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**Conversations in the tumor microenvironmentasion:
experimental models of fibroblast driven cancer cell inv**
Mehta; P.P.

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Conversations in the tumor microenvironment:

Experimental models of fibroblast
driven cancer cell invasion

Pranav Mehta

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Conversations in the tumor microenvironment: Experimental models of fibroblast driven cancer cell invasion

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Promotores:

Prof. Dr. Ir. P. ten Dijke

Dr. P. E. Boukany (Delft University of Technology, the Netherlands)

Leden promotiecommissie:

Prof. Dr. M. J. Goumans

Prof. Dr. E. Danen

Prof. Dr. J. Prakash (University Medical Center Radboud, the Netherlands)

Dr. A. Rwei (Delft University of Technology, the Netherlands)

Dr. M. Barnhoorn

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Dedication

*For my parents, who asked for a doctor and got one,
eventually, in the most inconvenient way possible.*

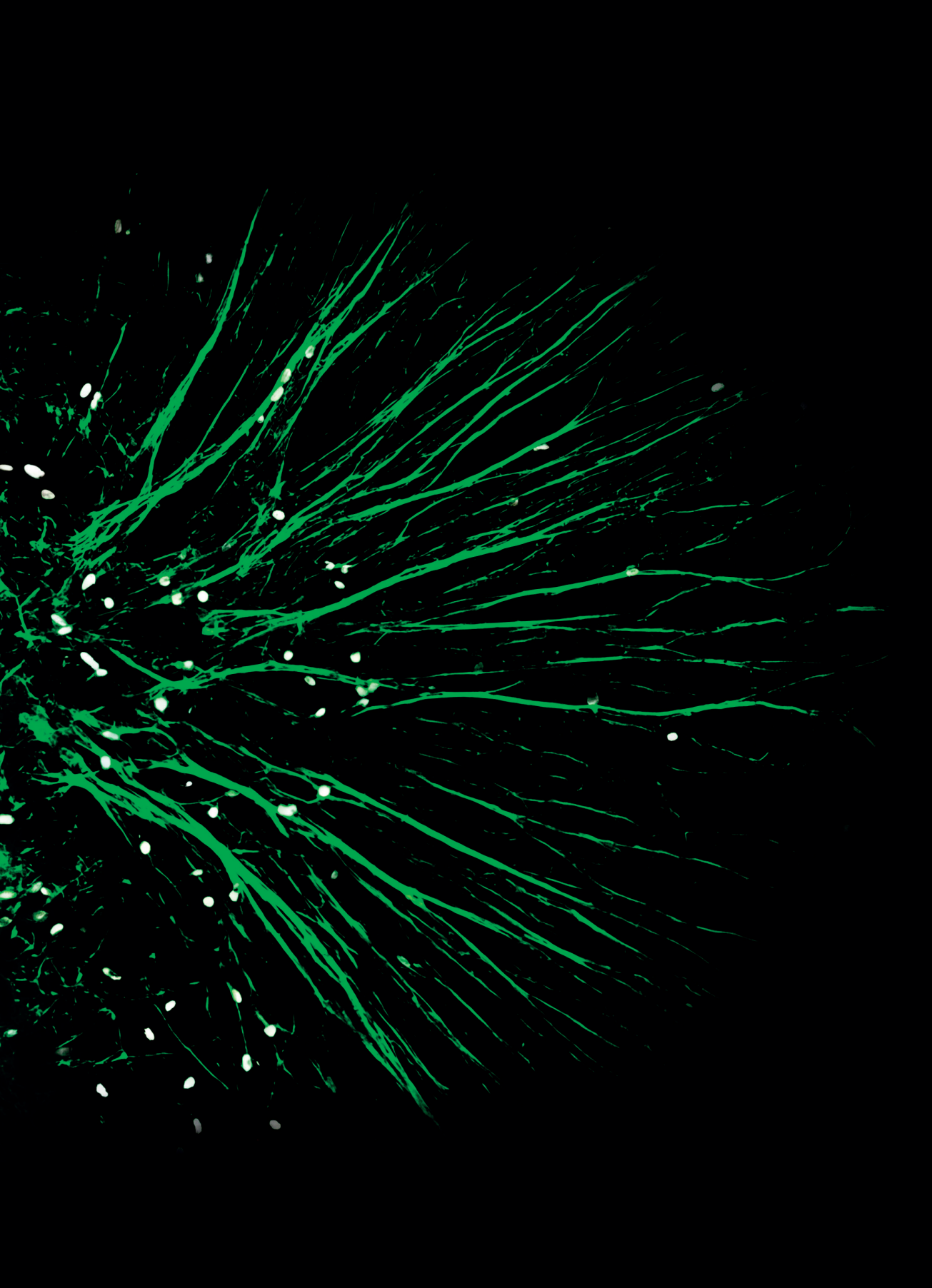
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List of Abbreviations

Abbreviation	Full term
2D	Two-dimensional
3D	Three-dimensional
ALK	Activin-like kinase
α -SMA	Alpha-smooth muscle actin
APCAF	Antigen-presenting cancer-associated fibroblast ATCC American Type Culture Collection
BB94	Batimastat
BLEB	Blebbistatin
CAF	Cancer-associated fibroblast
CAV	Caveolin
CM	Conditioned medium
CXCL-12	C-X-C motif chemokine ligand 12 DDR Discoidin domain receptor
E/M	Epithelial / mesenchymal
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EMP	Epithelial–mesenchymal plasticity
EMT	Epithelial–mesenchymal transition
FAK	Focal adhesion kinase
FITC	Fluorescein isothiocyanate
Gal	β -galactosidase
GelMA	Gelatin methacryloyl
GFP	Green fluorescent protein
H&E	Hematoxylin and eosin Heterospheroids Heterogenous spheroids
hTERT	Human telomerase reverse transcriptase iCAF Inflammatory cancer-associated fibroblast
IFF	Interstitial fluid flow
IFP	Interstitial fluid pressure
IGF	Insulin-like growth factor
IL-6	Interleukin-6
IP	Pixel Intensity
LOX	Lysyl oxidase
MAPK	Mitogen-activated protein kinase
MAT	Mesenchymal–amoeboidal transition

Abbreviation	Full term
MET	Mesenchymal–epithelial transition
MMP	Matrix metalloproteinase
myCAF	Myofibroblastic cancer-associated fibroblast NK cells
	Natural killer cells
NSCLC	Non-small cell lung cancer
PCK	Pan-cytokeratin
PEI	Polyethylenimine
PI3K/AKT	Phosphoinositide 3-kinase / AKT signaling pathway PSR
	Picrosirius red
RFP	Red fluorescent protein
ROCK	Rho-associated coiled-coil containing protein kinase TAMs
	Tumor-associated macrophages
TBST	Tris-buffered saline with Tween-20 TEMTIA
	The EMT International Association TFs
	Transcription factors
TGF- β	Transforming growth factor beta
TME	Tumor microenvironment
TPMT	Transmitted photomultiplier tube TRITC
	Tetramethylrhodamine isothiocyanate
TRPM7	Transient receptor potential melastatin 7 ULA
	Ultra-low attachment
VEGF	Vascular endothelial growth factor
VF	Void fraction
VIM	Vimentin
Y27	Y-27632
ZO	Zonula occludens



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.

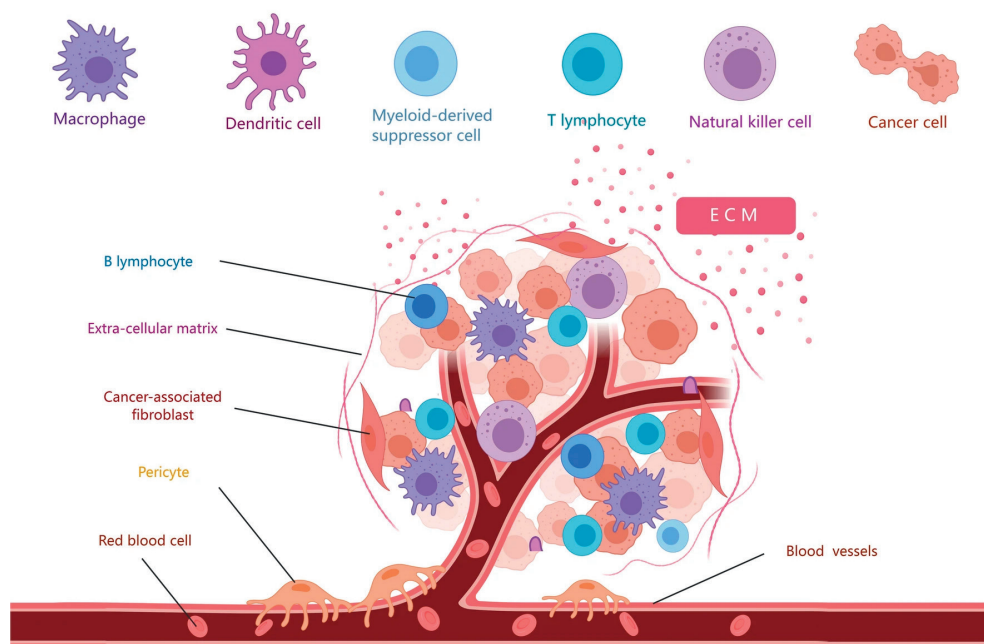


Figure 1. Cellular and structural components of the tumor microenvironment. Illustration depicting stromal, immune, and vascular elements including macrophages, dendritic cells, myeloid-derived suppressor cells, T lymphocytes, natural killer cells, B cells, CAFs, pericytes, ECM components, and associated 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.

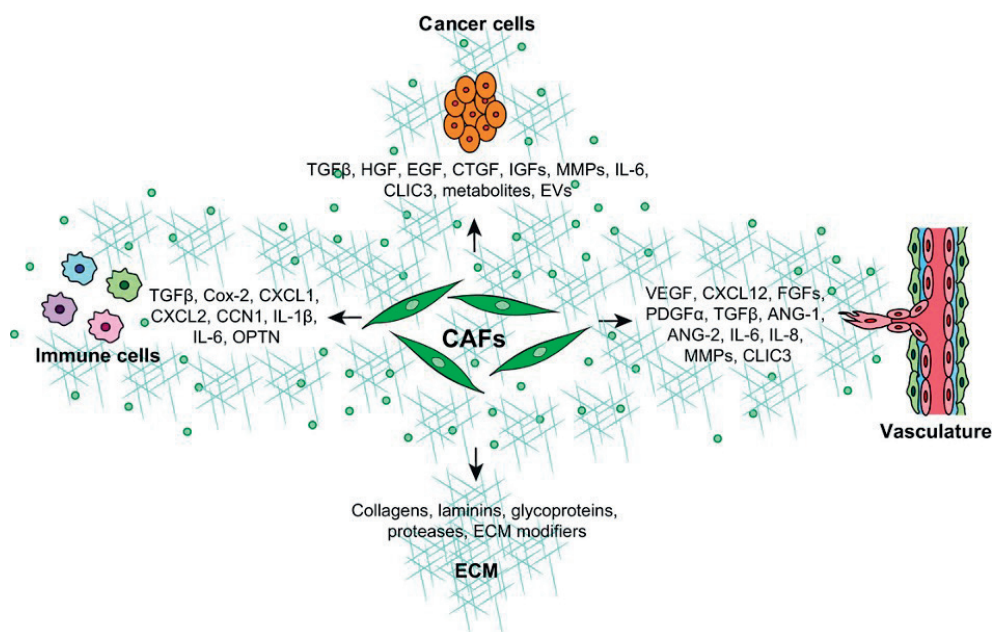


Figure 2. Crosstalk between cancer cells, CAFs, immune populations, vasculature, and the ECM. CAFs act as central signaling nodes within the TME, producing ECM components such as collagens, laminins, and glycoproteins, as well as proteases, cytokines, and chemokines including TGF- β and CXCL12. Reciprocal 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 (DDR), 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

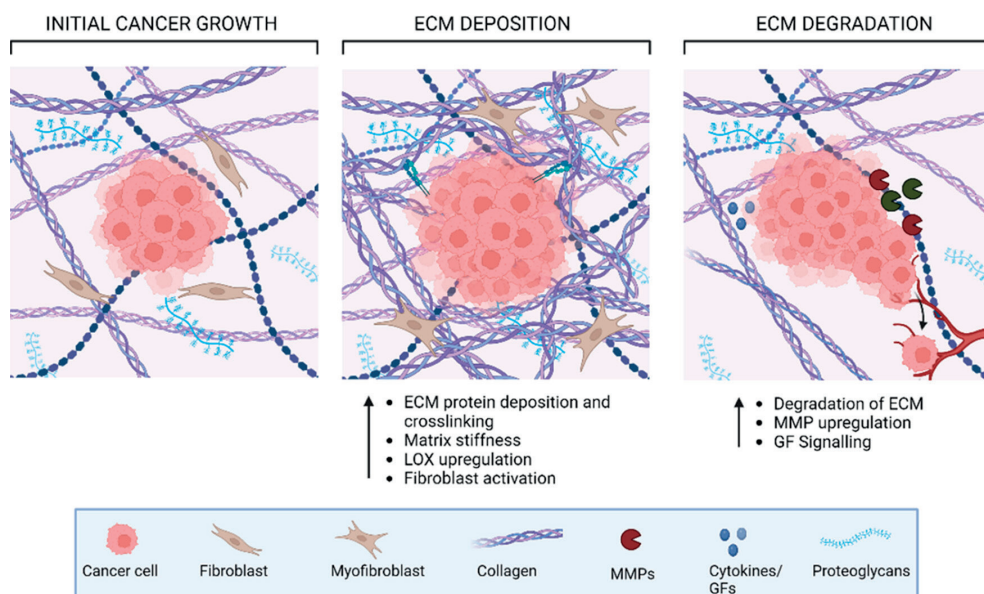


Figure 3. ECM remodeling during tumor progression. Schematic illustrating the dynamic changes in the extracellular matrix (ECM) accompanying tumor development. Left: initial tumor growth occurs within an intact ECM network. Middle: ECM deposition phase, characterized by increased collagen production, 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- β 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.

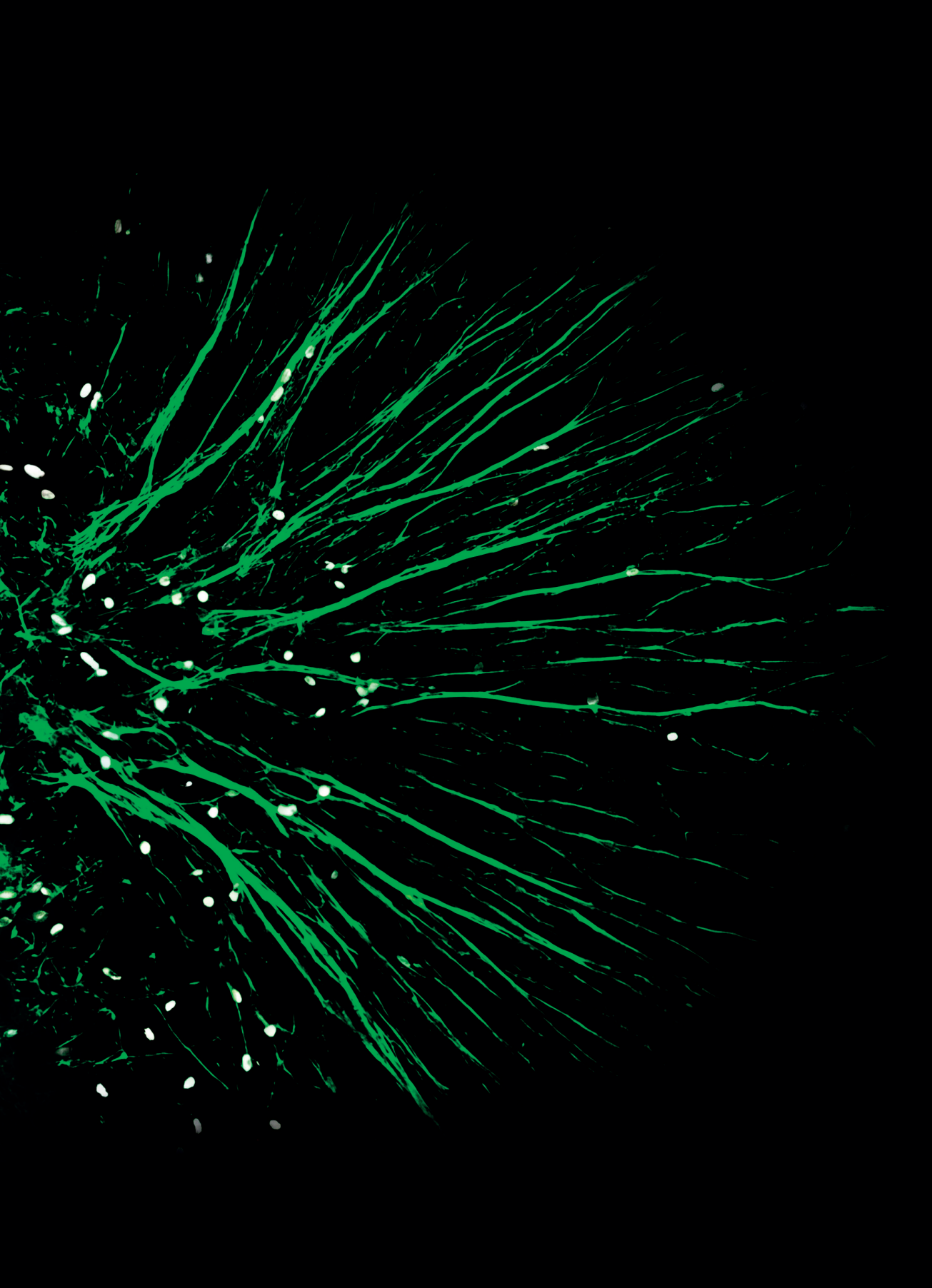
Together, these chapters provide a spatio-temporal framework for understanding how CAFs orchestrate tumor invasion through coordinated physical and molecular mechanisms. By integrating 3D modelling, live-cell imaging, and signaling analysis, this thesis aims to refine our mechanistic understanding of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

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Chapter 2

Inter-Spheroid Proximity and Matrix Remodeling Determine Cancer Associated Fibroblast Mediated Cancer Cell Invasion

Pranav Mehta^{1,2}, Ankur Deep Bordoloi^{2,5,†}, Cor Ravensbergen³, Ma. Kristen H. David², Wilma Mesker⁴, Gerrit Jan Liefers⁴, Peter ten Dijke^{1,†}, Pouyan E. Boukany^{2,†}

¹Department of Cell and Chemical Biology and Oncode Institute, Leiden University Medical Center, Leiden, The Netherlands

²Department of Chemical Engineering, Delft University of Technology, Van der Maasweg 9, 2629 HZ, Delft, The Netherlands

³Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands

⁴Department of Surgery, Section Surgical Oncology, Leiden University Medical Center, Leiden, the Netherlands

⁵Aix-Marseille Université, CNRS, Centrale Méditerranée, IRPHE, UMR 7342, 13384 Marseille, France

[†]Corresponding authors,

Email address: ankur-deep.bordoloi@univ-amu.fr (Ankur Deep Bordoloi);
p.ten_dijke@lumc.nl (Peter ten Dijke); p.e.boukany@tudelft.nl (Pouyan Boukany).

Abstract

Breast cancer is the most commonly diagnosed malignancy worldwide, with molecular subtypes following distinct clinical trajectories. While Luminal A breast cancers are typically indolent, a subset enriched in α -smooth muscle actin (α -SMA)-positive cancer-associated fibroblasts (CAFs) exhibits aggressive behavior, facilitating tumor invasion. However, the biophysical mechanisms by which CAFs drive invasion and extracellular matrix (ECM) remodeling remain unclear. In addition, the temporal and spatial dynamics of CAF interactions with the collagen matrix and cancer cell spheroids remain unknown, raising the question of whether these processes follow a deterministic sequence or occur stochastically. To address this, we conducted histological analysis of Luminal A tumors, which revealed variation in CAF, cancer cell, and ECM organization at tumor boundaries. To assess the impact of CAF on cancer cell invasion, we use a 3D *in-vitro* model co-embedding 19TT breast CAF and MCF7 luminal breast cancer spheroids within a three-dimensional (3D) collagen-I hydrogel and performed time-lapse imaging. We demonstrate that inter-spheroid distance critically determines 19TT CAF-induced MCF7 spheroid behavior. Moreover, we showed that CAF-mediated collagen matrix remodeling and degradation precedes observed MCF7 spheroid disruption and is critical in promoting cancer cell spheroid expansion and cell dissemination. While broad-spectrum matrix metalloproteinase inhibition suppressed CAF-driven collagen degradation and MCF7 spheroid expansion, it did not prevent ECM remodeling, CAF migration, or single-cell dissemination of cancer cell spheroids. Furthermore, a complementary heterospheroid model revealed similar ECM remodeling and invasion dynamics despite altered cellular arrangement of cancer cells and CAFs. Our findings enhance our understanding of the relationship between CAF activity and collagen matrix remodeling processes that promote cancer cell invasion, providing insights into the potential therapeutic benefits of targeting CAFs in breast cancer treatment.

Keywords: breast cancer, Cancer-Associated Fibroblasts (CAFs), collagen, extracellular matrix (ECM), matrix remodeling, cancer cell invasion, heterogeneous spheroids

Introduction

Breast cancer is one of the most commonly diagnosed cancers worldwide and comprises distinct molecular subtypes characterized by specific biomarkers and clinical features [1]. These subtypes arise from complex intra- and inter-cellular interactions driven by genetic mutations, epigenetic modifications, and dynamic crosstalk between cancer cells and their heterogeneous tumor microenvironment (TME) [2]. It has been established that Luminal A breast cancer - the most prevalent subtype - generally follows a more indolent course and has a favorable prognosis. However, a subset of Luminal A tumors associated with α -smooth muscle actin (α -SMA)-positive cancer-associated fibroblasts (CAFs) in the TME exhibits significantly aggressive behavior, leading to distant metastasis and relapse [3].

Within the TME, CAFs, along with other cellular components such as endothelial cells, immune cells, and the extracellular matrix (ECM), collectively constitute the tumor stroma. It has been shown that the stromal-to-tumor ratio serves as an independent prognostic indicator, with a higher stromal fraction correlating with increased tumor aggressiveness and poorer patient outcomes [4-6]. However, substantial variability across studies highlights the complexity of the stromal contributions to tumor progression [4-9]. These discrepancies likely stem from the dynamic and reciprocal interactions between cancer cells and their stromal counterparts, leading to extensive reorganization of cellular composition and ECM architecture during tumorigenesis [10,11]. Consequently, these processes create a heterogeneous TME characterized by spatially distinct features, underscoring the need for a more refined analytical framework to accurately assess the stromal contributions to disease progression. The inherent heterogeneity of the TME results in significant phenotypic diversity among cancer cells and CAFs, leading to unique functional signatures and cellular characteristics that vary across different regions of the TME [12].

In Luminal breast cancers, CAFs actively promote tumor progression by reprogramming cancer cells, which enhances therapeutic resistance, disrupts cancer cell metabolism, and increases cancer cell motility [13]. Direct physical interactions between CAFs and cancer cells further sustain these changes [13,14]. CAFs also exert considerable regulatory effects on the collagen ECM through mechanisms that modulate collagen cross-linking, deposition, and degradation [15]. Through secretion of matrix metalloproteinases (MMPs) and contractility-driven fiber realignment, CAFs both degrade and remodel the collagen ECM [16-20]. This biomechanical remodeling leads to the aggregation of thin collagen fibrils into thicker, aligned bundles - often termed "collagen highways" - which serve to facilitate and guide cancer cell migration and invasion [21].

As the TME undergoes temporal evolution, its ECM is gradually remodeled into a tumor-facilitating matrix due to the biophysical and biological activities of CAFs and cancer cells. These cells can modulate the biophysical and biochemical properties of the collagen ECM, leading to increased matrix stiffness, which is a notable characteristic of breast malignancy [22,23]. *In-vitro* models support these findings, showing that aligned collagen fibers increase matrix stiffness and enhance tumor cell migration efficiency by promoting directional persistence [24]. Together, these structural and mechanical changes promote tumor progression and therapeutic resistance [25]. Yet, the role of CAFs in indolent breast cancers, characterized by non-migratory cancer cells, and the mechanisms that may precipitate invasion and eventual metastasis in these settings remain unresolved. Specifically, the effects of CAF-induced matrix remodeling, along with its reciprocal influence on CAF-cancer cell interactions in indolent breast cancer, are not well defined. Recent advances in breast cancer research have emphasized that local ECM architecture at the TME boundary, along with the spatial organization of cancer cells and CAFs, significantly affects patient outcomes [26-30]. To address this, we studied the behavior of luminal breast cancer spheroids in collagen matrix and ECM remodeling in the presence of breast cancer CAF spheroids. To mimic the heterogeneous architecture of breast tumors, we employed both dual-spheroid and heterospheroid assays. The dual-spheroid system captured the spatial compartmentalization often observed in breast cancers, where CAF-rich stromal regions are partially or fully separated from epithelial tumor clusters [31,32]. By varying inter-spheroid distance, this system allows us to probe how stromal-epithelial proximity regulates invasion dynamics. Complementarily, we also considered a heterospheroid-model emulating intermixed epithelial-stromal regions, common in CAF-rich tumors. Our experimental findings enhance the understanding of the relationship between CAF activity and collagen matrix remodeling processes that promote cancer cell invasion, providing insights into the potential therapeutic benefits of targeting CAFs in breast cancer treatment.

Materials & Methods

Cell culture and reagents

The human Luminal A epithelial breast cancer cell line MCF7 was purchased from American Type Culture Collection (ATCC). The human telomerase reverse transcriptase (hTERT)-immortalized breast 19TT CAFs have been previously described [33]. These cells were originally derived from invasive ductal carcinoma tissue and have been reported to constitutively express α -SMA in culture, representing a stable and pre-activated CAF population [33,34]. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), High Glucose (Sigma) containing 4.5 g/L glucose, l-glutamine but without sodium pyruvate and supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% antibiotic-

antimycotic solution (Gibco). Cells were frequently tested for the absence of mycoplasma and checked for authenticity by short tandem repeat (STR) profiling.

Conditioned medium experiments

To generate conditioned medium (CM), 2×10^5 19TT-CAF cells were seeded per well in a 12-well plate and cultured in 1.5 mL of complete DMEM (composition as described in Section 2.1). After 48 hours, the supernatant was carefully collected and filtered through a 0.22 μm Millex™ PVDF syringe filter (Sigma-Aldrich) and stored at 4°C until further use. For stimulation experiments, MCF7 spheroids were embedded in 3 mg/mL collagen gels and treated with 200 μL of 19TT CAF CM. Control spheroids were cultured in standard medium as described above (Section 2.1).

Pharmacological Inhibition

Spheroids were treated with Batimastat (BB94, 30 μM ; Selleckchem), E64 (10 μM ; Tocris 5208), Blebbistatin (Bleb, 10 μM ; Abcam ab120425), or Y-27632 (Y27, 10 μM ; Tocris 1254). Batimastat powder was dissolved to 30 mM stock solution. E64 and Y-27 powders were dissolved to 1 mM stock solutions, and Blebbistatin to a 5 mM stock solution, all stored at -20°C until use. Working concentrations were prepared fresh by diluting stock solutions into culture medium according to the manufacturer's instructions. Vehicle controls were included.

Lentiviral Transduction

Constitutive expression of fluorescent markers was accomplished via lentiviral transduction. MCF7 cells were labeled with plv-mCherry, and 19TT CAFs were labeled with pLenti CMV H2B green fluorescent protein (GFP) Hygro (656-4). pLenti CMV GFP Hygro (656-4) was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid #17446; <http://n2t.net/addgene:17446>; RRID:Addgene_17446). Lentiviruses were produced by co-transfecting cDNA expression plasmids with helper plasmids pCMV-VSVG, pMDLg-RRE (gag/pol), and pRSV-REV into HEK293T cells using polyethylenimine (PEI). Cell supernatants were harvested 48 hours after transfection and stored at -80°C. Cells were labeled by infecting for 24 hours with respective lentiviral supernatants diluted 1:1 with cell culture medium and 5 ng/mL of polybrene (Sigma-Aldrich). 48 hours after infection, 19TT CAFs were placed under Hygromycin-B (Thermo Fisher Scientific) selection, and MCF7 cells were placed under Neomycin (A1720, Sigma-Aldrich) selection. The fluorescently labeled cells were cultured with 100 $\mu\text{g}/\text{mL}$ of respective antibiotics to obtain stable fluorescent cell lines and maintain selection pressure.

Spheroid seeding and characterization

MCF7 and 19TT CAF spheroids were seeded in Corning Elplasia 96-well Round Bottom Ultra-Low Attachment (ULA) Microcavity Microplates (Corning, 4442). Briefly, cells were

trypsinized (Trypsin-EDTA, Gibco™, 25200056) and resuspended to a concentration of 1×10^6 cells/ml. 40×10^3 (500 cells per microwell, 79 microwells) cells were seeded to produce 79 spheroids. Spheroids were ready after 48 hours of culture. MCF7 spheroids ($200 \pm 25 \mu\text{m}$ diameter) and 19TT CAF spheroids ($125 \pm 25 \mu\text{m}$ diameter) were used for invasion studies. MCF7 spheroids were cultured in DMEM, High Glucose (Sigma) containing 4.5 g/L glucose and l-glutamine but without sodium pyruvate and supplemented with 10% FBS (Sigma) and 1% antibiotic-antimycotic solution (Gibco). For 19TT CAF spheroids, a high-viscosity culture medium was made using methylcellulose as described previously [35]. Briefly, a 20% methylcellulose solution was made by sterilizing 6 g of methylcellulose in an autoclave. This was subsequently mixed with 250 ml of DMEM High Glucose. Once methylcellulose was dissolved, an additional 250 ml of DMEM High Glucose and 55ml FBS was added, and the solution was mixed overnight at 4°C. The following morning, the solution was centrifuged at 5000g for 2 hours at 4°C, and the supernatant was stored at 4°C until use.

19TT CAF - MCF7 heterogeneous spheroids (heterospheroids) were generated using the hanging drop method. Briefly, cells were trypsinized (Trypsin-EDTA, Gibco™, 25200056) and resuspended to a concentration of 1×10^3 cells/ml. Cell lines were mixed and diluted to a final concentration of 1000 cells / 30 μL , consisting of 500 cells of each cell line. As described above, a high-viscosity culture medium, made using 20% methylcellulose (M0512, Sigma), was used to culture cells. 30 μL droplets containing 1000 cells were cultured 48 hours before the fully formed spheroids were harvested for experiments.

Preparation of collagen hydrogels

Collagen type-1 hydrogels were prepared on ice to prevent polymerization. Briefly, collagen (Corning™, 354249) was mixed with 10x Reconstitution Buffer (200 mM HEPES + 262 mM sodium bicarbonate) and 10x DMEM (Gibco™, 12100061) in an 8:1:1 ratio, adjusted to pH 7.4 using 0.5M NaOH, and diluted with sterile Milli-Q water to the required concentration (3 mg/ml) [36]. The hydrogel was incubated at 4°C for 20 minutes, followed by 37°C for 40 minutes.

Spheroid Invasion Experiments

A thin 100 μL collagen bed (3 mg/ml) was created and allowed to gel for the invasion experiment (as mentioned in section 2.4). This was followed by harvesting 19TT CAFs and MCF7 spheroids, mixing them with freshly prepared collagen, embedding them on top of the collagen bed, and incubating them as stated above. The culture medium was added after incubation, and the device was set up for time-lapse imaging. Initial imaging was performed 1 hour after embedding (or immediately post collagen gelation). This time point was designated as T_0 for all subsequent analyses in this study. The culture medium was refreshed every 48 hours for experiments running until 96 hours. Analysis

was limited to spheroids (heterogeneous or dual-spheroid systems) that were at least 400 μm away from other spheroids. This ensured that no additional interactions would occur within the region of interest (ROI) for the experiment duration.

Time-lapse Confocal and Reflection Imaging

Confocal fluorescence and reflection microscopy were performed using the Zeiss LSM-980 integrated with a live cell incubator. Temperature was maintained at 37°C and CO_2 at 5% during microscopy measurements. Images were collected at 2-hour intervals and analyzed using ImageJ2 (v2.14, National Institute of Health, USA) and custom Matrix Laboratory (MATLAB) scripts. For image acquisition, EC Plan-Neofluar 10x/0.3NA M27 (Zeiss) air objective was combined with 13mW 488 nm and 543 nm lasers for fluorescence imaging. During acquisition, the digital gain was set to 1, digital offset 0, laser power to 0.2%, and master gain at 650V. Appropriate band-pass filters were used to prevent spectral overlap between GFP and mCherry channels. A 488 nm laser with a detection range of 476 nm to 502 nm was used for reflection microscopy. The master gain was set to 600 V, and 0.2% laser power was used during reflection imaging. The reflection images in the figures were collected with equivalent gain settings and presented with equivalent digital contrast, brightness, and levels. All images were acquired at an optical zoom of 1.0, with a field of view of 848.53 μm x 848.53 μm with 2048x2048 pixels. Z-stacks were acquired at 20 μm step size for a total range of 400 μm .

Image Processing

Carl Zeiss Image (CZI) files were extracted and saved as ‘.tiff’ files after image acquisition using custom ImageJ scripts. Before analysis, these files were processed, during which mean noise was subtracted from images, and fluorescent channels (GFP and mCherry) were checked for spectral overlap.

Circularity and Change in Area of MCF7 Spheroids.

MCF7 spheroid images were extracted and saved as image sequences using custom ImageJ scripts. Maximum Z-projections were created to find the maximum diameter of the MCF7 spheroids. These maximum projections were then utilized with custom MATLAB scripts to binarize and threshold images to identify MCF7 spheroid edges. In-built regionprops function was subsequently used to measure the change in area and circularity of spheroids. To calculate the shortest distance between MCF7 and 19TT CAF spheroids, image sequences in the Z-plane at T_0 were saved for each spheroid. Weighted centroids in three-dimensional space were computed based on the maximum spheroid area at each Z-plane. The 3D centroid-to-centroid distance was then determined. Using a nearest-neighbor algorithm, the coordinates of the intersection between the centroid-connecting line and the spheroid boundary were identified. These boundary coordinates for both spheroids were then used to calculate the shortest inter-spheroid distance.

Migration Analysis

CAF spheroid migration was calculated by measuring the maximum cross-sectional spheroid area at T_0 (A_0) and during its culture within the collagen matrix (A_t). The invasive index was then defined as $\delta A = (A_t/A_0) - 1$ to quantify the level of invasion for different spheroids.

Collagen Fiber Orientation, Void Fraction and Brightness Characterization

Collagen fiber images obtained via reflection imaging were analyzed using ImageJ and customized MATLAB scripts. Collagen planes corresponding to the central spheroid plane were selected in ImageJ for further analysis. Spheroid area and position are segmented from the image using Gaussian filtering ($\sigma = 2$ pixels), morphological operations and Otsu's thresholding [37]. OrientationJ's vector field analysis is used to determine collagen fibers' local orientation and isotropy relative to the absolute x-axis [38]. The size of the gaussian kernel was set to 10 μm [38,39]. As shown in Fig. S1, the resulting orientation mask was analyzed in conjunction with intensity thresholded collagen masks using custom MATLAB scripts to identify, transform, and subsequently calculate the collagen fiber alignment relative to the centroids of the corresponding spheroids, providing a detailed spatial understanding of collagen architecture relative to spheroid positioning. To quantify collagen degradation, we extended on our MATLAB script and used the intensity thresholded collagen masks to identify void spaces between collagen fibers based on relative pixel intensity values and calculate the void fraction (V_f) of the collagen plane. These V_f values were then normalized to the V_f value at T_0 . To quantify the change in collagen fiber brightness (pixel intensity values), we summed up all pixel values of the collagen fibers at each time point (I_p) and normalized them by T_0 .

Tissue Sectioning, Imaging and Analysis.

Tissue Processing, Histochemical Staining, and Digitization of Human Breast Cancer

Formalin-fixed, paraffin-embedded tumor tissues were sectioned into four μm slides and processed sequentially, including deparaffinization with xylene, rehydration through an ethanol gradient, histological staining, air drying at room temperature, and mounting with a xylene-based medium (Permount; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). For this study, a tissue block containing the most invasive tumor region, used for Tumor (T)-status assessment, was selected to prepare serial sections. These sections were stained with hematoxylin and eosin (H&E) and Picrosirius red (PSR). PSR staining involved treatment with 0.1% Direct Red 80 dye in saturated picric acid (Sigma-Aldrich, Merck, Darmstadt, Germany) for 60 minutes, followed by rinsing in two changes of 0.5% glacial acetic acid (Avantor, Inc., Radnor Township, Pennsylvania, USA). Stained slides were digitized in brightfield mode using a Panoramic 250 slide scanner

(3DHISTECH, Budapest, Hungary) at 200× magnification (0.39 µm/pixel) and saved as proprietary .mrxs files.

Fluorescent Whole-Slide Imaging of Picrosirius Red-Stained Tissue Sections

Picrosirius red-stained tissue sections were digitized in fluorescent mode using a Panoramic 250 slide scanner (3DHISTECH, Budapest, Hungary) at 200× magnification (0.39 µm/pixel) and saved as proprietary MIRAX (.mrxs) files. Tissue autofluorescence was captured using a Fluorescein isothiocyanate (FITC) filter cube (excitation: 460-488 nm, emission: 502-547 nm) with an exposure time of 800 ms. The fluorescence emission of the fibrous ECM stained by Sirius red was recorded using a Tetramethylrhodamine isothiocyanate (TRITC) filter cube (excitation: 532-554 nm, emission: 573-613 nm) with an exposure time of 44 ms.

Immunohistochemistry

For multiplex immunofluorescence, tissue sections were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval using citrate buffer (pH 6.0). To minimize non-specific binding, sections were incubated with 5% goat serum for 10 minutes at room temperature. Primary antibodies were applied overnight, including anti-αSMA (clone 1A4, DAKO, M0851, 1:300 dilution) and pan-cytokeratin (AE1/AE3, DAKO, M3515, 1:300 dilution). The next day, sections were incubated with Alexa Fluor-conjugated secondary antibodies: 647 goat anti-mouse IgG2a (Life Technologies, A21241, 1:200 dilution) and 546 goat anti-mouse IgG1 (Invitrogen, A21123, 1:200 dilution). All antibody incubations were performed for 30 minutes at room temperature in the dark. Finally, nuclei were counterstained with Hoechst 34580 (Sigma-Aldrich, 63493, 1:2000 dilution) for 5 minutes.

Statistical Analysis

All graphs shown in this work were produced using GraphPad Prism. Statistical analyses were run within the software, and significance between groups was measured using unpaired t-tests or ANOVA based on experiment variable dependencies. Statistical significance between more than two groups was assessed using ANOVA tests. Probability values less than 0.05 were deemed significant ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

Results

Cancer cell and CAF distribution, and collagen morphology observed in luminal breast tumor sections.

To explore the heterogeneity of the TME and the organization of the ECM in relatively indolent breast cancers, we analyzed tissue sections from Luminal A breast cancer samples. Our focus was on the collagen architecture and cellular organization at distinct

tumor boundaries (Fig. 1 A-C). The analysis of multiple tumor boundaries across four samples revealed significant variability in the collagen fiber organization in the TME ECM. Some tumor boundaries displayed a thick collagen layer that separated the TME from adjacent healthy tissues, while others were characterized by infiltrative collagen fiber projections extending into the surrounding tissue. Based on these structural differences, we classified tumor boundaries as either non-infiltrative or infiltrative tumor boundaries (Fig. 1B-C). Among four tumor samples, CAF01 and CAF02 showed both infiltrative and non-infiltrative boundaries, CAF03 displayed only non-infiltrative margins, and CAF06 exhibited only infiltrative margins. The tumor shown in Fig. 1 (Tumor ID: CAF01) featured both infiltrative and non-infiltrative fronts, while the remaining sections are presented in Fig. S2. Infiltrative tumor fronts were marked by highly organized collagen fibers running perpendicularly to the tumor boundary and forming tracks or protrusions that extended into adjacent mammary fat tissue (Fig. 1A, blue arrows). In contrast, non-infiltrative fronts showed collagen fibers predominantly aligned in parallel to the tumor border, delineating a continuous interface between tumor and surrounding tissue (Fig. 1A, red arrows).

Immunofluorescent staining for pan-cytokeratin (pCK), an epithelial marker identifying tumor cells, and α -SMA, a marker of activated fibroblasts (CAFs), revealed marked differences in stromal composition and organization between infiltrative and non-infiltrative tumor boundaries. In infiltrative regions (Fig 1C, bottom panels), tumor cells appeared as dispersed clusters intermingled with α -SMA-positive fibroblasts, which were prominently localized at the tumor-stroma interface. Notably, both CAFs and collagen fibers (Fig 1C, top panels) displayed aligned orientation along the apparent invasion paths of the tumor cells. In contrast, non-infiltrative boundaries (Fig. 1B) displayed cohesive tumor fronts where both collagen fibers and α -SMA-positive fibroblasts were predominantly aligned perpendicular to the tumor boundary. Rather than promoting invasion, this perpendicular alignment appeared to reinforce a well-demarcated and continuous interface between the tumor and surrounding stroma.

Despite these qualitative insights, several fundamental questions remain. A key challenge is understanding how spatio-temporal modifications of the collagen ECM induced by CAFs affect the structural integrity and dissemination of cancer cells. This evaluation is complex and cannot be directly assessed from tissue samples alone. These observations highlight the need for dynamic *in vitro* assays capable of separating contractile remodeling from enzymatic degradation and resolving how CAF spatial distribution influences ECM remodeling and cancer cell dissemination over time.

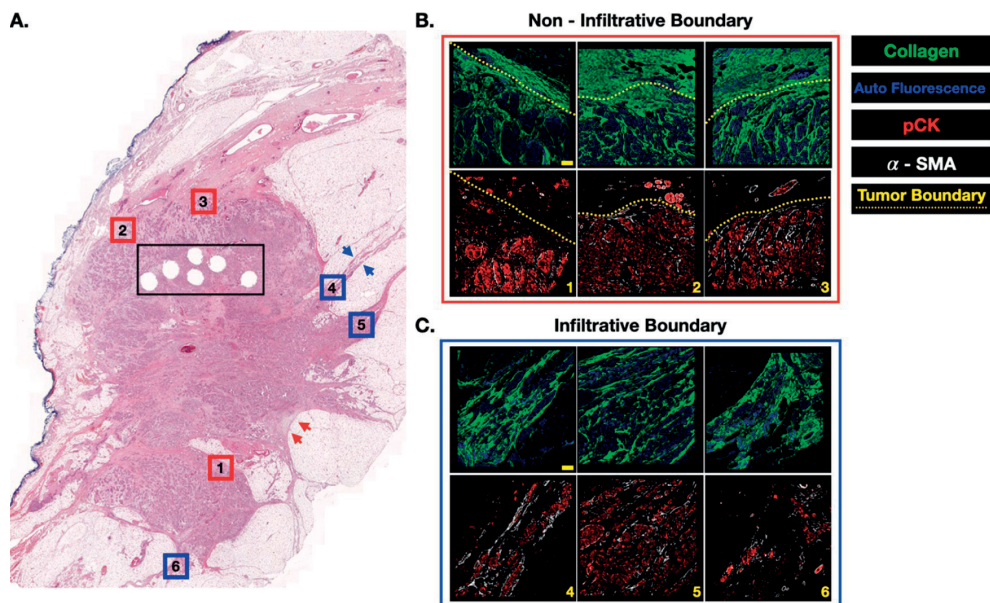


Figure 1. Infiltrative and Non-Infiltrative TME boundaries can be identified based on cellular organization and collagen architecture. (A). H&E Stained tissue section of Luminal A tumor. Holes visible at the center of the tumor (black box) are biopsy sections. ROIs boxed in red and blue indicate non-infiltrative and infiltrative tumor boundaries, respectively. Red arrows indicate a non-infiltrative tumor boundary creating a distinct boundary between the tumor core and breast tissue. Blue arrows indicate an infiltrative tumor boundary with collagen fingers protruding into the breast tissue. (B). Fluorescent scans of non-infiltrative tumor boundaries. On top (red) is the collagen organization at the tumor boundary (yellow dotted line), and below are the scanned sequential tissue slides indicating α -SMA (CAF, white) and pCK (tumor cells, red) organization at the tumor boundary. Scale Bar = 100 μ m. (C). Fluorescent scans of Invasive tumor boundaries. On top (red) is the collagen organization at the tumor boundary, and below are the scanned sequential tissue slides indicating α -SMA (CAF, white) and pCK (tumor cells, red) organization at the tumor boundary.

19TT CAFs mediates ECM remodeling and the subsequent MCF7 spheroid expansion and dissemination.

To investigate the impact of distinct cellular organization at infiltrative and non-infiltrative tumor fronts on intercellular interactions and ECM remodeling, we co-embedded 19TT CAF and MCF7 cancer cell spheroids within a 3D collagen matrix (3 mg/ml). This assay configuration enabled us to analyze key features observed in tissue sections, such as the interspersed cellular architecture, orientation of collagen fibers, and loose tumor clusters that could interact with 19TT CAF cells, while allowing for kinetic visualization of ECM remodeling (See Fig 2A, B, D). In this co-culture system, 19TT CAF spheroids maintained direct access to the collagen matrix and were able to engage with MCF7 tumor spheroids through short-distance migration. Fig. 2(B) and movies 1, 2 and 3 illustrate the spatial

organization between 19TT CAF and MCF7 spheroids compared to a single MCF7 spheroid embedded in the collagen matrix. Single MCF7 spheroids displayed gradual nearly uniform expansion over the entire 48 hour period. However, when 19TT CAF spheroids were seeded less than 200 μm apart from MCF7 spheroids, we observed non-uniform expansion and eventual collapse of MCF7 spheroids. In several samples, we also observed single-cell dissemination from MCF7 spheroids (Fig 2D, MCF7, 48 hours $d < D_c$). Conversely, when the inter-spheroid distances (d) were greater than 200 μm , MCF7 spheroids exhibited significantly less, yet non-uniform expansion after 48 hours. Based on the distribution of dA (change in MCF7 spheroid area) with respect to distance from 19TT CAF spheroids, we observed a distinct separation between conditions $d < D_c$ and $d \geq D_c$ where D_c (200 μm) was the average MCF7 spheroid diameter (Fig. S3A). In addition, we stimulated MCF7 spheroids with CAF conditioned medium, and we did not observe any significant spheroid expansion compared with the unstimulated (control condition) MCF7 spheroids (Fig. S4).

To estimate the overall expansion of MCF7 spheroids relative to its initial area was calculated as $dA = (A_{48} - A_0)/A_0$. As shown in Fig 2(C), for MCF7 spheroids embedded alone in the collagen matrix, we observed a change in the spheroid area of 1.1 after 48 hours. For 19TT CAF spheroids seeded at a distance $d < D_c$, the mean change in MCF7 spheroid area was 4.1 compared to 1.6 when 19TT CAF spheroids separated by distances $d > D_c$ (Fig. S3B).

Based on the dynamics captured in Fig. 2B (movies 1, 2 and 3), we identified three key time dependent interaction patterns (stages) involving 19TT CAFs with both the ECM and MCF7 spheroids. During the first stage (between 4 and 10 hours), 19TT CAF spheroids mechanically engaged with the collagen ECM, remodeling thin fibers into thicker, radially aligned bundles. Image analysis across multiple Z-planes revealed that 19TT CAFs drew collagen fibers from various focal planes, bundling them into radially aligned fibers within the central plane of the spheroids (Fig. S5). This matrix remodeling process likely contributed to increased collagen fiber pixel intensities and deformation of adjacent MCF7 spheroids (Fig. 2B, $d < D_c$) [39,40]. The transition from stage one to stage two was marked by the onset of single-cell dissemination from 19TT CAF spheroids into the surrounding collagen matrix, beginning between 10 and 12 hours and overlapping slightly with the remodeling stage. Disseminating CAFs migrated preferentially into regions that had already undergone mechanical remodeling, where fibers were radially aligned and thickened relative to the unperturbed matrix (Fig. S6). This invasion ($t > 10$ hours) was accompanied by progressive matrix degradation leading to formation of central voids and increasing the void fraction of the collagen matrix. The third stage, beginning around 24 hours was characterized by direct interactions between migrating 19TT CAFs and MCF7 spheroids. The timing of this stage varied with inter-spheroid distances: closer pairs engaged earlier, while those further apart engaged later. Functionally, this phase was

marked by non-uniform expansion of MCF7 spheroids, collapse into CAF-generated voids, and frequent single-cell dissemination (Fig. 2D). We further examined the MCF7 expansion

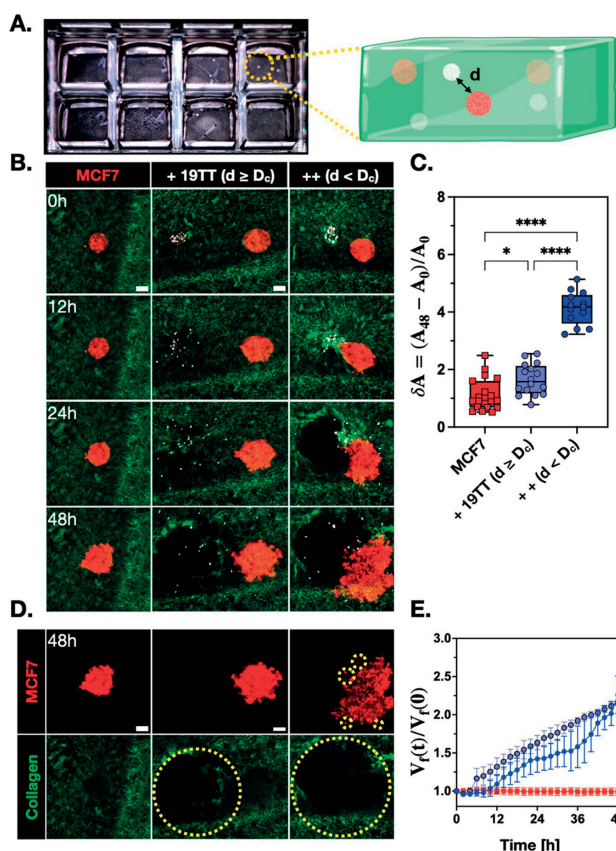


Figure 2. 19TT CAF - mediated disruption of MCF7 spheroids is dependent on inter-spheroid distance. (A). Ibidi 8-well glass-bottom slide with a 3D collagen matrix containing multicellular tumor spheroids. A zoomed-in schematic represents multicellular luminal MCF7 breast cancer and CAF spheroids embedded in a 3D collagen matrix, separated by a distance "d." (B). Representative fluorescence images of MCF7 mCherry-labeled cancer cell spheroids (red) and 19TT H2B GFP-labeled CAF spheroids (white) taken at 0-, 24-, and 48-hours post-embedding in a 3 mg/ml rat-tail collagen matrix (green). Spheroids were separated by distances of (ii) 450 μm and (iii) 150 μm . (C). Quantification of the MCF7 spheroid area was performed under three conditions: when embedded alone ($n = 19$, $N = 3$), with 19TT CAF spheroids within 200 μm ($n = 13$, $N = 3$), and with 19TT CAF spheroids at distances greater than 200 μm ($n = 16$, $N = 3$) after 48 hours (* $p < 0.05$, **** $p < 0.0001$). (D). Overview of (1) single-cell dissemination (yellow circles) observed from MCF7 spheroids and (2) collagen matrix integrity (degraded collagen area in yellow circles) under three conditions: (i) MCF7 alone, (ii) M7 + CAF, $d \geq DC$, and (iii) M7 + CAF, $d < DC$, after 48 hours. (E). Void Fraction ($V_f(t)/V_f(0)$) Quantification of the collagen hydrogel measured from 0 to 48 hours and normalized to its initial value ($V_f(0)$) for MCF7 (red), MCF7 + CAF, $d \geq DC$, (light blue) and (iii) MCF7 + CAF, $d < DC$ (dark blue).

during these three stages of 19TT CAF and ECM interactions by computing the time-wise evolution $dA = A_t/A_0$. We found that although the MCF7 spheroid expansion is initiated during the mechanical remodeling stage (4 - 10 hours), it is significantly enhanced during the subsequent ECM degradation ($t > 10$ hours) (Fig. S3B).

To quantify matrix degradation, we calculated the void fraction (v_f) of the collagen matrix through image analysis, normalized to the initial time point. Single MCF7 spheroids showed no detectable degradation, whereas co-cultures with 19TT CAFs displayed a steady increase beginning after ≈ 10 hours, doubling by 48 hours (Fig. 2E). Variability in degradation rates was greater at $d < D_c$ than at $d > D_c$, likely reflecting earlier 19TT CAF-MCF7 interactions at shorter distances, which could attenuate 19TT CAF-mediated collagen remodeling. In contrast, when seeded at greater distances, 19TT CAF spheroids would have been able to remodel the collagen matrix without such interference prior to engaging with MCF7 spheroids. These results confirm that inter-spheroid distance is a critical factor influencing MCF7 spheroid expansion, single-cell dissemination, and the rate of collagen matrix degradation. In the following sections, we will focus our analyses on the 19TT CAF and MCF7 spheroid system where $d < D_c$.

19TT CAF spheroid dissemination mediates collagen matrix remodeling and degradation

During 19TT CAF-mediated collagen remodeling, collagen fibers reoriented locally to align radially toward the center of the 19TT CAF spheroid (Fig. 3A, movie 4). To quantify this alignment, we identified and measured the angles of thick collagen fibers relative to the spheroid center (Fig. S1); values approaching 0° indicated stronger alignment. Over the first 10 hours (stage 1), collagen fiber alignment progressively intensified (Fig. 3A-B), with a pronounced peak at 0° by the end of the 10th hour. Concurrently, collagen brightness increased under identical imaging conditions. Pixel intensity analysis confirmed a steady rise during stage 1, consistent with fiber bundling (Fig. 3C). After ≈ 12 hours variability in collagen pixel intensities increased. Dissemination of 19TT CAF single cells (stage 2) began between 8 and 12 hours suggesting that the remodeled collagen matrix facilitated CAF migration and invasion. Minimal matrix degradation was detected during early matrix remodeling, as reflected by stable values of v_f during the initial 12 hours. At later times, the rate of collagen degradation increased, as evidenced by a steeper slope in the degradation curve (Fig. 3D). By contrast, MCF7-only spheroids showed no significant changes in void fraction or collagen alignment over time, consistent with a lack of intrinsic matrix remodeling capacity in luminal breast cancer cells (Movie 5, Fig 3A-D)[41].

To test whether collagen matrix remodeling was driven by contractility, we perturbed actomyosin pathways using a direct Myosin II blocker (Bleb) and Rho-associated kinase (ROCK) inhibitor [42,43]. Myosin II (Bleb, 10 μ M) and ROCK (Y27, 10 μ M) inhibition (Fig.

S7) confirmed the role of cellular contractility in these early stages. Myosin II inhibition markedly delayed bundling and suppressed early void formation, whereas ROCK

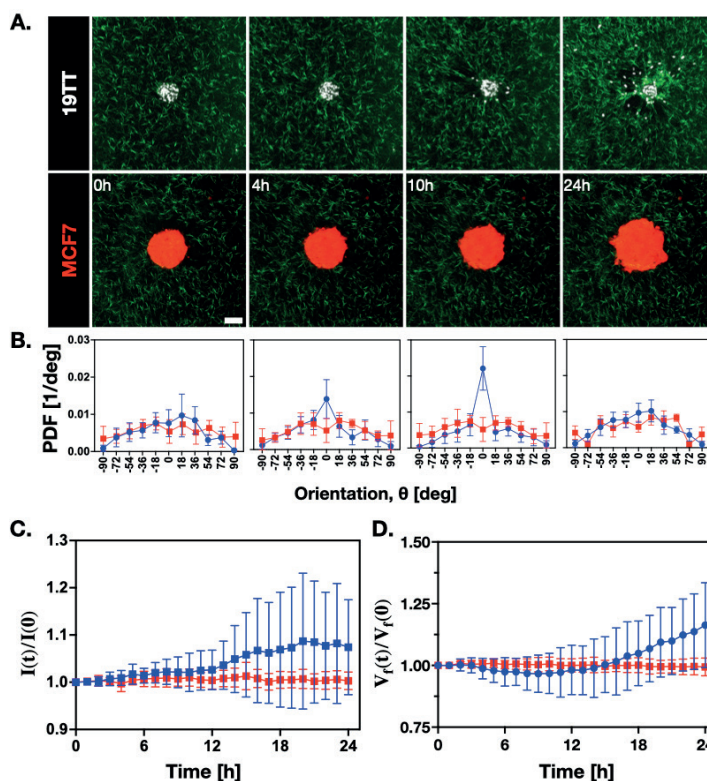


Figure 3. 19TT CAF spheroid-mediated matrix degradation is preceded by matrix remodeling and fiber reorientation. (A). Representative time-lapse images of 19TT CAF (white) and MCF7 tumor spheroid (red) in a collagen matrix (green). (B). Relative collagen fiber orientation concerning the CAF spheroid center in blue ($n=11$, $N=3$) and MCF7 spheroid center in red ($n=10$, $N=3$). Distribution of orientations from -90° to $+90^\circ$ is plotted on the x-axis and the probability density function (PDF) of orientations on the y-axis. Scale bar = $100 \mu\text{m}$. (C). Quantification of collagen VF during the first 24 hours of the experiment. MCF7 VF in red and CAF VF in blue. (D). Quantification of collagen fiber fluorescence intensities during the first 24 h. Mean pixel intensities of collagen fibers surrounding 19TT CAF and MCF7 spheroids are compared.

inhibition produced thinner but aligned fibers (Fig. S7B). Importantly, in both conditions CAFs continued to disseminate, and matrix degradation was observed at later times. These results indicate that contractile forces drive early matrix remodeling, while proteolysis sustains later degradation even when contractility is suppressed.

MMP Inhibition inhibits CAF-mediated matrix degradation but not CAF migration.

Next, we investigated the influence of collagen matrix degradation on 19TT CAF invasion and MCF7 spheroid expansion. To inhibit MMP-mediated matrix degradation, we used the broad-spectrum MMP inhibitor Batimastat (BB94) [44]. In comparison to the controls, BB94 treated 19TT CAF spheroids did not degrade the collagen matrix, resulting in no significant change in the void within the matrix over 24 hours (Fig. 4A, BB94 - movie 6, Veh Control - movie 7). Further quantification confirmed this observation: while V_f increased in control conditions, it remained nearly unchanged for CAF spheroids treated with BB94. Despite blocking matrix degradation, BB94 did not prevent collagen remodeling, as we observed

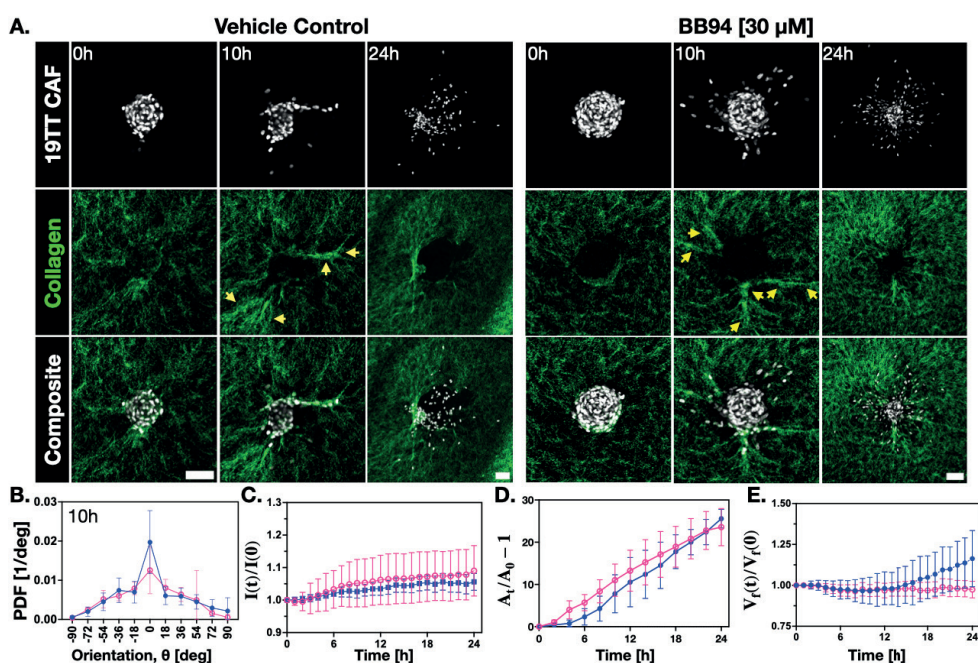


Figure 4. Broad-spectrum MMP inhibition with Batimastat (BB94, 30 μ M) attenuates collagen matrix degradation but fails to suppress 19TT CAF invasion or ECM remodeling.

(A). Representative images of 19TT CAF spheroids (white), surrounding collagen matrix (green), and composite overlays under vehicle control and broad-spectrum MMP inhibition (BB94, 30 μ M) conditions at 0 and 10 hours. A 24 hour overview highlights migrating CAF cells. Yellow arrows indicate reoriented collagen fibers in vehicle control and MMP inhibition conditions. (B). Probability density function (PDF) of relative collagen fiber orientation concerning the CAF spheroid center for BB94 treated (pink, $n = 8$, $N = 3$) and vehicle control spheroids (blue, $n = 8$, $N = 3$) after 10 hours. (C). Collagen fiber pixel intensities over 24 hours for control (blue) and BB94-treated spheroids (pink). (D). The invasive index of CAF spheroids was observed 24 hours post-embedding in the collagen matrix under vehicle control and MMP inhibition conditions. (E). Normalized collagen V_f comparing vehicle control and MMP inhibition conditions over 24 hours.

thick collagen fibers at 10 hours in both conditions. Orientation analysis likewise showed no major differences between BB94 and controls (Fig. 4B). Collagen pixel intensities also continued to rise under BB94, indicating preserved remodeling activity (Fig. 4C). To assess CAF motility, we calculated the invasive index ($I_v = A_t/A_0 - 1$), defined as the increase in spheroid cross-sectional area (A_t), to quantify migration dynamics. No significant difference was found between treated and control spheroids (Fig. 4D), showing that MMP inhibition had little effect on CAF invasion or single-cell dissemination. Consistent with expectations, MCF7 spheroids alone showed no remodeling under either condition (Fig. S8). Together, these findings confirm that MMP activity drives CAF-mediated degradation but is not required for CAF-driven remodeling or invasion.

We also tested Cathepsin inhibition using a cysteine protease inhibitor (E64, 10 μ M) [45]. This treatment marginally reduced collagen degradation compared to control (Fig. S9). The effect was modest compared to the near-complete suppression observed with MMP inhibition, suggesting that cathepsins may contribute to matrix clearance but are not the dominant drivers in this system.

MMP inhibition reduces MCF7 spheroid expansion but cannot prevent MCF7 cell dissemination.

After evaluating MMP inhibition in 19TT CAF spheroids, we expanded our analysis to the MCF7-19TT CAF system. Time-lapse imaging showed that MMP inhibition significantly reduced the expansion of MCF7 spheroids and prevented their disruption at the 48-hour mark compared to the vehicle controls (Fig. 5A-B, and see movies 8, 9). Despite this, MMP inhibition led to an observable increase in single-cell dissemination from MCF7 spheroids within the same timeframe (Fig. 5C). 19TT CAFs maintained their ability to remodel the collagen matrix under BB94 treatment, but MCF7 spheroids no longer displayed the deformations observed in the vehicle controls. In controls, CAF-mediated remodeling and subsequent degradation deformed the spheroid bulk before triggering expansion and dissemination. By contrast, under MMP inhibition, MCF7 spheroids released single cells isotropically - without prior bulk disruption - during the 48-hour window (Fig. 5C).

19TT CAF-MCF7 Heterogeneous spheroids display similar outcomes as dual-spheroid systems.

Heterospheroids were designed to model intermixed epithelial-stromal architectures observed in CAF-rich tumor regions, in contrast to the spatially separated dual-spheroid configuration [31,32]. Each heterospheroid contained ~500 MCF7 and 19TT CAFs each, with 19TT CAFs forming a central core surrounded by MCF7 cells (Fig. 6A). After embedding in collagen matrices, time-lapse imaging revealed that 19TT CAF cells remodeled the collagen matrix while largely remaining within the core of the heterospheroid in both control and BB94 treated conditions (Fig. 6A, movies 10, 11). Reoriented collagen bundles

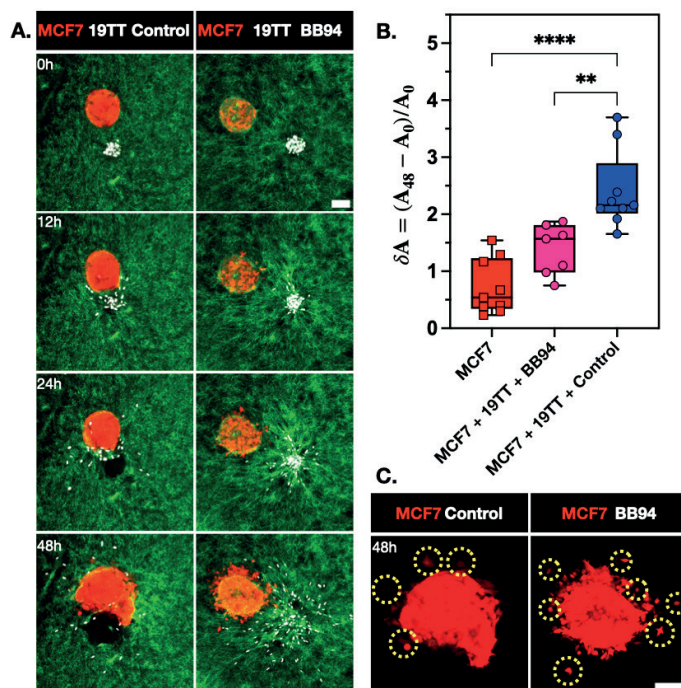


Figure 5. Broad-spectrum MMP Inhibition with Batimastat (BB-94, 30μM) of the CAF-MCF7 spheroid system reduces MCF7 spheroid expansion but does not prevent single-cell dissemination. (A). Representative time-lapse images of 19TT CAF spheroids (white) and MCF7 tumor spheroids (red) embedded in a collagen matrix (green) in vehicle control and MMP inhibition conditions. (B). Quantification of MCF7 spheroid area changes when seeded alone ($n = 9$, $N = 3$), vehicle control ($n = 9$, $N = 3$) and MMP inhibition conditions after 48 hours ($n = 7$, $N = 3$). (C). Representative images of MCF7 spheroids in vehicle control and broad-spectrum MMP inhibition conditions 48 hours post-embedding in a collagen matrix. Yellow circles highlight single cells disseminating from the spheroids.

were observed around the heterospheroid as early as 12 - 24 hours and remained visible at 30 hours coinciding with uniform spheroid expansion. Quantifying the relative orientation of collagen fibers in relation to the spheroid center (Fig. 6C) revealed comparable trends between the control and BB94 conditions, yet the degree of reorientation of the collagen fibers after 10 hours was markedly lesser compared to the dual-spheroid system (Fig. S10). The expansion of the heterospheroid over the first 48 hours (Fig. 6D) mirrored the behavior observed in the dual-spheroid system, although we noted a greater number of single disseminating cells. Importantly, broad-spectrum MMP inhibition produced results consistent with those observed in the dual-spheroid system, significantly restricting spheroid expansion without affecting collagen fiber reorientation or the dissemination of MCF7 single cells.

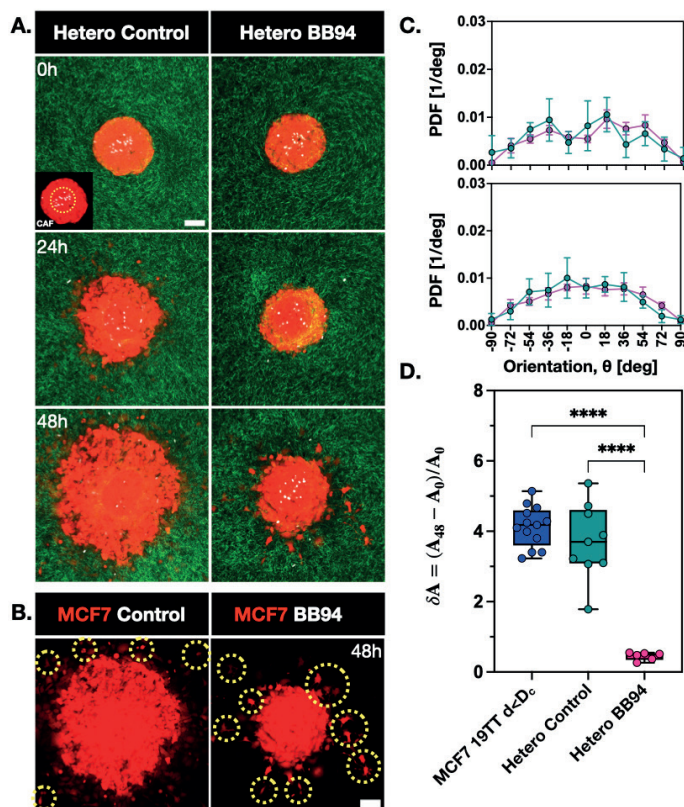


Figure 6. Heterospheroids (19TT CAF – MCF7) show similar expansion rates and single cell dissemination as MCF7 spheroids when seeded close to CAF spheroids. (A) Representative time-lapse images of heterospheroids composed of MCF7 tumor cells (red) and 19TT CAFs (white) embedded in a 3D collagen matrix (green). Shown are vehicle control and BB94 (30 μ M) treated spheroids. The inset highlights a heterospheroid, with 19TT CAF cells located centrally (yellow circle). Scale bar = 100 μ m. (B) Representative images of MCF7 cells in vehicle control and BB94-treated conditions 48 hours post-embedding in the collagen matrix. Yellow circles highlight single cells disseminating from the spheroids. (C) Collagen fiber orientation with respect to heterogeneous spheroid center at 0 and 24 hours, respectively. Control in green ($n = 9$, $N = 3$) and MMP inhibition in pink ($n = 6$, $N = 3$) (D) Quantifying heterogeneous spheroid area changes under vehicle control and MMP inhibition conditions after 48 hours.

Discussion

The TME is a dynamic and heterogeneous setting where CAFs play pivotal roles in promoting invasion through ECM remodeling and degradation. Previous work has largely correlated CAF activity and MMP expression with invasive behavior in patient samples or 2D assays [20,46], but has not resolved how these processes unfold over time in 3D or

how they differentially influence cancer cell outcomes. To establish patient relevance, we first analyzed infiltrative and non-infiltrative tumor fronts in luminal breast cancer tissues. Infiltrative regions showed pronounced collagen alignment, elevated α -SMA, and intermixed CAF - cancer cell clusters, whereas non-infiltrative regions displayed weaker collagen alignment, reduced α -SMA, and more segregated tumor - stromal compartments. These observations informed the design of our in vitro models, which can systematically dissect the spatiotemporal principles underlying CAF cancer ECM interactions.

Using a multi-spheroid 3D model coupled with time-lapse imaging, we demonstrate that CAF-mediated remodeling and degradation are temporally and mechanistically separable. During the first stage of matrix remodeling (4-10 hours), CAF spheroids bundled and reoriented fibrillar collagen into thick aligned fibers, thereby preparing the matrix for subsequent invasion and degradation. This early, contractility-driven remodeling facilitated deformation of adjacent MCF7 spheroids and, in many cases, early single-cell dissemination. Contractile forces exerted by 19TT CAFs also likely contributed to the increased pixel intensity of collagen fibers[39,40]. Following matrix reorganization, 19TT CAF migration commenced and was accompanied by progressive matrix degradation, as indicated by increasing void formation over the subsequent 24 hours. The protease-dependent matrix degradation was only detectable after $\sim$ 10-12 hours coinciding with increased void fraction and supporting bulk MCF7 spheroid expansion. However, the matrix degradation rate plateaued between 24 and 36 hours likely coinciding with 19TT CAF-MCF7 interactions. After 36 hours the degradation reaccelerated as 19TT CAFs migrated deeper into the matrix, having already disrupted the MCF7 spheroids. These observations highlight the spatiotemporal adaptability of 19TT CAFs in ECM remodeling to facilitate tumor invasion. Proximity of 19TT CAFs to MCF7 spheroids was a critical determinant of tumor expansion and dissemination, with spheroids located within one D_c of a 19TT CAF spheroid experiencing significantly increased expansion and single-cell dissemination compared to those positioned further away. Taken together, these data explicitly delineate the temporal and functional contributions of CAF-mediated ECM remodeling and degradation. Remodeling, defined as contractility-driven collagen bundling, occurred within the first 4-10 hours and facilitated MCF7 spheroid deformation and early single-cell dissemination. By contrast, degradation, detectable only after $\sim$ 10-12 hours, was protease-dependent and primarily supported bulk spheroid expansion.

Spatial organization further modulated these temporal dynamics. In dual-spheroid systems, remodeling and degradation occurred sequentially, whereas in heterospheroids, with intermixed CAFs and cancer cells - reflecting the intermixed epithelial-stromal architecture observed in CAF-rich tumor regions [31,32] - both processes occurred concurrently. These findings parallel research observations that epithelial-stromal

proximity accelerates invasion[7,47], while also providing mechanistic insight into how architectural context shapes invasion modes.

To inhibit matrix degradation, we employed broad-spectrum MMP inhibitor. MMP inhibition effectively prevented collagen degradation without impairing 19TT CAF motility or ECM remodeling. Under these conditions, MCF7 spheroid expansion was markedly reduced, but single-cell dissemination persisted or was even enhanced. This reinforces that bulk expansion depends on matrix degradation, whereas dissemination can proceed independently of it. Since MMP inhibition did not hinder CAF-driven remodeling, contractile forces likely create interstitial spaces that support dissemination. Further analysis confirmed the absence of matrix degradation in MMP-inhibited samples (Fig. 4E). Consistent with prior work, ECM architecture dictates migration mode: dense matrices promote single-cell dissemination, while looser matrices favor collective invasion [39,48-53]. Our results thus show that CAF-driven invasion operates through two separable mechanisms: contractility-dependent collagen remodeling and protease-mediated degradation. Collagen matrix remodeling supports single cell dissemination while matrix degradation supports bulk expansion and disruption of the cancer cell spheroid. This mechanistic dissection helps explain why MMP inhibitors have shown limited clinical efficacy and highlights the need to target both proteolytic and mechanical CAF activities. The differential effects of contractility and protease inhibition on collagen organization, void formation, and CAF behavior are summarized in Table S1, providing a concise overview of how each pathway contributes to distinct stages of matrix remodeling and invasion dynamics.

While our study emphasizes remodeling and degrading activities, CAFs also play broader roles *in vivo*, including ECM production, immune modulation, and metabolic support [12]. The 19TT CAF line used here represents a contractile, desmoplastic subtype particularly relevant to invasive tumor fronts. Future studies incorporating additional CAF subtypes and tumor contexts will be important to validate and expand these insights. Together these results also suggest that the cell dissemination observed under control conditions is a combination of both proteolytic and non-proteolytic migration modes. Additional future dissection of these routes is warranted to assess adhesion dependence, confinement sensitivity, and morphology-based kinematics (sphericity, cell speed, etc.). Beyond mechanistic insight, our findings also point to therapeutic strategies that disrupt CAF-mediated remodeling, such as combining inhibitors of actomyosin contractility with agents targeting collagen crosslinking enzymes like lysyl oxidase (LOX), which reduces matrix stiffening and tumor progression in preclinical models [54]. Such approaches could complement conventional MMP inhibition and provide a more comprehensive framework to mitigate CAF-driven invasion and resistance.

Conclusion

This study elucidates the spatiotemporal dynamics of CAF-driven ECM remodeling and its dual role in tumor invasion (focused on indolent breast cancer type), revealing valuable mechanistic insights into the interplay between stromal activity and tumor progression. Our findings demonstrate that CAFs orchestrate a sequential cascade of ECM reorganization, migration, and degradation, which collectively enable tumor spheroid disruption and dissemination. The initial phase of collagen fiber alignment and bundling by CAFs creates a permissive microenvironment for subsequent invasion, while MMP-mediated degradation facilitates bulk tumor expansion. Notably, MMP inhibition experiments revealed that ECM remodeling alone - via CAF contractility - suffices to generate interstitial spaces for single-cell dissemination, decoupling this process from matrix degradation. These results may also shed some light into the limited success MMP inhibitors have had in clinical settings as cancer therapeutics. Additionally, translating these insights into strategies to inhibit CAF-mediated matrix remodeling and subsequent CAF migration could offer novel avenues for metastasis prevention.

CRedit authorship contribution statement

Pranav Mehta: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Ankur Deep Bordoloi: Visualization, Validation, Software, Methodology, Formal analysis, Conceptualization, Writing – review & editing, Supervision. Cor Ravensbergen: Formal analysis, Data curation, Conceptualization. Ma.Kristen H. David: Data curation. Wilma Mesker: Resources. Gerrit Jan Liefers: Resources. Peter ten Dijke: Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. Pouyan E. Boukany: Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.actbio.2025.11.027](https://doi.org/10.1016/j.actbio.2025.11.027).

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Supporting Information

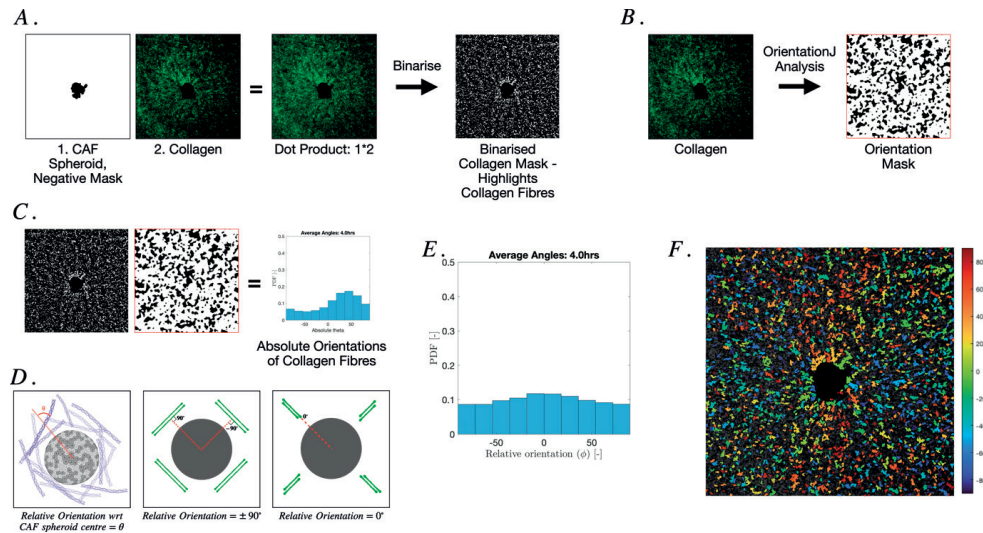


Figure S1. Analysis of CAF mediated matrix remodeling

- Dot product of CAF spheroid negative mask and collagen fiber image to produce collagen mask with CAF spheroid removed. This mask is further binarized to identify collagen fibers.
- Collagen fiber image is run through OrientationJ plugin (vector field analysis, gaussian kernel size 10) to produce orientation mask.
- Dot product of binarized collagen mask and orientation mask is used to obtain absolute orientations of collagen fibers (with respect to x-axis).
- Schematic of theta – relative orientation with respect to CAF spheroid center. Based on OrientationJ and ImageJ conventions, collagen fibers oriented tangentially are $\pm 90^\circ$ and fibers oriented radially are 0° .
- Histogram of relative collagen fiber orientation with its probability density function on the y-axis.
- Representative image of CAF spheroid and collagen channel overlaid with color-coded collagen fibers according to their relative orientation.

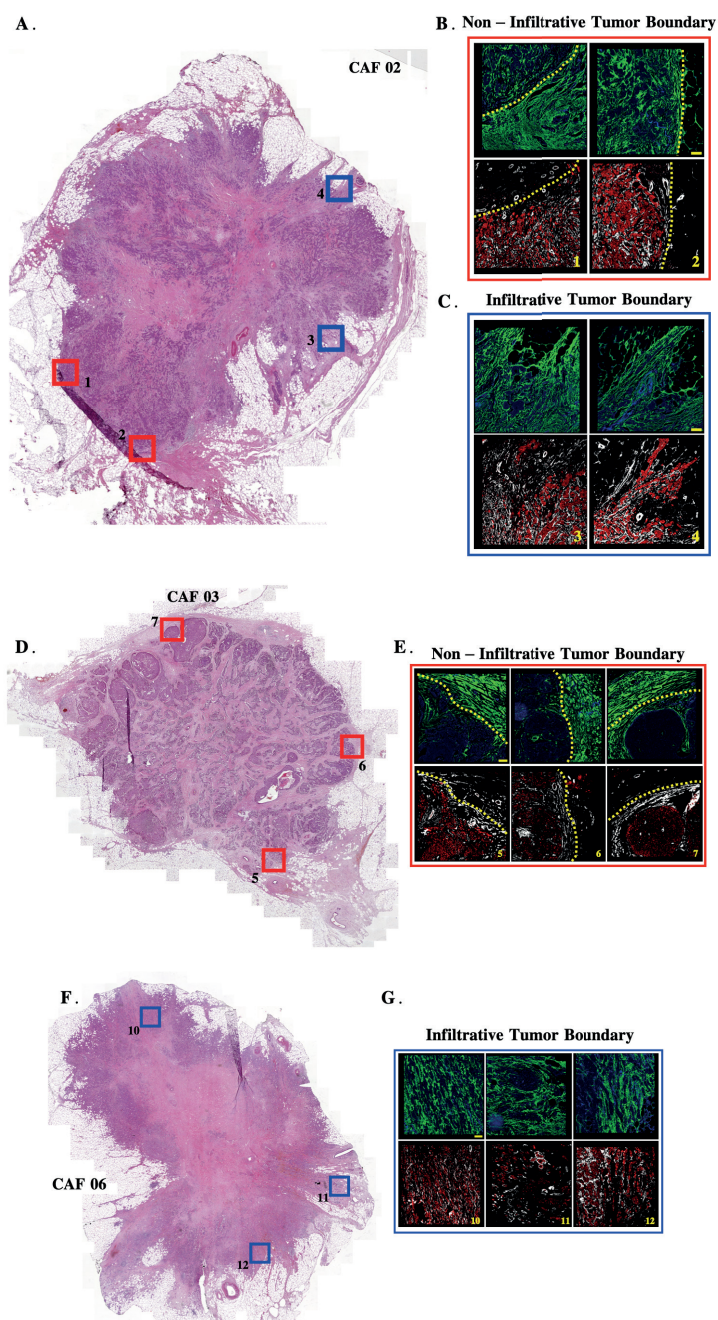


Figure S2. Tumor tissue section of Tumor ID CAF02, CAF03 and CAF06 with highlighted Tumor boundaries and their respective zoomed in ROIs.

A. Haematoxylin and Eosin (H&E) stained tissue section of Tumor ID CAF02 displaying both non-infiltrative (green) and infiltrative (blue) tumor boundaries.

- B. Picrosirius red (PSR) stained tissue sections depicting collagen fiber bundles at noninfiltrative tumor boundaries. Multiplex immunofluorescence imaging of pCK (Red) and α -SMA (White) depicting cancer cell and CAF organization at non-infiltrative tumor boundaries. Yellow lines indicate tumor boundaries.
- C. PSR stained tissue sections depicting collagen fiber bundles at infiltrative tumor boundaries. Multiplex immunofluorescence imaging of pCK and α -SMA depicting cancer cell and CAF organization at infiltrative tumor boundaries.
- D. H&E stained tissue section of Tumor ID CAF03 displaying only non-infiltrative tumor boundaries.
- E. PSR stained tissue sections depicting collagen fiber bundles at non-infiltrative tumor boundaries. Multiplex immunofluorescence imaging of pCK (Red) and α -SMA (White) depicting cancer cell and CAF organization at non-infiltrative tumor boundaries indicated by the yellow dotted line.
- F. H&E stained tissue section of Tumor ID CAF06 displaying only infiltrative tumor boundaries.
- G. PSR stained tissue sections depicting collagen fiber bundles at infiltrative tumor boundaries. Multiplex immunofluorescence imaging of pCK and α -SMA depicting cancer cell and CAF organization at infiltrative tumor boundaries.

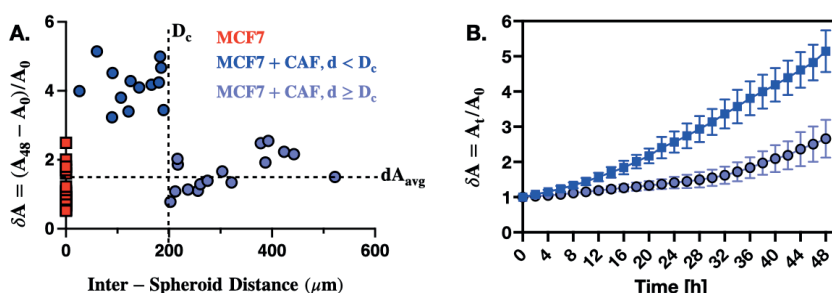


Figure S3. MCF7 spheroid expansion as a function of inter-spheroid distance.

- A. Relative expansion of MCF7 spheroids after 48 hours plotted against inter-spheroid distance (μm). MCF7 spheroids cultured alone (red) are compared with co-cultures containing 19TT CAFs located at distances below ($d < D_c$, dark blue) or above ($d \geq D_c$, light blue) the critical interaction distance $D_c = 200 \mu\text{m}$. Dashed lines denote D_c and the average expansion (dA_{avg}).
- B. Time-resolved expansion dynamics of MCF7 spheroids in the presence of CAFs for $d < D_c$, $d \geq D_c$. A pronounced increase in expansion rate is observed beyond 10 hours for $d < D_c$ co-cultures. Error bars represent mean $\pm$ SD.

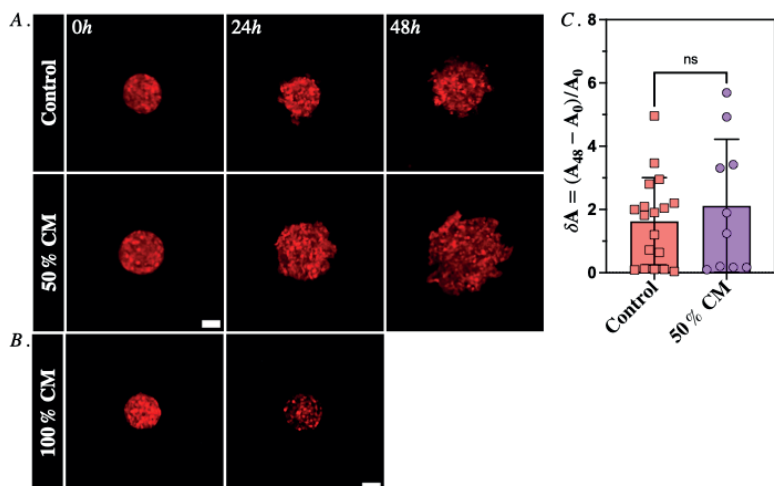


Figure S4. Stimulation by CAF Conditioned Medium (CM) does not have a statistically significant effect on MCF7 spheroid expansion.

- A. M7 spheroids embedded in collagen matrices were stimulated with CM obtained from CAF culture. As shown in the figure, 50% CM (1:1 dilution of conditioned medium in culture medium) had little effect on M7 spheroid expansion and no effect on M7 single cell dissemination compared to negative controls.
- B. The use of 100% CM on M7 spheroids resulted in significant cell death after 24 h and thus were excluded from further analysis.
- C. Quantification of M7 spheroid expansion ($n = 18$, $N = 3$ for control, $n = 9$, $N = 3$ for 50% CM) showed, on average, a slightly higher peak for M7 spheroids stimulated with CM but results were highly inconsistent with M7 spheroids control also showing similar behaviour ($p = 0.7116$).

Scale Bar = 100 μ m

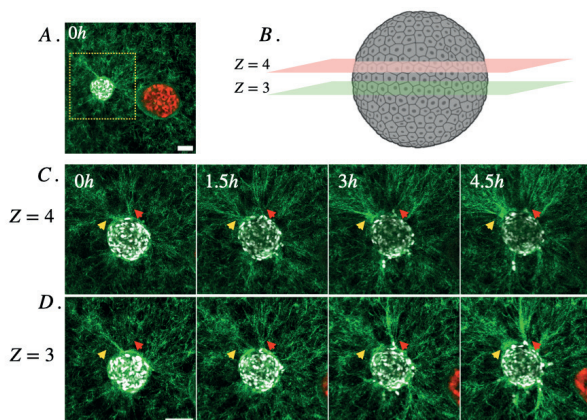


Figure S5. CAF spheroids aggregate collagen fibers from multiple focal planes to create thick collagen bundles.

- A. Overview of 19TT-CAF and MCF7 cancer cell spheroids embedded in collagen matrix at T_0 . Yellow square highlights the ROI corresponding to C and D.
- B. Visualization of consecutive Z focal planes 3 and 4. Each slice is $20\mu\text{m}$ thick.
- C. CAF and Collagen visualized at $Z = 4$. Yellow arrows correspond to the collagen fibers that are pulled into the imaging plane at $Z = 4$ while red arrows show collagen fibers that are pulled out of the $Z = 4$ focal plane.
- D. CAF and Collagen visualized at $Z = 3$. Yellow arrows correspond to the collagen fibers that are pulled out of the focal plane at $Z = 3$ while red arrows show collagen fibers that are pulled out of the $Z = 3$ imaging plane.

Scale Bar = $100\mu\text{m}$

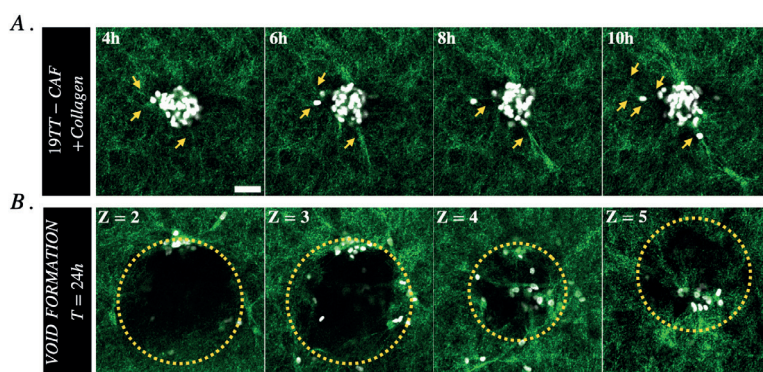


Figure S6. 19TT-CAF spheroids remodel and subsequently degrade the collagen matrix.

- A. Time-lapse reflection images (0–10 hours) of 19TT-CAF spheroids embedded in collagen (3 mg/ml). Yellow arrows indicate remodeled collagen fibers and subsequent CAF migration along these aligned tracks.
- B. Z-planes ($Z = 2 - 5$; Z-step size (voxel thickness) = $20\mu\text{m}$) acquired after 24 hours show progressive void formation within the collagen matrix (yellow dashed circles). The largest void corresponds to the plane with maximal CAF activity ($Z = 3$). Scale bar = $100\mu\text{m}$.

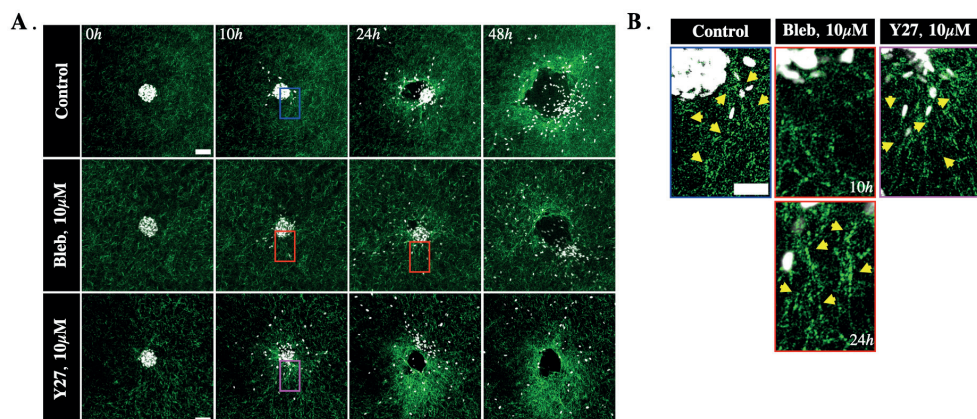


Figure S7. Actomyosin contractility is required for early collagen bundling.

- A. Time-lapse reflection images (0 - 48 hours) of 19TT-CAF spheroids embedded in collagen (3 mg/ml) under control, Blebbistatin (10 μ M), or Y-27632 (10 μ M) treatment. Representative frames at 10, 24, and 48 hours are shown. Collagen bundling and void formation are evident in control spheroids, delayed under Blebbistatin, and preserved but thinner under Y-27632. Scale bar = 100 μ m.
- B. Zoomed insets from boxed regions in A (control = blue, Y-27632 = pink, Blebbistatin = red) at 10 hours highlight collagen fiber bundling (yellow arrowheads). Realigned collagen fibers are visible at 10 hours, but absent in Blebbistatin, at 10 hours in the control and Y-27632 groups but lacking in the Blebbistatin group. In Blebbistatin, bundles emerge only at 24 hours (inset). Scale bar = 50 μ m.

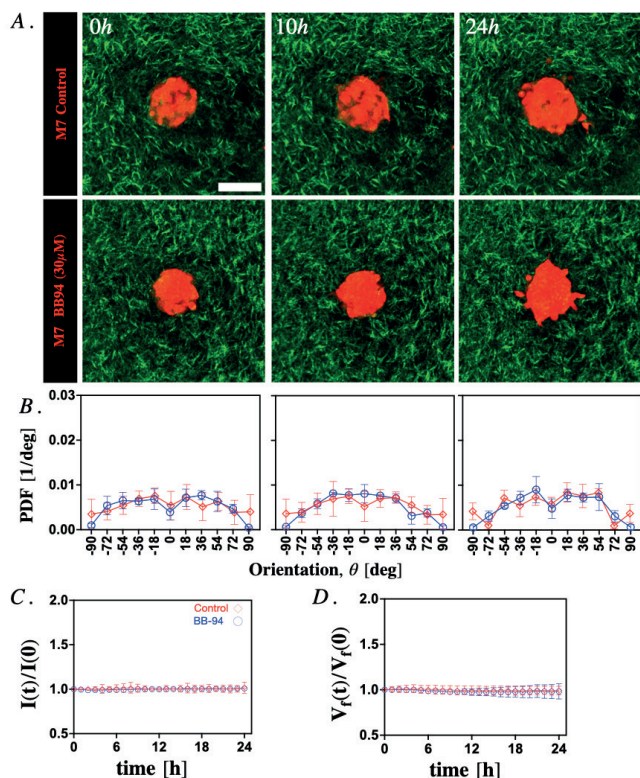


Figure S8. M7 spheroids embedded in collagen matrix treated with broad spectrum MMP inhibitors (BB94, 30 μ M)

- Representative time-lapse images of Vehicle Control and BB94 treated MCF7 spheroids in collagen matrix at 0, 10 and 24 hours.
- Relative orientation of collagen fibers with respect to MCF7 spheroid centroid at time 0, 10 and 24 hours. Veh Control ($n = 12$) and BB94 treated ($n = 5$) graphs are in Red and Blue respectively.
- Pixel Intensity of collagen fibers over 24 hours. Veh Control ($n = 12$) and BB94 treated ($n = 5$) graphs are in Red and Blue respectively.
- $V_f(t)/V_f(0)$ of collagen ECM over 24 hours. Veh Control ($n = 12$) and BB94 treated ($n = 5$) graphs are in Red and Blue respectively.

Scale Bar = 100 μ m

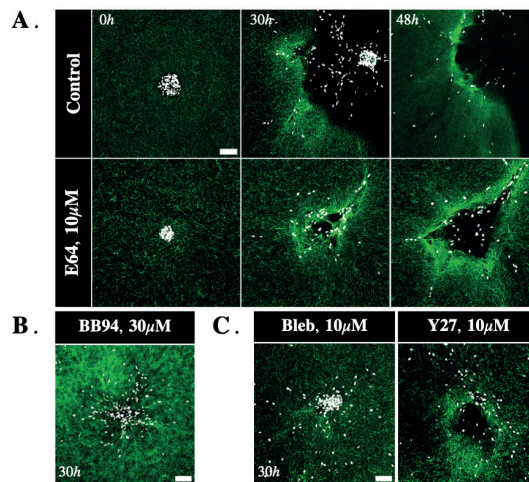


Figure S9. 19TT-CAF migration persists despite protease or contractility inhibition.

- A. Time-lapse reflection images (0–48 hours) of 19TT-CAF spheroids embedded in collagen (3 mg/mL) under control and E-64 (10 μ M) treatment.
- B. Representative frames at 0, 30, and 48 hours are shown. Scale bar, 100 μ m.
- C. Representative 19TT-CAF spheroid at 30 hours under BB-94 (30 μ M). Broad-spectrum MMP inhibition suppressed collagen degradation but did not block CAF dissemination. Scale bar, 100 μ m. Representative spheroids at 30 h under Blebbistatin or Y-27632. Inhibition of non-muscle myosin II or ROCK did not impair CAF motility.

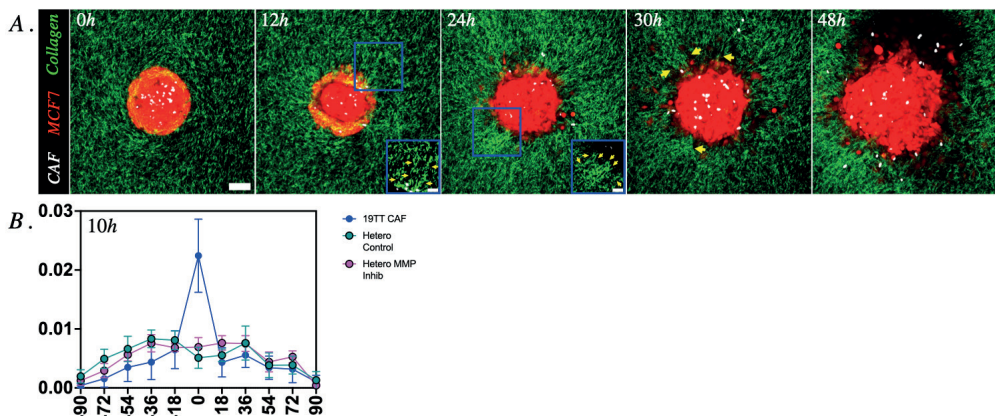


Figure S10. 48 hours timelapse of a heterospheroid embedded in 3 mg/mL collagen.

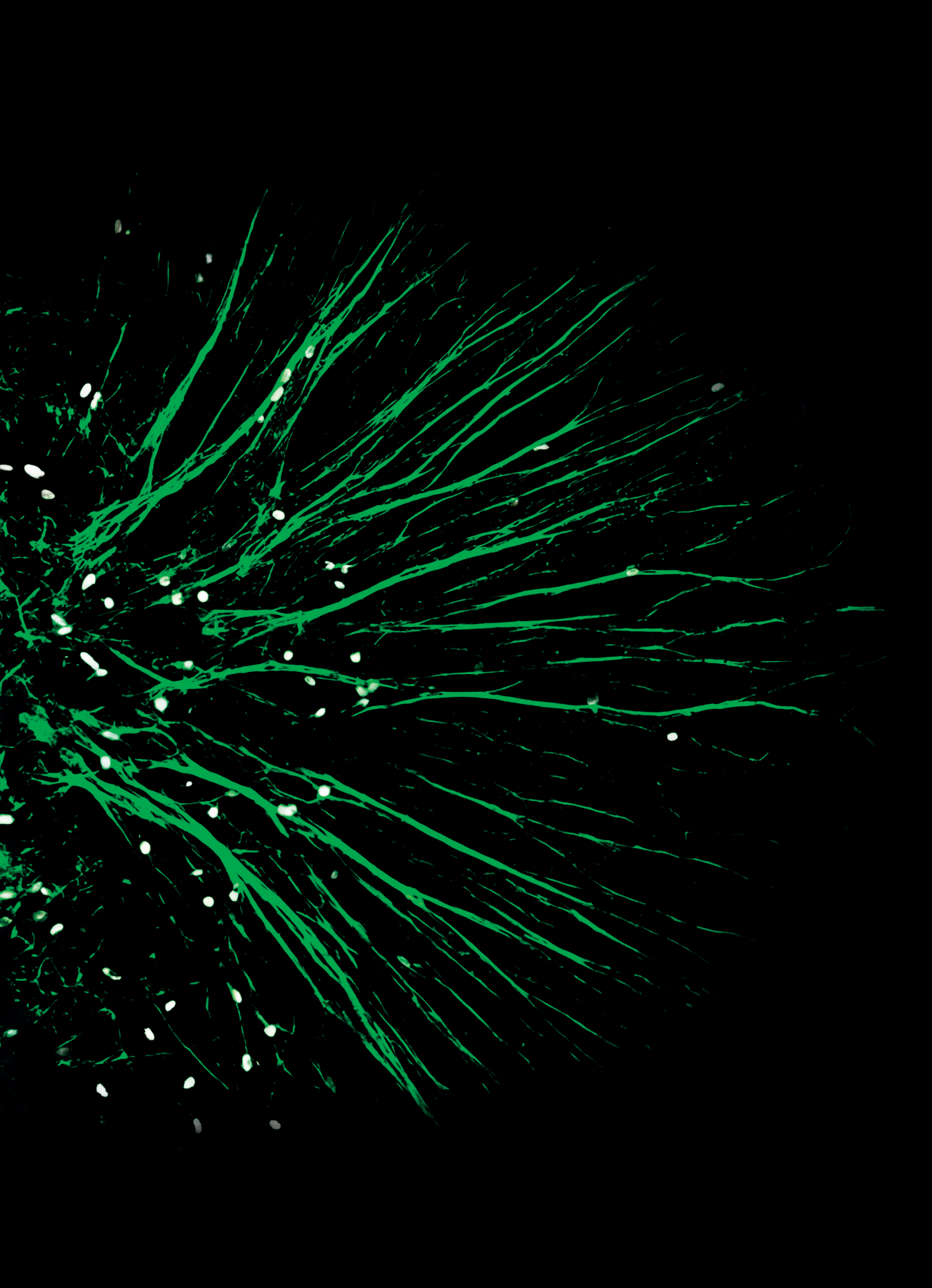
- A. 19TT CAF cells in White, MCF7 cells in Red and Collagen in Green. Yellow arrows in inset images highlight the reoriented collagen fibers around the heterospheroid at 12 and 24 hours. Reoriented collagen bundles, visible at 30 hours, are highlighted with yellow arrows.
- B. Collagen fiber orientation after 10 hours with respect to heterospheroid centroid (green = control, pink = MMP) and 19TT CAF (blue) spheroid embedded in 3 mg/mL collagen gel.

Table S1. Summary of inhibitor-specific effects on collagen remodeling, degradation, and CAF dissemination.

Assay	Collagen remodeling (t = 4 – 10 h)	Collagen degradation (t > 10 h)	19TT-CAF dissemination
Control	Robust bundling, aligned fibers	Prominent void formation	Progressive dissemination
Blebbistatin	Delayed onset and weak bundling	Still observed, comparable to controls	Dissemination is still observed, marginally more than in controls
Y27632	Bundling persists, comparable to controls	Still observed, comparable to controls	Dissemination present, comparable to controls
Batimastat	Bundling persists, comparable to controls	Blocked – No void formation	Dissemination present, comparable to controls
E64	Bundling persists, comparable to controls	Void formation still occurs, similar to controls	Dissemination present, comparable to controls

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Chapter 3

Visualizing dynamic changes during TGF- β -induced epithelial to mesenchymal transition

Abhishek Sinha^{1*}, Pranav Mehta^{1,2*}, Chuannan Fan¹, Jing Zhang¹, Dieuwke L Marvin¹, Maarten van Dinther¹, Laila Ritsma¹, Pouyan E. Boukany² and Peter ten Dijke^{1†}

¹ Oncode Institute, Department of Cell and Chemical Biology, Leiden University Medical Center; Leiden, The Netherlands

² Department of Chemical Engineering, Delft University of Technology, van der Maasweg 9, 2629 HZ Delft, the Netherlands

*These authors contributed equally.

†Address for correspondence:

Peter ten Dijke,
Dept. Cell and Chemical Biology,
Leiden University Medical Centre, Eindhovenweg 20,
2333 ZC Leiden, The Netherlands.
Tel: +31 71 526 9271; Email: p.ten_dijke@lumc.nl

Abstract

Epithelial to mesenchymal transition (EMT) is crucial during embryonic development, tissue fibrosis, and cancer progression. Epithelial cells that display a cobblestone-like morphology can undergo a switch to mesenchymal-like phenotype, displaying an elongated spindle shape or a fibroblast-like morphology. EMT is characterized by timely and reversible alterations of molecular and cellular processes. The changes include loss of epithelial and gain of mesenchymal marker expression, loss of polarity, increased cell migratory and invasive properties. Epithelial cells can progress unevenly during this transition and attain hybrid E/M states or metastable EMT states, referred to as epithelial cell plasticity. To gain a deeper insight into the mechanism of EMT, understanding the dynamic aspects of this process is essential. One of the most prominent factors to induce EMT is the cytokine transforming growth factor- β (TGF- β). This chapter discusses molecular and cellular techniques to monitor TGF- β -induced signalling and EMT changes in normal and cancer cell lines. These methods include measuring the TGF- β -induced activation of its intracellular SMAD effectors proteins and changes in epithelial/mesenchymal marker expression and localization. Moreover, we describe assays of cell migration and dynamic reorganization of the actin cytoskeleton and stress filaments that are frequently part of the TGF- β -induced EMT cellular response.

Keywords: EMT, EMP, TGF- β , mesenchymal, metastasis, cell migration

Introduction

Epithelial to mesenchymal transition (EMT) is an essential cellular process during mammalian development, whereby tightly coupled cobblestone-shaped epithelial cells display a substantial change in their morphology, polarity, and motility to acquire a more motile fibroblast-like appearance [1]. This change is accompanied by a loss of epithelial and gain of mesenchymal marker expression [2] (Fig 1). EMT plays a pivotal role in physiological processes such as embryogenesis and wound healing and is reactivated in pathological processes such as tissue fibrosis and cancer cell invasion and metastasis. EMT is a dynamic and reversible process, and a reversal of the cell state (from mesenchymal to epithelial) is called mesenchymal to epithelial transition (MET). The dynamic cell behaviour and plasticity often result in most cells acquiring hybrid epithelial-mesenchymal (E/M) characteristics. This phenomenon led to The EMT International Association (TEMTIA) proposing the term epithelial-mesenchymal plasticity (EMP) to underscore these E/M mixed phenotypic states [3]. This plasticity emphasizes the occurrence of hybrid E/M states and metastable EMT states [4] leading to express epithelial features while also displaying mesenchymal markers.

The cytokine transforming growth factor- β (TGF- β) is an important driver of EMT [5]. TGF- β elicits its cellular responses by specific binding to cell surface TGF- β type I and type II receptors (i.e., T β RI and T β RII) that are endowed with intrinsic serine/threonine kinase activity. Upon TGF- β -induced heteromeric complex formation the T β RII kinase trans-phosphorylates T β RI which subsequently induces the phosphorylation of receptor-regulated SMAD2 and SMAD3. This phosphorylation occurs at the two most carboxy-terminal serine residues in SMAD2 and SMAD3 [6]. Antibodies recognizing the C-terminally phosphorylated SMAD2 are often used to investigate if cells are responsive to TGF- β [7]. Upon phosphorylation, SMAD2 and SMAD3 form heteromeric complexes with SMAD4, which translocate into the nucleus [8]. These nuclear SMAD complexes act as transcription factors (TFs), and in cooperation with other DNA binding TFs and co-activators and co-repressors regulate target gene expression [9]. SMAD3 and SMAD4 bind directly to DNA to a so-called “CAGA” containing motif. Multimerization of such motif in front of a minimal promoter is sufficient to make the artificial transcriptional reporter construct highly sensitive to and selective for TGF- β /SMAD transcriptional activation [10]. A frequently used method to interrogate the TGF- β -induced transcriptional activation in cultured cells is to transfect or infect them with a CAGA₁₂-Luciferase transcriptional reporter construct. Alternatively, the luciferase can be replaced by fluorophores like enhanced Green Fluorescent Protein (eGFP) or Td-Tomato (Marvin DL et al, in Preparation) [11]. The intensity of luciferase luminescence or fluorescence signal is used to measure the level of transcriptional activation that occurs.

Activated SMAD complexes stimulate the expression of EMT-inducing TFs (EMT-TFs), such as SNAIL, SLUG, ZEB1/2 and TWIST1/2 [12]. These EMT-TFs mediate the repression of epithelial markers such as E-CADHERIN and Zona Occludens (ZO) and increase the expression of mesenchymal markers such as N-CADHERIN, α -smooth muscle actin (α SMA) and VIMENTIN [13]. E-CADHERIN, a transmembrane protein that directly interacts with cadherins of neighbouring cells, interacts with various regulatory proteins, notably β -CATENIN - which plays a vital role in stabilizing adherens junctions [14]. In the nucleus, β -CATENIN increases the transcription of EMT-inducing factors *SLUG*, *TWIST*, *FIBRONECTIN* and Matrix Metalloproteinases (MMPs). Canonical TGF- β signalling pathways promote the expression of Fascin, which is subsequently stabilized by the Rho GTPases [15]. Fascin, an actin-bundling protein, is implicated in many cancers such as breast, colon, gastric, and oral squamous cell carcinomas. It promotes metastasis by forming stable F-actin bundles and thus playing a critical role in forming filopodia, which subsequently regulates the invasiveness of cells [16].

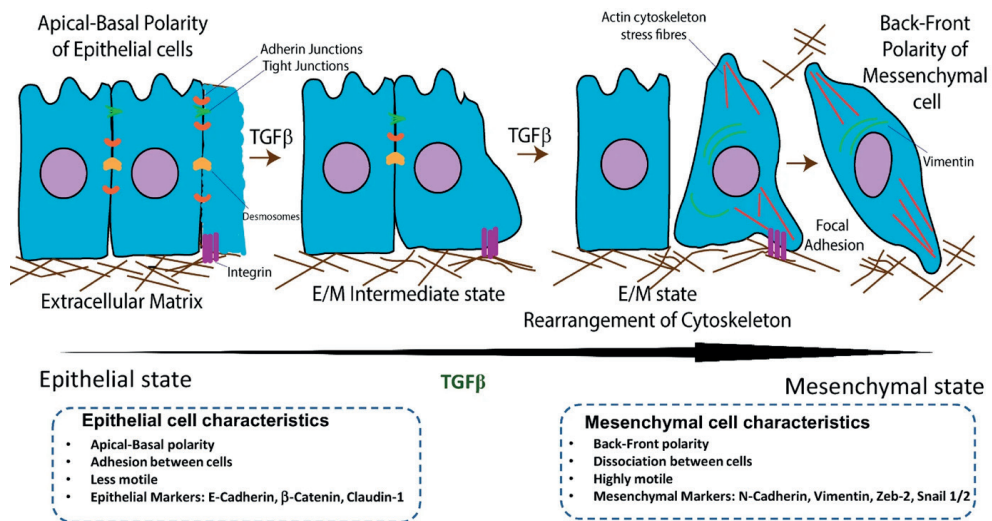


Figure 1 Schematic diagram of epithelial to mesenchymal transition (EMT). The intermittent and high plasticity of this process is highlighted. Characteristic changes of this transition process, such as change of polarity (from apical-basal to back-front), are shown. The cytokine TGF- β is a main driver of EMT.

TGF- β is also able to activate the non-SMAD pathways that contribute to EMT. These include but are not limited to the phosphatidylinositol 3-kinase (PI3K)/AKT, RHOA GTPase signalling, and p38 MAPK pathways [17-19]. Activation of RHO GTPases RAC and CDC42 are crucial to stimulate cell motility and invasiveness [20]. RHO GTPases can also be indirectly activated in a SMAD-dependent manner by upregulation of guanine exchange factor (GEF) NET1 that subsequently activates RHO [21].

Protrusions like filopodia such as protostomes, invadopodia and, lamellipodia are also a characteristic of EMT and are linked to invasion [22]. These protrusions are formed and maintained by change in the composition, orientation, and dynamics of cytoskeletal components. Cytoskeletal intermediate filaments (IFs), mediated by the induction of exchange factor activation of RHO kinase, also undergo significant compositional change as epithelial cells undergo EMT. Cells undergoing EMT initiate the expression of VIMENTIN (VIM) IFs, a downstream target of the canonical TGF- β signalling pathway (Figure 1) [23]. This dramatic change in intermediate filaments composition has led to VIM expression becoming a frequently used mesenchymal marker.

The above described molecular and cellular changes can be observed in two frequently used models for TGF- β -induced EMT, which are NMuMG, a mouse mammary gland epithelial cell line, and A549, a human lung adenocarcinoma cell line [24,25]. This chapter discusses experimental techniques that can be used to ascertain the dynamic alterations that occur upon TGF- β induced intracellular SMAD signalling and EMT. Methods to demonstrate different EMT markers' expression changes, changes in cell motility, shape, and cytoskeletal properties will be presented. The mouse mammary gland NMuMG and human lung adenocarcinoma A549 cell lines are used as examples. Whereas the TGF- β -induced SMAD2 phosphorylation peaks in most cells at 45 minutes to one hour and thereafter subsides, the TGF- β induced transcriptional response becomes more prominent after multiple hours of stimulation. TGF- β -induced changes in cell morphology, migration, changes and epithelial and mesenchymal markers become apparent in NMuMG and A549 cells after 1-2 days of treatment. TGF- β and other cytokines have been shown to induce (or synergize in) EMT responses in multiple other cell lines. Moreover, endothelial cells can also have a similar transition, termed endothelial to mesenchymal transition [26]. The procedures detailed here can also be applied (with slight modifications) for these cell types and other EMT stimuli.

Materials

2.1 Cell Lines:

1. Mouse mammary gland epithelial cells NMuMG cell line (ATCC®-CRL-1636).
2. Human lung adenocarcinoma A549 cell line (ATCC®-CCL-185)
3. A549 cells expressing red fluorescent tagged Vimentin (RFP-VIM) (ATCC®-CCL-185EMT)

2.2 Cell Culture:

1. Culture Medium: Dulbecco's Modified Eagle medium supplemented with 10% Fetal bovine serum (FBS), and 100u/ml penicillin and 0.1 mg/ml streptomycin (Pen/Strep).

2. Sterile phosphate buffer saline (PBS): For 1 litre, 8g NaCl, 0.2g KCl, 1.44g Na₂HPO₄, 0.24g KH₂PO₄. pH 7.2 adjusted with 1N HCl
3. Trypsin and Ethylenediaminetetraacetic acid (EDTA) in PBS: 0.25% Trypsin, 0.02% (0.53 mM) EDTA solution in PBS pH 7.4 solution.
4. Ligand Buffer: 4 mM HCl, 0.1% bovine serum albumin (BSA).
5. TGF-β3 [Gift from Andrew Hinck, University of Pittsburgh] (5μg/ml) stock prepared in ligand buffer.
6. Cell culture incubator at 37°C with 5% CO₂ level.

2.3. Reporter and Constructs

1. pGL3-CAGA₁₂ vector for CAGA-luciferase assay [27].
2. pLV-(CAGA)₁₂-eGFP construct was used to generate lentivirus mediated stable transfectant of (CAGA)₁₂-eGFP containing NMuMG cells (Marvin DL et al. Under preparation)
3. pCMV-β-galactosidase (gal) construct [28].

2.4 Western Blotting:

1. Lysis buffer: 20mM Tris-Cl pH 7.4, 75mM NaCl, 1% NP40, 0.1% Sodium Dodecyl Sulfate, 0.5% sodium deoxycholate containing a protease inhibitor cocktail.
2. Western blotting membrane washing buffer: Tris-buffered saline [20mM Tris-Cl pH 7.4, 150 mM NaCl] with 0.1% Tween 20 (TBST)
3. Blocking buffer: 5% non-fat dry milk in TBST.
4. Protein estimation: DC Protein Assay Reagent (BioRad)
5. Colorimetry Measurements (protein estimation or other luminescence assays): Victor Multilabel Plate Reader (Perkin Elmer)
6. Polyvinylidene Difluoride (PVDF) membrane.
7. Antibodies: E-Cadherin (BD Bioscience, 610181), N-Cadherin (BD Bioscience, 610920), Vimentin (Cell Signaling, #5741), phospho-SMAD2 antibody (raised antibody against phospho-SMAD2)[7], GAPDH (Merck Millipore, MAB374).
8. Clarity Western Enhanced Chemiluminescence substrate (BioRad)
9. Western Blot Imaging: ChemiDoc Imaging System (BioRad)

2.5 Real time Polymerase Chain Reaction (Q-PCR):

1. cDNA synthesis: Deoxynucleotide triphosphates (dNTPs) (Invitrogen), Revert Aid First Strand cDNA Synthesis Kit (Thermo Fischer Scientific).
2. Nucleic Acid Concentration measurement: NanoDrop 2000/2000c Spectrophotometers (Thermo Scientific)
3. RT-qPCR: GoTaq Q-PCR MasterMix (A600X, PROMEGA). Hard-Shell 384 well Q-PCR plates (BioRad) are used.
4. Primers (Amplification of EMT marker genes): See Table 1.

5. CFX connect Real time PCR detection system (BioRad)

2.6 Cell Staining:

1. Fixing: Formaldehyde solution, Triton X-100.
2. Staining: 4'-diamidino-2-phenylindole (DAPI) containing mounting medium (Vector Laboratories), Alexa Fluor 555 secondary antibody (Thermo Scientific) and Alexa Fluor 488 Phalloidin (Thermo Scientific).
3. Sterile glass slides and coverslips.
4. Confocal imaging microscope SP8 (Leica).

2.7 Wound Healing/scratch assay:

1. PBS: (pH 7.2).
2. 96 well cell culture plates compatible with scratch maker (Sartorius).
3. Automated scratch making system (Sartorius/Essen Bioscience).
4. Imaging: IncuCyte® incubator imaging system (Sartorius/Essen Bioscience).

Table 1. Sequence of primers used in the Q-PCR analysis of genes in mouse NMuMG cells.

Sl No.	Primer name	Purpose	Sequence 5'- > 3'
1	<i>Cdh1</i> Fwd	Forward primer for <i>Cdh1</i> Q-PCR	ACCAAAGTGACGCTGAAGTC
2	<i>Cdh1</i> Rvse	Reverse primer for <i>Cdh1</i> Q-PCR	GAGGATGTACTTGGCAATGG
3	<i>Snail</i> Fwd	Forward primer for <i>Snail</i> Q-PCR	CAGCTGGCCAGGCTCTCGGT
4	<i>Snail</i> Rvse	Reverse primer for <i>Snail</i> Q-PCR	GCGAGGGCCTCCGGAGCA
5	<i>Zeb2</i> Fwd	Forward primer for <i>Zeb2</i> Q-PCR	TTCTGCAAGCCTCTGTAGCC
6	<i>Zeb2</i> Rvse	Reverse primer for <i>Zeb2</i> Q-PCR	TTCTGGCCCCATTGCATCAT
7	<i>Gapdh</i> Fwd	Forward primer for <i>Gapdh</i> Q-PCR	TGGCAAAGTGAGATTGTTGCC
8	<i>Gapdh</i> Rvse	Reverse primer for <i>Gapdh</i> Q-PCR	AAGATGGTGATGGGCTTCCCG

Methods

Before analysing TGF- β induced EMT, it is recommended to check if cells respond to TGF- β treatment. To do this, we can perform the following experiments:

- a) TGF- β -induced SMAD2 phosphorylation,
- b) SMAD3/SMAD4-dependent CAGA₁₂-luciferase activity,
- c) SMAD3/SMAD4-dependent CAGA₁₂-eGFP activity.

A positive result in the above assays only examines the activation of the canonical TGF- β /SMAD signalling pathway. However as discussed above, it is possible for TGF- β to also act via non-SMAD (SMAD-independent) pathways, and this can contribute to the EMT response. Thus, TGF- β activity on activation of non-SMAD signalling pathways can also be tested, before examining EMT. TGF- β induced EMT can be examined by:

- a) Measuring transcriptional changes of EMT target genes,
- b) Quantifying changes in protein expression of genes encoding for epithelial and mesenchymal markers,
- c) Cell morphology analysis,
- d) Analysing the cell migratory behaviour.

TGF- β -induced SMAD activation

Analysis of activation of SMAD2 phosphorylation by western blotting

1. Seed 10 mm culture plates containing DMEM medium with NMuMG cells (1×10^6 cells per plate) and allow to attach at the bottom surface by overnight incubation at 37°C cell culture incubator.
2. Replace the medium the following day with fresh DMEM containing either TGF- β (5 ng/ml) or ligand buffer.
3. Harvest cells at specific time points in accordance following the method detailed below.
4. Transfer plates to ice, takeout the culture medium and wash with 1ml of cold PBS.
5. Aspirate the PBS, then add ice-cold lysis buffer. Let the plates sit for 20 minutes on ice to complete the lysis reaction.
6. Scrape cells off the bottom surface of the dish using a plastic cell scraper, then gently transfer the cell lysate into a 1.5 ml microfuge tube.
7. Centrifuge at 12×10^3g at 4°C for 15 minutes and transfer the supernatant to a fresh microfuge tube.
8. Measure the protein concentration of the lysates using a protein quantification assay.
9. Load equal amounts of protein (25 μ g of total protein from cell lysate) from each sample into the wells of 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and run it at a constant voltage of 100V.
10. Once the run is complete, transfer the protein from the SDS-PAGE gel to a pre-soaked (methanol) polyvinylidene difluoride (PVDF) membrane at a voltage of 100V for 1.5 hours minutes at 4°C. Alternatively, nitrocellulose membranes can also be used.
11. Protein transfer to the membrane can be verified (before blocking) using Ponceau S.
12. Block the membrane for 1 hour at room temperature or overnight at 4°C using the blocking buffer.
13. Wash the membrane with TBST for 5 minutes.
14. Incubate the membrane with primary antibody prepared in blocking buffer for overnight at 4°C or at room temperature for 1.5-2 hours.

15. Wash the membrane thrice for 5 minutes each in TBST.
16. Incubate the membrane with the appropriate conjugated secondary antibody in the appropriate blocking buffer (follow manufacturer's instructions) at room temperature for 1 hour.
17. Wash the membrane three times for 5 minutes each in TBST.
18. For signal development, incubate 1ml of western Enhanced Chemiluminescence (ECL) substrate (BioRad) for 1 minute.
19. The signals can be detected using the ChemiDoc Imaging System (BioRad). Protein expression varies between cell lines and is also dependent on the treatment. Thus, the exposure time must be optimized for different blots. Figure 3B shows the change in expression of EMT markers post TGF- β treatment. (Note 1,2)
20. For statistically significant results, perform three independent repeats. Band intensities can be measured using Image-J software

TGF- β /SMAD-induced activation of CAGA₁₂-luciferase transcriptional reporter activity

1. Harvest mammalian/mouse cancer cells at 80% confluency using trypsin-EDTA. (Note 3)
2. Count the harvested cells and resuspend them to create a solution containing 1×10^5 cells/ml of culture medium.
3. Mix the suspension thoroughly and seed 2ml of the solution in a 6-well plate.
4. Incubate cells, at 37°C and 5% CO₂, overnight.
5. Transfect the incubated cells in each well with 500 ng of TGF- β /SMAD3 inducible (CAGA)₁₂ luciferase transcriptional reporter construct (pGL3-CAGA₁₂) and 200 ng of the β -galactosidase (bgal) expression construct (pCMV-bgal) using Lipofectamine 3000 reagent and following manufacturers instruction.
6. Incubate overnight.
7. Replace the reporter-containing culture medium with culture medium now containing either TGF- β (5ng/ml) or ligand buffer.
8. Incubate for 24 hours.
9. Aspirate culture medium and wash cells twice with PBS.
1. Lyse cells in each well by using 200 μ l of lysis buffer (see composition in 2.4). Keep the 6-well plate on ice for 20 minutes to complete the lysis reaction.
2. Collect the cell lysates in a 1.5ml microfuge tube using cell scrapers and spin in a microcentrifuge at 12×10^3 g at 4°C for 20 minutes.
3. Transfer supernatant to a fresh microfuge tube and measure the protein concentration of the extract.
4. Transfer 50 μ l (1 mg/ml concentration of protein) lysate to a 96-well plate, add the luciferin substrate (Sigma, Cat No. L9504) and measure luciferase activity using a plate reader (Victor, Perkin Elmer).

5. In a separate assay, measure β -galactosidase activity similarly.
6. Normalize luciferase activity to β -galactosidase activity to get quantitative change in CAGA-luciferase expression.

TGF- β /SMAD induced activation of CAGA₁₂-eGFP transcriptional reporter activity

1. Seed 1×10^4 NMuMG cells in 100 μ l of DMEM, stably expressing CAGA-eGFP, to a 96 well clear bottom black culture plate.
2. Allow the cells to adhere to the bottom surface by incubating in a 37°C incubator for 8-12 hours.
3. Replace media with media containing TGF- β (5ng/ml) or ligand buffer and transfer the plate to the IncuCyte life cell imager.
4. IncuCyte live cell imager can acquire images while the cells are maintained in a temperature-controlled CO₂ incubator.
5. Imaging can be performed with various time intervals.
6. Alternatively, after addition of media containing TGF- β (5 ng/ml) or ligand buffer the plates can be incubated in normal incubator and at specific time points the fluorescence intensity can be checked at fluorescence plate reader.
7. A representative result is presented in Figure 2: CAGA-eGFP signal is examined after addition of different concentrations of TGF- β at 0 h and 24 h in IncuCyte imaging platform and quantified using IncuCyte S3 software. But the same can be assayed for prolonged time in the same platform to understand the dynamics of expression change of eGFP with time using the same platform.

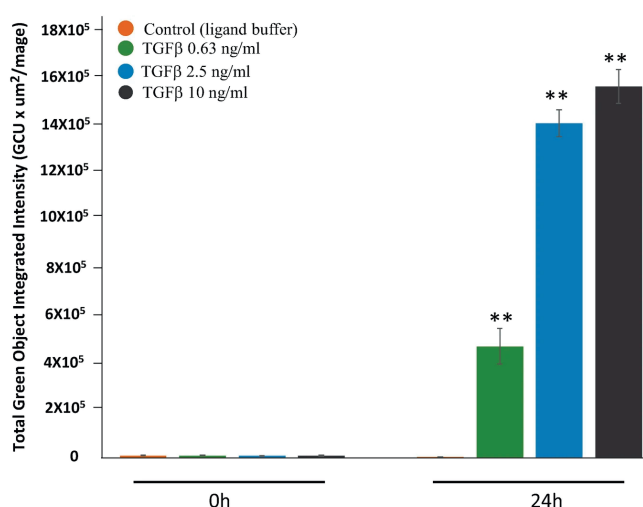


Figure 2. TGF- β /SMAD-induced transcriptional response in NMuMG cells. TGF- β /SMAD induced transcriptional response is monitored in NMuMG cells that stably express the eGFP fluorescent marker under transcriptional control of multimerized 5'-CAGA-3' Smad3/4 binding sites coupled to a minimal promoter. Three different concentrations of TGF- β were used, and eGFP levels were measured at 0h and 24h timepoints.

Real-time Reverse transcriptase PCR for TGF- β target genes and EMT markers

The experiment protocol is described with NMuMG cells. (*Note 4*)

1. Culture cells as described in Section 3.1.1 and allow them to attach overnight.
2. Next morning, treat cells with TGF- β (5ng/ml) or ligand buffer and incubate for 24 hours. (*Note 5*)
3. Discard the medium from each well and wash the cell pellet with 1ml of PBS.
4. Isolate RNA from cultured cells using NucleoSpin RNA isolation kit (Macherey-Nagel). Follow manufacturer's protocol.
5. Measure the RNA concentration using a micro-volume spectrophotometer.
6. Prepare cDNA using First strand cDNA synthesis kit. Follow manufacturer's protocol.
7. Using ten-fold diluted cDNA, prepare a reaction mixture containing specific forward and reverse primers of target genes (See Table 1 to check primer sequences) and GoTaq QPCR master-mix..
8. With the reaction mixture prepared, set up a Q-PCR.
9. Reaction conditions for the Q-PCR: Initialization at 95°C for 2 minutes; denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds and, extension at 80°C for 20 seconds with 40 cycles to be repeated.
10. For statistically significant results, perform three independent repeats as described in Figure 3A.
11. The results can be analysed using the Gene Set Enrichment Analysis (GSEA) 4.0.3 software (Broad Institute).

TGF- β -induced changes in EMT markers using western blot analysis.

1. Culture cells as described in Section 3.1.1, and isolate protein extracts as mentioned in the same section followed by western blotting.
2. For EMT marker protein expression analysis, use anti-E-cadherin, anti-N-cadherin, anti-Vimentin and anti-GAPDH primary antibodies.
3. Subsequent steps are also similar as Section 3.1.1.

Changes in the expression of EMT marker genes in response to TGF- β are shown in Figure 3B.

TGF- β -induced cell migration analysed using wound healing/scratch assay

1. For this experiment special types of scratch resistant 96 well plates are used, and real time imaging is conducted using IncuCyte® life cell imaging system. Images are taken every 2 hours for 2.5 days.
2. Seed 3×10^4 A549 cells in 96 well scratch resistant plates and add 100 μ l of culture medium to each well. (*Note 6*)

3. Incubate overnight (37°C and 5% CO₂), to allow the cells to adhere to the surface.
4. Aspirate medium and add 100µl of fresh culture medium containing 0.5% serum and antibiotic mix in each well.
5. Create a wound in the wells using an automated scratch maker.
6. After wound creation, aspirate culture medium and wash the wells with 50µl of prewarmed sterile PBS. (*Note 7*)
7. Aspirate the PBS (taking away all suspended cells).
8. Mix TGF-β (5ng/ml) or ligand buffer with fresh culture medium (DMEM containing 0.5% FBS and antibiotic mix) and add 100µl to each well.
9. Place the plate in the IncuCyte imaging system, maintaining growth conditions at 37°C with 5% CO₂ level inside the system. Setup a time-series, taking images in either brightfield or fluorescence based on the requirement.
10. The width of the wound can be calculated with the help of in-built software (IncuCyte S3) and the wound healing pattern can be graphed across different time points as shown in Figure 3C. The wound healing kinetics can also be presented in form of videos. A representative video taken using the IncuCyte live cell imaging system, showing change in cell shape and size upon TGF-β treatment is presented as **Video S1**. Images of wound healing are shown at Figure 3D for 48h time point.

TGF-β-induced induction of Vimentin in A549 VIM-RFP cells

A549 cells expressing Red Fluorescent Protein (RFP) labelled Vimentin are used for this experiment. This assay can be applied for other cell lines expressing different IF markers labelled with fluorescent tags.

1. Seed 1 x 10⁴ cells to 96 well culture plates containing fresh culture medium. (*Note 8, 9*)
2. Incubate overnight to allow the cells to adhere to the surface.
3. Following incubation, stimulate cells with TGF-β (5ng/ml) or ligand control and place the 96-well plate in the IncuCyte.
4. Create a time-series imaging sequence to capture images every 2 hours for 3 days with brightfield and in red fluorescence (TRITC) filters.
5. Analyse the results looking for changes in red fluorescence protein (RFP)-Vimentin expression for cells with and without TGF-β stimulant (Figure 4).
6. Time dependent changes of RFP-Vimentin expression can also be presented in the form of videos as shown in **Video S2 & S3**.

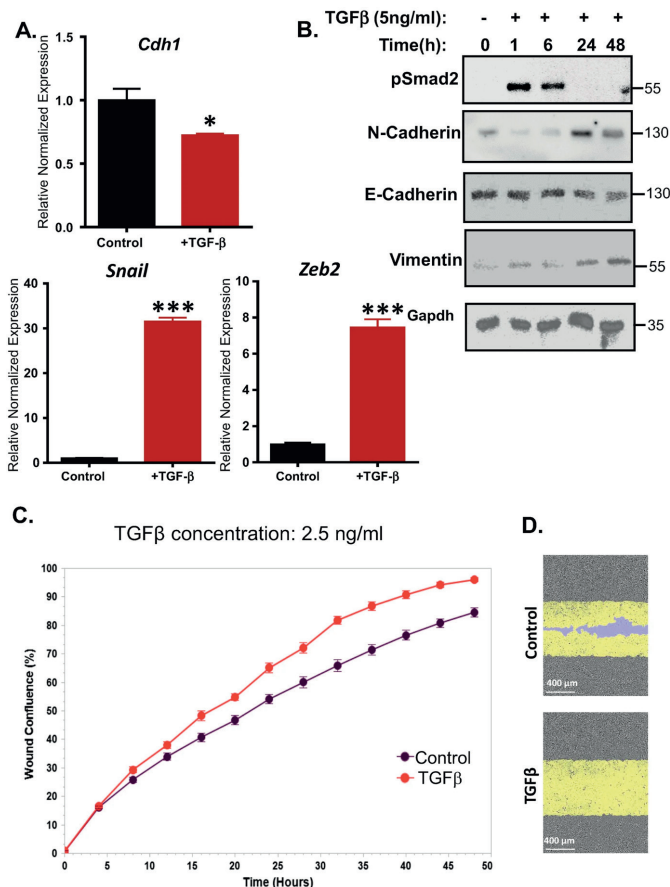


Figure 3. TGF- β induced changes in Smad2 phosphorylation, epithelial and mesenchymal marker expression, and cell migration of NMuMG cells.

- (A) Q-PCR analysis of expression levels of *Cdh1* (E-Cadherin), *Snail*, and *Zeb2* mRNA in NMuMG cells upon stimulation with TGF- β for 24 h. Figure adopted from Zhang et al. 2020 (<https://www.jove.com/v/61830/studying-tgf-signaling-tgf-induced-epithelial-to-mesenchymal>). The results are expressed as the mean $\pm$ s.d., $n = 3$. Student's t -test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.
- (B) Western blot analysis of expression levels of phospho-Smad2, N-Cadherin, E-Cadherin, Vimentin in response to TGF- β (5ng/ml) stimulation of NMuMG cells. GAPDH was included as the protein loading control.
- (C) Migration of A549 cells treated with either ligand buffer (Control) or TGF- β (2.5ng/ml).
- (D) Representative image of scratch at 48h time points of the same experiment as described in panel C. Grey: Cells, Yellow: scratch healed; Purple: final wound remaining.

TGF- β -induced changes in EMT marker localization using indirect immunofluorescence and cytoskeleton reorganization by direct fluorescence staining

For this experiment, NMuMG cells treated with either TGF- β or ligand buffer are stained at specific time points. The experimental protocol is as follows.

1. Place sterile 18 x 18 mm glass slips in 6-well plates (One coverslip in each well). (Note 10)
2. Seed 5 x 10⁴ cells in each well of the plate containing 2ml culture medium.
3. Incubate overnight to ensure cells adhere to the coverslip.
4. Following incubation, remove the coverslips and place them in a fresh 6-well plate.
5. Stimulate cells with a fresh culture medium containing either TGF- β (5ng/ml) or ligand buffer.
6. Incubate cells for two days.
7. Aspirate culture medium after two days and wash twice with prewarmed PBS.
8. Add 1ml of formaldehyde and incubate for 20 minutes at room temperature to fix cells.
9. Wash the cells twice with PBS.
10. Permeabilize cells using 0.1% Triton X-100 solution for 10 minutes at room temperature.
11. Aspirate the Triton solution, wash the cells once with PBS and block with 3% BSA solution in PBS for 45 minutes to 1 hour at room temperature.
12. Wash the cells twice with PBS.
13. Incubate the cells with anti-E-Cadherin antibody (1:1000 in PBS) by placing 100 μ l of antibody solution on top of coverslip.
14. Incubate for 1 hour at room temperature.
15. Remove the primary antibody solution and wash the slides three times with PBS (5 minute each wash).
16. Add Alexa Fluor 555 tagged secondary antibody solution in PBS (1:500 dilution) on the coverslips.
17. During the same time, stain the cells by adding Alexa Fluor 488 Phalloidin (1: 1000 dilution in PBS) on the coverslips and incubate the coverslips at room temperature for 1 hour in a dark cabinet in presence of both the fluorophore tags.
18. Wash the cells thrice with PBS.
19. Remove the coverslip from the 6 well plate and mount them onto fresh slides using a mounting medium.
20. Use a confocal microscope to image and compare the fluorescence intensity of the cells.
21. Actin reorganization was evident when 48h TGF- β -treated cells were compared with ligand buffer-treated cells (Figure 5).

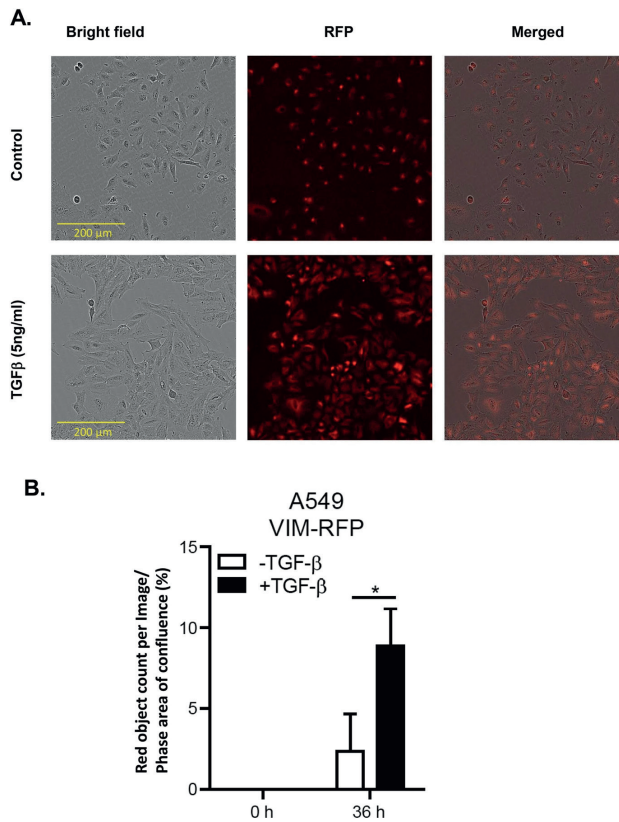


Figure 4. TGF- β induced morphological change and expression of VIMENTIN in A549 lung adenocarcinoma cells in which RFP is inserted into the endogenous locus of VIMENTIN.

- (A) A549 cells were stimulated with TGF- β (5 ng/ml) and changes in cell morphology were visualized by bright field images and appearance of RFP signal, indicative of Vimentin expression, is measured using a fluorescent microscope. Imaging was performed in an automated IncuCyte platform and the changes in cell morphologies and vimentin expression was evident from fluorescence images.
- (B) The expression of VIMENTIN-RFP was quantified using IncuCyte S3 software and signal levels of VIM-RFP were plotted at 0h and 36h time points. Results were analysed using One-way ANOVA followed by Tukey's HSD test (n=4) * represents $p < 0.1$

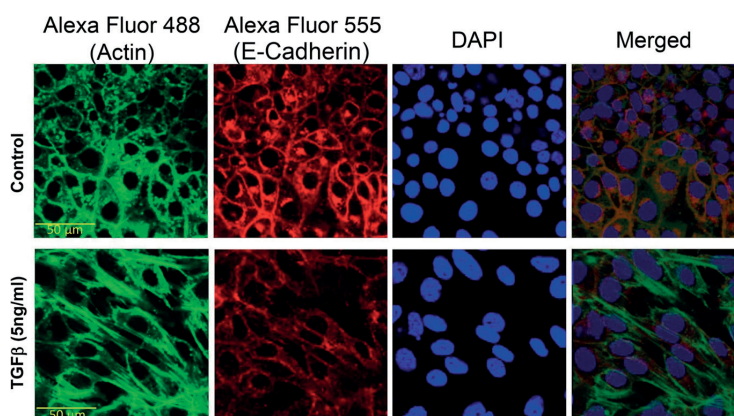


Figure 5 Changes in F-actin cytoskeleton arrangements and E-Cadherin expression in NMuMG cells treated with TGF- β . Confocal imaging of NMuMG cells treated with [ligand buffer or TGF- β (5ng/ml)] for 48hours and stained with Phalloidin (Alexa Fluor 488), anti-E-cadherin antibody (Alexa Fluor 555 tagged secondary antibody) and DAPI (nuclear staining). Actin cytoskeletons are stained in green, nucleus in blue and E-Cadherin in red colour.

Notes

1. As a positive control during PAGE running, cell lysate from cells transfected with constitutively active ALK5 (pcDNA3 with ALK5T204D) and SMAD2 can be used [27]. These cells will express high pSMAD2 levels. By taking the same aliquot along, this can serve as an internal standard to confirm that transfer of proteins from gel to PVDF membrane has occurred properly, compare the results from different experiments, and understand where the pSMAD2 signal runs on the western blot.
2. As a positive control during PAGE running, cell lysate from cells transfected with constitutively active ALK5 (pcDNA3 with ALK5^{T204D}) and SMAD2 can be used [29]. These cells will express high pSMAD2 levels. By taking the same aliquot along, this can serve as an internal standard to confirm that transfer of proteins from gel to PVDF membrane has occurred properly, compare the results from different experiments, and understand where the pSMAD2 signal runs on the western blot.
3. Depending on the gel thickness (0.8 mm/1mm/1.5 mm), expression levels of the target proteins, the sensitivity of an antibody, and the number of wells in the PAGE, one can vary the amount of protein to be loaded. The protein weight (25μg) stated here is used for the ten-well page with a thickness of 1.5 mm. Although we ran the PAGE at constant voltage, in some cases, it is preferable to run at much lower voltage till the dye front crosses the stacking gel and then increase the voltage to 100 volts.

4. Different immortalized cell lines vary in their growth and cell division kinetics. Similarly, their response to TGF- β also varies. For gene induction activity, usually serum-starved cells are used to obtain strong response. If cells need to be monitored for extended periods, serum can be added; it can also promote the TGF- β induced EMT response. For growth inhibition or stimulation also different serum concentrations are required. Very high serum concentration may compromise the observation of growth arrest/apoptotic or proliferation response. Similarly, the robustness and duration of TGF- β response are also time-dependent. In some cell lines, the reporter activity is induced rapidly (within 6 hours) and long lasting, but in others it can take longer or may be transient.
5. As discussed previously (*Note 3*) TGF- β response for certain cell lines requires low concentration of serum into the medium. Similarly, optimum TGF- β concentration also varies for different cell lines. For some cell lines transcriptional response can be observed at much lower concentration of TGF- β (1-2.5 ng/ml).
6. For some cell line the change in transcriptional response can start very fast within an hour, compared to 24 hours as we shown in our representative result with NMuMG cells. Hence standardization of optimum time points is necessary before conducting large-scale transcriptional response studies.
7. The cells must be close to 100% confluency to ensure optimal wound creation and examination of wound healing dynamics. Depending on the cell line, the number of cells seeded will vary, necessitating standardization before proceeding with the experiment.
8. Post wound creation, it is imperative to clear the cell debris for accurate measurements and imaging. To ensure this occurs, wash the 96-well plate two times with PBS.
9. 96-well plates in an IncuCyte live-cell imager are used in the protocol to visualize RFP-Vimentin expression changes at differing time points. Alternatively, this experiment can be conducted with various tissue culture plates in any microscope with a red fluorescence filter.
10. For microscopy experiments, it is recommended to seed fewer cells and begin observations at 50-60% confluency. Overcrowding can compromise the experiment as it affects cell shapes and sizes, making EMT visualization difficult.
11. It is preferable to use gelatin coated coverslips for cell attachments, as cells do not attach well on normal glass surface. Otherwise normal glass coverslips need to be

placed on 6 well plates, coat surface with 600 μ l of gelatin solution, incubate them for 1 hour at 37°C, aspirate the unattached solution and dry the coverslips by keeping the plates open in a hood. It will take 15-25 minutes for the coverslips to dry. After that the coverslips can be used immediately or can be kept for future experiments for 4 months.

Acknowledgments

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Conflict of Interests

The authors declare no conflict of interest.

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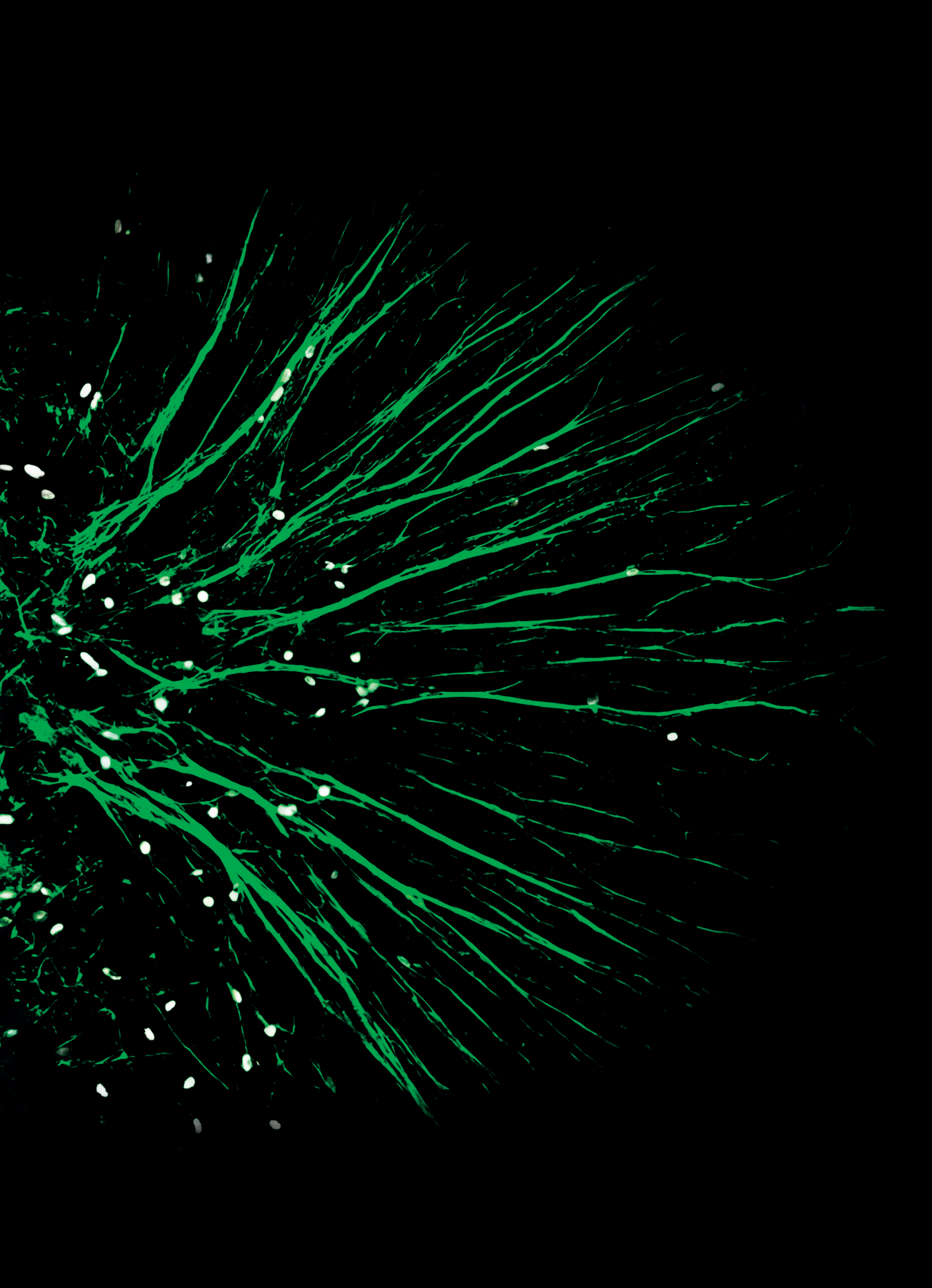
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Video S1 Changes in cell shape, size and morphology of NMuMG cells upon addition of TGF- β in DMEM medium containing 10% serum. The images were taken at an interval of 1 hour for 36 hours in IncuCyte live cell imaging platform and presented in the form of a video.

Video S2 and S3 A549 cells (with VIM-RFP) treated with either ligand buffer (S2) or TGF- β (S3) for 48 h and the changes in cell morphology and VIMENTIN expression are presented through these videos.



Chapter 4

Hidden TGF- β signaling in fibroblast lung cancer crosstalk

Conditioned medium (CM) induces dissemination of lung adenocarcinoma spheroids via receptor signaling without measurable TGF- β ligand



Pranav Mehta^{a,b}, Zaid Rahman^b, Ankit Sinha^b, Kristen David^b, Gerard van der Zon^a, Pouyan E. Boukany^b, Peter ten Dijke^a

^aDepartment of Cell and Chemical Biology and Oncode Institute, Leiden University Medical Center, Leiden, the Netherlands

^bDepartment of Chemical Engineering, Delft University of Technology, Van der Maasweg 9, 2629 HZ, Delft, the Netherlands

Abstract

Paracrine signaling from stromal fibroblasts is a key regulator of lung cancer invasion, but its signaling requirements in three-dimensional (3D) matrices are incompletely defined. In this chapter, we examined the effect of lung fibroblast-derived CM on A549 lung adenocarcinoma spheroids embedded in GelMA hydrogels. MRC5 CM induced pronounced single-cell dissemination from A549 spheroids in compliant (3% w/v) matrices, while control spheroids remained compact and viable. Dissemination was completely abolished by inhibition of TGF- β type I receptors (ALK4/5) using SB-431542 and significantly reduced by pan-TGF- β neutralization, whereas fibroblast-induced spheroid expansion was suppressed only by ALK4/5 inhibition. Reporter assays and sensitive detection approaches showed no detectable active or total soluble TGF- β in MRC5 CM. Together, these results demonstrate that fibroblast-CM promotes single-cell dissemination of lung adenocarcinoma spheroids through a requirement for ALK4/5 signaling, despite the absence of detectable soluble TGF- β in the CM.

Keywords: lung cancer; TGF- β ; Conditioned medium; cancer cell invasion; GelMA

Introduction

Lung cancer remains a major contributor to global cancer-related mortality, with non-small cell lung cancer (NSCLC) representing the majority of diagnoses and characterized by early and aggressive metastatic behavior [1]. Increasing evidence shows that tumor progression is not dictated solely by cancer cell-intrinsic programs but emerges from reciprocal signaling with the tumor microenvironment, particularly with stromal fibroblasts. Through extracellular matrix remodelin, secretion of soluble factors, and modulation of tissue mechanics, these fibroblasts in close proximity of cancer cells can reprogramme cancer cells toward invasive phenotypes. These stromal cues have been implicated in promoting epithelial–mesenchymal transition (EMT), single-cell dissemination, and enhancing metastatic potential across multiple solid tumors [2].

Among the signaling pathways mediating fibroblast–tumor crosstalk, TGF- β has a central and context-dependent role in lung cancer progression, where it promotes EMT, cytoskeletal remodelin, and invasion at advanced disease stages [3]. TGF- β is structurally and functionally related to activin, which signal via type I receptors of the activin receptor-like kinase (ALK)5 and ALK4, respectively. Activated ALK4 and -5 phosphorylate SMAD2/3 to drive transcriptional programmes associated with invasion and EMT. Pharmacological inhibition of these receptors using the small-molecule inhibitor SB-431542 provides a widely used approach to functionally investigate pathway dependence by blocking ALK4/5 kinase activity and therefore, further downstream SMAD signaling [4]. Importantly, inhibition of ALK4 and -5 defines a dependence on ALK4/5-mediated signaling rather than on a specific ligand [4].

In three-dimensional (3D) culture systems, the relationship between paracrine signaling, matrix properties, and invasive behavior is further complicated by spatial confinement, mechanical resistance, and local signal amplification. Under these conditions, functional activation of signaling pathways can occur in the absence of robust bulk readouts of soluble ligand, challenging ligand-centric interpretations of paracrine control. As a result, receptor-level perturbation provides a critical tool for defining signaling dependencies in matrix-embedded tumor models.

Here, we investigated the MRC5 lung fibroblast CM-induced dissemination of A549 lung adenocarcinoma cells using a 3D GelMA spheroid model. Invasive response of A549 cells upon MRC5 CM stimulation was abolished by inhibition of ALK4/5 signaling using a small molecule kinase inhibitor and significantly attenuated by neutralization of TGF- β activity, despite the absence of detectable soluble TGF- β in the CM. By combining live-cell imaging with quantitative invasion and reporter assays, this study defines a fibroblast-driven, TGF- β type I receptor-like-dependent signaling axis that promotes early single-cell dissemination in a 3D lung cancer model.

Methods

Cell Culture

Wild-type A549 human male lung adenocarcinoma cells (CCL-185, ATCC) and MRC-5 lung fibroblasts (CCL-171, ATCC) were obtained from ATCC. A549-VIM-RFP-CAGA12-GFP dual reporter cells were kindly provided by Yifan Zhu (Department of Cell and Chemical Biology, LUMC) [5]. The CAGA12-GFP reporter system has been functionally validated in multiple independent studies [6], [7]. Human hepatocellular carcinoma HepG2 cells (HB-8065™, ATCC) were obtained from ATCC and stably transduced to express the CAGA₁₂-GFP reporter as previously described[7]. All cell lines were cultured in DMEM (Gibco, #41965039) supplemented with 10% FBS (Gibco) and 1% Antibiotic–Antimycotic solution (Gibco) under standard conditions (37 °C, 5% CO₂). Cells were used between passages 5 and 15, routinely passaged at 80–90% confluency, sub-cultured two to three times per week, regularly screened for mycoplasma contamination, and authenticated by STR profiling.

A549 Spheroid Generation

A549 spheroids were generated in Corning™ Elplasia™ 96-well round-bottom plates with an ultra-low attachment (ULA) surface (Corning) to prevent cell–substrate adhesion and promote cell–cell aggregation. Wells contained 79 micro-wells, each seeded at 500 cells (total 4.0×10^4 cells per well). Spheroids were cultured for 4 days to a mean diameter of $200 \pm 30 \mu\text{m}$ before use[8].

Generation of MRC5 CM

1×10^5 MRC-5 fibroblasts were seeded per well in 12-well plates and maintained for 3 days in standard culture medium as mentioned above in subsection 2.1. The CM was then collected, and passed through a $0.45 \mu\text{m}$ filter to remove cell debris. This CM was used immediately, either undiluted or diluted with standard culture medium for stimulation experiments.

Preparation and cross-linking of GelMA hydrogels

Gelatin methacryloyl (GelMA; 300 g bloom, ~60% degree of substitution; Sigma-Aldrich) was prepared at 3% or 5% (w/v) in Dulbecco's phosphate-buffered saline (DPBS; Gibco) with lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP; Sigma-Aldrich) at a LAP:GelMA mass ratio of 1:16. Solutions were incubated at 37°C in a water bath for ~2 h until fully dissolved. Hydrogels were cross-linked by exposure to 365 nm UV light (Spectroline, serial no. 1832066) for 45s.

Invasion Assays

A549 spheroids were suspended in 3% (w/v) GelMA and polymerized in Ibidi 8-well μ -Slides as described above. Each hydrogel was overlaid with 200 μ L culture medium and imaged using a Zeiss LSM 980 confocal microscope equipped with a live-cell incubation chamber (37 °C, 5% CO₂). Medium was refreshed every 48 hours. Images were acquired at 24 hour intervals. Brightfield images were acquired using transmitted light (TL) illumination on the Zeiss LSM 980. The transmitted light was detected using the transmitted photomultiplier tube (TPMT) detector and master gain was set to 350V, digital gain set to 1.0 and digital offset to 0. Green fluorescence protein (GFP) images were acquired using an EC Plan-Neofluar 10 $\times$ /0.3 NA M27 air objective with a 13 mW 488 nm laser (0.2% power, master gain 650 V, digital gain 1, digital offset 0). The field of view was 848.53 x 848.53 μ m² (2048 x 2048 pixels) at 1.0x optical zoom. Z-stacks were collected at 20 μ m steps over a 400 μ m range.

Image analysis

Spheroid area was quantified on day 0 and day 7 using ImageJ2 (v2.14, NIH). Maximum-intensity Z-projections were generated, and spheroid boundaries were manually outlined to measure projected area. Spheroid expansion was expressed as the ratio of day 7 to day 0 area. Disseminated single cells were manually counted in the same projections at both time points.

Pharmacological and antibody treatments

Recombinant TGF- β 3 (R&D Systems, 7666; Minneapolis, MN, USA) was prepared in 4 mM HCl containing 0.1% recombinant bovine serum albumin (BSA) and used at a final concentration of 1 ng/mL unless stated otherwise. For TGF- β receptor inhibition, spheroids were pre-treated with SB-431542 (10 μ M; Sigma-Aldrich) for 1 hour before imaging. For TGF- β ligand neutralization, MRC5 CM was incubated for 30 min at room temperature with the pan-TGF- β neutralizing antibody 1D11 (10 μ g/mL; R&D Systems) or its isotype-matched negative control 13C4 at the same concentration, before addition to spheroid cultures.

CAGA12-GFP reporter & 2D TGF- β assay

To assess TGF- β levels in fibroblast-derived conditioned medium, HepG2 CAGA12-GFP reporter cells were seeded in 96-well plates (#3595, Corning) and exposed to graded concentrations of recombinant TGF- β 3 or MRC5 conditioned medium. Live-cell imaging was performed at 2-hour intervals using the Incucyte® S3 system with brightfield and GFP fluorescence acquisition (488 nm laser, 300 ms exposure). Fluorescence intensity and cell confluency were quantified using Incucyte® S3 software, with data pooled from three independent wells. Signal-to-background (s/b) values were calculated by normalizing

fluorescence intensity to unstimulated controls at corresponding time points. Z' scores were calculated using the following formula:

$$Z' = \frac{3 \times (STDev_{pos} + STDev_{neg})}{|AVG_{pos} - AVG_{neg}|}$$

For the fluorescence reporter assay, A549 CAGA₁₂-GFP cells (1x10⁵ per well) were seeded in 12-well plates and incubated overnight to allow adhesion. The following day, cells were washed with PBS and stimulated with either recombinant TGF-β1 (10 ng/mL) or MRC5 CM in a total volume of 1.5 mL culture medium per well.

Graphing and Statistical Analysis

All data were analyzed and plotted in GraphPad Prism (v10, GraphPad Software). Results are presented as mean ± SD from ≥3 independent experiments unless stated otherwise. Comparisons were performed using two-way ANOVA with post hoc tests or nonparametric t tests as appropriate. *P* values < 0.05 were considered significant.

Results

MRC5 CM induces single-cell dissemination of A549 CAGA-GFP cells in 3D GelMA matrices

A549 CAGA₁₂-GFP reporter cells embedded in 3% (w/v) GelMA hydrogels exhibited uniform spheroid expansion over 7 days in control medium, with no detectable dissemination (Fig. 1A). In contrast, exposure to 100% CM derived from MRC5 lung fibroblasts induced a pronounced single-cell dissemination phenotype, characterized by numerous cells migrating away from the spheroid core into the surrounding matrix (Fig. 1A, MRC5 CM). Quantification revealed a significant increase in disseminated single cells in CM-treated cultures compared with control (*P* < 0.001; Fig. 1B). Spheroid expansion, assessed as the ratio of spheroid area at day 7 to day 0, was modestly increased in CM relative to control (*P* > 0.05; Fig. 1C).

The magnitude of dissemination depended on the concentration of fibroblast-derived CM. Treatment with 10% MRC5 CM had no measurable effect on dissemination or spheroid expansion (Supporting Fig. 1). Increasing CM concentration to 30% induced limited single-cell dissemination by day 5, which remained difficult to quantify due to the early stage of migration (Supporting Fig. 2). Thus to enable robust and reproducible quantification of dissemination, all subsequent experiments were therefore performed using 100% MRC5 CM. Matrix composition further modulated response to fibroblast signals. When A549 spheroids were cultured in a stiffer 5% (w/v) GelMA matrix, MRC5 CM promoted spheroid expansion and compaction but failed to induce single-cell dissemination over the same

time frame (Supporting Fig. S3). Based on these observations, 3% (w/v) GelMA was selected as a permissive matrix condition for analysing fibroblast-induced dissemination.

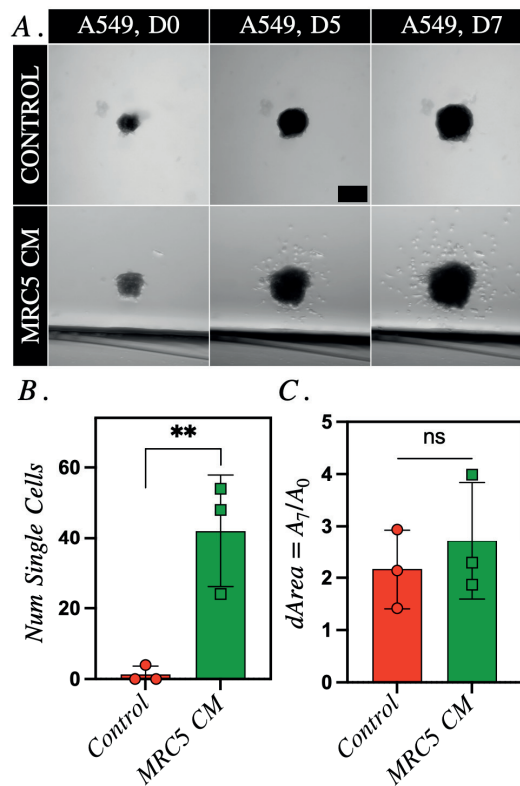


Figure 1. MRC5 CM induces single-cell dissemination in A549 spheroids embedded in 3% (w/v) GelMA.

- Brightfield maximum-projection images (Day 0, Day 5 and, Day 7) of A549 spheroids cultured in control medium or MRC5 CM. Spheroids increased in size under both conditions; however, CM stimulation induced numerous rounded single cells migrating away from the spheroid by Day 7. Scale Bar = 200 μ m.
- Quantification of single cells per condition. Data represents mean $\pm$ SD (control: 1 ± 2.3 , CM: 42 ± 16 , $n = 3$ biological replicates, ≥ 2 technical repeats each). $**P < 0.01$, two-tailed unpaired t-test.
- Spheroid expansion over 7 days, expressed as the ratio of spheroid area at Day 7 to Day 0. Data represents mean $\pm$ SD (control: 2.2 ± 0.8 , CM: 2.9 ± 1.1 , $n = 3$ biological replicates, ≥ 2 technical repeats each). $P > 0.05$, two-tailed unpaired t-test.

TGF- β type I receptor inhibition abolishes fibroblast-induced dissemination

CM-induced dissemination was abolished upon pharmacological inhibition of TGF- β type I receptor activity using SB-431542, restoring a compact spheroid morphology (Fig. 2A, CM + SB). Disseminated cell counts in the SB + CM group were reduced to baseline control levels ($P < 0.0001$ vs CM alone; Fig. 2B). In addition to suppressing dissemination, SB-431542 treatment also reduced spheroid expansion to levels significantly below those observed in control cultures, indicating an additional suppressive effect on spheroid growth (Fig. 2C) [9].

To assess whether dissemination was driven by TGF- β activity in the CM, CM was treated with the pan-TGF- β -blocking antibody 1D11, a potent inhibitor of all three isoforms[10]. 1D11 treatment significantly reduced single cell dissemination to a similar extent as SB-431542 ($P < 0.001$ vs CM; Fig. 2B) but had little effect on spheroid expansion, which remained comparable to untreated CM ($P > 0.05$; Fig. 2C). These results indicate that dissemination in this model is dependent on TGF- β receptor signaling, whereas fibroblast-driven spheroid expansion is largely TGF- β -independent.

Absence of detectable TGF- β ligand in MRC5-CM

To test for the presence of soluble TGF- β activity, A549 CAGA₁₂-GFP reporter cells were stimulated with TGF- β 3 (10 ng/mL) ligand or MRC5 CM, followed by quantification of GFP signal after 18 hours. TGF- β 3 stimulation induced a robust increase in reporter intensity, whereas CM elicited no change relative to control (Fig. 3A).

To further assess the presence of soluble TGF- β activity, active and total TGF- β 3 levels in MRC5 CM were measured using a high-sensitivity reporter assay, with HepG2 CAGA₁₂-GFP reporter cells used as positive controls. Neither active nor total TGF- β 3 was detected above the assay's lower limit in CM, whereas recombinant TGF- β 3 at concentrations as low as 0.05 ng/mL produced a clear reporter response (Fig. 3B). Together, these data demonstrate that fibroblast-CM promotes single-cell dissemination through a TGF- β type I receptor-like -dependent signaling requirement that occurs in the absence of detectable soluble TGF- β 3.

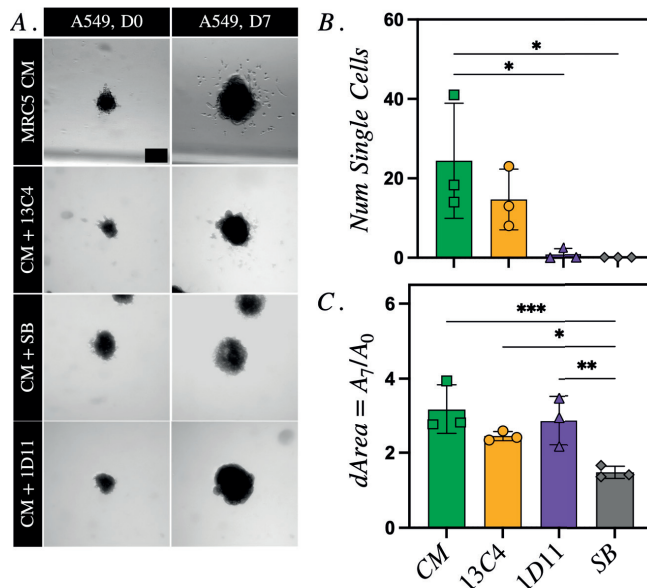


Figure 2. TGF- β and TGF- β type I receptor inhibition prevents single-cell dissemination in A549 spheroids.

- A. Brightfield maximum-projection images (Day 0 and Day 7) of A549 spheroids cultured in control medium or MRC5 CM. CM stimulation increased spheroid size and induced single-cell dissemination, whereas treatment with SB-431452 or neutralization of TGF- β in CM using 1D11 prevented dissemination. Scale Bar = 200 μ m.
- B. Quantification of single cells per condition on Day 7. Data represents Mean $\pm$ SD (CM: 24 $\pm$ 14, 13C4: 15 $\pm$ 8, 1D11: 1 $\pm$ 1, SB: 0 $\pm$ 0, n = 3 biological replicates, $\geq$ 2 technical repeats each). * P < 0.05
- C. Spheroid expansion over 7 days, expressed as the ratio of spheroid area at Day 7 to Day 0. Data represents Mean $\pm$ SD (CM: 3.2 $\pm$ 0.7, 13C4: 2.4 $\pm$ 0.1, 1D11: 2.9 $\pm$ 0.7, SB: 1.5 $\pm$ 0.1, n = 3 biological replicates, $\geq$ 2 technical repeats each). *** P < 0.001, ** P < 0.01, * P < 0.05. No statistical significance was found upon comparing CM group to 13C4 and 1D11 groups. Statistical significance was determined by two-way ANOVA (factors: treatment $\times$ replicate) with Tukey's post-hoc.

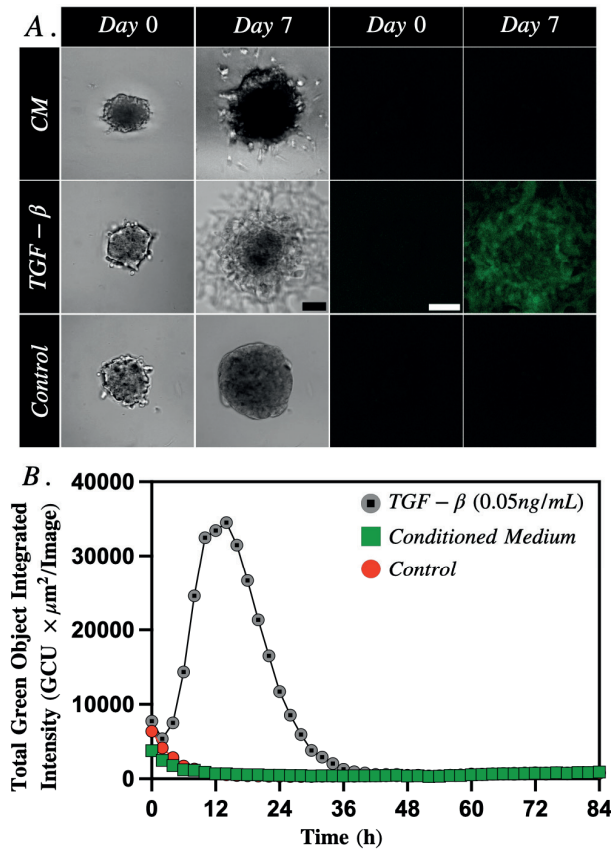


Figure 3. MRC5 CM lacks detectable TGF- β activity.

- A. Brightfield and GFP fluorescence images of A549 CAGA-GFP spheroids embedded in 3% (w/v) GelMA on day 0 and day 7 following stimulation with recombinant TGF- β 1 or MRC5 CM. TGF- β 1 (10 ng/mL) treated spheroids exhibit pronounced invasion and increased GFP reporter activity, whereas control and CM-treated spheroids show minimal changes.
- B. Fluorescence reporter assay confirming absence of measurable TGF- β in MRC5 CM, with standard (0.05 ng/mL) producing a robust concentration-dependent signal.

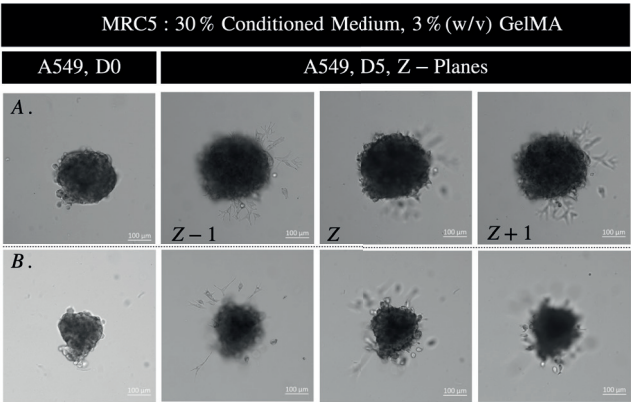
Discussion

Our results demonstrate that CM obtained from MRC5 fibroblasts promotes single-cell dissemination of A549 lung adenocarcinoma spheroids in 3D GelMA matrices, despite the absence of detectable soluble TGF- β in the CM of MRC5 cells. Dissemination was completely abolished by pharmacological inhibition of ALK4/5 signaling using SB-431542 and significantly reduced by neutralization of TGF- β activity with the pan-TGF- β antibody 1D11, whereas fibroblast-driven spheroid expansion was suppressed only by SB-431542.

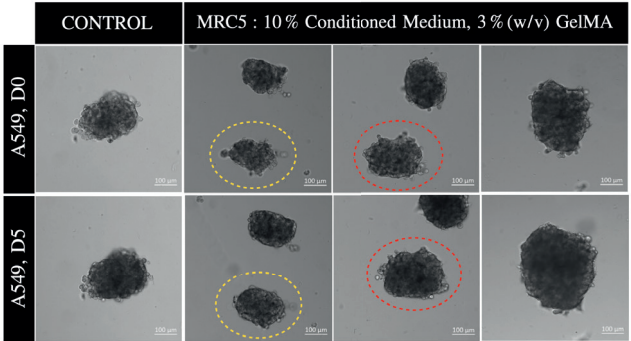
Together, these findings establish a clear requirement for TGF- β type I receptor like signaling in fibroblast-induced dissemination, while indicating that distinct receptor-dependent mechanisms underlie dissemination and spheroid growth. The ability of TGF- β type I receptor kinase inhibitor and TGF- β neutralizing antibody inhibit A549 spheroid cell invasion induced by MCR5 CM, but the inability to detect soluble TGF- β in CM suggests that dissemination is TGF- β dependent, but is enhanced by other soluble factors that are present in the CM. Our finding that the small molecule ALK4/5 inhibitor, which targets both TGF- β type I and Activin type I receptors, produced a more pronounced inhibition of dissemination than 1D11, which neutralises TGF- β but not activin, suggests that activin signalling from MRC5 CM may contribute to the observed dissemination phenotype. Together, this work reveals that fibroblast-derived paracrine cues can drive early single-cell dissemination of lung adenocarcinoma spheroids through a TGF- β -like dependent signaling in the absence of detectable soluble TGF- β . Further work will be required to define the factors with which TGF- β like signals cooperate to induce A549 cell spheroid invasion.

Several mechanistic explanations may account for TGF- β receptor activation in the absence of detectable soluble ligand. First, TGF- β may be present in the CM at concentrations below the detection threshold of standard assays, yet sufficient to activate receptors under permissive conditions. Second, latent TGF- β complexes may be activated locally at the cell surface through integrin-mediated or protease-mediated mechanisms, bypassing the need for freely diffusible active ligand [11], [12]. Third, other ALK4/5 ligands present in fibroblast CM, including activins and Growth Differentiation Factor (GDF) family members, may activate overlapping downstream signalling cascades, consistent with the more pronounced inhibition observed with SB-431542 compared to the TGF- β -specific antibody 1D11 [13]. Finally, crosstalk from integrin-FAK or other mechanosensing pathways may transactivate Smad-dependent signalling independently of canonical ligand-receptor engagement [14]. Distinguishing between these possibilities will require proteomic characterisation of the MRC5 secretome combined with targeted ligand depletion and phosphoproteomic approaches.

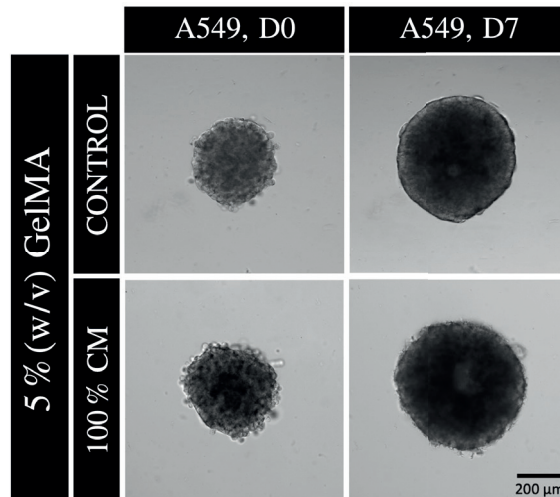
Supporting Figures



Supporting Figure 1. A549 spheroids embedded in 3% GelMA (w/v) displaying no invasive activity on Day 5 after stimulation with 10% MRC5 CM. Yellow and Red circles highlight respective spheroids on Day 0 and Day 5.



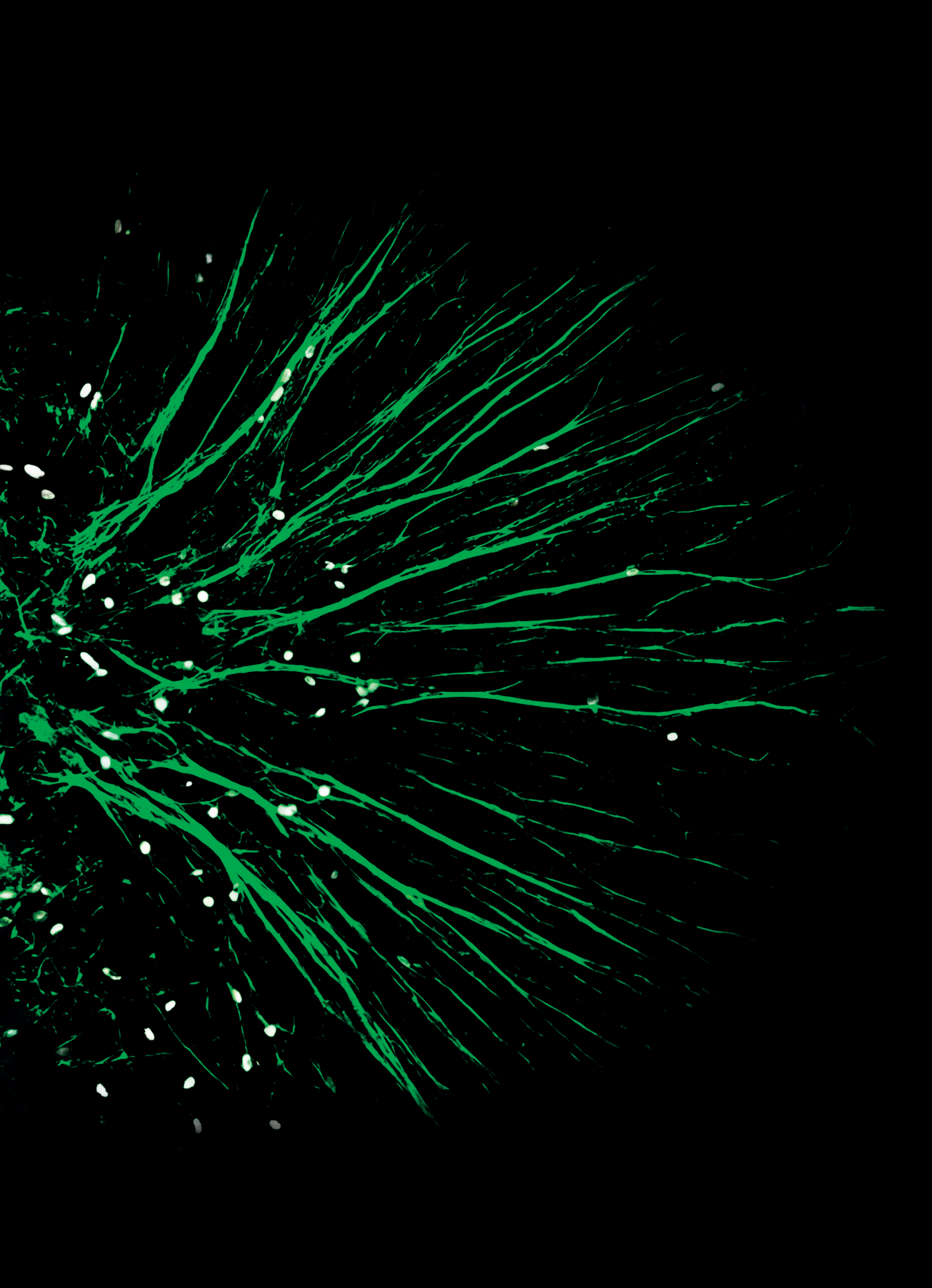
Supporting Figure 2. (A) & (B) Multiple Z-planes of A549 spheroids embedded in 3% GelMA (w/v) displaying invasive activity on Day 5 after stimulation with 30% MRC5 CM. Manual quantification revealed, on average, 9 cells beginning to disseminate away from the spheroid in both (A) and (B) by end of Day 5.



Supporting Figure 3. A549 spheroids in 5% GelMA (w/v) showing no invasion upon stimulation with 100% MRC5 CM after 7 days.

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Chapter 5

Microfluidics meets 3D cancer cell migration



Pranav Mehta,^{1,2,3} Zaid Rahman,^{2,3} Peter ten Dijke^{1,*} and Pouyan E. Boukany^{2,*}

¹Department of Cell and Chemical Biology and Oncode Institute, Leiden University Medical Center, Leiden, The Netherlands

²Department of Chemical Engineering, Delft University of Technology, Van der Maasweg 9, 2629 HZ, Delft, The Netherlands

³These authors contributed equally to the work

Abstract

An early step of metastasis requires a complex and coordinated migration of invasive tumor cells into the surrounding tumor microenvironment (TME), which contains extracellular matrix (ECM). It is being appreciated that 3D matrix-based microfluidic models have an advantage over conventional in vitro and animal models to study tumor progression events. Recent microfluidic models have enabled recapitulation of key mechanobiological features present within the TME to investigate collective cancer cell migration and invasion. Microfluidics also allows for functional interrogation and therapeutic manipulation of specific steps to study the dynamic aspects of tumor progression. In this review, we focus on recent developments in cancer cell migration and how microfluidic strategies have evolved to address the physiological complexities of the TME to visualize migration modes adapted by various tumor cells.

Microfluidic modeling of cell invasion

Metastasis, the spread of cancer cells away from the primary solid tumor and into the body's healthy tissues, eventually leading to the formation of secondary tumors causing organ failure, is responsible for over 90% of cancer-related deaths [1]. Metastasis requires the complex and coordinated migration of invasive tumor cells into the surrounding TME containing ECM. The TME plays a critical role in the early stages of metastasis, dictating cancer cell motility, invasion, and spread into neighboring tissues [2]. Key extracellular determinants of cancer cell migration include the biophysical and biochemical cues provided by the ECM, interstitial flow (IF), and cancer cell-TME interactions, including the interplay with cancer-associated fibroblasts (CAFs).

Conventional tumor models previously used to study these cell motility mechanisms lie on either end of the spectrum: on one end, macroscopic in vitro models are easy and simple, but poorly recapitulate the local TME characteristics, including biomechanical forces, IF, fluid shear stress, and ECM remodeling. On the other end, animal models can mimic the pathophysiological complexity of tumors, but they are expensive, time-consuming, and still have a degree of error due to the innate differences between animal and human physiology. The use of microfluidics in 2D and 3D models has become an attractive alternative to overcome these challenges. Going a step further, microfluidics can more closely mimic the TME by incorporating various physiologically relevant biophysical and biological cues with precise spatiotemporal control (Figure 1, Key figure) [3,4]. Microfluidic devices also permit real-time imaging of cellular dynamics, enabling them to probe biophysical, biochemical, and (epi)genetic activities present in healthy and diseased multicellular tissues [5]. The field of microfluidics continues to extend the development of tumor models ranging from tumor cancer spheroids to cancer-on-a-chip models with relevant TME and (patho)physiological conditions [6,7].

Microfluidic platforms have been used for the study of the metastatic microenvironment, tumor-stroma interactions, and the TME in cancer metastasis and therapy [8–11], cancer extravasation in response to biophysical and chemical cues [12,13], single-cancer-cell separation, detection, and migration [14], biophysical studies of cancer cells at the single-cell level [15], modeling cancer immune interaction [16], and development of tumor/organ-on-a-chip in personalized cancer therapy [17,18]. However, a critical perspective on microfluidic approaches for mechanistic studies of cancer cell migration into surrounding TME, for example, how cancer cells adapt their migratory behavior through different TME, is still missing. Here, we discuss how cancer cells adapt their migration strategies through different and dynamically changing TMEs, and how novel microfluidics platforms have evolved to unravel the mechanisms of collective cancer cell migration through a tunable TME. We discuss how microfluidics is an effective tool to mimic the metastatic

microenvironment for mechanistic investigations (Figure 1). We then discuss the underlying migration mechanisms adopted by cancer cells in a physiologically relevant TME model. We focus on lung and breast cancer cells as they are well studied but remain two of the most diagnosed cancers worldwide in terms of incidence and mortality due to the complexity of the TME [1,2]. Next, we highlight the role of tumor heterogeneity, ECM, interstitial and shear flow as biomechanical stimuli, CAFs, and the cytokine, transforming growth factor beta (TGF- β) as relevant biochemical cues to emphasize tumor invasion properties. Finally, we discuss challenges and new directions in applying 3D matrix-based microfluidic devices in personalized medicine and cancer therapy.

Highlights

Microfluidic cancer cell migration models enable an integrated assessment of how cell-cell and cell-matrix interaction, matrix stiffness, interstitial and shear flow, hypoxia and cytokine, and metabolic gradients affect dynamic cancer cell migration.

While many in vitro static cell migration models fail to mimic the complexities and biomechanical features of the tumor microenvironment (TME), incorporation of microfluidics allows us to investigate cancer cell migration through the TME and invasion under well-controlled pathophysiological conditions associated with tumor progression events.

Tumor cells can elicit distinct migratory behavior in the form of single or collective cell migration modes in response to extracellular biochemical and biophysical cues.

Microfluidics in collective cell migration

Both biophysical and cellular cues alter tumor cell responses (from migration modes to the level of invasiveness) inside the dynamic TME, resulting in the dissemination of cancer cells from the primary tumor. 3D in vitro tumor models such as heterogeneous or coculture multicellular spheroid models are widely used to investigate cancer cell migration and invasion [10]. Static (no-flow) 3D cell culture models have been used to examine cell migration through semipermeable membranes or to study the biological response to specific chemoattractants or drugs [13]. Moreover, a programmable and multifunctional 3D cancer cell invasion platform has been recently developed that dynamically releases mature TGF- β to induce invasive migration of cancer cells by assembling functionalized micro-buckets and a tunable ECM that emulates a more complex TME during cancer invasion [19]. A major advantage of using 3D microfluidic platforms is that different cell

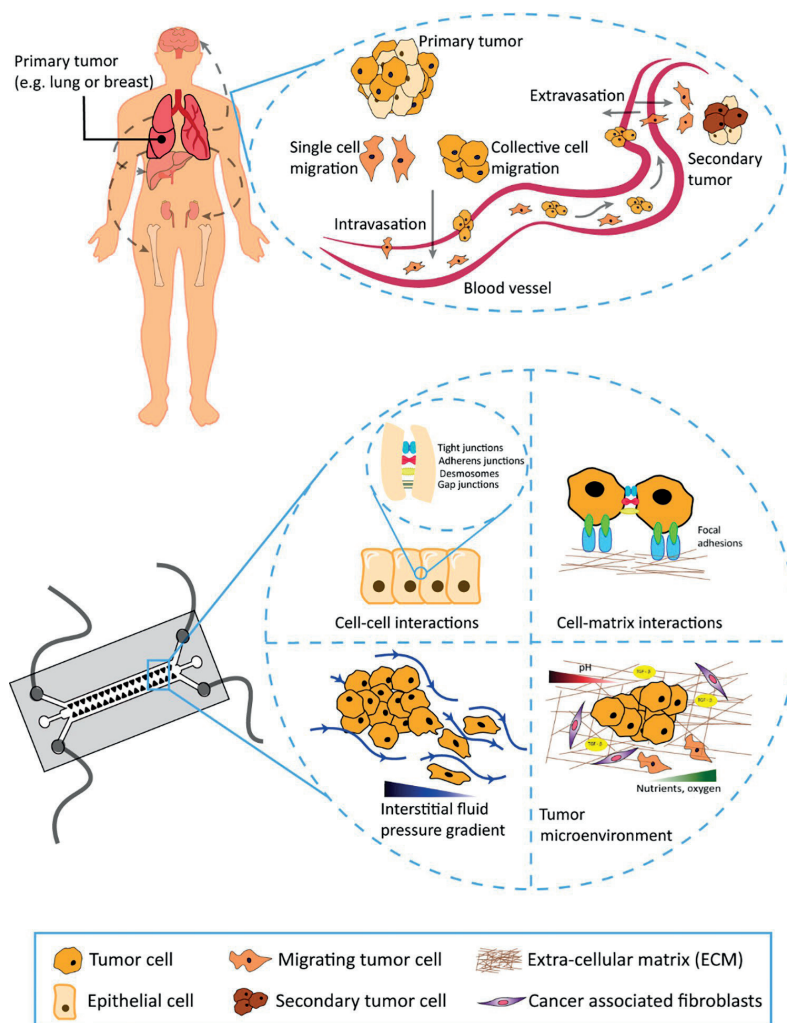


Figure 1. Key figure. Schematic of primary tumor (lung or breast) metastasis to different parts of the body and application of microfluidics to study tumor invasion.

Top: invasive cancer cells metastasize from a primary solid tumor (such as the lung or breast) to different parts of the body such as the brain, liver, adrenal glands, and bones. Primary tumors disseminate into a single or cluster of migrating tumor cells with adaptive migration morphology. These disseminated tumor cells can then enter the blood vessel, known as intravasation. Tumor cells travel through the blood vessel and exit at a distant location, known as extravasation. The tumor cells that survive and adapt to the new surrounding microenvironment have the potential to form a secondary tumor (e.g., in the brain). This chain of events is known as the metastatic cascade, where external factors such as biochemical and biomechanical cues influence the migratory behavior of cells from the primary tumor. Bottom: schematic of a microfluidic device highlighting the important applications used to study various critical biological phenomena [ranging from cell/cell and cell/extracellular matrix (ECM) interactions to interstitial flow within tumor microenvironment (TME)] associated with the metastatic cascade. Microfluidics

gives us the advantage of real-time imaging to study cell–cell and cell–matrix interactions by quantifying biophysical properties such as changes in levels of adhesion proteins. A microfluidic device also provides desirable control to include external forces such as shear stress or interstitial flow, usually referred to as promigratory factors that influence cancer cell migration behavior. Last, recent developments in microfluidic models have focused on building a physiologically relevant TME that can provide a comprehensive understanding of the migratory behavior of specific cancer cell types.

types can be cocultured long term in a controlled microenvironment, thus more closely resembling physiology *in vivo*. Through real-time visualization and the ability to study specific biological cues, 3D microfluidic platforms allow the characterization of individual cellular responses, providing insight into intercellular communication. For example, 3D matrix-based microfluidic models incorporated the use of endothelial cell (EC)-covered microchannels and spatiotemporally controlled ECM compartments consisting of stromal cells, tumor-associated macrophages (TAMs), CAFs, and immune cells to recreate intratumor heterogeneity [13,20,21]. Other 3D microfluidic models explored the use of patient-derived cells [9,22], response of natural killer (NK) or immune cells [23], and drug delivery for drug screening applications [24,25]. Researchers have also been able to reconstitute *ex vivo* a human tumor ecosystem by coculturing four distinct cell populations (cancer, immune, endothelial, and fibroblasts) in a microfluidic device in 3D. Using this platform to screen for drug interactions and mechanisms, researchers elucidated cancer–immune–CAF cell interactions, illustrating the long-time scale (several hours) signaling cascade between cancer and immune cells and CAFs that antagonized the effects of the drug.

3D microfluidic models create a more effective and powerful platform to track intermediate steps to differentiate between single and collective cell migration mechanisms in cancer models with a dynamic interplay of multiple (dynamically phenotypically changing) cell types [26] (Box 1). Various microfluidic models have been developed to investigate metastasis-related phenomena, such as cancer cell migration, epithelial-mesenchymal transition (EMT), cell invasion, intravasation, and extravasation. In the following sections, we take a closer look at the microfluidic models developed for lung and breast cancers highlighting microfluidic modeling parameters and improved biological outcomes. Furthermore, these (3D) microfluidic devices have been adapted to investigate the impact of TME complexity on a wide variety of tumor invasions. An overview of 3D microfluidic models for various cancer types modeled for different tumor progression events and with chemotherapeutic applications is provided in Table 1.

Microfluidic models incorporating biophysical and biochemical cues

Biophysical cues, including IF, fluid pressure, and ECM stiffness, play a central role in directing cell movement, migration, and invasion inside the TME. For tumor cells to extravasate into circulation and distinct tissues, they interact by altering their mechanical properties to adjust to the physical changes of the ECM. The ECM architecture goes through constant remodeling, additionally influenced by interstitial fluid flow (IFF) and interstitial fluid pressure (IFP). Tumor cells respond to these biophysical cues by mechanotransduction pathways, which in a dynamically controlled manner, result in cell migration and invasion. For example, increased ECM stiffness activates the EPHA2/ LYN complex, thereby triggering EMT in breast cancer [27]. Increasing collagen density in the ECM diminishes collective cell migration in breast cancer cells [28]. Matrix porosity can also promote single to collective transitions such as cell jamming, the switch between the two being determined by cellular adhesion to the ECM and the extent of actomyosin contractility of the cytoskeleton [28,29]. Confinement of cells in tissues can also cause structural changes in cells. Cells deform into small unstable blebs so they can squeeze through the confinement [30,31]. This change can cause damage to the nuclear envelope, leading to DNA damage and aberrant epigenetic regulation [32]. As such, confinement and changes in matrix porosity can lead to mesenchymal-to-amoeboid transition (MAT) [31,33].

Collagen fiber alignment in conjunction with matrix stiffness regulates cell migration characteristics, such as speed and persistence, and it also affects their ability to migrate collectively [34]. This property is relevant for cell migratory behavior in a 3D matrix environment compared with cells that prefer directional migration on 2D stiffer substrates. Traditional 3D in vitro models, such as Boyden Chambers, and heterogeneous tumor spheroid invasion assays compared with in vivo models are inexpensive, require less expertise to operate, and can better recapitulate cell-cell/ cell-ECM interaction when embedded in hydrogel matrices [35]. Cell migration behavior observed using 3D cell culture and 2D microfluidic models have provided insights into more guided cell trajectory in response to a biochemical signal or to analyze cellular response by mimicking vascular networks using micro-confinement geometries [36,37]. In conventional 2D/3D culture models, cancer cells are frequently grown on top or within synthetic ECM as monolayers. However, they fail to capture the behavior of cell-cell and cell-matrix interactions present in the 3D cellular architecture of a tumor tissue [38]. These classical in vitro culture models lack the ability to include fluid flow or shear stress that drives tissue deformation and transport across vascular endothelium, all observed in an in vivo TME [4]. Due to these limitations, these models fall short in recreating relevant physical cues, chemokine signals, or hypoxic gradients present in the TME.

Microfluidic models can incorporate biochemical and biomechanical factors by replicating tumor stroma in a controlled microenvironment. Recently, a microfluidic assay was employed to study the role of collagen densities and the biochemical signal, TGF- β , on the migratory behavior of H1299 spheroids [established from a non-small cell lung cancer cell line (NSCLC)] (Figure 2A) [39]. This programmable platform provides controllable conditions to correlate migrating mechanisms from single to collective migration with varying degrees of micro-confinement. Increasing collagen density inhibited single cell migration and promoted clustered migration patterns, whilst TGF- β stimulation promoted cell detachment and single cell migration. Furthermore, migrating cells in high-density collagen matrices showed an increase in strand-like collective motility on encountering TGF- β (Figure 2Aii and iii)) [39]. Jamming transitions under static conditions have also demonstrated that high tissue density or increased confinement can regulate collective migration by driving EMT in epithelial cancer cells via mechanotransduction pathways [28,39]. When large cell populations encountered enriched matrices, they moved as multicellular sheets or strands, forming weak cell-cell junctions due to EMT. Moreover, the matrix was proteolytically degraded to create migration tracks. It has been proposed that cooperation between homotypic cancer cells increases migration efficiency and directional persistence and minimizes the energy costs needed compared with individual cell migration, and improves cancer cell survival when invading into neighboring tissues [28,39,40].

The influence of mechanical confinement and substrate topology are important factors in determining the role of tumor heterogeneity in migratory behavior during cancer cell invasion and progression [37,41,42] (Box 2). To investigate such complex tumor-stroma interactions, a novel 3D bio-microfluidic platform was designed to implement microchamber arrays that replicate the hollow mammary glands that are a part of the tumor microstructures [43]. MCF-10A (normal breast epithelium) cell cultures in the microchamber arrays resulted in the formation of cellular aggregates that covered the entire microchamber area. These aggregates with tight cellular adhesions led to the formation of lumen-like structures mimicking epithelial cells that surround the basement membrane. Cocultures of MCF-10A with MDA-MB-231 (a highly invasive triple-negative breast cancer cell line), however, inhibited cluster formation [43]. The inability of MCF-10A cells to form tight cell adhesions was attributed to secretion of matrix-metalloproteases (MMPs) by MDA-MB-231 cells. MMPs mediated the cleavage of E-cadherin on MCF-10A cells, which resulted in poor cell-cell adhesion that prevented cluster formation. The microfluidic channels allow for gradients of biochemical signals, that is, a range of concentrations of MMP inhibitors induced dose-dependent effects on MCF-10A cluster formation. The model, equipped with microchambers surrounded by collagen hydrogel, mimicked the TME and showed the importance of surrounding cell structures (i.e., epithelial layer) and basement membrane. The microfluidic model recapitulated the

characteristic property of lumen-like structures around the basement membrane that prevented metastatic cells from intravasating into deeper ECM areas.

Box 1. Migration dynamics and epithelial-mesenchymal plasticity (EMP)

Cell migration can be broadly classified into two modes, individual or single-cell migration and collective cell migration. Morphologically and functionally, single-cell migration can be further classified into amoeboidal and mesenchymal movement. Amoeboid single migrating cells often display structures referred to as plasma membrane blebs, defined by a bulky and rounded morphology, and these blebs are an extension of the plasma membrane caused by local disruption of the membrane actin cortex interactions, high intracellular hydrostatic pressure, and enhanced contractility [66,67]. Single cells displaying a mesenchymal migration mode, moreover, display actin-rich protrusions and rely on the proteolytic degradation of the surrounding ECM [68,69].

Collective migration occurs when cells maintain their intercellular junctions and thus migrate jointly as an integrated group. The collective morphology and dynamics of cohesive motility are strongly dependent on the type and stability of their intercellular junctions and extracellular tissue conditions [67]. Collective migration in cancer is characterized by the expression of homophilic cell-cell junctions and epithelial morphologies within the migrating cluster [70]. Protrusive cells at the leading edge, termed leader cells, are connected to the rest of the cluster and have been shown to coordinate multicellular sheet/strand migration [71]. Collectively migrating cancer cells display structural ECM remodeling, further promoting migration and metastasis [72]. Cellular plasticity, the ability to repress or activate specific gene expression and modulate the activity of gene products to allow for optimal migration mechanics or survival, is crucial for collective cell migration. This plasticity allows migrating cancer cells to integrate various mechanical and chemical cues around them and adjust their direction, speed, and mode of migration [67].

Epithelial cancer cells can undergo a switch to a mesenchymal phenotype, a process termed epithelial-to-mesenchymal transition (EMT) [73]. New studies show that EMT is a dynamic plastic process, where cells can acquire different hybrid intermediate states called partial EM states (p-EMT), a process referred to as EMP [74,75]. In such states, cells retain various epithelial features while also expressing EMT markers such as N-cadherin and vimentin intermediate filaments [67]. Cells undergoing complete EMT (c-EMT) transcriptionally suppress the epithelial markers such as the transmembrane protein E-cadherin [74].

Table 1. 3D microfluidic models to study cancer cell migration and anticancer therapeutic approaches in different tumor types.

Cancer type	Microfluidic model highlights	Biological observations/ outcome	Limitations	Refs
Breast cancer				
Type: tumor invasion Cells: SUM-159 breast cancer cells and CAF coculture	3D microfluidic based organotypic model. Focuses on tumor invasion by stromal activation. Coculture of SUM-159 and patient-derived fibroblasts to model patient-specific TME. Allows for tumor–stroma crosstalk.	RNA-seq is applied to profile the transcriptome of breast cancer cells with CAFs to delve deeper into molecular mechanisms in tumor–stroma bidirectional crosstalk. Breast cancer invasion enhanced in the presence of CAFs, is mediated by the expression of glycoprotein nonmetastatic B (GPNMB) in breast cancer cells.	Patient-derived CAFs heterogeneously express varying levels of specific biomarkers. Two out of three patient-derived CAFs showed tumor-promoting behavior. No defined single or set of biomarkers was defined owing to CAF heterogeneity.	[83]
Type: hypoxia-driven tumor migration Cells: HUVECs, MCF-10A, MDA-MB-231, MCF-7 and normal human lung fibroblasts (NHLFs)	Microfluidic model consisting of vascular networks to quantify breast cancer extravasation when exposed to different oxygen concentrations. Device with three-gel channels, alternated with media channels. An advantage in the study of different cell lines and conditions of normoxia and varying hypoxia levels in short-term cultures.	Increase in hypoxia inducible factor (HIF)-1 α protein levels in hypoxic conditions were linked to elevated levels of metastatic potential. The increase in aggressive phenotype under hypoxic conditions is independent of the malignancy of different cell lines.	Effect of hypoxia on cell proliferation and viability can be different in cells lines of varying malignancy based on short-term or long-term exposure to hypoxic conditions. More studies are needed to understand the signaling pathways responsible for hypoxia-induced increased metastatic potential.	[84]
Type: tumor extravasation Cells: MDA-MB-231 breast cancer cells, monocytes	A 3D vascularized microfluidic model that provides high-resolution visualization of intravascular migration, transmigration, and differentiation of monocytes through human vasculature. Characterization of tumor cell extravasation in the presence of monocytes.	Undifferentiated monocytes present with MDA-MB-231 cancer cells in intraluminal vasculature reduced tumor cell extravasation. Heterogeneity of cancer cell in extravasation mediated by monocyte type was observed. This difference was attributed to the role of myosin IIA in monocyte cell motility.	Future studies should examine media conditioned by cells in 3D compared with 2D system conditioned medium. More studies need to be performed on monocyte homeostasis and extravasation cascade in response to flow. Incorporation of other immune cells.	[85]
	Physiological differences between inflammatory and patrolling monocyte extravasation patterns were measured by the platform.			
	Potential to study effects of monocytes on tumor progression.			

Lung cancer

Type: tumor migration Cells: H1299 lung adenocarcinoma cancer cells	A 3D matrix-based microfluidic device with mixed hydrogel (collagen, Matrigel) compositions to investigate impact on migration.	Hydrogel composition mimicking the TME. A disorganized basement membrane at the front of cancer invasion was mimicked using an ECM containing hydrogel.	Inconsistency with results [86] on migration speed and morphology with increasing matrix stiffness and pore sizes.
	Quantitative image analysis to measure H1299 lung cancer migration in different hydrogel (ECM) compositions.	An increase in migration speed in collagen-Matrigel hydrogel compared with collagen only was observed because of increased stiffness and pore size.	Difficult to identify the exact composition of <i>in vivo</i> ECM based on specific cancer types and their location.
		Hydrogel composition was identified as an important determinant for cell migration.	
Type: tumor migration and anticancer therapy response Cells: A549 lung cancer cells, miR-497 exosomes.	A 3D microfluidic device in which A549 lung cancer cells and HUVECs are cocultured that is capable of delivery and controlled regulation of miRNAs via exosomes to explore therapeutic potential.	Significant inhibition of A549 cell migration and reduced sprouting of HUVECs was observed in the presence of miR-497 exosomes.	The model does not consider the inclusion of additional cell types such as CAFs and/or immune cells and hypoxic gradients. [87]
	The model permitted the study of the role of VEGF concentration gradient on angiogenic sprouting. miRNA therapeutics combined with microfluidics for a predictive, low-cost development tool for targeted cancer therapy.	Exosomes containing miR-497 suppressed tumor growth and expression of associated genes.	The role of ECM composition in the lung TME and the mechanical strain on the lung remains uncharacterized.

Pancreatic ductal adenocarcinoma (PDAC)

Type: heterogeneous tumor invasion Cells: pancreatic cell (PCC) line	A complex and heterogeneous PDAC model was developed by embedding a duct of genetically engineered PCCs surrounded by collagen matrix. Precisely controlled intratumoral heterogeneity was engineered to enable heterogeneous invasion characteristics as a response to TGF- β 1 on EMT and local invasion.	Confirmation of TGF- β 1 promoting EMT and local invasion in different PCC lines. Study revealed complex interaction between different cancer cells making them more aggressive and invasive. Model can be used to study interaction between heterogeneous patient-derived cancer and stromal cells.	The microfluidic model falls short to incorporate CAFs and other ECM components as a part of pancreatic TME. [88]
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Table 1. Continued.

Cancer type	Microfluidic model highlights	Biological observations/ outcome	Limitations	Refs
Type: tumor progression Cells: PANC-1 pancreatic cells and pancreatic stellate cells	Microfluidic models using pancreatic stellate cells (PSCs) to study their role in cancer progression and drug resistance. Coculture of pancreatic tumor spheroids (made of PANC-1 cells) with PSCs in 3D collagen matrix to mimic <i>in vivo</i> TME to visualize EMT and quantify chemoresistance.	Expression of EMT markers such as vimentin, TIMP1, interleukin (IL)-8, and TGF-β was higher in cocultured PANC-1 spheroids compared with monocultures. PANC-1 cells also showed greater cell motility. EMT leads to drug resistance, mediated by the TME components such as stromal cells and their interactions with cancer cells.	Contradicting results when using different CAFs targeting strategies raises an issue of intratumoral CAF heterogeneity.	[89]
Prostate cancer				
Type: chemotherapeutic response Cells: DU 145 prostate cancer cells	Microfluidic platform representing a 3D TME for rapid evaluation of chemotherapeutic drugs on different cancer cells. Multiple chambers equipped with perfusion channels allows screening of multiple drugs to determine the right combination in a patient-specific manner.	The proposed platform enabled the differentiation between drug susceptible, drug tolerant/resistant cancer cells in less than 12 hours. Impedance measurement of cells seeded in gel provided information on cell growth, morphology, and cell density.	Polydimethylsiloxane (PDMS)-based microfluidic devices have the disadvantage of being porous to molecules less than 500 Da. These molecules can be absorbed creating a difference in the concentration of drugs.	[90]
	Integration of electrical sensing modality with microfluidics to measure electrical (impedance) response of a cancer cell upon exposure through dynamic drug delivery in a 3D matrix environment.	Potential to study patient-derived samples for personalized medicine in a rapid, low-cost approach for real-time drug screening analysis.		
Melanoma				
Type: tumor migration Cells: fibroblast and keratinocyte	Equipped with air walls to pattern cells without using conventional hydrogel barriers. Consists of circular chambers with narrow connections.	The presence of dermal fibroblasts and keratinocytes led to changes in melanoma cell morphology and growth pattern.	The chemokine secretion analysis method does not explicitly identify the cell type responsible for secreting the chemokines.	[91]
	Ability to coculture melanoma cells with keratinocytes and dermal fibroblasts. Air walls dissolve progressively and allows cellular crosstalk and migration.	The proposed analysis demonstrated upregulation of multiple secreted factors involved in tumor progression.	Cellular crosstalk can involve multiple mechanisms such as exosomes, RNAs, or mechanical forces that were not investigated.	

Microfluidic models incorporating hydrodynamic flow

Cells can sense IF and fluid shear stress (or pressure) and change cellular behavior [44]. Fluid flow and shear stress dynamically influence activation or inhibition of a series of mechanosensitive molecules, such as insulin growth factor (IGF)-2, vascular endothelial growth factor (VEGF), Rho-associated kinase (ROCK), and caveolin (CAV)-1. IF and shear flow are crucial migratory factors for cancer cell invasion [45,46]. Flow can trigger increased migration of cells via activation of surface receptors, such as the chemokine receptor CXCR4 and CCR7 [47]. Autologous chemotaxis, induced by a transcellular chemokine gradient, is responsible for driving directional cell migration in the presence of IF [48]. The most significant effect of IF, elevated IFP and solid stresses, leads to constricted and leaky vasculature, which impacts on drug delivery and the collapse of these vessels, which eventually causes hypoxia. These biophysical factors are important in inducing tumor migration events, which are not addressed in conventional in vitro culture models. The first step is to introduce biomimetic and natural matrices to recapture the native architecture of the ECM. To address physical stresses in the TME, a biomimetic ECM must be equipped with perfusion (or relevant IF rates). In recent years, the development of 3D matrix-based microfluidic devices as 3D in vitro cell migration platform addresses the limitations to integrate biophysical cues. Such studies have highlighted the role of 3D microfluidics and the importance of mechanical forces from ECM remodeling and IF in cancer cell migration events [45,49,50].

Microfluidic models can generate flow rates and shear stress mimicking those found within tumor tissues. For example, increased interstitial and shear flow can lead to morphological and epigenetic changes that can result in a more aggressive behavior of breast cancer cells [45,49]. Recent microfluidic models have highlighted the importance of flow-induced hydrodynamic shear stress on EMT in A549 (lung epithelial adenocarcinoma) cells cultured in a 3D microfluidic platform for several days [51]. Striking phenotypic changes were observed in A549 cells within the device when stimulated with shear flow compared with static cultures (no flow). Shear flow and IFP in solid tumors have been implicated in reducing the efficacy of cancer therapeutics [52]. Indeed, when the effect of flow on the efficacy of drugs on A549 cells was investigated, distinct cytotoxic effects were observed compared with static models, highlighting the role of flow in response to drugs [51]. A downregulation of E-cadherin and an increase in vimentin and N-cadherin expres-

sion was observed, which is indicative for EMT, a mesenchymal cell phenotype that is frequently associated with therapy resistance [51]. This dynamic microfluidic model serves as a drug screening tool for different cancer types.

IF-induced downregulation of cadherins has also been implicated in increased tumor invasion as shown in a heterogeneous spheroid model consisting of MDA-MB-231 and MCF10A cells embedded in a collagen type I matrix (Figure 3B) [49]. In no-flow experiments, the heterogeneous spheroid does not show any disintegration, and only peripheral MDA-MB-231 cells migrate (Figure 3Bii and iii). When IF was applied, the reduced E-cadherin expression on MCF-10A cells led to the dissociation of MDA-MB-231 cells from the spheroid core (Figure 3Bii and iv). Designed to operate under a flow rate of 2 $\mu\text{m/s}$, lower than the elevated IF rates observed in animal models (up to 9 $\mu\text{m/s}$) or human patients (up to 55 $\mu\text{m/s}$), this platform highlights the importance of biophysical parameters in the TME [49]. This effect is a characteristic of morpho-logical change that occurs if cells switch from a mesenchymal to an amoeboid migration mode. In cells, structural rearrangements are determined by time-dependent adhesion interactions and long-range hydrodynamic interactions along with actin remodeling. In the presence of flow, amoeboid motility is triggered by the lack of molecules such as the fibronectin, which fails to form long-lived adhesions with collagen fibers [53] (Box 1).

Shear flow is a key regulator of cancer cell intravasation. Cancer cells preferentially intravasate in areas of low shear flow as high shear flows can destroy the cells [54,55]. A microfluidic platform was developed to investigate the mechanism of intravasation and the way in which cells detect shear flow. Cells migrated inside longitudinal microchannels with an orthogonal channel such that cells would encounter an active fluid flow to mimic intravasation [55]. The shear stress sensed by cells due to fluid flow activated the transient receptor potential melastatin 7 (TRPM7), which promoted the influx of extracellular calcium ions and led to the reversal of migration direction via RhoA/Myosin-II and CDC42 pathways [55]. This study shows that cancer cells with high TRPM7 expression are not able to intravasate into blood vessels, thereby reducing the probability of metastasis [55]. Interstitial and shear flow studies using microfluidic models have improved our understanding of the migratory behavior of cells in the presence of flow and cellular plasticity as influenced by mechanical stimuli.

Microfluidic models incorporating cell–cell interactions

As cells migrate collectively into healthy tissues, the dynamic reciprocity between the secreted cytokines and their cell surface receptors governs cell migration. Intercellular signaling cascades between tumor cells and other non-cancerous cells (CAFs, stromal

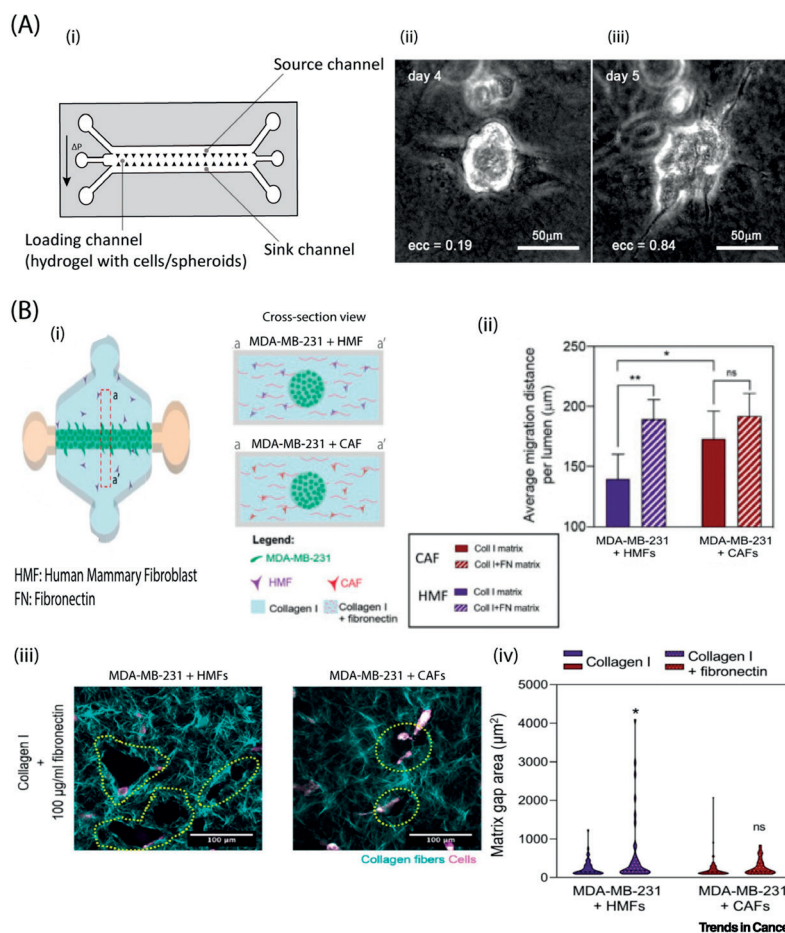


Figure 2. 3D matrix-based microfluidic models with physiologically relevant tumor microenvironment (TME) features. (Ai) A microfluidic device with a three-channel geometry. The source and the sink channel ensure proper hydration and study the impact of interstitial flow (IF) on cell migration. This model is very commonly used to study the effect of different cytokines on cell behavior. (Aii) Bright field image of transforming growth factor beta (TGF- β)-treated H1299 cells in a cluster-like morphology after 4 days, (Aiii) same cluster with strand-like morphology after 5 days [39]. (Bi) A 3D matrix-based microfluidic coculture model to recapitulate the TME with incorporation of fibronectin in collagen matrix and human mammary fibroblasts (HMFs) or cancer-associated fibroblasts (CAFs), (Bii) quantitative analysis of the average migration distance of metastatic cells (MDA-MB-231) from the edge of the lumen after 48 hours when cultured in the presence of HMFs or CAFs and in the presence of collagen without or with fibronectin. (Biii) Second harmonic generation (SHG) imaging of collagen fibers depicted in cyan and cells in magenta. Degradation of the collagen matrix (matrix gap area) in the presence of HMFs and CAFs highlighted by yellow broken line [63]. (Biv) Quantification of matrix gap areas for the conditions in (Biii) using violin plot representation [63]. (Aii) and (B) are reprinted and adapted from [39,63] licensed under CC by 4.0. * denotes a significant difference for $P \leq 0.05$ and ** for $P \leq 0.01$.

Box 2. CAFs and ECs in the TME

Cancer is a highly heterogeneous disease caused by multiple distinct (epi)genetic alterations in epithelial cells [76]. During cell proliferation, intratumoral heterogeneity can arise due to genetic, epigenetic, or proteomic changes in different cells present within a tumor [76]. In addition, cell-extrinsic factors, such as the local varying TME, hypoxia in the tumor core, residing and infiltrating immune cells, ECs, and CAFs, can contribute to intratumoral heterogeneity [76]. In addition to cancer cells, TME cells display heterogeneity in function, behavior, and origin. For example, the term 'CAFs' describes all activated fibroblasts found within the TME, regardless of their location, phenotype, or function [58]. Recent data show distinct CAF subtypes and subpopulations within the tumor that appear as cancer progresses over time [57,77]. These CAF subtypes have unique roles within the TME and are plastic, and one subtype can differentiate into another subtype [57,58]. Forming heterophilic cadherin junctions with cancer cells to lead collective invasion, CAFs can also intricately deposit fibronectin to remodel and produce a fibronectin-rich ECM to promote migration [61]. Intratumoral heterogeneity and signaling cascades between cells (including tumor cells, immune cells, and CAFs) are exacerbated by the presence of growth-induced stresses, IFP, and flow. This further activates mechanotransduction signaling pathways within the TME and promotes intercellular crosstalk between CAFs, immune cells, and tumor cells by creating chemokine gradients [78,79]. Hypoxia is prevalent in the tumor core and is a key driver of angiogenesis, one of the most vital stages of cancer progression [80]. In adults, ECs are quiescent and have low proliferation rates; however, cancer tumors can induce angiogenesis to sustain themselves, grow and eventually metastasize [81]. In cancer cells, the activation of oncogenes and mutations in tumor suppressor genes cause increased expression of VEGF which in turn activates ECs through paracrine signaling and stimulates their migration, proliferation, and ultimately results in angiogenesis [81]. There exists a high degree of heterogeneity within ECs obtained from the TME [81,82]. Combining microfluidics with hydrogel scaffolds to incorporate CAFs or ECs with cancer cells provides a unique platform to investigate cell migration characteristics, the role of CAFs/ECs within the TME, and other stages of the metastatic cascade such as tumor dissemination, intravasation, and extravasation.

cells, and immune cells) further contribute to tumor progression events [2,56]. CAFs are frequently one of the most prominent and active components in the TME. While their origins remain obscure, recent data suggest that distinct subpopulations of CAFs exist within the tumor and their roles evolve and change as the disease progresses [57,58]. CAFs, in some cancers, have been shown to lead to collective migration from the front by forming heterophilic junctions with cancer cells [59–61]. Through secreted cytokines, epigenetic reprogramming, and signaling cascades within the TME, CAFs have been implicated in ECM remodeling, the creation of a hypoxic microenvironment, and the alteration of the metabolic state of tumor cells, resulting in an acidic microenvironment [28,60–62]. Tumor angiogenesis is key to cancer progression and metastasis. The creation of a hypoxic TME causes the secretion of VEGF, a key angiogenic factor. VEGF activates ECs through paracrine signaling and stimulates cell migration and proliferation of ECs, eventually resulting in the induction of angiogenesis.

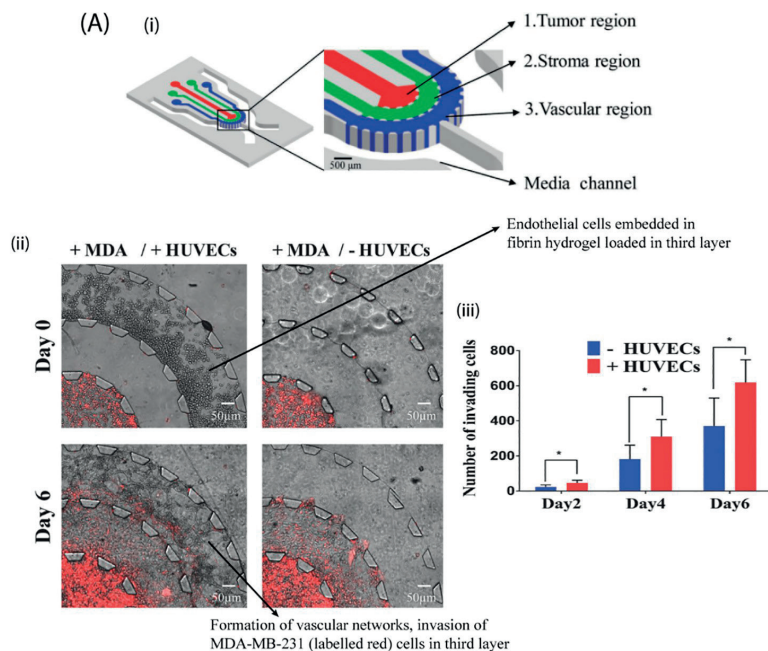
3D microfluidic models have been developed to recapitulate TME heterogeneity by introducing CAFs as ECM remodeling components that secrete MMPs. Stromal cells play a key role in the dynamic nature of the TME with an active influence on remodeling, which controls physical properties such as stiffness, viscoelasticity, and pore size of ECM [34]. For example, a 3D matrix-based microfluidic platform was developed to colocalize MDA-MB-231 embedded in fibronectin-rich collagen matrix as a lumen-like geometry with fibroblasts to recreate a 3D tumor-stroma model (Figure 2B). The microfluidic model incorporates human mammary fibroblasts (HMFs) and CAFs, thereby assessing the impact of cancer-stromal crosstalk on the migratory behavior of MDA-MB-231 cells [63]. In the first set of studies, fibronectin-rich matrix showed a qualitative increase in the number of cells migrating, regardless of the type of fibroblast present (Figure 2Bii). A second set of studies were performed to observe the effect of HMFs and CAFs on the surrounding matrix degradation due to the secretion of MMPs shown in Figure 2Biii and iv. Matrix degradation was quantified based on the gaps formed that are indicative of structural deformation as assessed by fluorescence imaging. In a TME consisting of fibronectin-rich collagen embedded with HMFs, invasive human breast cancer (MDA-MB-231) cells migrated faster and for longer distances. The fibronectin-rich matrix signals HMFs to produce and secrete MMPs, which are responsible for ECM degradation [63]. These results indicate the influence of biochemical factors inside the TME (i.e., fibroblasts and ECM protein compositions) that directly induces matrix degradation, which results in a biomechanical response. Therefore, microfluidics, with its extensive modeling parameters, allows the incorporation of different cancer cell types and other TME-related biological components to recreate key components of a tumor model. The model provides a platform to study the effect of different chemokines and a combination of matrix materials for a comprehensive understanding of certain metastatic events.

A model developed to simultaneously study cell invasion and intravasation integrates a 3D multilayered microfluidic platform in the tumor stroma with MDA-MB-231 cancer cells embedded in a collagen hydrogel. In this model, MDA-MB-231 cells had increased migratory behavior, and they invaded the stromal region in the presence of vascular networks, which mimicked *in vivo*-like capillaries on day 0 compared with day 6 (Figure 3Aii) [64]. The unique advantage of this model is the spatial control on the 3D tumor, stroma, and the vascular network region that allows for the formation of *in vivo*-like capillaries by human umbilical vein endothelial cells (HUVECs) embedded in relevant biomimetic ECM matrices. The presence of HUVECs led to the enhanced invasion of MDA-MB-231 cells and the degree of invasiveness was quantified as shown in Figure 3Aiii. The microfluidic design serves as a novel platform to study interactions and biological mechanisms between cells and their TME for invasion and intravasation studies. While the collagen and fibrin matrices used in the study are physiologically relevant, the adaptability of microfluidics

enables the incorporation of patient-derived primary tumor cells and decellularized ECM to increase the accuracy of 3D matrix-based microfluidic models [9].

Outstanding questions

- How can we adopt microfluidic models further to replicate nearly all of the complexities of the TME?
- Will the incorporation of microfluidics in multicellular 3D models enable us to recapitulate the multiple steps of the metastatic process, from initial invasion into adjacent tissue, intravasation, survival in circulation, and extravasation to outgrowth in distant tissue?
- What next steps are needed for microfluidic models to be utilized in routine clinical studies for drug screening and personalized cancer therapy?



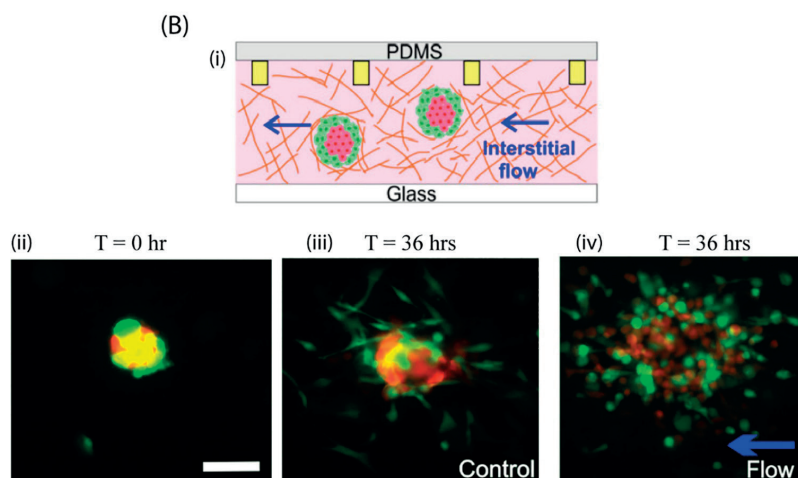


Figure 3. 3D microfluidic models to study and investigate invasion and intravasation events. (Ai) Schematic of multilayered tumor-stroma microfluidic model. The microfluidic model consists of three layers: 1. tumor region: consisting of MDA-MB-231 cells embedded in collagen hydrogel, 2. stromal region: to introduce tumor-stroma crosstalk, 3. vascular region: endothelial cells embedded in fibrin to produce vascular networks. (Aii) MDA-MB-231 breast cancer cells (red fluorescence) invade the stromal region from the tumor region with simultaneous formation of vascular networks by the end of day 6. (Aiii) Quantitative analysis of MDA-MB-231 breast cancer cells invading into the tumor stromal region in the presence and absence of human umbilical vein endothelial cells (HUVECs) [64]. * indicates a significant difference for $P < 0.05$. (Bi) cross-sectional view: channel filled with collagen hydrogel embedded with spheroids of cocultured MCF-10A normal breast cells and MDA-MB-231 breast cancer cells. (Bii–iv) (enlarged) fluorescence images of MDA-MB-231 cells (in green), MCF-10A (in red), scale bar 100 μm . (Biii) Control experiments (no-flow) do not promote spheroid dissociation, only peripheral MDA-MB-231 cells invaded outwards. (Biv) after application of 36 hours of interstitial flow, tumor dissociation is induced and is demonstrated by the disintegration of the spheroid core [49]. (A) is reproduced from [64] with permission from © 2018 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (B) is reprinted and adapted from [49] licensed under CC by 4.0. Abbreviation: PDMS, polydimethylsiloxane.

Concluding remarks

3D matrix-based microfluidic models allow for more precise physiological representation and recreation of the TME, enabling us to determine the dominant mechanism of cancer cell migration events under conditions mimicking more closely the aspects of in vivo situation. By combining relevant physicochemical parameters in a tumor-stroma model, the extent of specific roles of ECM, tissue heterogeneity, chemical signals, biomolecules, and mechanical forces can be explored. In recent years, microfluidic models were able to critically interrogate the influence of biochemical and biomechanical factors in tumor progression events. In addition, the advantage of real-time imaging makes this platform highly suitable to visualize dynamic processes and distinguish between single or collective

migratory behavior displayed by tumor cells. The ability to select synthetic biomaterials that can be tuned for properties such as stiffness and pore size and to promote cell–matrix interactions gives an added advantage in decoupling interdependent factors for an in-depth revelation to interpret cell migration mechanisms.

This review focuses on recent advances in microfluidic models to study the TME and the migration mechanisms of cancer cells (mostly breast and lung cancer) involved in tumor metastasis events, such as dissemination and invasion. Based on the type of tumor and influencing factors, including CAFs, ECM, TGF- β , and IF, the modes of cancer cell migration differ from each other. These emerging results demonstrate the importance of microfluidic models to determine preclinical therapeutic responses. By consolidating complex features of TME inside microfluidics, we can implement crucial physical and chemical cues (associated with different cancer types) that may enable more relevant drug screening platforms than could be achieved using static models. In addition, it has been confirmed that the increase of vascular density leading to enhanced mechanical forces during tumor growth can drastically diminish the delivery of drugs and oxygen supply at the inner region of the tumor [65].

There exists an opportunity to replace animal models preceding clinical trials for drug screening and anticancer therapeutics in the next decade by establishing specific quality controls for such 3D in vitro models. To fully identify the potential of 3D-microfluidic approaches in cancer metastasis modeling and clinical trials, several key challenges and issues must be confronted (see Outstanding questions). To achieve this vision, microfluidics needs to undergo considerable upgrades by integrating it with cutting-edge and complementary research tools such as 3D bioprinting, engineered tissue constructs, artificial intelligence, and computational modeling. This integration will create next-generation robust microfluidic models with a vision for personalized therapies by using patient-derived cells and mimicking patients' in vivo TME conditions. In the future, 3D microfluidic models will become more refined and equipped with automation functionalities and artificial intelligence algorithms to automatically identify crucial factors in tumor progression events, improving our understanding of cancer invasion and discovering a new class of drugs for cancer treatment. Therefore, microfluidics will become an ideal analytical tool to systematically investigate these complex and coupled features. Moreover, various types and combination of drugs can be tested to select the most suitable therapeutics for each cancer patient.

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Declaration of interests

No interests are declared.

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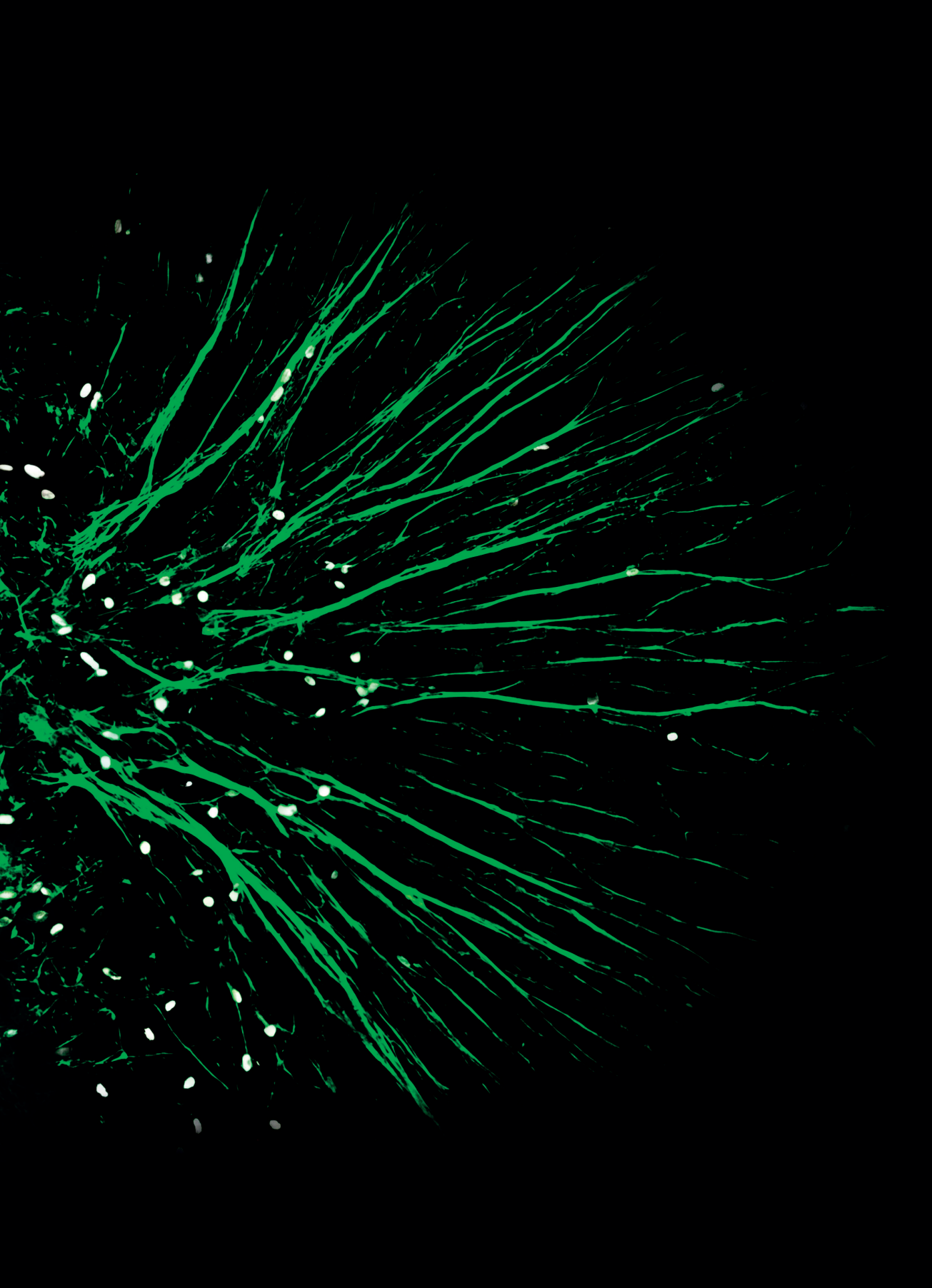
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Chapter 6

Conclusion & Discussion



Integration With Current Knowledge

The findings in my PhD thesis contribute to refining concepts in tumor – stroma biology. First, the spatial dependence of CAF influence advances earlier work showing CAFs promote invasion through contact-dependent and paracrine mechanisms[1], [2]. Our results show that mechanical force propagation through collagen has a finite spatial reach, complementing earlier matrix mechanics studies and providing a functional explanation for spatially restricted invasion fronts observed in vivo [3], [4].

Second, the demonstrated distinction between mechanical and proteolytic remodeling expands existing MMP-centric models, indicating that proteolysis is key to spheroid expansion, whereas non-proteolytic CAF remodeling of collagen promotes cell migration and dissemination. Additionally, our findings reinforce that mechanical anisotropy alone can generate invasive paths[5], [6].

Third, the methodological framework outlined in Chapter 3 supports current models of EMT as a dynamic and reversible continuum [7]. By assembling techniques to monitor SMAD activation, epithelial and mesenchymal marker dynamics, cytoskeletal reorganization, and migration responses, the chapter provides practical tools for interrogating the temporal and phenotypic plasticity associated with TGF- β -induced EMT. These experimental approaches are well suited to studying the heterogeneous and metastable EMT states that have been increasingly recognized as major contributors to tumor progression and metastatic competence [8].

Fourth, the observation that fibroblast CM induces significant single-cell dissemination of lung adenocarcinoma spheroids despite undetectable soluble TGF- β suggests that TGF- β receptor dependent responses can arise from the combined action of multiple fibroblast derived factors. These results show that distinct paracrine inputs present in fibroblast CM may act synergistically to drive a dissemination phenotype, even in the absence of detectable levels of a single dominant ligand.

Finally, the microfluidic review highlights an ongoing shift toward engineered models capable of imposing spatial, temporal, and mechanical control over tumor – stroma interactions. Together, the findings across Chapters 2 – 5 support an integrated view in which tumor invasion emerges from the interplay of CAF driven matrix mechanics, ECM architecture, EMT plasticity, and noncanonical modes of stromal signaling. *The figure below provides an integrated schematic overview of the myriad mechanisms through which fibroblasts orchestrate tumour invasion.*

Fibroblast Mediated Cancer Cell Invasion: Thesis Overview

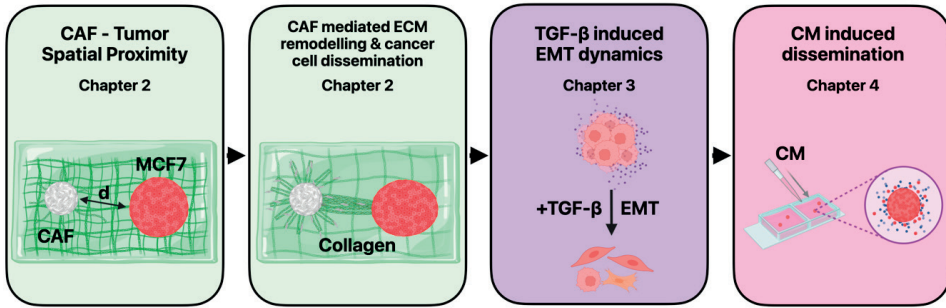


Figure 1. Fibroblast-mediated tumour invasion: thesis overview. CAF-tumour spatial proximity determines the onset and directionality of collective invasion through distance-dependent mechanical force propagation in collagen matrices (Chapter 2, left). CAF-mediated ECM remodelling generates thick and aligned collagen fiber tracks and invasion-permissive channels that direct cancer cell dissemination (Chapter 2, centre-left). TGF- β stimulation of cancer cells induces dynamic and heterogeneous epithelial-to-mesenchymal transition at the single-cell level, captured through quantitative live-cell imaging and morphometric analysis (Chapter 3, centre-right). Fibroblast-conditioned medium drives single-cell dissemination of lung adenocarcinoma spheroids through TGF- β receptor-dependent signalling, in the absence of detectable soluble ligand (Chapter 4, right). Together, these findings position CAFs as active orchestrators of a spatially and biochemically coordinated invasion programme. Created in BioRender. Mehta, P. (2026).

6

Limitations

Matrix Composition

The 3D models used in this thesis rely primarily on type I collagen hydrogels, which, while widely used for studying invasion, capture only a subset of the biochemical and mechanical properties of native tumor ECM. Collagen I represents the fibrillar backbone of many tumors, but in vivo matrices also contain laminins, fibronectin, hyaluronan, elastin, and basement membrane derived proteoglycans that contribute to nonlinear elasticity, viscoelastic relaxation, and spatial variations in stiffness [9], [10]. These additional components may modulate integrin engagement, receptor clustering, and mechanotransduction. Furthermore, collagen hydrogels lack the degree of enzymatic crosslinking and lysyl oxidase (LOX) dependent stiffening observed in breast tumors, processes known to amplify invasion by altering fiber tension and pore architecture[11]. Because CAF induced remodeling in our system occurs within a relatively homogeneous collagen network, the results may underestimate or oversimplify the complexity of matrix guided invasion in vivo.

In essence, while collagen provides a tractable model to isolate CAF – cancer cell interactions, it does not fully recapitulate the mechanical heterogeneity or biochemical cues that shape invasion in native tumors.

CAF Heterogeneity

CAFs are not a uniform population; they encompass inflammatory (iCAF), myofibroblastic (myCAF), antigen presenting (apCAF), and matrix remodeling subtypes, each exerting distinct influences on cancer behavior [12], [13]. The 19TT breast CAF line used in this thesis displays features characteristic of myCAFs, more specifically a subtype that is S100A4+, displaying α -SMA expression, contractility, and strong ECM remodeling capacity[14], [15]. As a result, the invasion phenotypes observed here reflect the behavior of a contractile, matrix remodeling CAF subtype and may not generalize to iCAFs, which are more cytokine driven and less mechanically active [15].

Additionally, CAFs in vivo exist in spatially organized niches and gradients that influence their phenotype; the standardized culture conditions used here may dampen or reshape this heterogeneity [2]. *Similarly, the use of a single CAF and cancer cell line, 19TT and MCF7 respectively, reflects a specific contractile stromal subtype paired with a luminal breast cancer phenotype, and limits the generalizability of the invasion phenotypes observed to other CAF subtypes, breast cancer subtypes, or cancer types more broadly.* Therefore, while the models reveal core mechanistic principles, they cannot account for the full functional diversity of CAF populations across patients or tumor regions.

Missing Immune and Vascular Components

Tumor invasion is shaped not only by fibroblasts and ECM architecture but by crosstalk with immune and endothelial cells. Macrophage secreted tumor necrosis factor alpha (TNF- α), Interleukin-6 (IL-6), and epidermal growth factor (EGF) can synergize with CAF derived signals to promote EMT and collective invasion; conversely, cytotoxic immune cells can suppress invasion through direct or paracrine mechanisms [16], [17]. Endothelial cells and pericytes also influence matrix deposition and tension through VEGF driven angiogenic remodeling. Importantly, perfusion and interstitial flow present in vivo generate pressure gradients that modulate collagen alignment, chemokine distribution, and integrin activation[18].

The in vitro systems used in this thesis isolate CAF – cancer cell interactions, which is advantageous for mechanistic clarity but excludes emergent behaviors arising from multicellular interactions. This limitation is particularly relevant for Chapter 4, where paracrine signaling, when considered in isolation, may differ in magnitude or direction when competing or compensatory immune derived signals are present in vivo.

In Chapter 4, fibroblast CM induced TGF- β receptor dependent dissemination of A549 spheroids despite the absence of measurable active TGF- β in standard detection assays. This raises the possibility that low abundance or context dependent forms of TGF- β signaling can drive invasion even when ligand levels fall below conventional detection thresholds. The observation that SB-431542 effectively suppressed dissemination indicates that TGF- β receptor activity is required, suggesting that TGF- β itself, or a functionally equivalent activator, remains present in a form capable of initiating signaling. Importantly, the lack of strong reporter activation does not rule out such activity; reporter constructs typically capture sustained or high amplitude SMAD signaling, whereas CM driven dissemination may rely on subtle, transient, or spatially restricted receptor activation that is not faithfully reflected in global reporter output.

Several mechanistic explanations remain viable. First, TGF- β may be present in concentrations below assay sensitivity yet still sufficient to activate receptors under permissive biochemical conditions. Second, the CM may contain cofactors that potentiate receptor responsiveness, such as ECM fragments, proteases, cofactors, or cytokines that lower the threshold for receptor activation. Third, integrin mediated or protease mediated modulation of latent TGF- β cannot be excluded, although the absence of strong reporter activity suggests that if such activation occurs, it is limited in magnitude or duration.

Without molecular characterization of the CM, the precise mechanism remains unresolved. However, the data strongly support the conclusion that fibroblast derived factors can render tumor cells responsive to extremely low level or weakly activated TGF- β signaling, sufficient to promote dissemination even in the absence of detectable ligand. Future proteomic, phosphoproteomic, and secretome analyses will be required to delineate whether this response reflects low abundance ligand based signaling, context enhanced receptor sensitivity, or a more complex mode of stromal signal integration.

Need for In Vivo Validation

Although 3D in vitro models offer precise control over cellular organization and matrix properties, they cannot fully recapitulate the complexity of living tissues. Tumors in vivo are shaped by immune infiltration, vascular dynamics, mechanical constraints, and fluctuating metabolic gradients factors known to influence invasion and therapy response [16], [19]. These limitations mean that key mechanisms described in this thesis, including distance dependent matrix remodeling and low level TGF- β receptor activation, require confirmation in more physiologically integrated systems such as organotypic cultures, ex vivo tumor slices, xenografts, or genetically engineered mouse models [20], [21]. Such studies will determine whether the mechanistic principles identified here operate within the heterogeneity and evolving microenvironments characteristic of intact tumors.

Future Research Directions

Advanced ECM Engineering

The collagen-only matrices used in this thesis isolate mechanical remodeling mechanisms but do not capture the biochemical and architectural diversity of native ECM. Future models should incorporate composite ECM systems that combine collagen I with fibronectin, laminin, hyaluronan, and basement-membrane proteoglycans, each contributing unique ligand receptor interactions and nonlinear mechanical behaviors [9], [22], [23]. Beyond biochemical complexity, mechanical properties can be refined by introducing LOX-mediated cross-linking to establish tumor-like stiffness gradients, employing viscoelastic or stress-relaxing hydrogels to examine how CAF contractility adapts to time-dependent mechanical cues, and designing aligned or micropatterned fiber architectures to enable controlled interrogation of CAF-driven anisotropy. Together, these engineered matrices would permit a more rigorous and physiologically relevant analysis of how CAFs remodel heterogeneous ECM environments and would test whether the distance-dependent remodeling observed in simplified collagen systems persists in more complex stromal contexts.

Mechanistic Dissection of Ligand Independent Activation

Chapter 4 shows that fibroblast-derived CM induces TGF- β receptor dependent dissemination below levels of detectable active ligand, indicating that low-level or context-dependent activation may drive the response. To resolve the underlying mechanism, future work should characterize the molecular components of the CAF secretome by profiling extracellular vesicles, testing for latent TGF- β activation through integrin-blocking or protease assays, and assessing redox or proteolytic activities that may modulate receptor sensitivity. Complementary phosphoproteomic analyses could help distinguish signaling patterns associated with ligand-dependent versus ligand-independent activation [24], [25], [26]. Together, these studies will provide insight into CM induced dissemination and whether it represents a distinct activation mode or reflects an underappreciated form of contextual signal amplification. These mechanistic studies will reveal whether CM mediated dissemination represents a novel activation route or an underappreciated form of contextual signal amplification.

Single Cell and Spatial Mapping of CAFs

The findings in Chapter 2 highlight robust CAF-mediated remodeling but do not resolve which CAF subtypes drive these behaviors. Given the well-established heterogeneity of CAF populations and their distinct roles in invasion, immune modulation, and therapeutic response [12], [15], future work should integrate single-cell RNA sequencing to classify CAFs into contractile, inflammatory, antigen-presenting, or matrix-modifying subtypes, together with spatial transcriptomics or multiplexed proteomics [23] to map their

distribution relative to remodeling zones and invasive fronts. Complementary CRISPR-based or pharmacologic perturbations targeting key regulators of CAF function would help determine the causal contributions of these subtypes. Such approaches will clarify whether distance-dependent invasion arises from general fibroblast activity or is driven by specific, functionally specialized CAF subsets.

Immune Integrated and Vascularized 3D Models

The absence of immune and vascular components in the current models limits their physiological relevance. Future work should incorporate microfluidic organ-on-chip systems with perfusable endothelial channels, which can reproduce vascular shear stress, nutrient and oxygen gradients, and leukocyte trafficking to more accurately model tumor vascular crosstalk [25]. In parallel, co-culture models including macrophages, neutrophils, or T cells would allow systematic testing of how immune-derived cytokines and cell-cell interactions modulate CAF remodeling, EMT dynamics, and context-dependent TGF- β receptor signaling [16]. Finally, organoid stromal hybrid systems, combining patient-derived tumor organoids with primary CAFs, endothelial cells, and defined ECM components, could capture patient-specific heterogeneity and multi-lineage interactions [27]. Together, such immune-integrated and vascularized 3D models would enable assessment of whether the matrix-guided invasion observed in Chapter 2 and the CM driven dissemination in Chapter 4 persist, are attenuated, or are reshaped within a fully interactive tumor microenvironment.

In Vivo Validation

Ultimately, the mechanistic insights proposed in this thesis require validation in living tissues. Orthotopic implantation of CAF cancer cell mixtures could determine whether spatial proximity in vivo reproduces the distance-dependent invasion patterns and matrix anisotropy observed in vitro. Metastatic mouse models may clarify whether low-level or context-dependent TGF- β receptor activation influences dissemination efficiency or metastatic tropism, while intravital imaging [20] would allow real-time visualization of CAF dynamics, ECM remodeling, and EMT state transitions within intact tissue. Complementary genetically engineered models targeting CAF-specific remodeling enzymes or secreted factors would help establish causality. Together, these approaches will test whether the spatiotemporal framework identified here –mechanical guidance, EMT plasticity, and paracrine activation operate as a generalizable mode of microenvironment-driven invasion in vivo.

Concluding Remarks

This thesis demonstrates that tumor invasion emerges from **multiscale interactions between CAF mechanics, ECM architecture, EMT plasticity, and unconventional paracrine signaling**. By integrating spatial, temporal, and mechanical perspectives, the work reframes CAFs as active organizers not merely supporters of the invasive microenvironment. The conceptual framework developed here contributes to a broader understanding of metastasis as a system level phenomenon shaped by microenvironmental context.

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Summary

This thesis examines how CAFs regulate tumor invasion through coordinated mechanical, biochemical, and spatial mechanisms. Using 3D in vitro models, time-lapse imaging, and pharmacologic perturbations, the findings reveal several interconnected principles that reshape stromal control paradigms in cancer biology.

Chapter 2 demonstrates that CAF – tumor spatial proximity is a primary determinant of invasion onset and organization. This observation complements in vivo studies showing that stromal architecture and fibroblast distribution define invasive niches[1], [2]. By co-embedding CAF and tumor spheroids at controlled distances, we show that CAFs remodel collagen I through fiber alignment, compaction, and tension propagation, consistent with prior demonstrations of fibroblast driven ECM contractility [3], [4], [5], [6]. This distance dependent ECM remodeling forms anisotropic fiber tracks that direct collective cancer cell migration, supporting earlier mechanobiology findings on ECM anisotropy and migration guidance [4], [7], [8]. Importantly, pharmacologic inhibition of MMPs reduced collagen degradation and spheroid expansion but did not abolish CAF migration or low level dissemination, echoing recent work distinguishing “mechanical remodeling” from “proteolytic remodeling” [9]. Heterospheroid models confirmed that CAFs generate similar ECM dynamics even when spatial order differs, demonstrating the robustness of matrix directed invasion.

Chapter 3 focuses on methods to investigate EMT and its dynamic regulation by TGF- β signaling. EMT is now recognized as a spectrum rather than a binary switch[10]. Using live cell imaging, we systematically outline molecular and cellular techniques to monitor TGF- β induced EMT in normal and cancer cell lines. It describes how to assess SMAD activation, changes in epithelial and mesenchymal marker expression and localization, alterations in cell morphology and polarity, dynamic reorganization of the actin cytoskeleton and stress fibers, as well as migration assays. Together, these methods provide a practical toolbox for probing EMT dynamics and epithelial plasticity in vitro and are well suited to interrogate heterogeneous or metastable EMT states described in recent literature [11].

In chapter four we found that conditioned media of lung fibroblasts supports invasion of lung adenocarcinoma spheroids in a TGF- β receptor signaling dependent manner, but in the absence of detectable TGF- β ligand. While this thesis does not establish the molecular basis of this activation, the findings are consistent with broader literature suggesting that receptor signaling strength can be shaped by the biochemical context of secreted factors. These results underscore the possibility that fibroblast derived cues can potentiate signaling pathways even when ligands are not detectable, revealing an additional layer of stromal influence on tumor cell behavior.

Finally, the microfluidic review highlights an ongoing shift toward engineered models capable of imposing spatial, temporal, and mechanical control over tumor – stroma interactions. Together, the findings across Chapters 2 – 5 support an integrated view in which tumor invasion emerges from the interplay of CAF driven matrix mechanics, ECM architecture, EMT plasticity, and noncanonical modes of stromal signaling. Collectively, this thesis shows that CAF mechanics, ECM architecture, EMT dynamics, and paracrine signaling operate as an integrated regulatory system that shapes tumor invasiveness.

Nederlandse Samenvatting



Dit proefschrift onderzoekt hoe CAFs tumorinvasie reguleren via gecoördineerde mechanische, biochemische en ruimtelijke mechanismen. Met behulp van 3D in vitro-modellen, time-lapse-beeldvorming en farmacologische verstoringen onthullen de bevindingen verschillende onderling verbonden principes die de paradigma's van stromale controle in de kankerbiologie herdefiniëren.

Hoofdstuk 2 toont aan dat de ruimtelijke nabijheid van CAF en tumor een primaire bepalende factor is voor het begin en de organisatie van invasie. Deze observatie vult in vivo-onderzoeken aan die aantonen dat de stromale architectuur en de fibroblast distributie invasieve niches definiëren [1], [2]. Door CAF en tumorsferoïden op gecontroleerde afstanden samen in te bedden, laten we zien dat CAFs collageen I remodelleren door middel van vezeluitlijning, compactie en spanningspropagatie – consistent met eerdere demonstraties van door fibroblasten gedreven ECM-contractiliteit [3], [4], [29], [30]. Deze afstandsafhankelijke ECM-remodelling vormt anisotrope vezelbanen die de collectieve migratie van kankercellen sturen, wat eerdere mechanobiologische bevindingen over ECM-anisotropie en migratiegeleiding ondersteunt [5], [13], [29]. Belangrijk is dat farmacologische remming van MMP's de collageenafbraak en de sferoïde-expansie verminderde, maar de CAF-migratie of verspreiding op laag niveau niet teniet deed, wat overeenkomt met recent werk dat onderscheid maakt tussen “mechanische remodelling” en “proteolytische remodelling” [31]. Heterosferoïde modellen bevestigden dat CAF's vergelijkbare ECM-dynamiek genereren, zelfs wanneer de ruimtelijke ordening verschilt, wat de robuustheid van matrixgerichte invasie aantoont.

Hoofdstuk 3 richt zich op methoden om EMT en de dynamische regulatie ervan door TGF- β -signalerings te onderzoeken. EMT wordt nu erkend als een spectrum in plaats van een binaire schakelaar [32]. Met behulp van live-cell imaging schetsen we systematisch moleculaire en cellulaire technieken om TGF- β -geïnduceerde EMT te monitoren in normale en kankercellijnen. Het beschrijft hoe SMAD-activering, veranderingen in de expressie en lokalisatie van epitheliale en mesenchymale markers, veranderingen in celmorphologie en polariteit, dynamische reorganisatie van het actine-cytoskelet en stressvezels, evenals migratieassays kunnen worden beoordeeld. Samen bieden deze methoden een praktische toolbox voor het onderzoeken van EMT-dynamiek en epitheliale plasticiteit in vitro en zijn ze zeer geschikt om heterogene of metastabiele EMT-toestanden te onderzoeken die in recente literatuur worden beschreven [8].

In hoofdstuk vier vonden we dat geconditioneerde media van longfibroblasten de invasie van longadenocarcinoom-sferoïden ondersteunen op een manier die afhankelijk is van TGF- β -receptor signalerings, maar in afwezigheid van detecteerbare TGF- β -ligand. Hoewel dit proefschrift de moleculaire basis van deze activering niet vaststelt, zijn de bevindingen consistent met bredere literatuur die suggereert dat de sterkte van receptor signalerings

kan worden gevormd door de biochemische context van uitgescheiden factoren. Deze resultaten onderstrepen de mogelijkheid dat signalen afkomstig van fibroblasten signaalroutes kunnen versterken, zelfs wanneer liganden niet detecteerbaar zijn, wat een extra laag van stromale invloed op het gedrag van tumorcellen onthult.

Tot slot benadrukt het overzicht van microfluidische technieken een voortdurende verschuiving naar gemanipuleerde modellen die in staat zijn om ruimtelijke, temporele en mechanische controle uit te oefenen over tumor-stroma-interacties. De bevindingen in de hoofdstukken 2 tot en met 5 ondersteunen samen een geïntegreerd beeld waarin tumorinvasie voortkomt uit de wisselwerking tussen door CAF's aangedreven matrixmechanica, ECM-architectuur, EMT-plasticiteit en niet-canonische vormen van stromale signalering. Dit proefschrift toont aan dat CAF-mechanica, ECM-architectuur, EMT-dynamiek en paracrine signalering functioneren als een geïntegreerd regulerend systeem dat de invasiviteit van tumoren vormgeeft.

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Curriculum Vitae



PRANAV MEHTA

Delft, Netherlands | pranav.mehta114@gmail.com | +31 6 489 25688 | [linkedin.com/in/pmehta12](https://www.linkedin.com/in/pmehta12)

PhD-trained life scientist with 5 years of experience independently designing, executing, and documenting research studies to rigorous quality standards. Skilled in protocol development, deviation identification, troubleshooting, and communicating scientific results to diverse stakeholders. Hands-on expertise in cell biology and molecular biology techniques. Experienced in SOP authorship, compliance, and cross-functional coordination.

EDUCATION

PhD, Chemical & Cell Biology / Product & Process Engineering

2021 – 2026

Leiden University Medical Center x Delft University of Technology

Research conducted in the laboratories of Prof. dr. Peter ten Dijke & dr. Pouyan E Boukany, investigating the interplay between cancer associated fibroblasts and cancer cells.

Thesis: Conversations in the Tumor Microenvironment: experimental models of fibroblast-driven cancer cell invasion

MSc, Chemical & Biomolecular Engineering

2018 – 2020

Johns Hopkins University, Baltimore, Maryland, USA

Research conducted in the laboratory of Prof. Konstantinos Konstantopoulos, investigating cancer cell invasion under micro and nanoscale confinement with a focus on cellular adaptations to high viscosity microenvironments.

Thesis: Viscosity Induced Cellular Adaptations for Increased Cell Motility

Bachelor of Engineering, Chemical Engineering

2014 – 2018

D.J. Sanghvi College of Engineering, University of Mumbai, Mumbai, India

Thesis: Design and fabrication of a spiral microfluidic device for size-based separation of bacterial cells from complex biological fluids including blood.

Cambridge International AS & A Levels

2012 – 2014

Vibgyor High, Mumbai, India

EXPERIENCE

Lead Researcher | LUMC x TU Delft

2021 - 2026

- Translated research objectives into clear, robust study protocols; designed and executed studies from conception through completion, ensuring documentation met rigorous quality standards throughout.
- Reviewed and interpreted experimental results across concurrent studies; identified deviations, coordinated troubleshooting with the laboratory team, and ensured corrective actions were properly documented and implemented.
- Executed hands-on laboratory work across mammalian cell culture, ELISA, FACS, Western blot, qPCR, lentiviral transduction, and 3D tumour models; provided scientific guidance and practical support to junior researchers during study execution.
- Managed data analysis and quality control across concurrent projects; prepared well-structured study reports and communicated results, progress, and deviations clearly to internal teams and external stakeholders.
- Served as single point of contact between LUMC and TU Delft, coordinating internally across biology, engineering, and clinical functions; communicated study content, progress, and outcomes to stakeholders with different scientific backgrounds.
- Established ML2-certified cell biology laboratory at TU Delft; co-authored biosafety SOPs and authored standard operating procedures for all core wet lab techniques; ensured compliance with regulatory and ethical standards across dual-institution research; familiar with GMP quality system principles.
- Led scientific writing across 4 peer-reviewed PhD publications; prepared technical reports and contributed to cross-functional documentation; presented findings at international conferences to scientific and clinical audiences.
- Resolved a critical methodological challenge by independently synthesising primary literature with current findings; coordinated the troubleshooting process and implemented a novel workflow adopted lab-wide.
- Maintained current awareness of scientific literature using PubMed; synthesised findings to inform study design, identify methodological improvements, and support QA and audit processes from a scientific and methodological perspective.
- Coordinated workload across multiple concurrent projects; mentored and advised graduate students and junior researchers on study design, experimental execution, and data interpretation; prioritised tasks effectively under time and performance pressure.

Graduate Researcher | Johns Hopkins University

2018 - 2020

- Designed experimental frameworks underpinning successful in vivo cancer dissemination studies; performed data QC and quantitative interpretation for cross-lab datasets.
- Developed automated MATLAB/FIJI pipeline for focal adhesion segmentation and quantification, mapping distribution, size, and turnover dynamics from formation through dissolution.
- Collaborated across engineering, biology, and imaging groups; contributed to a Nature (2022) publication.

Teaching Assistant, Thermodynamics | Johns Hopkins University

2019

- Led problem-solving sessions for engineering cohorts; simplified complex quantitative concepts for diverse student audiences.

Director & Senior Educator | eKids, Mumbai

2016 - present

- Designed and delivered science curriculum across Physics, Chemistry, Biology, and Mathematics for GCSE, AS/A2, and IBDP students through to undergraduate level; developed interactive strategies including analogy-based teaching, quizzes, and trivia to improve engagement across diverse learning profiles.
- Maintained continuous teaching output through MSc, PhD, and remote periods including full online delivery during COVID; contributed to institutional leadership as Director overseeing programme quality and educational effectiveness.

SELECTED PUBLICATIONS

- Mehta P. et al. **Inter-Spheroid Proximity and Matrix Remodelling Determine CAF-Mediated Cancer Cell Invasion.** Acta Biomaterialia (2025)
- Boot R.C., Mehta P. et al. **Cell Spheroid Viscoelasticity is Deformation-Dependent.** Scientific Reports (2024)
- Mehta P.P. et al. **Microfluidics Meets 3D Cancer Cell Migration.** Trends in Cancer (2022)
- Sinha A.*, Mehta P.* et al. (*equal contribution) **Visualizing Dynamic Changes During TGF-β-Induced EMT.** Methods in Molecular Biology, Springer (2022)
- Bera K., Mehta P. et al. **Extracellular Fluid Viscosity Enhances Cell Migration and Cancer Dissemination.** Nature (2022)

CERTIFICATIONS

Fundamentals of Finance, The Wharton School, University of Pennsylvania (Coursera)

Jan 2026

Introduction to Corporate Finance, The Wharton School, University of Pennsylvania (Coursera)

Jan 2026

TECHNICAL SKILLS & COMPETENCIES

Study Design & Management: Study protocol development, deviation identification and handling, troubleshooting coordination, study report preparation, QA audit support, SOP authorship; familiar with GMP quality system principles

Molecular Biology & Cell Systems: Mammalian cell culture (MCF7, MDA-MB-231, HEK293, primary CAFs), ELISA, FACS, Western blot, qPCR, SDS-PAGE, lentiviral transduction, stable cell line generation, 3D spheroid and ECM co-culture, confocal microscopy

Data Analysis & Reporting: MATLAB (custom pipelines), FIJI/ImageJ, Python, MS Excel; quantitative data analysis, structured report writing (4 peer-reviewed publications), technical documentation, scientific literature search (PubMed)

Client Communication & Coordination: Scientific communication to clients and stakeholders, study progress reporting, cross-departmental coordination (QA, lab, management), workload prioritisation across concurrent studies, researcher mentoring and guidance

Compliance & Quality: ML2 biosafety certification, SOP authorship and implementation, assay validation, data QC, deviation documentation; familiar with GMP quality system principles; MS Office, MATLAB, Python

Languages: English (fluent), Dutch (actively learning)

List of Publications

- **Mehta P**, Bordoloi AD, Ravensbergen C, David MKH, Mesker W, et al. Inter-spheroid proximity and matrix remodeling determine cancer-associated fibroblast-mediated cancer cell invasion. *Acta Biomaterialia*. 2025.
- Boot RC, van der Net A, Gogou C, **Mehta P**, et al. Cell spheroid viscoelasticity is deformation-dependent. *Scientific Reports*. 2024;14(1):20013.
- Sinha A, **Mehta P**, Fan C, Zhang J, Marvin DL, van Dinther M, et al. Visualizing dynamic changes during TGF- β -induced epithelial to mesenchymal transition. *TGF-Beta Signaling: Methods and Protocols*. 2022:47–65.
- **Mehta P**, Rahman Z, Ten Dijke P, Boukany PE. Microfluidics meets 3D cancer cell migration. *Trends in Cancer*. 2022;8(8):683–697.
- Bera K, Kiepas A, Godet I, Li Y, **Mehta P**, et al. Extracellular fluid viscosity enhances cell migration and cancer dissemination. *Nature*. 2022;611(7935):365–373.
- Si BR, Choudhury D, Bera K, Li Y, **Mehta P**, et al. Extracellular fluid viscosity regulates cancer cell invasion via KCC4-dependent signalling. *Science Advances*. *Under review*.