disproportionately high number of upregulated DEGs suggests the activation of a new set of genes that recapitulate new biological activities in 3D culture. Furthermore, mesenchymal induction resulted in 368 DEGs. Gene ontology analysis revealed that these DEGs can be broadly grouped into three clusters. Genes associated with angiogenesis, cell growth, cell-matrix adhesion and responses to mechanical stimulus and stress were more prominently elevated in 3D cultures than in 2D cultures. Interestingly, genes involved in apoptosis and calcium ion transport were suppressed in 3D culture, suggesting that cancer cells in 3D culture are intrinsically more resistant to apoptotic signals. Gene set enrichment analysis (GSEA) of the mechanotransduction gene set [190] revealed that mechanosignaling plays a pivotal role in the regulation of gene expression in 3D tumoroids and mesenchymal transition but not in 2D culture.

By overlapping the DEGs from the two main effects, 3D culture and mesenchymal transition, we revealed 74 common genes implicated and hence termed the "3D MES DEGs". Functionally, the 3D MES DEGs are primarily responsible for ECM remodeling, collagen metabolism, cellular motility, and cell–cell adhesion. Protein–protein interaction (PPI) network analysis revealed that many inducers and remodelers of ECM, such as TGFB1, serine protease inhibitors (SERPINs) and MMPs, ANGPTL4, Fibronectin 1 (FN1) and integrins, form a highly intertwined network, highlighting the importance of these genes in orchestrating mesenchymal transition in a 3D context. Our analysis identified potential anti-metastatic targets involved in the growth and mesenchymal transition of cancer cells in 3D culture, which deliberate disruption can yield beneficial clinical implications.

4.1.2 ANGPTL4 gene is hub gene in 3D mesenchymal signature

To uncover the underlying mechanisms, we examined the expression of the 74 "3D MES DEGs" established from our transcriptomic meta-analysis in our hydrogel-encapsulated 3D cancer cultures. We observed a significant influence of the surrounding stiffness on gene expression related to ECM remodeling and integrin signaling. Specifically, genes such as ITGA2, COL13A1, ANGPTL4, and LAMB3 displayed notable upregulation when cancer cells were encapsulated within a stiffer matrix. To identify key genes that regulate important aspects of 3D EMT, we performed a hub gene analysis of the PPI network (**Figure 9**). The top five hub genes based on Density of Maximum Neighborhood Component were TGFB1, SERPINB2, LAMB3, ANGPTL4 and COL22A1. TGF-β1, a well-established EMT inducer, has been shown to upregulate the expression of ANGPTL4 [191].

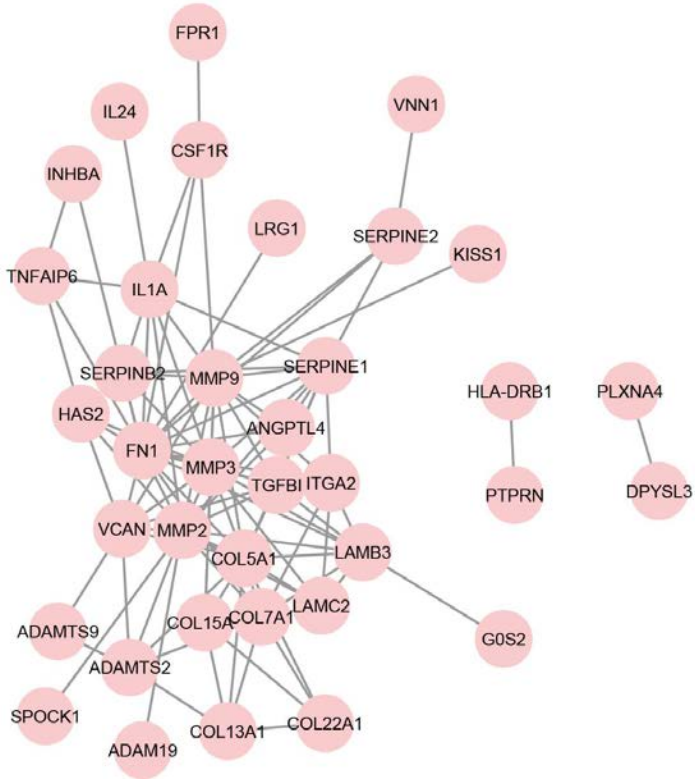

Figure 9: Protein–protein interaction (PPI) network of the 3D MES DEGs.

To assess the clinical relevance of these hub genes, we interrogated the PRECOG, Prognoscan cohort and TCGA databases [182]. ANGPTL4 was ranked highest for its prognostic value in cancer patients among the top five hub genes, ANGPTL

family and well-established oncogenes, thus implying a stronger association of ANGPTL4 expression with poorer cancer outcome. The stratification of the patients into high- and low- ANGPTL4-expressing tumors, as defined by the median expression of ANGPTL4, revealed that patients with high ANGPTL4-expressing cancers had a poorer prognosis and an overall shorter median survival time. In the PrognoScan database, cohort studies that reported overall and relapse-free survival, which had significantly corrected P values, were used in the analysis. All identified studies, except for the Stockholm cohort (GSE1456) [192], illustrated the association between high ANGPTL4 expression and poor patient outcomes. These findings suggest that ANGPTL4 is a potential anti-metastatic target.

4.1.3 Mechanoregulation of human ANGPTL4 gene

The expression of many activated genes in 3D culture is mechanosensitive, including the hub gene ANGPTL4. However, the mechanoregulation of the hub gene ANGPTL4 has not been thoroughly investigated. We first identified regulatory sites in the human ANGPTL4 gene. Two DNAse I hypersensitive sites (DHS 1-2) are potential regulatory sites in ANGPTL4, as revealed by data from various cancer cell lines in the ENCODE database. DHS1 corresponds to the proximal regulatory promoter, and DHS2 corresponds to the characterized peroxisome proliferator response element (PPRE) [193].

Proto-oncogene YAP and TAZ are master regulators of mechanotransduction in response to various physical cues, such as substrate stiffness and dimensionality, which regulate critical cellular functions and tissue homeostasis [194]. Since MKN74 is a YAP-dominant cancer cell line [195], quantitative ChIP was performed to examine the occupancy of these DHSs associated with YAP protein. Primers for Ch10 and CTGF were used as the negative control and positive control, respectively. Quantitative ChIP revealed that YAP was associated with DHS1, but not DHS2, of the ANGPTL4 gene. As matrix stiffness increased, the occupancy at DHS1 similarly increased.

4.1.4 ANGPTL4 deficiency attenuates EMT-augmented chemoresistance

ANGPTL4 has been recurrently highlighted in numerous studies for its role in highly aggressive oncogenic processes, including EMT, chemoresistance, anoikis resistance and metabolic reprogramming [110, 112, 191, 196, 197]. These ANGPTL4-mediated activities could empower cancer cells with metastatic capabilities. Notably, within the 3D architecture, ANGPTL4 emerges as a hub gene in the

transcriptomic landscape of cancer EMT, which is implicated in its involvement in mechano-signal transduction.

Consequently, we would like to investigate if ANGPTL4 is a potential anti-metastatic target. To simulate the biophysical environment encountered by cells in various body tissues for EMT induction tissues, we established two types of hydrogels, i.e., collagen-alginate and PEGDA-GelMA hydrogels, with tunable stiffness for 3D MKN74 cancer cell cultures. The collagen-alginate hydrogel comprises of interpenetrating network of alginate and type 1 collagen with matrix of different stiffness [198, 199]. Also, the PEGDA-GelMA hydrogel forms UV-inducible crosslinks with tunable stiffness of ~100-4000 Pa. The choice of stiffness were ~160Pa (denoted as 3D160) and ~1600Pa (3D1600) which corresponded to the stiffness of adipose tissues and liver, respectively [200]. Importantly, it also recapitulates the changes in biophysical properties of TME as the tumor progresses. Furthermore, by tuning the matrix stiffness to either 160 or 1600 Pa, we can also mimic the evolving matrix stiffness observed as tumors advance in their stages. Using the two hydrogels, we examined the EMT response of 3D MKN74 cancer cell cultures. After treatment with the EMT inducers, DMOG (mimics hypoxia) and TGF-β1, a higher expression level of Snai1 and ZEB-1 was detected in cancer cells in 3D1600, with concomitant downregulation of Erbb3, than cells in 3D160. Therefore, a more robust EMT was detected in a high stiffness matrix, compared to low stiffness matrix.

Next, we validated the expression profile ANGPTL4 in cancer cells undergoing EMT in 2D, 3D160 and 3D1600 conditions. The expression of ANGPTL4 was higher in 3D culture compared with 2D culture, which was further increased when stimulated with DMOG and TGF-β1. To ascertain a pivotal role for ANGPTL4 in 3D EMT, we blocked the function of ANGPTL4 using a neutralizing monoclonal antibody mAb11F6C4, which has previously been shown to neutralize the function of ANGPTL4 effectively [110, 112]. The co-treatment with mAb11F6C4 either abolished or diminished the changes in EMT-associated gene expression in 3D160 and 3D1600 compared with control. These observations from both the collagen-alginate and PEGDA-GelMA hydrogels consistently suggest that a stiffer TME elicits a more robust EMT response, which is attenuated by ANGPTL4 deficiency.

To understand the effect of matrix stiffness and ANGPTL4 on the well-recognized EMT-associated chemoresistance of cancer cells, we determined the mean IC50 of MKN74 for cisplatin and 5-fluorouracil (5FU), two common chemodrugs. A

higher IC50 for the two drugs was observed in cancer cells cultured in 3D compared with 2D culture. In addition, cells culture at 3D1600 were more resistant to the drugs than at 3D160. The IC50 of MKN74 further increased during 3D EMT, suggesting a greater EMT-associated enhanced chemoresistance at a stiffer matrix. Notably, immunoblocking of ANGPTL4 by mAb11F6C4 lowered the IC50 of MKN74 to chemodrugs in 3D160 and 3D1600 cultures. In summary, these observations suggest that ANGPTL4 deficiency reduces cell viability in cancer cell spheroids and attenuates EMT-augmented chemoresistance.

4.1.5 Matrix stiffness enhances EMT in vivo

In our in vivo study, MKN74 cells were combined with liquid PEGDA-GelMA (with LAP) and subcutaneously injected into mice before initiating UV-induced gelation. Our results revealed a ~4-fold increase in ANGPTL4 in MKN74-derived tumors with 3D1600 PEGDA-GelMA hydrogel compared to the 3D160 in vivo. These findings are in line with our in vitro results, as we observed elevated expression levels of EMT-associated transcription factors Snai1 and ZEB-1 in MKN74-derived tumors from the 3D1600 hydrogel. Additionally, we detected a concomitant downregulation of the epithelial Erbb3 gene compared to the 3D160 hydrogel in vivo. Zymography analysis revealed more MMP9 activity in tumor derived from 3D1600 than 3D160 hydrogels. Taken together, our data demonstrate that a stiffer matrix elicits more robust EMT response (**Figure 10**).

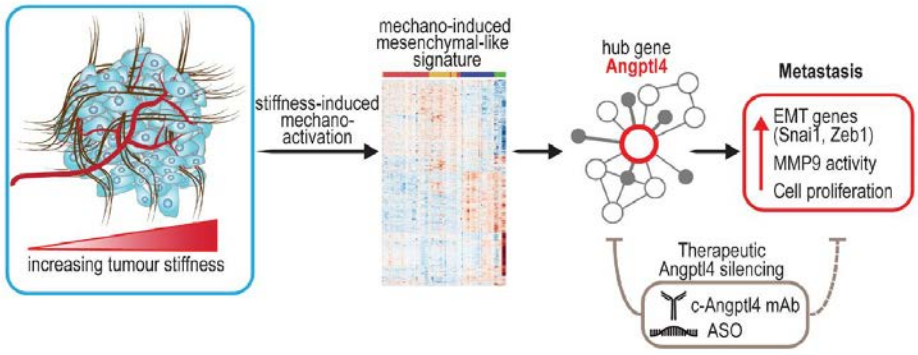

Figure 10: A schematic diagram illustrates the effect of TME stiffness in mechanoregulation of ANGPTL4, a hub gene within the 3D EMT gene signature. Our results revealed ANGPTL4 as a promising target to curtail cancer EMT in a 3D tumor architecture at physiological-relevant stiffness.

4.2 Paper II – Molecular dynamics of the recruitment of immunoreceptor signaling module DAP12 homodimer to lipid raft boundary regulated by PIP2

Immune evasion is a hallmark of cancer. T cells has been widely identified for their important roles in the adaptive immune response and the TME. However, less is known regarding the role of natural killer (NK) cells in the TME [201]. Furthermore, it was also widely studied that lipid rafts were involved in the T cell receptor signaling transduction but not in NK cells.

In this collaborative paper, we illustrate that phosphatidylinositol 4,5-bisphosphate (PIP2) lipids are positioned at the boundary of lipid rafts in our coarse-grained (CG) model of membrane organization (**Figure 11**) [202]. These negatively charged lipids attract DAP12 homodimers to the lipid raft boundary through interactions between the basic-rich areas and the signaling immunoreceptor tyrosine-based activation motifs (ITAMs) of DAP12 and PIP2. Moreover, our findings indicate that the interaction between proteins and lipids can be interrupted by the presence of Ca2+, which competes with DAP12 for binding to PIP2. Consequently, the cytoplasmic segment of the DAP12 homodimer separates from the membrane and returns to the nonraft region, exposing the ITAMs for subsequent downstream signaling. These discoveries offer essential insights into comprehending how signal transduction in NK cells is controlled by the microenvironment of the cell membrane.

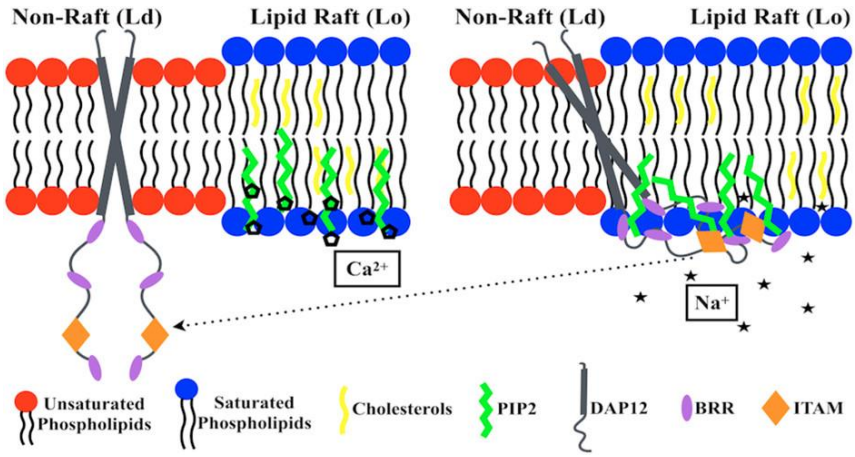

Figure 11: The graphical abstract of Paper II. PIP2 regulates the recruitment of immunoreceptor signaling module DAP12 homodimer to lipid raft boundary. Reprinted with permission from [202]. Copyright 2023, American Chemical Society.

4.3 Paper III – HTCA: a database with an in-depth characterization of the single-cell human transcriptome

Single-cell RNA-sequencing has become a widely utilized approach in recent years for analyzing individual cells in a population at the transcriptomic level. While attempts have been made to consolidate published single-cell data, a comprehensive characterization is still lacking.

Here, we introduce HTCA, an interactive database developed from over two million high-quality cells sourced from about 3000 single-cell RNA-seq samples and includes detailed profiles of some healthy adult tissues and their respective matching fetal tissues [203]. It serves as a platform for exploring transcription factor (TF) activities, TF motifs, gene signatures, enriched gene ontology terms, receptor-ligand interactions, and more across different cell types in fetal and adult tissues.

In addition to its pre-compiled data, HTCA includes user-friendly web-based analysis tools that offer adjustable parameters for a diverse range of interactive bioinformatics analyses. Moreover, it allows for the comparison of data from other experiments with its in-built datasets, enabling swift comparisons across multiple studies. Overall, HTCA serves as a comprehensive solution for quick and thorough analysis of multi-omics single-cell data encompassing diverse tissues and cell types.

For the central theme of this thesis, transcriptomic expression of ANGPTL4 across different organs and cell types in fetal and adult samples are presented using HTCA (**Figure 12**).

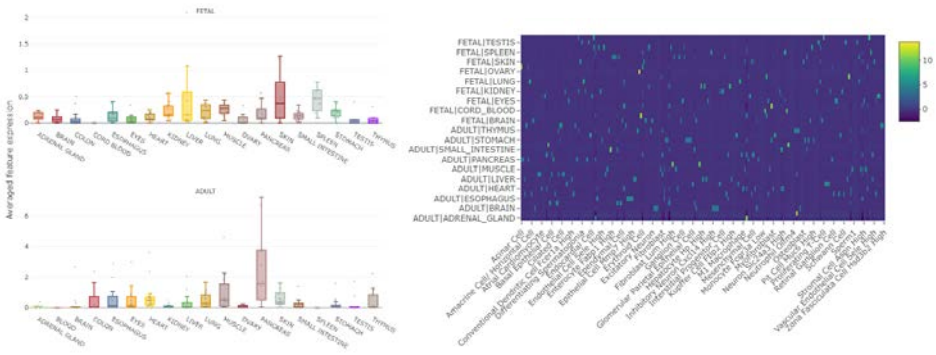

Figure 12: Transcriptomic expression of ANGPTL4 across different organs and cell types in fetal and adult samples presented using HTCA.

5 Discussion

5.1 Mechanosensitive gene signature related to metastasis

Many therapeutic strategies focused on the key biological regulators in EMT mechanisms, our study cemented the critical involvement of the not only the 3D biophysical TME of the cancer cells, but also the mechanical stress rendered by varying stiffness of the TME of the organs or tumor. This is highlighted in our meta-analysis of transcriptomes of cancer cells undergoing EMT across various 2D and 3D experiments, which revealed a distinct metastasis-related gene signature in 3D-EMT cultures. Notably, many genes in this 3D EMT gene signature exhibited a mechanosensitive expression profile, which may contribute to the ability of cancer cells to adapt to mechanical stresses, such as those present in the TME, and invade surrounding tissues. Indeed, 3D culture systems have been documented to induce cancer EMT, enhance stemness traits and promote chemoresistance in different cancer types [204-206]. These increased aggressive behaviors are, in part, mediated by mechanosensory such as ZEB1 and YAP1, activated via the interplay with ECM-binding integrins [207]. Furthermore, various secretory molecules like TGF-β1, ANGPTL4 and proinflammatory cytokines, known for their dual roles in mechano-signaling and EMT, are overexpressed in 3D cancer milieu [208]. It is also noteworthy that 3D EMT might manifest with a different set of biomarkers compared to the traditional 2D benchmarks, hinting at the intricacies of the third dimension in cellular behavior. In congruence with the meta-analysis, transcriptomes of cancer cells in collagen-alginate 3D EMT culture with varying stiffness revealed exacerbation of these distinct metastatic gene signatures in 3D culture models of higher stiffness. These data suggests that the varied stiffness of the organ-specific microenvironments is crucial in aggravating EMT signaling.

5.2 ANGPTL4 is an important hub gene

Several hub genes were identified within the 3D EMT gene signature which form a highly interconnected network, regulating key aspects of EMT and cancer progression. The top ranked hub genes include TGFB1, SERPINB2, LAMB3, ANGPTL4 and COL22A1. Transcriptome analysis of SerpinB2-deficient breast tumors showed that SerpinB2 deficiency delayed mammary tumor progression. SerpinB2 also regulates stromal remodeling and local invasion in pancreatic cancer [209, 210]. LAMB3 has been shown to mediates proliferative, invasive, and metastatic behavior of several cancer types, such as pancreatic and colorectal cancers [211,

212]. A direct role for COL22A1 in metastasis is unclear, however, it is involved in remodeling of the TME [213, 214]. TGF-β1, a well-established EMT inducer, has been shown to upregulate the expression of ANGPTL4, also a hub gene [191]. ANGPTL4 is involved in different processes in metastasis such as anoikis resistance, metabolic reprogramming and chemoresistance [110, 112, 197].

5.2.1 ANGPTL4 is a key player in cancer metastasis and growth

ANGPTL4 is a secreted protein that can undergo proteolytic cleavage to generate the N-terminal coiled-coil fragment (nANGPTL4) and the C-terminal fibrinogen-like domain (cANGPTL4) [215]. While nANGPTL4 is mainly responsible for the regulation of lipids, cANGPTL4 has been found to regulate cell migration by conferring anoikis resistance, promoting cancer growth and invasiveness and acting as a key player in fueling energy in cancer cells for EMT and metastasis [216-220].

The importance of ANGPTL4 in cancer metastasis and growth has been explored in many studies. Earlier work by Le Jan et al suggested that various perinecrotic tumors express ANGPTL4 under hypoxia, leading to the growth of nodules and vasculature [221]. Furthermore, the involvement of ANGPTL4 in metastasis can also be shown by its expression profile in tumors. Elevated expression of ANGPTL4 has been found across all epithelial tumors, and progression of these tumors correlates with ANGPTL4 expression [109]. Indeed, cANGPTL4 promotes metastasis to the lung by stimulating vascular leakiness within tumors [109].

Furthermore, a recent study on the proteolytically cleaved fragments of ANGPTL4 revealed that cANGPTL4 (C-terminal fragment) promotes cancer growth and metastasis, but nANGPTL4 (N-terminal fragment) prevents metastasis [222]. In our study, we focused on cANGPTL4 as a potential metastatic target. More mechanistic details on how cANGPTL4 is regulated and involved in metastasis will be discussed in the following sections.

According to our gene ontology analysis, 3D MES DEGs are mainly responsible for ECM remodeling, collagen metabolism, cellular motility, and cell–cell adhesion. All these characteristics of metastasis have been studied in ANGPTL4 in vitro. A major strength of our study is that we incorporated multiple cell lines in our analysis, and we found that ANGPTL4 is mechanosensitive, which is not cell specific. However, other than ANGPTL4, there are other hub genes that are also mechanosensitive. For example, TGF-β is mechanosensitive and has been found

to be involved in mechanobiological signaling in tissues and cells of the bone and cartilage [223].

5.2.2 ANGPTL4 is a key player in mechanosensing

We found that most 3D MES DEGs are mechanosensitive because our GSEA of the mechanotransduction gene set [190] revealed that mechanosignaling plays a pivotal role in the regulation of gene expression in 3D tumoroids and EMT but not in 2D culture. Previously, gene expression changes when cells cultured in 3D have been investigated, however little is known about their regulation. Furthermore, despite 3D tumoroid being a better model to mimic in vivo tumor biology, few studies investigated EMT in 3D and even less is known about how genes are regulated in 3D EMT, which may have major scientific and clinical impact.

In our results, we systematically revealed the 74 common genes implicated in both the 3D culture and EMT ("3D MES DEGs") using meta-analysis of 95 RNA-seq data from 14 cell lines in GEO. As our analysis suggests that most 3D MES DEGs are mechanosensitive, an important aspect is to identify key hub genes underlying the observed phenotype, i.e., 3D EMT and metastasis. We then performed a hub gene analysis of the PPI network and identified ANGPTL4 to be one of the top five hub genes based on the density of the maximum neighborhood component.

In addition, by interrogating the PRECOG, PrognoScan and TCGA database, we discovered that ANGPTL4 is the most clinically relevant hub gene, associated with poor clinical prognosis. In our 3D culture model, we recapitulate these findings that mechanotransduction of EMT signaling involved the increase expression of ANGPTL4. As 3D culture stiffness is increased, the expression of EMT biomarkers was further elevated, indicating that the physical properties of TME reshape the biological properties of the cancer cells. In particular, this effect aggravates EMT processes in tumor. Similarly, using a dynamic magneto-softening matrix, it was reported that matrix stiffness increases tumor malignancy, EMT and hypoxia. These malignant transformations could be halted or reversed with matrix softening [224]. Consistent with our clinical and transcriptomic analysis, targeting ANGPTL4 through ASO or mAb immunoneutralization successfully delayed EMT and suppressed tumor growth. The critical role of ANGPTL4 in the 3D TME and chemoresistance could potentially be a common trait in many cancer types as exemplified by our data from multiple cancer cell types including the gastric tubular adenocarcinoma (MKN74) and human bladder transitional cell carcinoma (UMUC3 and T24). This underscores the broader implications of targeting

ANGPTL4 as a potential treatment against cancer undergoing EMT beyond a singular cancer type. Therefore, ANGPTL4 is a potential target for antimetastatic therapy.

5.3 Regulation of ANGPTL4 expression

5.3.1 PPAR-mediated expression of ANGPTL4

Peroxisome proliferator-activated receptors (PPARs) are transcription factors in the nuclear hormone receptor superfamily [225]. There are three subtypes of PPARs, PPARα, PPARβ/δ and PPARγ, which can be activated by various ligands, such as free fatty acids and eicosanoids [226]. Although the three subtypes share significant homology, they have different biological functions and tissue distributions. PPARα regulates fatty acid catabolism and is elevated in tissues with elevated fatty acid oxidation, such as the heart, liver, skeletal muscle and brown adipose tissues [227]. PPARβ/δ is expressed in almost all tissues and is characterized by elevated lipid metabolism [228]. PPARγ plays an important role in regulating adipogenesis, glucose metabolism, fat storage and the expression of proinflammatory cytokines [225]. The expression of PPARγ is also elevated in white and brown adipose tissues.

When activated by a ligand, PPAR changes in conformation and translocates to the nucleus to form a heterodimer with the retinoid X receptor (RXR), another nuclear receptor [225]. The PPAR-RXR heterodimer then binds to a specific portion of the DNA, known as the PPRE, to regulate the expression of the target gene under various conditions [225, 227, 228]. Indeed, PPREs have been found in both the promoter and intron-3 regions of the ANGPTL4 gene (Figure 19) [229, 230]. For example, ANGPTL4 is a direct transcriptional target of PPARγ with a regulatory site located upstream of the ANGPTL4 transcription start site [229]. PPARγ-mediated expression of ANGPTL4 usually leads to cell proliferation, migration and angiogenesis in physiological and pathological processes [229].

5.3.2 TGFβ and hypoxia mediated expression of ANGPTL4

TGF-β is a cytokine that plays a pivotal role in various cellular functions, such as proliferation, differentiation, migration, apoptosis, and EMT [231]. While TGF-β1 was the hub gene with the highest connectivity in our PPI analysis, ANGPTL4 was the hub gene that was most clinically relevant. Indeed, both TGF-β1 and ANGPTL4 have been heavily studied in vitro and have been found to promote tumor invasiveness and metastasis [231]. Furthermore, high expression of ANGPTL4 defines patients

with poor prognosis in multiple types of cancer [232]. Interestingly, TGFβ also induces the expression of ANGPTL4 via the Smad signaling pathway [233]. Furthermore, under hypoxic conditions, HIF-1 upregulates the expression of ANGPTL4 to promote angiogenesis and metastasis. This further implies the major connectivity and importance of ANGPTL4 in cancer progression and metastasis.

5.3.3 YAP/TAZ regulation of ANGPTL4

In our study, we confirmed using quantitative ChIP that YAP/TAZ regulates the transcription of the ANGPTL4 gene via the promoter region (DHS1). YAP/TAZ-mediated ANGPTL4 expression in cells has also been suggested in earlier published works [177, 188]. In 2021, Cheng et al studied YAP-mediated ANGPTL4 expression in trophoblast cells and revealed that YAP activation was required for GPER-stimulated ANGPTL4 expression [177]. However, a direct causal relationship between YAP and ANGPTL4 was not established, as no experiment on the direct binding of YAP (or associated complexes) to the ANGPTL4 gene was performed. Therefore, there were no mechanistic details on how YAP/TAZ regulates ANGPTL4. It was also not known whether the YAP regulation of ANGPTL4 can be found in other types of cells.

In another earlier work on ovarian cancer cells, Yang et al found that ferroptosis can be promoted by TAZ in ovarian cancers by regulating ANGPTL4 and NOX, and hence, TAZ activation can be offered as a potential therapy for ovarian cancers [188]. In this study, TAZ (complex) was shown to bind to the promoter region and directly regulate the transcription of the ANGPTL4 gene in CAOV2 cells. However, there was no systematic study on whether YAP/TAZ binds to the intron-3 region of the ANGPTL4 gene, the other DHS site. The result from our study aligns with the results of this study that YAP/TAZ binds to the promoter region but not to intron-3 and regulates the transcription of the ANGPTL4 gene in MKN74 cells.

5.4 Limitations of study

Although our study aims to explore the role of ANGPTL4 in metastasis and offer an antimetastatic strategy, most of our work mainly focuses on EMT, which is only the initiation of metastasis in solid tumors. There are many other stages of metastasis that were not explored in detail during our in vivo study [12]. While it is realistically impossible to prognosticate when patients' tumors metastasize, there are methods that we can explore in vivo to precisely control (or accelerate) and study metastasis in the future. One example is to use a transgenic cancer cell line harboring a Snai1-ER transgene, which can provide direct initiation of EMT via 4

hydroxytamoxifen (4-OHT) in vivo and induce metastasis to the lungs in mice [234]. Additionally, a potential method to accelerate the stages of metastasis in vivo is to use a more aggressive cell line, such as the breast cancer cell line MDA-MB-231 [235]. Therefore, future studies should incorporate these in vivo methods to fully explore the potential of targeting ANGPTL4 during other stages of metastasis as an antimetastatic strategy.

6 Conclusions

In summary, we have identified 74 common genes implicated in both 3D culture and EMT, termed the 3D MES DEGs, via meta-analysis of 2D vs 3D culture RNA-seq data of various cancer cell types from the GEO database. By performing a PPI network analysis of the 3D MES DEGs and screening for clinical relevance of the top five hub genes in TCGA, PrognoScan and PRECOG, we showed that ANGPTL4 is the most clinically relevant hub gene. Indeed, we also found that cancer cells undergoing EMT have higher transcriptomic and protein expression levels of ANGPTL4.

Furthermore, we validated that ANGPTL4 is a good anti-metastatic target in vitro because treatments using an antibody against ANGPTL4 (mAb11F6C4) diminished the elevation of mesenchymal markers in cancer cells as well as reduced 3D spheroid formation. Similarly, treatment with an antibody against ANGPTL4 in in vivo orthotropic xenografting of noninvasive muscle bladder cancer and subcutaneous xenografting of MKN74 cells also reduced the growth of bladder tumor xenografts and MKN74 xenografts compared with the control, respectively. Mechanistically, we showed that ANGPTL4 expression is regulated by YAP/TAZ via the promoter region of the ANGPTL4 gene. Hence, ANGPTL4 deficiency can curb the growth of primary tumors and tumors undergoing EMT, fulfilling the criterion of an antimetastatic strategy.

Taken together, these findings reflect the dynamic control of the physical stiffness in tumor microenvironment in EMT signaling of cancer cells and the intricate involvement of ANGPTL4 as a key player. Our multi-facet analyses also indicates that ANGPTL4 is of high clinical importance with regards to advanced stage cancer, corroborating with our clinical understanding of tumors in patients [236-238]. In summary, our study demonstrated the effect of TME stiffness in mechanoregulation of ANGPTL4, a hub gene within the 3D EMT gene signature.

7 Points of perspective

Metastatic cancer continues to be the leading cause of mortality in cancer patients, primarily due to the lack of therapeutics targeting metastasis. In cancer, EMT has emerged as a critical mechanism key mechanism that promotes tumor progression, invasion, and metastasis. The cancer cells gain cellular plasticity during EMT, which facilitates their dissemination from the primary tumor, migration to other parts of the body, entry into the bloodstream or lymphatic system, and colonization at distant organs, leading to the formation of secondary tumors [239]. As a result, patients with tumor cells undergoing EMT manifest into increased tumor aggressiveness, decreased response to therapy, higher metastatic cancer, and cancer recurrence. Particularly, the presence of EMT markers in patients' primary and secondary tumors have been found to be associated with advanced cancers and severely declining clinical prognosis.

Despite the advancement of cancer therapeutics, very few therapeutics targeting EMT were developed and made it to the bedside [240]. Together with the high failure rate of treatments resulting in terminal metastatic cancer of patients, this highlights a major disconnect in the development anti-metastasis strategies. To effectively combat metastatic cancer, it is important to develop multi-pronged strategies that consider the changes in the TME, curb the growth of the primary tumor, and eradicate cancer cells undergoing EMT.

As a final point, the 3D culture systems are increasingly recognized as superior to traditional 2D cultures in replicating physiological and pathological cell behaviors. Critically, the physicochemical attributes of the 3D matrix, such as porosity, permeability, and viscoelasticity, can modulate cellular responses independent of biological factors. For instance, cancer cells can perceive the matrix pore size and rewire the mechano-signaling pathways to migrate out of their local environment [241-243]. Notably, recent research has spotlighted the role of matrix viscoelasticity - a characteristic inherent to many biological tissues - in governing cellular spheroid arrangement and tumor proliferation [244, 245]. The interplay between matrix stiffness and viscoelasticity shapes the proliferative capacity and motility of cells in 3D condition [245]. Thus, a deeper exploration into how various physicochemical properties of 3D matrices and their interactions impact cell behaviors is paramount. Bridging these gaps will provide a framework for refining 3D culture techniques.

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