Review

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Future Drug Discovery

The case for cancer-associated fibroblasts: essential elements in cancer drug discovery?

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Although cancer-associated fibroblasts (CAFs) have gained increased attention for supporting cancer progression, current CAF-targeted therapeutic options are limited and failing in clinical trials. As the largest component of the tumor microenvironment (TME), CAFs alter the biochemical and physical structure of the TME, modulating cancer progression. Here, we review the role of CAFs in altering drug response, modifying the TME mechanics and the current models for studying CAFs. To provide new perspectives, we highlight key considerations of CAF activity and discuss emerging technologies that can better address CAFs; and therefore, increase the likelihood of therapeutic efficacy. We argue that CAFs are crucial components of the cancer drug discovery pipeline and incorporating these cells will improve drug discovery success rates.

Plain language summary: Recent advances in cancer research have improved our understanding of disease progression; however, the number of drugs failing in clinical trials remains high and therefore, present a critical challenge for cancer drug discovery. Although the interactions of the tissue surrounding the tumor, the tumor microenvironment, are now considered key targets for new interventions in cancer, the role of microenvironment is largely absent in drug discovery pipelines. Here we explore the role of the most prominent cell type in the tumor microenvironment, cancer-associated fibroblasts (CAFs), in altering cancer therapy response and ultimately patient outcome. To provide new perspectives for future studies, we draw attention to key complications of CAF biology and highlight emerging technologies that could be used to address this. We believe including CAFs in drug discovery, whether for targeting cancer cells or the microenvironment, will allow for a better understanding of therapeutic efficacy and ultimately improve clinical outcome.

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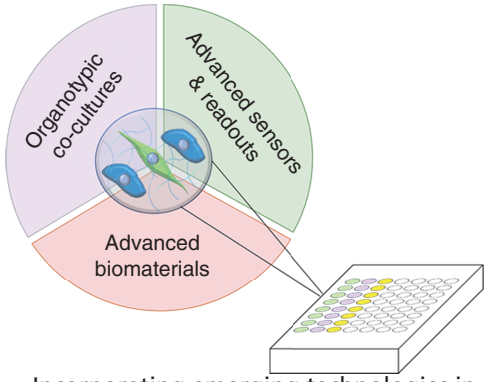
Graphical abstract:



Incorporating emerging technologies in cancer drug discovery to improve translational impact.

The tumor microenvironment (TME) is a complex landscape, composed of cellular and non cellular components; it is comprised of tumoral cells, as well as fibroblasts, endothelial cells, various immune cells and non cellular matrix proteins and ligands, collectively referred to as stroma. Cancer associated fibroblasts (CAFs) are one of the most abundant stromal components of the TME and have been demonstrated to play a prominent role in cancer pathogenesis [1–4]. CAFs are a highly heterogenous 'catch-all' description for several subpopulations of activated fibroblasts that function differently depending on their numerous precursors (i.e., tissue-resident fibroblasts, trans-differentiated endothelial or epithelial cells or bone marrow-derived mesenchymal stem cells) [5–8], and on the local microenvironmental context (i.e., hypoxia or distance to tumor) [9–11]. For example, CAF subpopulations can undergo metabolic reprogramming to provide a supportive niche for adjacent cancer cells [12]. Moreover, recent single cell studies have demonstrated the broad heterogeneity of CAF within individual tumors in mice or humans [13–17]. The evolving nature of CAF subpopulation compositions makes CAFs difficult to study in culture, as conventional culture methods can select and modify the subpopulations, therefore changing their functional behavior.

Activation of CAFs depends on tumor induced signals, including TGF-β, to contract, remodel and secrete extracellular matrix (ECM) proteins, ultimately altering the TME [6]. Many studies have established a link between patient outcome and CAF number, complexity or function [18–20]. CAFs improve organoid and cancer cell growth *in vitro* [11], as well as enhance invasion and migration of associated cancer cells during metastatic disease progression [21,22]. While the mechanisms of these actions remain unclear, they are likely to require a multidisciplinary understanding of the cancer ecosystem, as CAFs direct remodeling and stiffening of the ECM, phenotypes which have been correlated with breast cancer aggression and therefore patient outcome [23]. Fibroblast heterogeneity also contributes to promoting an immunosuppressive microenvironment [8] as well as metastatic progression [24]. Hence, CAFs may serve as a viable target for anticancer therapies.

Although our knowledge of CAF complexity in the TME is still evolving [25], targeting CAF mediated ECM changes and associated downstream signaling have become increasingly appealing strategies to modulate CAF cancer cell communication. However, identifying such targets has not yet translated into clinical benefit. For example, inhibitors of the CAF dependent hedgehog pathway, IPI-926, failed to recapitulate the overall survival benefits shown in mouse model trials [26–28] and paradoxically decreased patient survival when added to the standard of care [29]. While the reasons for this failure remain unclear, this example highlights the complex roles of CAFs in both stabilizing and supporting the TME.

In this review, we outline the current understanding of CAF biology with specific emphasis on their role in modulating cancer cell drug response. We then discuss the limitations of current models, as well as the complications of studying CAFs in conventional model systems. We conclude by proposing that specific features arising from the relationship between CAFs and cancer cells should be included in the next generation of drug discovery platforms and suggest technological approaches currently being developed that may be of value in this area.

CAFs modulate cancer drug efficacy

Recent reviews have summarized the results of therapeutic strategies focused on modulating CAF behavior [25,30–34]. The general lack of success in this area suggests that we do not yet fully understand the role of CAFs in altering

cancer responses, particularly to therapeutic strategies. It is therefore important to briefly review how CAFs are known to modulate cancer drug efficacy, as this will likely affect the design of drug screening platforms.

Collectively, CAF subpopulations modify therapeutic efficacy in several ways. First, CAFs are highly secretory cells, altering cancer cell phenotypes through paracrine cell-to-cell soluble signaling: modulating cancer cell stemness [35–37], increasing cancer cell epithelial to mesenchymal transition through TGF-β signaling [38], altering chemotherapeutic responsiveness [39–42], as well as immune evasion through production of chemokine CXCL12 [43–45] and TGF-β [46,47]. Further, a dense fibrotic stroma is also a common feature of immunotherapy resistant tumors, where signatures of TGF-β induced desmoplasia in the stroma is associated with restricted T-cell infiltration into the tumor [48]. Interestingly, dual targeting of TGF-β and immune checkpoint inhibitor, PD-1, is currently under clinical trial and showing some promise in improving the success of immune checkpoint therapies [49]. Second, existing therapies can often create fibrotic and tissue-stiffening side effects, which are thought to be mediated by CAFs. These fibrotic reactions are associated with overall worsened survival [23]. For example, the highly publicized B-Raf inhibitor used to treat advanced melanoma, activates stromal fibroblasts [50–52], while radiotherapy in general increases fibrosis [53]. Hence, CAF behavior may unintentionally be triggered by conventional therapies, in turn modulating the efficacy of said therapy. Taken together, these findings collectively suggest that CAFs are a crucial player in therapeutic response and ultimately in modulating patient outcome. Understanding the effect of therapeutic agents on CAF function and thus the TME, is evidently crucial for the development of new therapeutic strategies.

Current cancer models capturing CAF functionality

The biology of CAFs has been studied using a variety of strategies ranging from conventional 2D culture or histology sections, to mouse models and *ex-vivo* tissues slices [25]. Current consensus is that 3D models are essential for studying CAFs, as they support the formation of oxygen, nutrient and growth factor gradients similar to those that occur *in vivo* [54,55]. They also enable the formation of 3D spatial cellular organization, so that cells simulate bidirectional cell–cell and cell–ECM interactions critical for evaluating stroma-mediated effects on cancer development and progression [56]. Moreover, 2D tissue culture plastic presents mechanical and topographical cues that alter fibroblast behavior [57], and renders CAFs less secretory than in 3D [58]. Given this established knowledge, we will limit this section to 3D models that incorporate CAF activity for drug discovery.

Mouse models have been used extensively to build an understanding of CAF function *in vivo*. These models demonstrate that non-specific deletion of CAFs or fibrosis causes rapid tumor progression rather than suppression [4,59–61]. These findings outlined the foundation of future studies, promoting researchers to focus on altering CAF behavior rather than ablating CAFs altogether. Although useful, mouse models are far from a perfect system. In co-injection models, where human cancer samples are introduced into the mouse, host-derived fibroblasts will outgrow the co-injected CAFs, leading a study to focus on the interaction of mouse fibroblasts with foreign tissue [25]. With transgenic mouse models, activation of fibroblasts relies on a Cre-driver, yet no CAF-specific Cre driver exists, so intended CAF inhibition or ablation leads to off-target cellular effects. Moreover, they are extremely low-throughput and not a direct parallel to human disease progression.

Controlled *in vitro* studies of CAF biology are most commonly performed today using low throughput, 3D, collagen or Matrigel ECM scaffolds with tumor organotropic cultures. Mixing epithelial tumor organoids and fibroblast cells in 3D matrices supports CAF-induced improvements in organoid passaging capabilities and enhanced cellular growth through direct cell–cell contact [11,62–65], highlighting the symbiotic interactions between tumor cells and CAFs. Using an organotypic culture system where cells are seeded on top of a 3D matrix, CAFs have also been shown to enhance ECM remodeling in a manner that supports tumor cell invasion [21,22]. Moreover, conditioned media from CAF cultures can enhance tumor growth, invasion and resistance [41,66,67], without dynamic cell–cell interactions. The use of CAFs instead of normal fibroblasts in these systems is essential; in both premalignant and malignant mammary epithelial cells, CAFs promote epithelial to mesenchymal transition, while normal breast fibroblasts favour the maintenance of epithelial morphology and constrain metastasis [68], therefore altering therapeutic response.

While these organotypic approaches have defined key roles for CAFs in tumor biology, they are limited in throughput. To increase experimental throughput with minimal biological source material, microfluidic systems have been developed for CAF and cancer co-culture studies. These devices have been used to demonstrate increased cancer cell growth and invasion into physiologically-relevant matrices [69,70], as well as response to chemotherapy [71].

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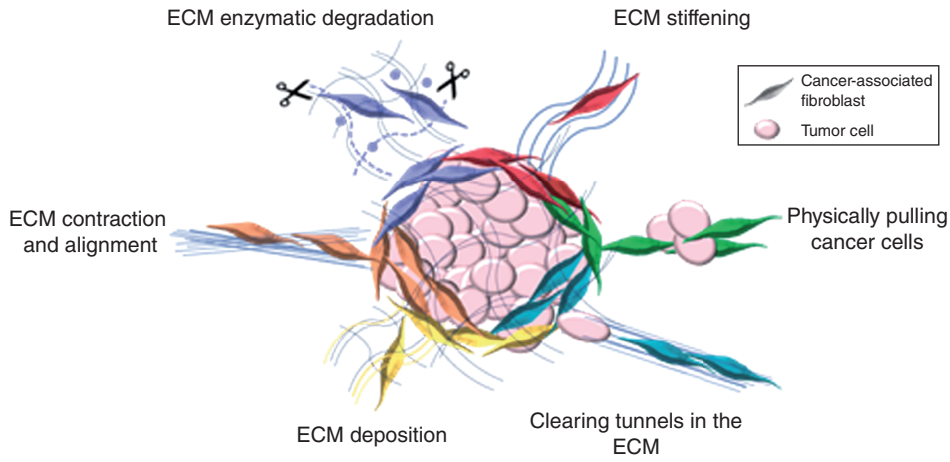

Figure 1. The dynamic, functionality of cancer-associated fibroblasts impacts extracellular matrix remodeling and cancer cell invasion.
Cancer-associated fibroblasts (CAFs) are a highly heterogenous population of cells, with distinct key features that impact cancer progression. CAFs are responsible for dynamically modulating the ECM, though contraction and alignment (orange), ECM deposition (yellow), matrix stiffening (red) and enzymatic degradation (purple). CAFs also play a key role in modulating tumor invasion by clearing tunnels in the ECM (blue) or physically pulling cancer cells through cadherins junctions (green). ECM: Extracellular matrix.

3D bioprinting is also gaining traction in studying CAF biology, as it allows the formation of precisely arranged cells within tissue-like structures, while simultaneously controlling the mechanical properties of the bio-printed ECM [72]. These specific capabilities allow the formation of realistic culture environments important for physiologically relevant CAF function. Printing lung cancer epithelial cells and CAFs in a physiologically relevant matrix stiffness [73], demonstrate that robust and manipulable *in vitro* models of human tumors can be bioprinted. Furthermore, Langer *et al.* successfully printed cancer cells, fibroblasts and epithelial cells, demonstrating that distinct microenvironments that differentially effect proliferation, ECM deposition and migration, can be recapitulated [74]. This demonstrates that these models can be used to interrogate complex tumor–stromal interactions in physiologically relevant and manipulable environment. However, the application of these as high-throughput screening methods, is limited by the availability of primary cells and cell detection methods (i.e., imaging techniques to decipher cell types).

These studies collectively demonstrate that the relationship between CAFs and cancer cells is both symbiotic and dynamic. While these studies have led to significant gains in the understanding of tumor biology, future drug discovery models need to better encompass the functionality of CAFs and their influence on tumor drug response. In this next section we aim to elucidate the key considerations for future drug studies.

Complications with CAFs: emerging questions for future studies

Given that CAFs play a complex role in tumor restraint and growth, drug discovery must incorporate CAFs. However, addressing the functionality of CAFs in such assays implies that a simple live/dead readout for drug screens is inadequate. Given the microenvironmental impacts outlined in the previous section, we believe that future cancer drug discovery must consider: the heterogeneity of CAF populations; their role in enhancing tumor invasion; and their contribution to dynamic tissue mechanics (Figure 1); all of which have been shown impact drug activity and response, and which cannot be easily recreated in standard drug screens. Here, we overview CAF functions that must be considered in future studies.

First, sufficient evidence for the presence and impact of CAF heterogeneity now exists in both *in vivo* and *in vitro* models [5,7,8,75–77] to support considering this complication in drug discovery (Figure 1). It is likely that the subtypes of CAFs are plastic, with capacity to transition between CAF states; activated fibroblasts are known to exhibit multipotency [78–80], and CAFs are dependent on the microenvironment to influence their subtype [81]. Öhlund *et al.* demonstrated the conversion of CAF subtypes based on their proximity to tumor cells. Additionally, the heterogeneity of CAF populations is induced and stabilized by CAF signaling [82]. Current studies focusing on

changes in CAF populations are limited, even though understanding the interconversion in response to therapies may be key for drug discovery.

Second, CAFs play a key functional role in tumor invasion by; secreting proteases that break down ECM to enable cancer cell motility [3,83,84], clearing tunnels in the ECM [22] and physically pulling cancer cells through heterotypic cell junctions (Figure 1) [85]. To understand drug efficacy on these CAF functional phenotypes, systems must track the movement of individual cells, a process that has been challenging to scale to high-throughput screens, while maintaining a suitable level of robustness. This is challenged by the fact that typical invasion assays follow cumbersome procedures [86,87] and have end point readouts with low signal to background ratio [88]. Moreover, they require precise, automated, multidimensional microscopy and analysis software, an expensive addition to these studies.

Finally, CAFs are mechanically competent cells that both respond to and change their physical environment, by remodeling the tumor ECM through contraction, secretion, crosslinking and aligning of the surrounding collagen and fibrillar proteins (Figure 1) [6]. Given the broadly-established impact of 3D tissue mechanics on biological function [89], this can significantly influence the direct response of cancer tumors to candidate therapeutics. Progressive deposition and remodeling of the ECM by CAFs is associated with disease transformation in human breast cancer [23] and *in vitro* analysis shows that changes in the ECM alter breast cancer aggression [90–94]. Moreover, remodeling also aligns, thickens and straightens ECM fibres, where signatures of this are an independent prognostic indicator of poor disease progression [95,96]. This remodeling, thickening and deposition of the ECM also contributes to an overall increase in tissue stiffening, shown in mouse models and human patient samples to foster tissue transformation and metastasis [23,97–99]. Although tissue stiffening is recognized as an important factor in cancer epithelial drug discovery [100–102] and promoting chemo resistance [103,104], current drug discovery models lack the dynamic interplay between CAFs and the ECM. In addition to stiffening the environment, aggressive tumors typically have dense and aligned ECM [23], providing highways for cells to invade and altering cancer cell signaling and behavior. We believe capturing these mechanical phenotypes arising from CAF inclusion is therefore critical to identifying better stromal targeted therapies and; thereby, improving patient outcome.

Moving forward: emerging technologies

Several emerging technologies from various fields might allow us to bridge the gaps between cancer drug discovery and patient benefit by incorporating CAFs. While there have been major advances in recent work aimed at targeting CAFs, the TME or cancer cells directly, implementing these approaches into next-generation high-throughput screening will improve overall drug efficacy. We highlight emerging strategies to improve the drug development process by: incorporating CAFs via high-throughput organoid co-cultures; conducting assays in matrices that consider realistic mechano- and biological elements; and integrating techniques designed to measure functional CAF behaviors in living cultures.

Organoid co-cultures

Patient cancer cell derived organoids have gained increased traction in drug discovery. While they have a 3D, functional ECM for the cancer cells to interact with, conventional cancer drug studies with organoids lack stromal CAFs. Since CAFs play a key role in reshaping the TME, we argue that the addition of CAFs to such organotypic cultures is essential. In fibrosis, by incorporating multiple cell types, a clear resemblance between the *in vitro* cultures and human disease pathophysiology is possible [105,106]. Similarly, in liver fibrosis *in vitro*, 3D multicellular tissues enable preclinical screening of antifibrotic drugs [107,108], further highlighting the importance of the microenvironment in drug screening. Multiple commercially available systems now exist for high-throughput multicellular, physiologically relevant *in vitro* assays. Some examples of these include; organ-on-a-chip systems for mechanically realistic lung–blood barriers [109]; 3D co-culture chips that support barrier integrity-, transport- and migration assays [110]; tissue culture force sensors to measure human heart health [111]; and bioreactors to model human pulmonary fibrosis [112]. The use of these assays would allow for the interrogation of complex biological questions involving cell–cell and cell–ECM interactions that would encompass the dynamic invasive and mechanical changes induced by CAFs.

Advanced biomaterials

Physical characteristics of the tissue ECM vary substantially *in vivo*, with changes in fiber length, thickness, density and organization. Given that these changes are induced largely by CAF remodeling, building models that recapitulate

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the mechanobiological elements of the surrounding TME will reduce the need for CAFs within the system. For example, the use of pre-aligned matrices [113] or fibroblast preconditioned matrices could be used for invasion assays incorporating the dense and aligned matrix highways seen in aggressive tumors [23]. Advanced biomaterial formulations that consider these factors may therefore capture the mechanical effect of CAFs, without the need to obtain and include live CAFs themselves. While this approach would not capture the dynamic interactions between CAFs and cancer cells, the ability to recreate this important phenotype may improve translational screening efficiency and translational realism while maintaining the assay robustness required for drug screening technologies.

Advanced biomaterials can be tailored *de novo* to present specific characteristics [114], or can be used in blended formulations to modify the properties of existing materials. For example, Matrigel is well-established in many organoid culture protocols, but is challenging to mechanically tune for specific applications. Interpenetrating polymer networks such as gelatin [115–117], hyaluronic acid [118–120] or alginate [102] may be used as a supporting network to modulate substrate stiffness to physiologically relevant levels, while avoiding any modifications to critically important ligand composition or density.

In addition to linear elastic modulations, physiological ECM also exhibits more complex material behaviors such as stress relaxation, or viscoelasticity, parameters proving to be critically important in designing matrices for drug discovery [89]. For example, it has been shown that use of soft substrates with stress relaxation in 3D, promotes cell spreading, fiber remodeling and focal adhesion formation [121–123], emphasizing the importance of incorporating physical cues from the ECM in regulating cellular phenotype and therefore drug response. Additionally, human breast tumor samples exhibit ECM plasticity [124], a permanent deformation of the ECM. Given that, fibroblasts can produce stresses large enough to permanently deform the biomaterials [125,126], incorporating these forces is critical to recapitulating the effect of CAF functionality, even in their absence.

The contribution of the ECM is more than just mechanical, and due to the diverse range of proteins, proteoglycans, growth factors and other enzymes, it presents a wide range of biological cues to the cells. The use of decellularized ECM (dECM) is well studied in idiopathic pulmonary fibrosis, where it activates myofibroblasts [127] and alters fibroblast gene expression [128]. By implementing this in gut models for intestinal fibrosis it increases the fidelity of disease modeling [129] and the throughput of drug screening [130]. However, the systematic use of dECM is not ideally suited for highly systematic drug screening processes in the context of cancer; biological material available for such assays is limited to the size of the excised tumor and tends to largely vary in composition from patient to patient. While it has proven reliable in other systems, the use of dECM may therefore only be relevant in the context of personalized cancer therapeutic screenings.

Advanced readouts

If CAFs primarily modulate tumor response via mechanical activity, studies to assess the extent to which CAFs remodel the ECM, exert mechanical forces and mechanically tune their surroundings will grow in importance. Emerging microscale-engineered technologies that allow quantitative measurements of mechanical changes in tissues, may prove an effective tool in understanding the changes made to the environment by CAFs, to better understand and ultimately simulate their activity. Several recently developed technologies can provide insight into fibroblast behavior at this extremely local length-scale. Asmani *et al.* developed a 3D fibrotic microtissue array, in which 3D-cultured fibroblasts remodel the surrounding matrix to deform micro-engineered pillars that anchor the matrix to the substrate. Analyzing the deformation of these pillars provides readouts of forces generated by the CAFS, and therefore enable quantification of fibrosis and drug efficacy testing [87]. This fundamental premise has recently been expanded toward developing dispersible microfabricated sensors that can be applied in a variety of culture contexts to quantify cell-generated mechanical forces [131,132], mechanical compressive forces [133], residual tissue elasticity [134] and other mechanical properties of tissues [135,136]. Reducing the size and accessibility of these sensors may hence prove quite valuable in understanding the CAF contributions to the surrounding matrix at the cellular level, to better understand tissue dynamics in response to therapy.

Conclusion

It is evident that tumors can no longer be viewed as static clumps of cancerous cells; the complex and dynamic interactions with the surrounding TME play a key role in altering cancer cell response to therapy and therefore patient outcome. Accumulating work suggests improved strategies could be possible by targeting CAFs; however, the disconnect between drug discovery and clinical benefit remains. Therefore, we believe carefully assessing the impact of cancer cell or TME targeted therapies on the mechanical and functional forces within the TME, prior to

clinical translation, is critical for narrowing the translational gap. We propose the use of organotypic co-cultures, advanced biomaterials and various force sensors as technological advances that will be instrumental in improving the drug discovery pipeline. Accommodating CAFs and CAF modulated ECM in this process, has the potential for physiologically relevant discoveries, bridging the gap between bench-side and clinical benefits.

Future perspective

Unfortunately, nearly 25 years after the initial studies showing the physiological relevance of 3D culture systems [56,137,138], the vast majority of drug discovery pipelines are still in 2D. This is likely due to the ease of culture, growth and biochemical testing in 2D systems as well as the relative success of these technologies in identifying useful molecules. However, it would appear that the low-hanging fruit of easily identifiable therapeutic molecules have already been identified, as evidenced by the dwindling number of novel therapeutic discoveries, despite increases in economic resources allocated to this problem [139]. Furthermore, the poor clinical translatability for many seemingly-promising drugs suggests that 2D systems are no longer sufficient in this area, and that more complex 3D culture systems will be required to identify next-generation therapeutics. Moving forward, we believe advanced technologies will bridge this gap, in improving the physiological relevance of such assays, while also improving the ease of setting up, operating and analyzing information from 3D assays; such as those outlined in this review. Moreover, we anticipate the inclusion of immune cell populations will become more and more important, as numerous recent studies have demonstrated a key role for immune cells in tumor progression [23,44,48,49,140]. Given that there are small subsets of patients who respond to immune checkpoint inhibitors [141], perhaps other microenvironmental factors dictate patient responsiveness to checkpoint blockade. Recent findings are demonstrating that CAFs may be critically important in these microenvironmental feature sets that drive immune evasion [8,43,46,47,142–147], and so these advanced discovery systems may better pair patient populations with successful therapeutics. The consideration of immune cell infiltration in these future drug discovery models will be significant. In our view, the ongoing collaborations between engineers and biological scientists will be fundamental in the successful building and implementation of new models and technology for future drug discovery.

Executive summary

Background

- Cancer-associated fibroblasts (CAFs) are one of the most abundant stromal components of the tumor microenvironment, playing a prominent role in cancer pathogenesis.
- Clinically targeting CAFs has become an increasingly appealing approach for cancer therapies, yet a disconnect between drug discovery and clinical benefit remains.

CAFs modulate cancer drug efficacy

- CAFs modulate cancer drug efficacy through: secretions; extracellular matrix remodeling; and off-target activation by conventional chemotherapies.

Current models capturing CAF functionality

- Current 3D models: mouse models; organotypic co-culture models; 3D bioprinting demonstrate that the relationship between CAFs and cancer cells is dynamic and that these functionalities are missing from current drug discovery.

Complications with CAFs: emerging questions for future studies

- In order to improve cancer therapy efficacy, future drug discovery must capture: the heterogeneity of CAF populations; their role in enhancing tumor invasion; and their contribution to tissue mechanics.

Moving forward: emerging technologies

- Several emerging technologies from various fields have the potential to improve the drug development by incorporating CAFs.
- We propose the use of: high-throughput organoid co-cultures; conducting assays in matrices that consider realistic mechano- and biological elements; and integrating techniques designed to measure functional CAF behaviors in living cultures.

Conclusion

- Given that the complex and dynamic interaction of the tumor and surrounding microenvironment play a key role in altering response to therapy, we believe that studying the impact of therapies on the mechanical and functional forces within the tumor microenvironment is critical for narrowing the translational gap.

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- **Demonstrates the importance of CAFs in tumor progression, and outlines future studies focused on deactivating CAFs, rather than depleting them.**

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- **This pioneering study demonstrates that stromal induced remodeling at the tumoral boundaries plays a prominent role in cancer cell invasion and; therefore, patient outcome.**

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