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# Cancer associated fibroblasts: is the force the path to the dark side?

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The most abundant cell type in the tumor microenvironment are cancer-associated fibroblasts (CAFs). CAFs play an important role in tumor growth and progression. Besides direct communication with cancer cells via secreted molecules or cell–cell adhesions, CAFs also indirectly affect cancer cell behavior by remodeling the extracellular matrix (ECM). Here, we summarize recent findings on the distinct mechanisms that CAFs use to modify ECM, specifically, their proteolytic versus force-dependent activity. We then review the consequences of CAF force transmission on the physico-chemical properties of the matrix, focusing on the deposition of new matrix components, and the alteration of the organization and stiffness of the ECM. CAFs promote tumor invasion by creating the roads cancer cells use to escape the tumor mass. However, there is also evidence that CAFs can prevent invasion, possibly by forming a physical barrier around the tumor edge. We discuss the controversial role of CAFs in tumor progression.

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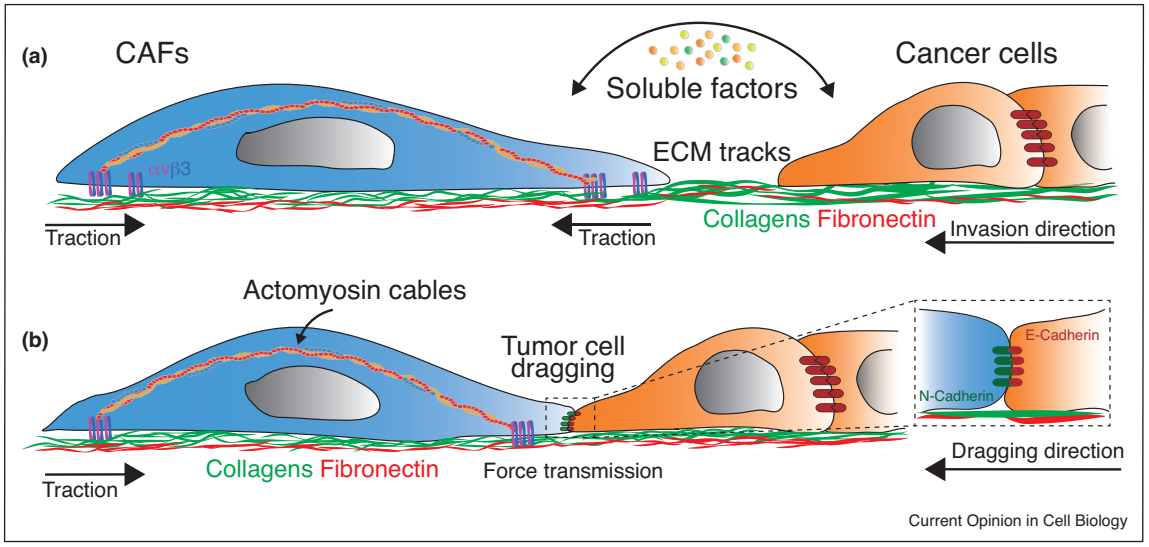
## Introduction

In tumors, cancer cells are not alone. They are intercalated with myriad cell types, which together form the tumor microenvironment. During formation of carcinomas, cancer-associated fibroblasts (CAFs) accumulate close to tumor cells and generate a specific extracellular matrix (ECM) in a process known as a desmoplastic reaction. A range of structures become embedded in this ECM, including blood and lymphatic vessels, made of endothelial cells and pericytes, and the tumor associated immune component, established by T-lymphocytes and B-lymphocytes, dendritic cells, NK cells, macrophages and others. Tumor cells actively interact with these cell types in many different ways and such interactions determine the dynamics of tumor behavior over time.

CAFs constitute a heterogeneous population of mesenchymal cells whose origin and specific characteristics remain unclear. Among potential CAF origins are recruitment of bone marrow mesenchymal stem cells, differentiation from adipose stem cells, or conversion from tumor or endothelial cells through a process of epithelial/endothelial to mesenchymal transition (EMT/EndMT) [1–3]. However, EMT/EndMT are controversial CAF sources that still need to be further explored. To date, the best-documented source of CAFs is the activation of 'normal' resident fibroblasts. Soluble factors produced by tumor cells such as leukemia inhibitory factor (LIF) [4], CCL2 [5] or oncostatin M [6], drive fibroblasts activation through JAK/STAT signaling. In addition, the relocalization of cell-matrix receptor integrin α5β1 from the membrane to endosomes leads to the same phenotype [7]. Once activated, CAFs acquire a series of particular features such as the expression of αSMA, PDGFR, FSP1 or FAP, and increased cell contractility. Those, together with the absence of epithelial, endothelial or immune markers are currently used for the identification of CAFs. However, CAF-specific markers are still lacking. The development specific functional tests together with the analysis of the absence of driver oncogenic mutations could serve as platforms to determine which fibroblasts can be considered as bona fide CAFs.

CAFs play a variety of roles in tumor development. For example, CAFs at the vicinity of tumors feed cancer cells through nutrient-rich exosomes [8]. Reciprocal signaling of CAFs with cancer cells promote cancer cell survival and proliferation [2,9]. CAFs as well interact with tumor immune cells, generating a pro-tumorigenic inflammation through cytokine production, and creating at the same time an immunosuppressive environment that limits antitumor immunity [2,10]. Cancer cell invasion is one of the hallmarks of tumor progression as it determines tumor spreading, impacting dramatically patient survival. CAFs influence the invasion of cancer cells in multiple ways (Figure 1). They secrete growth factors, such as HGF and PDGF, that increase the migration of tumor cells [11,12••]. CAFs also lead cancer cell invasion in a contact-mediated fashion [13,14••]. By establishing heterotypic contacts with cancer cells via N-E cadherins, CAFs pull cancer cells out of the tumor bulk [14]. Finally, CAFs excavate passageways in the ECM that cancer cells use to disseminate [13,15••,16].

**Figure 1**




Interactions between cancer cells and CAFs during tumor invasion. **(a)** Production of soluble factors by CAFs and cancer cells has a bidirectional effect. Factors produced by CAFs promote cancer cell migration, and at the same time, cancer cells secrete molecules that stimulate CAF activation. Once activated, CAFs actomyosin contractility generates force that is transmitted to the ECM via integrins. Rearrangement of matrix components leads to collagen and fibronectin fibers alignment. This creates ECM tracks that cancer cells use to invade. **(b)** CAFs can also establish direct contacts with epithelial cancer cells. These contacts are heterotypic, formed by CAF's N-cadherin and cancer cell's E-cadherin. CAFs generated force can be transmitted to cancer cells through those contacts, dragging tumor cells out of the tumor bulk.
For simplification, CAFs and cancer cells were depicted contacting the ECM only ventrally, but on a 3D *in vivo* scenario, ECM contact will be present both ventrally and dorsally.

The molecular changes that CAFs undergo during tumor progression determine their function. For instance, gain of proteolytic activity endows CAFs the capacity to degrade the ECM. At the same time, fibroblasts activation increases their contractile properties and consequently their ability to generate force. This turns CAFs into masters of ECM remodeling. Altogether, CAF acquired functions result in a dramatic change in chemical and physical properties of the tumoral ECM with consequences for tumor cell behavior. Here, we review recent advances in understanding the distinct mechanisms that CAFs use to modify ECM, focusing on their proteolytic versus force-dependent activity, and how this affects cancer cell properties.

## Running with scissors: CAF-driven proteolytic ECM degradation

The ability of cancer cells to invade has traditionally been attributed to their capacity to degrade surrounding ECM. Tumor cells form actin-based protrusions called invadopodia, which are enriched in matrix metalloproteinases (MMPs) that cut the fibers in the matrix. This opens a passage in the surrounding stroma through which cancer cells move [17,18]. However, CAFs, but not normal fibroblasts, can also form invadopodia and use MMPs to degrade the ECM [17]. Invadopodia are formed upon Twist1 translocation into the nucleus, which upregulates the expression of the actin-binding protein palladin (isoform 4) [19], contributing to the activation of a small GTPase Cdc42 [20]. CAFs also form degradative protrusions that differ from classical invadopodia. These tubular/reticular structures do not contain actin or cortactin and are independent of Cdc42 activity, but are still MMP positive [21], and so could be used by CAFs to degrade the ECM. Whether CAFs forming invadopodia differ in their origin from CAFs lacking these invasive structures is still unknown. Although it is appealing to hypothesize that invadopodia-rich CAFs could directly derive from tumor cells undergoing EMT, this needs to be explored. Lineage tracing experiments could help addressing this question.

The expression of MMPs in CAFs is regulated by a number of different factors. For instance, under hypoxic conditions, reactive oxygen species (ROS) produced by the metabolic activity of tumor cells [22] downregulate MMP-3 in CAFs [23]. Hypothetically, this could result in degraded matrix at the tumor edges that are well-oxygenated, and intact matrix on poorly vascularized and likely hypoxic tumor necrotic cores. But, this still needs to be experimentally tested.

Transforming growth factor beta (TGFβ) is another example of a regulator of MMPs in CAFs. For instance, it is required for the maintenance of MMP-9 activity [24]. In turn, binding of MMP-9 to lysil hydroxylase 3 (LH3)

activates TGFβ signaling [25], possibly creating a positive feedback loop.

Besides MMPs, CAFs also express other proteases [15]. Fibroblast activation protein (FAP) is a dipeptidyl peptidase, collagenase and gelatinase [26], which is often used as a marker to distinguish CAFs from normal fibroblasts. In fibrosis, FAP contributes to tissue normalization through collagen clearance and matrix turnover in collaboration with MMPs [27]. FAP is also involved in the immunosuppressive activity of CAFs. $FAP^+$ CAFs produce the chemokine CXCL12, which coats cancer cells and repels cytotoxic T cells. Removal of $FAP^+$ CAFs or inhibition of CXCL12 leads to the accumulation of T cells in tumors, increasing tumor response to checkpoint inhibitors such as PD-L1 [28]. Depletion of $FAP^+$ CAFs also reduces the number of immunosuppressive cells, such as regulatory T cells, myeloid-derived suppressor cells and tumor-associated macrophages. This improves the antitumor efficacy of cytotoxic T cells by delaying their functional exhaustion [29]. Interestingly, anti-FAP antibodies have been used to develop targeted therapies against the tumor stromal compartment, showing a therapeutic response [30].

## A show of force: CAFs mechanical ECM remodeling

Fibroblasts in their resting state are poorly contractile. However, in response to an external tissue injury or biochemical changes of an established homeostasis they become activated and start expressing αSMA [31]. Those changes turn fibroblasts into myofibroblasts, highly contractile cells that have an enhanced capacity to remodel the ECM, facilitating wound healing [10]. Once the wound is closed, myofibroblasts die or revert back to their resting state [32].

In tumors, the stimuli that drive fibroblast activation are persistent over time, preventing them from undergoing apoptosis, and generating a progressive accumulation of highly contractile CAFs that share many characteristics with myofibroblasts in wounds (Figure 2). Signals from the tumor microenvironment activate the Rho-ROCK-Myosin II pathway and αSMA incorporation into actomyosin cables, increasing CAF contractility. αSMA expression is also modulated by FAP [33]. As a result, CAFs generate forces that, once transmitted to the matrix, lead to its remodeling at different levels. At the biochemical level, CAFs alter the molecular composition of the matrix by increasing the deposition of new matrix components. At the mechanical level, CAFs affect the physical properties of the matrix by modifying its organization and stiffness [13,16,34••,35].

### Matrix deposition

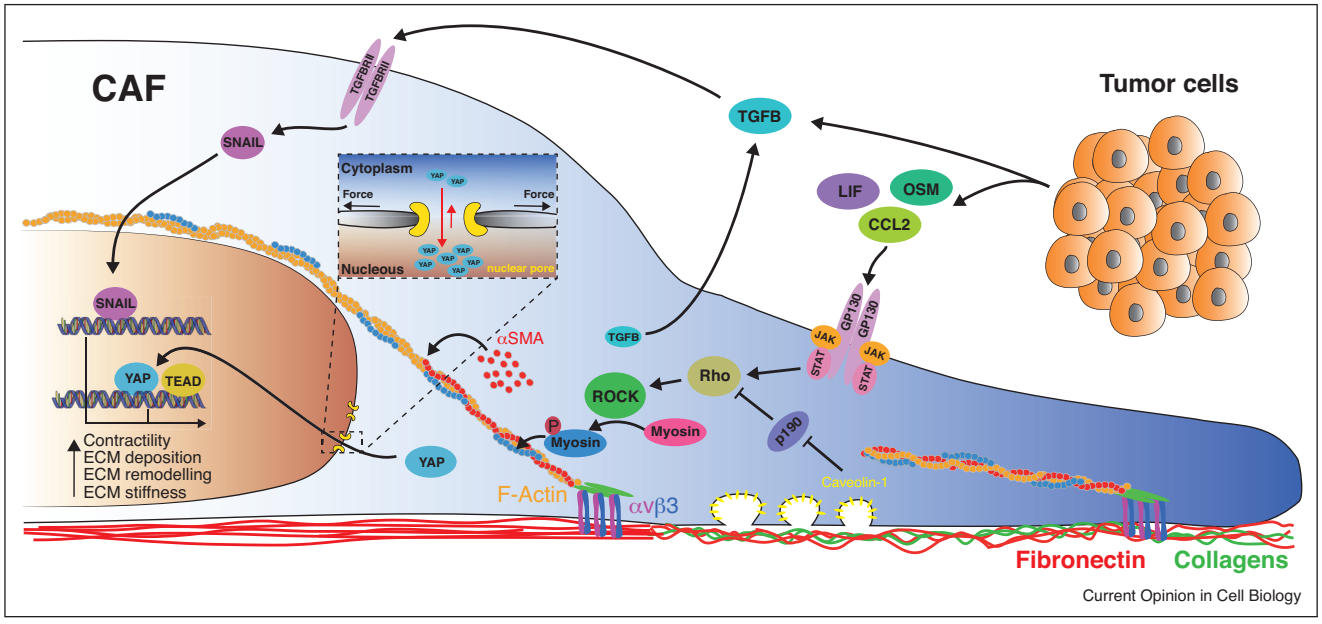
One of the hallmarks of CAFs is their ability to produce high amounts of ECM proteins, including fibronectin and its oncofetal variants (EDA-FN) as well as different types of collagens [15,16,36]. In fibrosis, fibroblasts first deposit fibronectin, generating supracellular structures that are under tension. This creates a feed-forward loop that keeps fibroblasts in their activated state through YAP nuclear translocation and αSMA expression [37••]. As the fibrotic tissue matures, more fibroblasts get recruited and other ECM components are produced. Specifically, the deposition of type I collagen is increased, leading to a decrease in the fibronectin/collagen I ratio. Once the collagen I network becomes cross-linked, fibronectin cables are relaxed and fibroblasts resume quiescence [37••] (Figure 3). In tumors, ECM remodeling is continuous [38–40], which probably affects the distribution of relaxed and tense fibronectin areas. This hypothesis could be tested using the recently developed bacterial-derived probe FnBPA5, which allows the visualization of fibronectin tensional states in tumor slices or in living animals [41]. Whether varied distribution of fibronectin properties within tumor ECM affects CAF characteristics and function remains to be determined.

### ECM topology reorganization

Tumor ECM varies in structure, composition and biophysical characteristics when comparing the dense, 2D sheet-like configuration of the basement membrane, to the 3D fibrillar network of stromal ECM. Basement membranes are mainly composed of laminin and type IV collagen networks, whereas interstitial stromal ECMs are rich in type I and III collagens and fibronectin [42,43].

By exerting forces on the ECM, CAFs alter its native topology. At the initial stages of tumor progression, CAFs mechanically remodel the tumor basement membrane in an MMP-independent manner [15]. By physically pulling and stretching the basement membrane, CAFs possibly push aside ECM fibers, enlarging the gaps in the basement membrane, so allowing cancer cells to squeeze through (Figure 4). Thus, CAFs use force to promote cancer cell invasion through the basement membrane. CAFs also use force to remodel the stromal ECM. Using actomyosin contractility, CAFs straighten out collagen fibers, aligning them parallel to their long axis [7,16,34••,35,36]. Interestingly, proteases like FAP can regulate how fibroblasts remodel the ECM. For instance, FAP overexpression in normal fibroblasts generates CAF-like anisotropic ECM's, which promotes tumor cell invasion [33]. Altogether, this generates a gradual change in ECM organization during tumor progression. Seminal work from Dr Keely's group, based on second harmonic generation microscopy revolutionized the field of ECM alignment, setting the foundations for further studies. In particular, Keely's group proposed that tumor ECM organization could be classified using the so called 'tumor associated collagen signatures (TACS)' which directly correlate with patient's prognosis [44,45]. In normal tissues, collagen fibers are wavy and isotropically oriented

**Figure 2**




Signaling pathways involved in CAFs' contractility regulation. Assembly of actin and myosin into actomyosin cables is the main contractility driver. CAFs also incorporate αSMA into those cables further increasing contractility. This process is regulated at different levels. Soluble factors produced by cancer cells (LIF, OSM, CCL2) or the formation of caveolae, regulate Rho/ROCK pathway activity, which tunes the levels of myosin phosphorylation and thus, its function. Cell generated force promotes nuclear pore stretching, favoring YAP nuclear translocation. Other transcription factors such as Snail1 are activated through TGFβ signaling produced both by CAFs and cancer cells. Transcriptional activity of Snail1 and YAP leads to a further reinforcement of cell contractility, matrix deposition, remodeling and stiffening.

(TACS-1). In tumors, they become straightened out, anisotropic and oriented either parallel to the tumor boundary (TACS-2) in non-invasive tumors or perpendicular to the tumor edge (TACS-3) in invasive tumors (Figure 4). ECM realignment is associated with changes in collagen composition, and highly aligned TACS-3 signatures display a higher rate of collagen type I/type III ratio due to a reduction in type III collagen content [46]. Whether matrix alignment specifically happens at low collagen III areas, or if ECM composition changes later after realignment is an important point that still needs to be addressed. CAFs follow the same orientation relative to the tumor boundary than ECM fibers, suggesting that they could be the masters of this ECM organization. However, this still needs to be formally demonstrated.

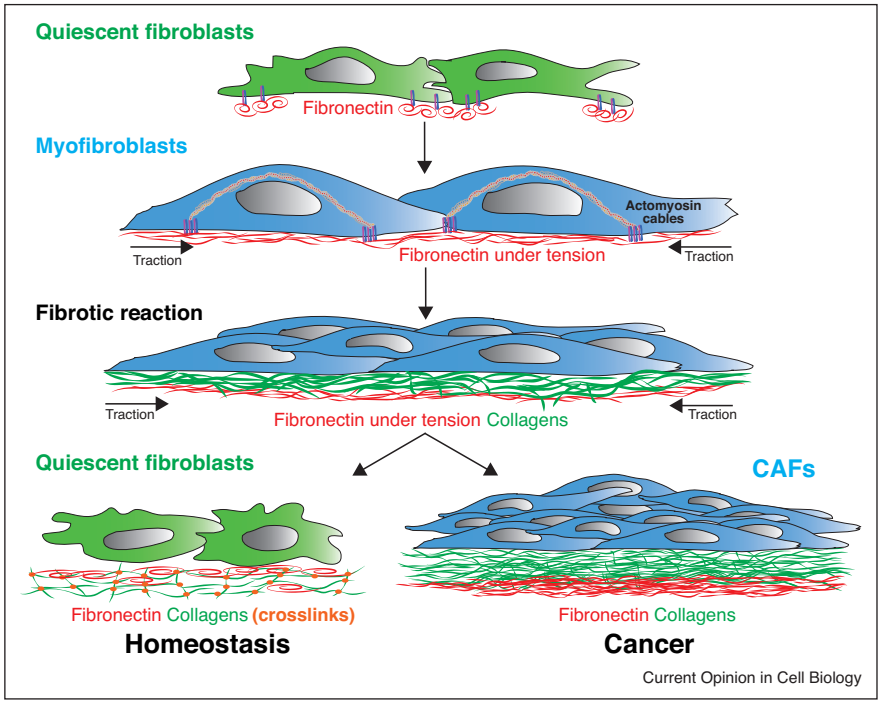
Fibronectin fiber assembly is also dependent on forces exerted by CAFs. Fibrillogenesis is driven by cell-surface receptors, αvβ3 [16] or α5β1 [47] integrins, localized in specialized cell-matrix adhesions called fibrillar adhesions [48]. Hic-5, a paxillin-family member, regulates the formation of fibrillar adhesions through its mechanosensitive interaction with tensin1 [49,50]. Aligned collagen fibers decorated with fibronectin and other ECM molecules generate tracks that cancer cells exploit to disseminate [13,16,35,36] (Figure 4). Normal fibroblasts, which are less contractile, cannot straighten ECM fibers and consequently do not promote cancer cell directional migration [16,35] and proliferation [34].

## ECM stiffening

An immediate consequence of contractility-driven ECM remodeling is the alteration of matrix stiffness. CAFs stiffen stromal ECM by bundling collagen filaments and cross-linking collagen networks [34,51] by producing lysyl-6-oxidase (LOX) [52]. LOX covalently cross-links collagen fibers by oxidatively deaminating-specific lysine and hydroxylysine residues in telopeptide domains [53]. CAFs also produce lysil hydroxylase 2 (LH2), which increases the ratio of hydroxylysine/lysine aldehyde-derived collagen crosslinks, generating stiffer ECMs [54–57].

CAFs stiffen environments in response to several mechanosensitive pathways. These generate a positive feedback loop that reinforces CAF activation. The initial regulators of force transmission are integrin-based adhesions, which link the ECM to the actin cytoskeleton [58]. Increases in ECM stiffness change the force-loading rate at focal adhesions, resulting in the stretching of talin1 and the recruitment of vinculin [59]. This leads to the activation of pathways such as FAK, RhoA or Src that further increase CAF contractility [58].

**Figure 3**




ECM remodeling during wound healing, fibrosis and cancer. On steady state fibroblasts are quiescent. Tissue injury leads to their activation turning them into contractile myofibroblasts. During early steps of wound healing, myofibroblasts secrete high amounts of fibronectin. Myofibroblasts transmit forces to the ECM through integrins that stretch fibronectin fibers generating a network under tension. At the later stages of the fibrotic reaction, collagens are secreted and cross-linked. This releases tension from the fibronectin network, leading to a reversion of myofibroblasts to their resting state. In cancer, tissue disorganization could prevent fibronectin tensional release, favoring continuous CAF activation.

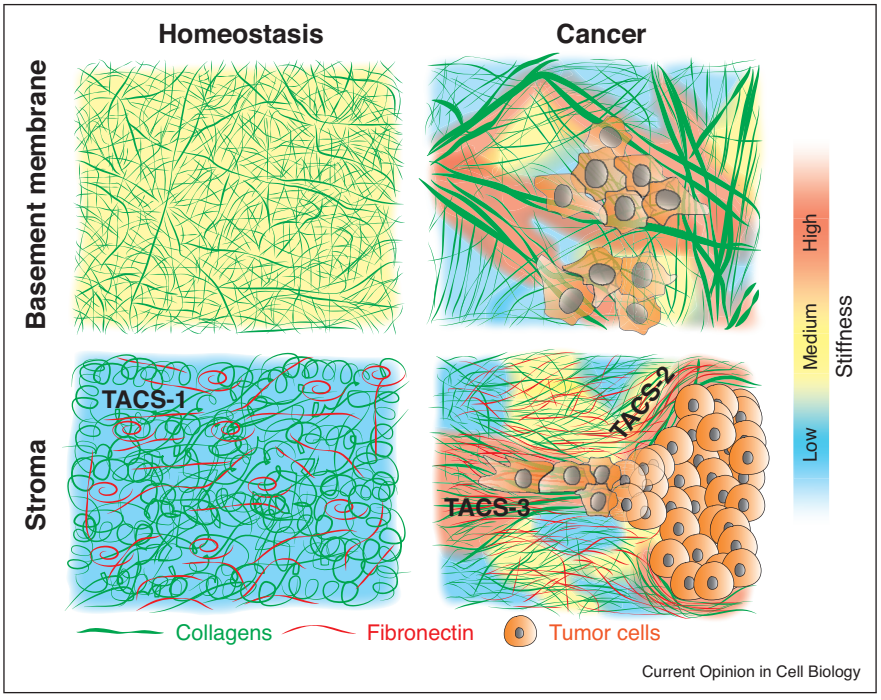
Stiff matrices also promote the activity of YAP and TAZ transcriptional regulators [60,61]. In non-activated fibroblasts, YAP is excluded from the nucleus and so remains inactive. On rigid substrates, the generated force favors YAP nuclear import by reducing the mechanical restriction of nuclear pores [62••] (Figure 2). Thus, in response to stiffness, YAP translocates to the nucleus, where it activates a series of genes that regulate cell contractility and matrix remodeling [34,61]. In pancreatic cancer, poorly differentiated tumors with impaired TGFβ signaling are characterized by highly rigid stroma with activated integrin-dependent signaling and YAP [63]. Other transcription factors are also mechano-responsive. For example, on stiff matrices, the activation of ROCK/Erk2 pathways promotes Snail1 nuclear localization, triggering transcriptional activity that leads to a fibrogenic response in CAFs [64].

CAFs also respond to environmental stiffness through plasma membrane invaginations called caveolae. Caveolin-1, the main component of caveolae, regulates cell contractility by modulating the activity of the Rho-inhibitor p190 [65] (Figure 2). Caveolin-1 is overexpressed in CAFs in different human tumor types [65]. However, a recent meta-analysis involving more than 2000 breast cancer patients, found an inverse correlation between Caveolin 1 expression in CAFs and patient prognosis [66]. Heterogeneity within the tumor microenvironment, and between patients and cancer types might explain this variability, signifying at the same time a need for sample analysis standardization.

Regardless of the signaling pathway used, the common feature is that CAFs respond to changes in matrix stiffness by increasing cell contractility, which maintains them in their activated state. This, in turn, reinforces ECM alignment and stiffness, generating an invasion-permissive environment for tumor cells. Indeed, one consequence of matrix stiffening is the unbinding of Twist1 from its cytoplasmic partner G3BP2 in cancer cells. It translocates into the nucleus, where it activates an EMT transcription program, resulting in invasion and metastasis [67]. Increased tumor stiffness is linked to poor disease prognosis [63,68]. Taken together, these studies show that CAF remodeling stiffens the stromal ECM.

Surprisingly, CAF remodeling has the opposite effect on basement membranes, making them globally softer [15]. Under homeostatic conditions, the distinct molecular composition and organization of basement membranes

**Figure 4**




Remodeling of the basement membrane and stromal ECM in cancer. Homeostatic basement membranes are highly organized structures that have higher stiffness than stromal ECM. Stromal ECM display curly collagen fibers intermingled with other ECM components such as fibronectin. This structure typically define TACS-1 collagen-associated signature. In tumors, CAFs remodel basement membrane structures, leading to the formation of large collagen bundles, and at the same time opening holes through which cancer cells can squeeze. CAFs also remodel stromal ECM's, bundling collagen and fibronectin fibers, parallel to the tumor edge in non-invasive tumor areas (TACS-2) or perpendicular where cancer cells invade (TACS-3). CAFs introduces stiffness heterogeneity in both the basement membrane and stromal ECM.

make them significantly stiffer than stromal ECM [69,43]. Maintenance of this stiffness requires the continuous and regulated production of ECM proteins and their precise assembly into highly organized networks. In healthy tissues, normal fibroblasts regulate basement membrane properties [43], but their role is likely supplanted by CAFs in tumors. The resulting misbalanced production of basement membrane building blocks and uncontrolled mechanical modifications could result in decreased stiffness. Softer CAF-modified basement membrane is, however, still stiffer than CAF-modified stromal ECM [15,34,61,70] despite these differences in global stiffness, a common feature of CAF-modification of the stroma and the basement membrane is the introduction of stiffness heterogeneity [15,71] (Figure 4). Stiff areas probably result from filament crosslinking, while soft areas could arise from filament clearance or breakage. Cancer cells could exploit this heterogeneity either to squeeze between loose fibers or to use aligned fibers as highways that give them the directional cues.

## Conclusions

In summary, recent studies have shown that mechanical forces developed by CAFs influence the growth and dissemination of cancer cells. CAFs can affect cancer cell invasion directly by pulling and dragging cancer cells out of the tumor bulk. More commonly, however, CAFs influence invasion indirectly by remodeling the ECM that surrounds cancer cells. Remodeling of the ECM includes deposition of new ECM components, ECM stretching, crosslinking, aligning, bundling, and stiffening. These ECM modifications are a direct consequence of high contractility, the feature that fibroblasts acquire when transforming into CAFs.

Different populations of CAFs can be found within the same tumor. For example, in breast cancer, four CAF subgroups can be discerned-based on the expression of Integrin β1, αSMA, FSP1, FAP, PDGFR and Caveolin 1 [72]. In lung cancer, RNAseq identified five CAF subtypes differing mainly in the production of collagen types and in the expression of genes related to myogenesis [73]. Similarly, in pancreatic cancer, different types of CAFs were found, some expressing αSMA (myCAFs) and others IL6 (iCAFs) [74••]. CAF heterogeneity could result from their multiple origins, the distinctive signals CAFs receive from different tumor areas, or the different substrate properties they feel. For example, CAFs could receive different signals from well-oxygenated areas close to blood vessels compared to hypoxic areas located

further away. However, the effect that hypoxia has on fibroblasts activation is contradictory. This could be explained by the effect that hypoxia has on normal fibroblasts versus CAFs, but also on different CAFs subpopulations, leading to their activation [75] or inactivation [76]. This is reviewed in details in [77]. This intrinsic CAF variability could directly translate into the role they play within a tumor. Indeed, despite many reports favoring a pro-tumorigenic role for CAFs, there are a few reports showing the opposite. αSMA$^+$ CAFs (myCAFs) surround tumor cells [74••], and their ablation in pancreatic tumors increased tumor spreading, reducing mouse survival due to the induction of an immunosuppressive phenotype [78]. This suggests that the accumulation of highly contractile CAFs around the tumor could provide a physical barrier constraining tumor expansion. Overproduction of ECM around tumor clusters most likely also reduces ECM porosity, hindering the passage of cancer cells through ECM pores. However, cancer cells could potentially overcome this physical barrier by increasing their nuclear deformability-coupled to nuclear envelope and DNA damage repair mechanisms [79–81].

The fact that CAFs increase in numbers as tumors progress and their role in stimulating cancer cell invasion, suggests a more complex scenario. Possibly, a fine-tuning of contractility during tumor progression might dictate when and how CAFs switch from a tumor-restrictive to a pro-tumorigenic phenotype. The uncontrolled increase in contractility could possibly transform good guys into bad guys. Thus, understanding how CAF contractility is regulated is of therapeutic interest. Finally, as CAFs are the major organizers of the ECM, they could be responsible for the change in ECM topology, from TACS-1/2 to TACS-3, favoring cancer cell dissemination. The development of stromal 'normalization' therapies would, in combination with standard drugs, potentially impact tumor response and patient survival.

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