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

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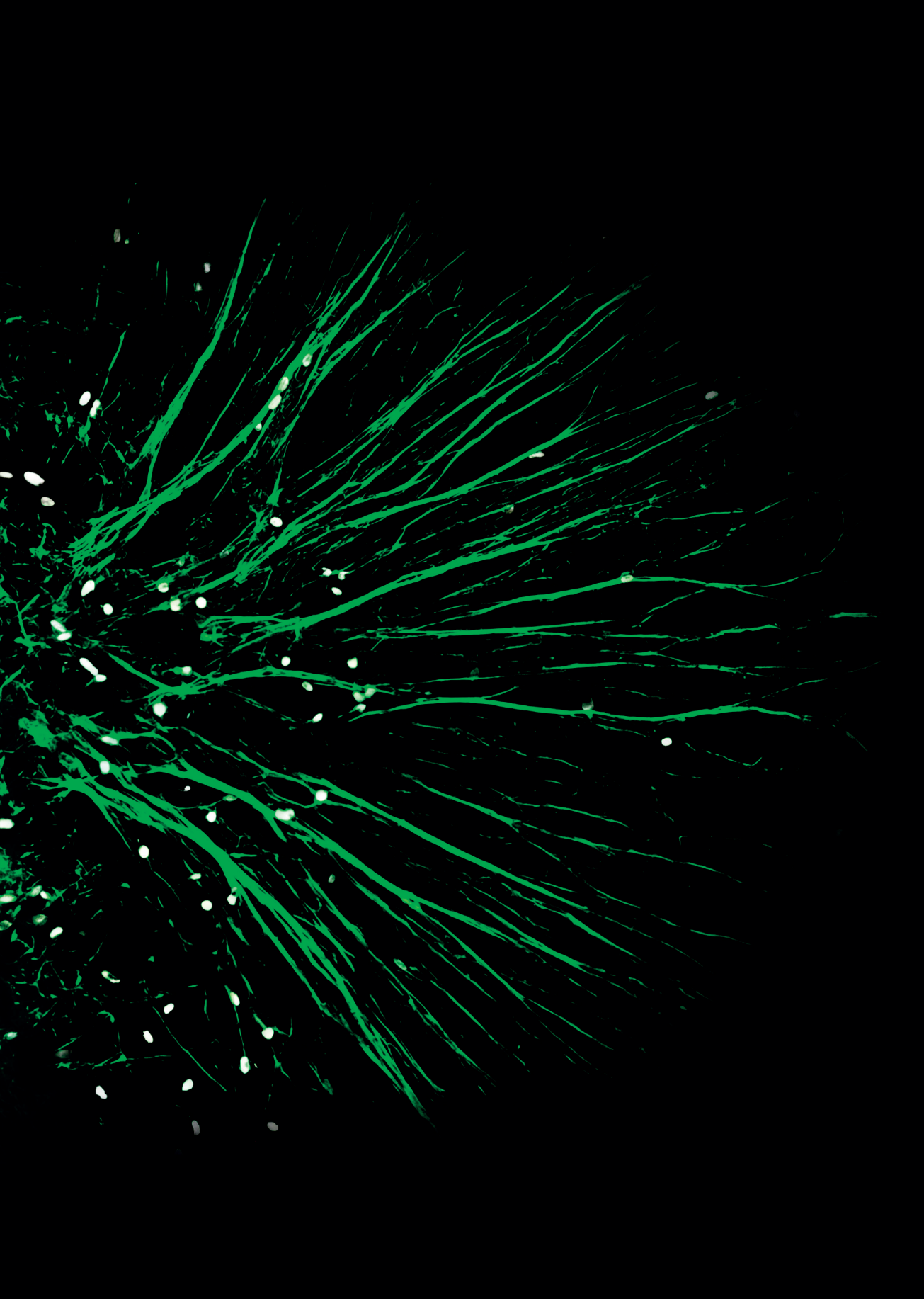

Chapter 6

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.*

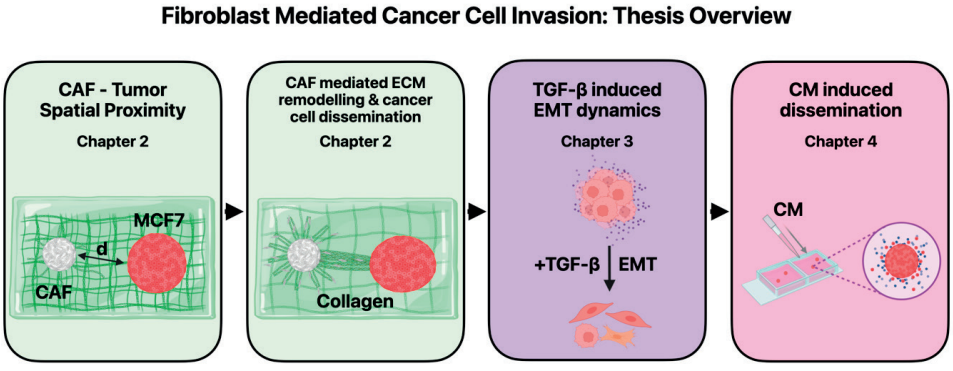

Figure 1. Fibroblast-mediated tumour invasion: thesis overview. *CAF-tumour spatial proximity determines the onset and directionality of collective invasion through distance-dependent mechanical force propagation in collagen matrices (Chapter 2, left). CAF-mediated ECM remodelling generates thick and 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).*

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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