Conversations in the tumor microenvironmentasion: experimental models of fibroblast driven cancer cell inv

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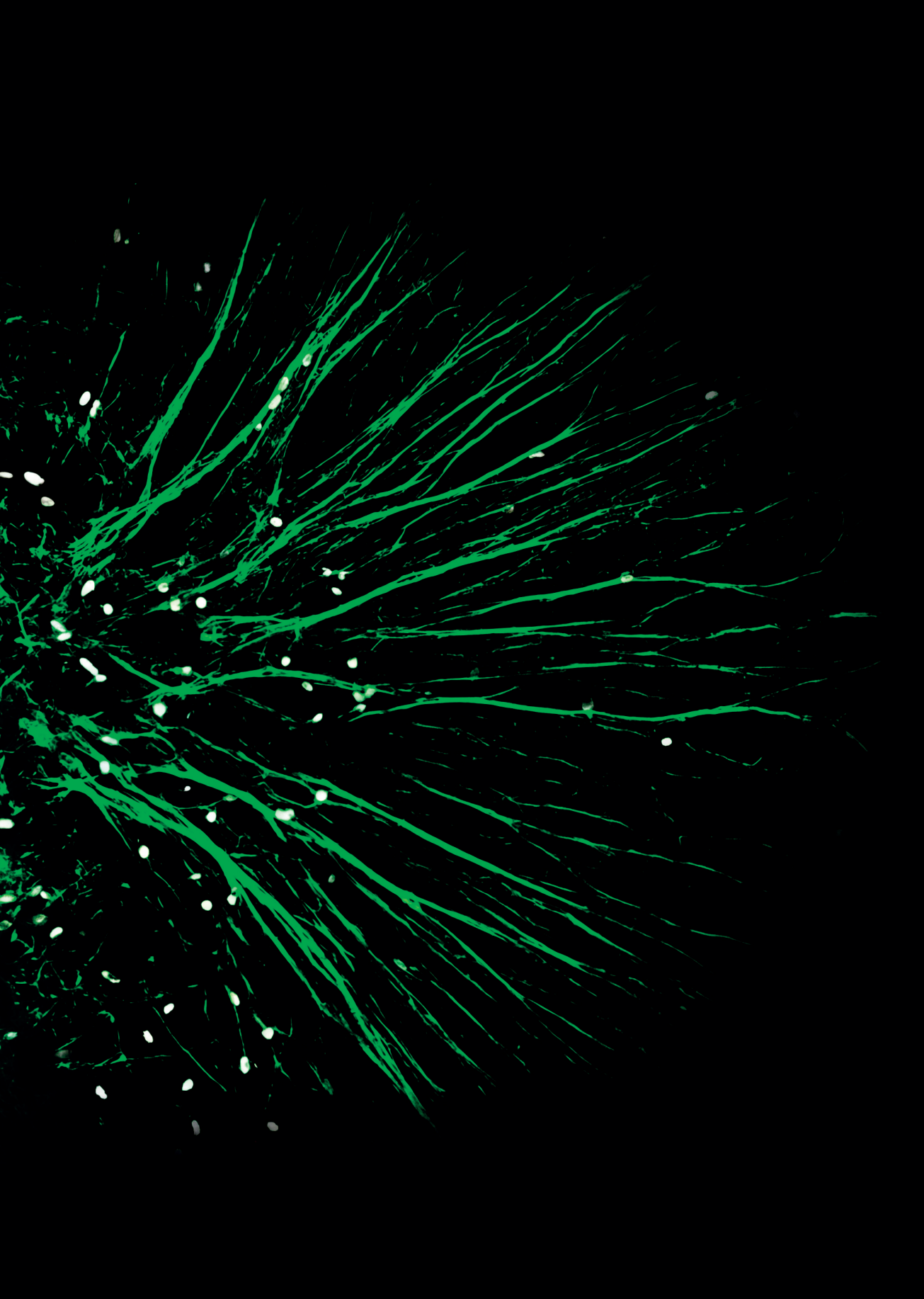

Chapter 1

General introduction

Cancer is a multifaceted disease that develops through the accumulation of genetic mutations and the influence of its surrounding tissue environment. As tumor cells proliferate, they reorganize and recruit neighboring stromal and immune cells, giving rise to a complex network referred to as the tumor microenvironment (TME) [1], this compartment surrounds and supports tumor cells throughout disease progression. The TME consists of fibroblasts, endothelial cells, immune cells, extracellular matrix (ECM) components, and soluble factors that together support tumor growth and enable invasive behavior culminating in metastasis [2], [3]. Rather than serving as a passive structural support, the TME evolves along with the tumor. Stromal cells respond to cancer cells-derived signals by altering matrix composition, releasing cytokines and growth factors, and modifying tissue mechanics[4], [5]. These reciprocal interactions create selective pressures that shape tumor cell behavior, promoting adaptation, invasion, and resistance to therapy. Thus, malignant progression emerges not only from genetic instability within cancer cells but also from continuous biochemical and mechanical communication with the surrounding stroma.

A central aim of modern cancer research is to decipher how this interplay between tumor and stroma drives invasion. Among the stromal constituents, fibroblasts, and particularly their activated forms known as cancer-associated fibroblasts (CAFs), have emerged as major regulators of tumor architecture and invasive behavior[6]. The following section introduces the organization and cellular diversity of the TME, providing the foundation for understanding CAF function within this evolving landscape.

The Tumor Microenvironment (TME)

After tumor initiation, cancer cells coexist within a continuously adapting TME that dictates every stage of disease progression. Far from a uniform scaffold, the TME is spatially and functionally heterogeneous, composed of diverse stromal, immune, and vascular elements whose coordinated activity governs tumor behavior[3]. The cellular and structural complexity of the TME is schematically illustrated in Figure 1, highlighting the close spatial organization of cancer cells with fibroblasts, immune populations, vasculature, and the surrounding extracellular matrix.

This heterogeneity arises through two interconnected processes: Intrinsically, genetic and epigenetic variability within tumor cells generates subpopulations with distinct phenotypes and invasive capacities[7]. Extrinsically, gradients in oxygen, nutrients, cytokines, and matrix stiffness impose selective pressures that further diversify both tumor and stromal compartments[8], [9]. The result is a mosaic of micro-niches, each supporting specific modes of proliferation or invasion.

Mechanical and biochemical factors cooperate to sustain this diversity. Variations in ECM composition, organization, and cross-linking regulate how cells attach, exert traction, and remodel their surroundings through integrin-mediated adhesion complexes and actomyosin contractility [5], [10]. Stromal cells, particularly fibroblasts and infiltrating immune populations, actively modify the ECM by depositing fibrillar proteins, secreting proteases, and reorganizing collagen alignment[4]. These processes generate mechanical anisotropy and directional guidance cues that influence collective invasion and metastatic dissemination[11].

Among stromal constituents, fibroblasts are especially influential. Activation of stromal fibroblasts into CAFs further increases microenvironmental heterogeneity through the production of matrix proteins, proteolytic enzymes, and cytokines that modify both biochemical composition and tissue mechanics [12]. Deciphering how such multi-scale heterogeneity emerges and how CAFs sustain it is central to understanding tumor invasion and metastasis. The next section focuses on CAFs, outlining their origins, phenotypic diversity, and dual roles as structural and signaling regulators within the TME.

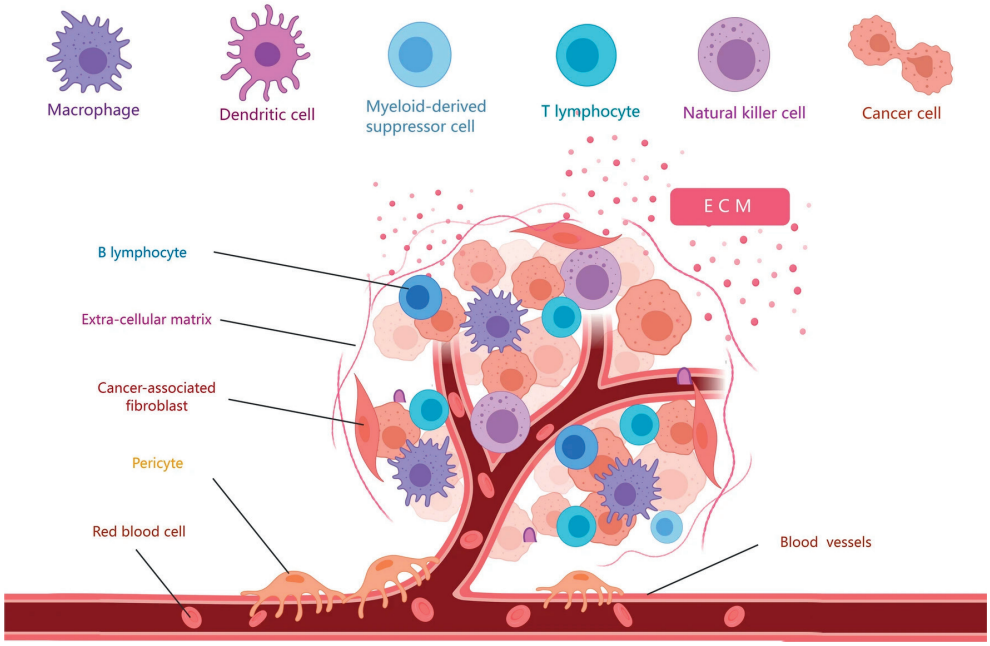

Figure 1. Cellular and structural components of the tumor microenvironment. *Illustration depicting stromal, immune, and vascular elements including macrophages, dendritic cells, myeloid-derived suppressor cells, T lymphocytes, natural killer cells, B cells, CAFs, pericytes, ECM components, and associated vasculature. These components collectively influence tumor growth, immune regulation, and invasion. Adapted from Springer Nature (doi: 10.1007/s12094-024-03697-w).*

CAFs in the TME

Sustained exposure to tumor-derived growth factors, inflammatory cytokines, and altered mechanical conditions drives the activation of stromal fibroblasts into CAFs, a heterogeneous and plastic population that supports tumor progression[6], [12]. CAFs arise from multiple sources such as resident fibroblasts, mesenchymal stromal cells, pericytes, and, in some contexts, epithelial or endothelial cells undergoing mesenchymal transition which later converge on an activated phenotype characterized by increased contractility, elevated ECM production, and a pro-secretory profile [13], [14].

Functionally, CAFs promote tumor progression through two principal routes. First, they remodel the ECM by depositing fibrillar collagens and fibronectin, aligning existing fibers, and generating contractile forces that increase local stiffness and create anisotropic tracks that facilitate directional invasion [10], [15]. CAFs also secrete a broad range of growth factors, chemokines, and proteases, including transforming growth factor beta (TGF-β), interleukin-6 (IL-6), C-X-C motif chemokine ligand 12 (CXCL12), and matrix metalloproteinases (MMPs), which promote tumor cell motility, sustain epithelial-to-mesenchymal transition programmes, and influence immune and endothelial cell behavior [16], [17]. These diverse mechanical and signaling functions position CAFs as central regulators of tumor-stromal communication, integrating inputs from cancer cells, immune populations, and the vasculature, as illustrated in Figure 2. These actions establish a reciprocal loop in which cancer cells maintain CAF activation, preserving a pro-invasive stromal niche.

Among the factors secreted by CAFs, TGF-β occupies a particularly central role. It functions both as a primary driver of fibroblast activation and as a potent inducer of epithelial-to-mesenchymal transition (EMT) in cancer cells, reprogramming epithelial cells toward a mesenchymal, invasive phenotype through canonical Smad-dependent signalling and a range of non-canonical pathways[18], [19]. Crucially, TGF-β signalling is dynamic and heterogeneous at the single-cell level, with individual cells within the same population responding differently depending on receptor expression, co-factor availability, and local matrix context[20]. This heterogeneity means that bulk-level measurements of TGF-β activity can mask the complexity of the underlying response, motivating the need for real-time, single-cell approaches to capture EMT as it unfolds.

Single-cell analyses reveal that CAFs segregate into functionally distinct subsets, commonly described as contractile, ECM-remodeling myofibroblastic CAFs and more secretory, cytokine-producing inflammatory CAFs, often occupying different spatial zones within tumors[16]. This division highlights that stromal regulation of

invasion is not uniform but distributed across CAF subtypes with complementary mechanical and signaling roles. Because many of these CAF-driven effects operate through modification of the ECM such as its composition, organization, and mechanical properties. As such, the ECM itself must be considered an active regulator of invasion. The next subsection therefore, examines ECM composition and mechanics in cancer.

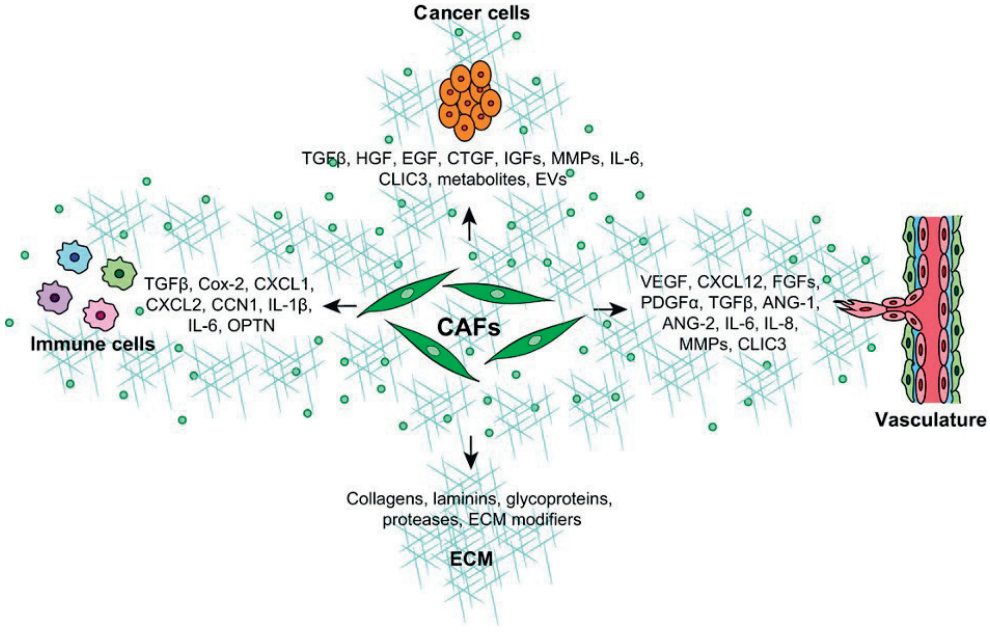

Figure 2. Crosstalk between cancer cells, CAFs, immune populations, vasculature, and the ECM. *CAFs act as central signaling nodes within the TME, producing ECM components such as collagens, laminins, and glycoproteins, as well as proteases, cytokines, and chemokines including TGF-β and CXCL12. Reciprocal interactions with cancer cells, immune infiltrates, and endothelial cells reinforce tumor-promoting signaling and regulate angiogenic responses. Adapted from Wiley Analytical Science (doi: 10.1002/pmic.201700167).*

The ECM in Cancer

The ECM forms the structural and biochemical scaffold within which all cellular components of the TME are embedded. In healthy tissues, ECM composition and organization are tightly regulated to maintain mechanical integrity and control cell behavior. During tumor progression, however, this homeostasis is lost. Cancer cells and stromal components, particularly CAFs, cooperatively remodel the ECM, generating a microenvironment that promotes invasion, angiogenesis, and therapeutic resistance [4], [5]. The progressive nature of these ECM alterations, spanning deposition, stiffening, and subsequent degradation, is schematically illustrated in Figure 3.

Tumor-associated ECM is primarily composed of fibrillar collagens (types I, III, and V), fibronectin, laminins, and proteoglycans such as hyaluronan and decorin. Enzymatic cross-linking mediated by proteins including lysyl oxidase (LOX) alters matrix stiffness and fiber organization [10], [21], leading to changes in cellular behavior.

Quantitative measurements have revealed that these mechanical changes are substantial and clinically meaningful. Normal mammary tissue exhibits stiffness values in the range of 0.1 kPa to 0.2 kPa, whereas tumor-associated stroma is about two orders of magnitude stiffer, with local regions of dense collagen crosslinking becoming most pronounced in late-stage disease [9], [22]. These changes are not passive consequences of tumor growth but are actively driven by CAFs, which generate contractile forces in the range of nanonewtons through actomyosin activity, upregulate collagen remodelling, bundling fibrillar collagen into aligned fibers, and upregulate collagen crosslinking to stiffen the surrounding matrix. Critically, this CAF-driven mechanical remodeling has direct clinical correlates: increased tumor stiffness detected by elastography imaging associates with higher grade, lymph node involvement, and poorer prognosis in breast and pancreatic cancers [23], [24]. The three stages illustrated in Figure 3 therefore reflect a progressive structural transformation of the ECM, spanning from an intact network, through CAF-driven stiffening and fiber alignment, to MMP-mediated degradation. This coordinated mechanical program culminates in the creation of invasion-permissive channels within the tumor stroma.

Increased ECM stiffness and fiber alignment also promotes integrin clustering, focal adhesion formation, enhanced actomyosin contractility, and directional migration [24], [25]. In parallel, degraded or porous regions of the matrix provide invasion channels that allow collective and single-cell dissemination[26], [27]. Remodeling of the ECM occurs through a combination of deposition, degradation, and reorganization. CAFs are key effectors of this process, continuously producing and aligning collagen fibers while secreting matrix metalloproteinases (MMPs) that locally degrade ECM to facilitate cell movement [15]. The resulting mechanical anisotropy creates guidance tracks that orient invasion and enable the transfer of forces across multicellular clusters. Tumor cells exploit these remodeling events to migrate collectively or individually depending on the matrix's viscoelastic and topological context [28].

ECM remodeling also regulates intracellular signaling. Matrix-associated ligands engage integrins, discoidin domain receptors (DDRs), and related adhesion receptors, activating downstream pathways such as focal adhesion kinase (FAK), SRC family kinases, and mitogen-activated protein kinase (MAPK) cascades that support invasive and survival phenotypes [4], [29]. In addition, the ECM functions as a reservoir for growth factors including TGF-β, vascular endothelial growth factor (VEGF), and epidermal growth factor

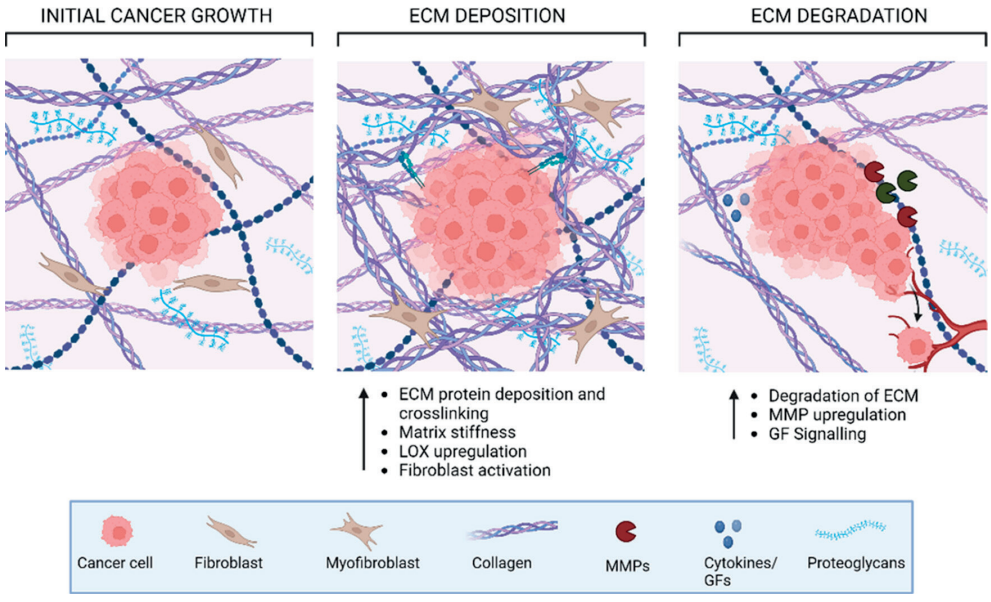

Figure 3. ECM remodeling during tumor progression. *Schematic illustrating the dynamic changes in the extracellular matrix (ECM) accompanying tumor development. Left: initial tumor growth occurs within an intact ECM network. Middle: ECM deposition phase, characterized by increased collagen production, crosslinking, matrix stiffening, lysyl oxidase (LOX) activity, and fibroblast activation. Right: ECM degradation phase, marked by elevated matrix metalloproteinase (MMP) activity, proteolysis, and enhanced growth-factor signaling that facilitates invasion. Adapted from ACS Omega (doi: 10.1021/acsomega.4c04442).*

(EGF), which are sequestered and released in a context-dependent manner, thereby shaping paracrine communication between tumor and stromal cells [30].

Together, these structural and biochemical alterations transform the ECM from a passive scaffold into an active participant in tumor progression. Its constant remodeling generates mechanical heterogeneity and directional cues that integrate with soluble signaling to regulate invasion. Understanding these interactions requires experimental systems that capture both spatial organization and dynamic remodeling in a physiologically relevant manner. The next section therefore discusses 2D and 3D model systems, their comparative advantages, and how 3D models provide a controlled yet realistic platform to study tumor–stroma interactions.

In Vitro Experimental Models

Experimental cancer models have evolved to better capture the spatial, mechanical, and biochemical complexity of the TME. While traditional two-dimensional culture systems

have been instrumental in defining core cellular processes, they lack the architectural and dynamic features of solid tumors. Three-dimensional culture models address these limitations by preserving cell-cell and cell-matrix interactions, enabling mechanistic studies that more closely reflect in vivo behavior [31], [32].
In 2D monolayers, cells grow on rigid plastic substrates with uniform access to nutrients and oxygen. This environment fails to reproduce the gradient-driven conditions, variable stiffness, and mechanical confinement experienced by cells in tissue. As a result, 2D cultures often display altered polarity, cytoskeletal organization, and gene expression, leading to differences in proliferation, migration, and drug response compared with their in vivo counterparts [33]. Despite these limitations, 2D systems remain valuable for high-throughput screening and reductionist analyses of isolated molecular pathways.

The transition to 3D culture systems marked a paradigm shift in tumor biology. In 3D matrices or multicellular spheroids, cells experience spatial confinement, differential oxygenation, and integrin engagement on all surfaces, conditions that induce more physiologically relevant behaviors [34]. Such systems recapitulate key aspects of tumor architecture, including the formation of proliferative outer layers, quiescent interiors, and necrotic cores. Importantly, 3D models allow the study of invasion in response to matrix stiffness, topography, and fiber alignment; factors that are absent or oversimplified in 2D.

A major advantage of 3D cultures lies in their tunability. By embedding tumor cells within natural matrices such as type I collagen, Matrigel, or fibrin, or within synthetic hydrogels like gelatin methacryloyl (GelMA), researchers can modulate matrix composition, crosslinking, and degradation kinetics to mimic tissue-specific mechanics [35], [36]. Co-culture systems incorporating fibroblasts, immune cells, or endothelial cells further enable reconstruction of multicellular interactions within a controlled environment. For example, the introduction of fibroblasts into 3D matrices has elucidated how stromal contractility and matrix remodeling guide collective invasion [37], [38], [39]. Recent developments have integrated microfluidic and organ-on-chip platforms with 3D cultures to reproduce spatial gradients and dynamic fluid flow, offering greater control over nutrient diffusion, shear stress, and mechanical loading[40]. These platforms bridge the gap between static in vitro models and complex in vivo systems, permitting live imaging of invasion, metastasis, and drug response under physiologically relevant conditions.

While animal models remain valuable for studying systemic aspects of tumor biology, this thesis deliberately employs in vitro systems to address questions that require precise mechanistic control. In vivo models introduce confounding variables such as immune activity, hormonal milieu, and systemic metabolism, which make it difficult to isolate the contribution of individual stromal or matrix components to invasion [34]. Real-time imaging of invasion dynamics at single-cell resolution remains technically constrained in living

animals, and inter-animal biological variability introduces noise that can obscure subtle mechanical or signaling effects [41]. The 3D in vitro and microfluidic systems used in this work were therefore chosen not as surrogates for the whole organism, but as purpose-built platforms for dissecting specific CAF-ECM-tumour cell interactions with the spatial and temporal resolution that in vivo models cannot readily provide.

Thus, 3D and micro-engineered model systems have become indispensable tools for dissecting tumor–stroma interactions. They retain experimental control while approximating the mechanical and spatial properties of living tissue. *Several questions nonetheless remain unresolved: how the physical positioning of CAFs relative to tumor cells determines the onset and directionality of invasion; how CAF-driven matrix remodeling translates mechanical anisotropy into directional guidance cues; how TGF-β reprograms individual cancer cells toward invasive phenotypes and whether this can be captured dynamically at single-cell resolution; and whether fibroblast-derived paracrine signals can drive invasion independently of detectable ligand activity.* The next section builds on this foundation to outline the research questions and scope of this thesis, which employs 3D models to investigate how spatial proximity, matrix remodeling, and paracrine signaling collectively regulate cancer cell invasion.

Thesis Overview and Scope of Investigation

This thesis investigates how mechanical and signaling interactions between tumor cells and the stroma coordinate cancer cell invasion. Building upon the biological principles outlined in the preceding sections, the work employs 3D model systems to dissect the influence of cancer-associated fibroblasts (CAFs), extracellular matrix (ECM) remodeling, and paracrine signaling on tumor progression. The overarching aim is to integrate spatial, mechanical, and biochemical perspectives to better understand how stromal context drives invasive behavior.

Research Questions

The following research questions guide this thesis:

Q1: How does the spatial proximity between CAFs and cancer spheroids influence the initiation, organization, and collective dynamics of cancer cell invasion within 3D matrices?

Q2: How does CAF-mediated matrix remodeling alter ECM architecture and mechanical anisotropy to direct collective invasion?

Q3: How can real-time imaging and quantitative analysis of TGF-β–induced epithelial-to-mesenchymal transition (EMT) capture the dynamic and heterogeneous nature of this process at the single-cell level?

Q4: How do fibroblast-derived soluble factors modulate cancer cell dissemination through receptor-mediated pathways, and can these effects occur independently of measurable ligand presence?

Collectively, these questions address the spatial and temporal complexity of tumor–stroma interactions and identify potential regulatory mechanisms linking physical proximity, biochemical signaling, and matrix mechanics.

Chapter 2 – CAF-Mediated Cancer cell Invasion in 3D Collagen Matrices

This chapter investigates how inter-spheroid proximity and CAF mediated ECM remodeling coordinate the initiation and directionality of collective invasion in 3D collagen systems. Using dual-spheroid assays combining tumor and CAF spheroids at controlled distances, the study reveals how fibroblast contractility and matrix alignment define invasion trajectories. These findings establish a mechanical framework linking stromal positioning and collagen anisotropy to cancer cell guidance and cooperative motility.

Chapter 3 – Visualising Dynamic Changes During TGF-β-Induced EMT

This chapter introduces a methodological platform for capturing the temporal dynamics of EMT in live cells. Using fluorescence-based imaging and quantitative morphometric analysis, it delineates morphological transitions, signaling kinetics, and cell-state heterogeneity during TGF-β stimulation. The resulting framework provides the analytical foundation to visualise and interpret dynamic processes underlying epithelial plasticity, which is key to understanding how signaling networks reprogramme cells for invasion.

Chapter 4 – Paracrine Signaling Between Fibroblasts and Lung Cancer Spheroids

This chapter explores the effects of secreted factors from fibroblasts on the invasion of cells from lung carcinoma spheroids embedded in GelMA matrix. By stimulating A549 spheroids with fibroblast-conditioned medium in GelMA hydrogels, we demonstrated that soluble factors can activate receptor-level signaling and induce dissemination even in the absence of detectable TGF-β ligand. These results highlight the complexity of stromal communication and suggest a cooperation between fibroblast-derived TGF-b and other secreted factors in inducing lung cancer cell invasion.

Chapter 5 – Microfluidic and Engineered Models of 3D Cancer Cell Migration (Review)

This review synthesises advances in microfluidic technologies for studying 3D cancer cell migration and stromal interactions. It discusses how micro-engineered systems can replicate spatial gradients, matrix confinement, and dynamic perfusion, enabling precise control of mechanical and biochemical cues. The chapter situates emerging organ-on-chip models within the broader context of mechanobiology and tumor ecology, outlining their potential to refine in vitro modelling of metastasis.

Together, these chapters provide a spatio-temporal framework for understanding how CAFs orchestrate tumor invasion through coordinated physical and molecular mechanisms. By integrating 3D modelling, live-cell imaging, and signaling analysis, this thesis aims to refine our mechanistic understanding of the tumor–stroma interface and identify potential avenues for therapeutic intervention.

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