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Review article

# Cancer-associated fibroblasts in tumor microenvironment – Accomplices in tumor malignancy

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ABSTRACT

There is much cellular heterogeneity in the tumor microenvironment. The tumor epithelia and stromal cells co-evolve, and this reciprocal relationship dictates almost every step of cancer development and progression. Despite this, many anticancer therapies are designed around druggable features of tumor epithelia, ignoring the supportive role of stromal cells. Cancer-associated fibroblasts (CAFs) are the dominant cell type within the reactive stroma of many tumor types. Numerous previous studies have highlighted a pro-tumorigenic role for CAFs via secretion of various growth factors, cytokines, chemokines, and the degradation of extracellular matrix. Recent works showed that CAFs secrete $H_2O_2$ to effect stromal-mediated field cancerization, transform primary epithelial cells, and aggravate cancer cell aggressiveness, in addition to inflammatory and mitogenic factors. Molecular characterization of CAFs also underscores the importance of Notch and specific nuclear receptor signaling in the activation of CAFs. This review consolidates recent findings of CAFs and highlights areas for future investigations.

## 1. Introduction

In 1889, Stephen Paget proposed that "seeds" (cancer cells) preferentially grew in the conducive "soil" (microenvironment) of select organs [1]. To date, the reciprocal communication between the "seed" and the "soil" has been proven to be the case whenever the tumor growth and metastasis were examined [2]. However, extensive research combining studies of the "seed" and "soil" was not pursued, largely because of the molecular revolution and the discovery of oncogenes and tumor suppressors in the "seed". Indeed, the prevailing paradigm of carcinogenesis for the last 50 years, which focused on the "seed", is the Somatic Mutation Theory (SMT) [3–5]. It postulates that somatic cells accumulate genetic abnormalities as the primary cause that confers a selective advantage to cancer cells. This change is supposed to account for the entire complex process of formation, growth, and metastasis of the tumor. This theory has guided the entire field of cancer research to cancer cell genomics, and the design of cancer treatments around druggable features of tumor epithelia, ignoring the supportive role of the stromal components. Over the years, an alternative theory that underscored the importance of "soil" has developed – the Tissue Organization Field Theory (TOFT) [3,6–8]. It suggests that neoplasia arises from a disorder of the three-dimensional microenvironment of a tissue and that drives the cell toward transformation, not from a cell gone awry by mutation or by other mechanisms. This involves the disruption to the normal communication between the stroma and the epithelium.

The extraordinary capacity of cancerous cells to mutate and thus evade almost any therapeutic intervention underpins the lethality of the disease and has proven impervious to many decades of effort to overcome it. In retrospect, Paget's "seed-and-soil" approach to cancer, along with SMT and TOFT, demands a serious examination of cancer cells as they exist in their natural habitats. During the past two decades, it has become unequivocally evident that tumor development is not a cell-autonomous process but rather depends on the intricate reciprocal interplay of tumor cells with their local and distant environments [9–11]. The composition and transformation of cells in the tumor microenvironment (TME) depend on genetic and environmental factors [12,13]. This has a direct influence on anti-tumor therapy. It is increasingly clear that the TME which any pre-neoplastic cell inhabits plays an important and perhaps even essential role in tumor progression, and the cells making up that niche are of course much more genetically and phenotypically stable than those of the tumor itself

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**Table 1**
Biomarkers of CAFs from various tumor types.

| Tumor Types | α-Smooth Muscle Actin (α-SMA) | Fibroblast Activation Protein-α (FAP-α) | Podoplanin | Others |
|---|---|---|---|---|
| Breast carcinoma - Ductal carcinoma (DC), Lobular carcinoma (LC) | High expression in DC and LC linked to poor relapse-free survival (RFS) and overall survival (OS) [31,44–46]. | Low expression in DC associated with poor RFS and OS [31,47]. | Presence in DC and LC CAFs associated with poor RFS and OS [31,48,49]. | |
| Colorectal carcinoma - Adenocarcinoma (ADC) | High expression in ADC associated with poor RFS and OS [31,50–52]. | High expression at ADC tumor center or in metastatic tumor associated with RFS and OS [31,50,53,54]. | Absent in ADC associated with poor RFS [31,50,55]. | High expression of Fibroblast specific protein 1 (FSP1) and Vimentin in ADC associated with poor RFS and OS [31,50,56]. |
| Esophageal carcinoma - Squamous cell carcinoma (SCC), ADC | Presence in ADC and SCC CAFs associated with poor OS [31,57,58]. | | Presence of podoplanin-positive CAFs in ADC associated with poor RFS and OS [31,59]. | Presence of FSP1 and PDGFR in SCC linked with poor disease-free survival (DFS) and OS [31,57]. |
| Head and neck carcinoma - SCC | High stromal αSMA expression associated with poor OS [31,60–66]. | | | High expression of Fibroblast growth factor receptor-1 (FGFR-1) in fibroblasts at invasive front of oral SCC linked to poor OS [67–69]. |
| Lung carcinoma - Non-small-cell lung carcinoma (NSCLC) | High expression in NSCLC associated with poor OS [31,70,71]. | High expression in NSCLC associated with OS [31,72]. | Presence of podoplanin-positive CAFs in NSCLC associated with poor DFS, RFS and OS [31,73–79]. | High expression of transforming growth factor-β1 (TGF-β1) in NSCLC associated with poor OS [70]. |
| Pancreatic carcinoma - ADC | High expression in ADC tumor stroma associated with poor RFS and OS [31,80]. | High expression in ADC associated with poor RFS and OS [31,81]. | Presence of podoplanin-positive CAFs in ADC associated with poor RFS and OS [31,82]. | |

Note: This list is non-exhaustive.

[14,15]. Furthermore, therapies which target the niche are likely to be effective against a broad range of cancers – overcoming some of the economic difficulties resulting from the often-limited market for drugs against cancers of a specific origin, and markedly improving therapeutic options for patients with refractory cancers. Indeed, therapy that aims to shape the tumor milieu and consecutive communication conduits must address the complex pathophysiology of tumor more adequately, in order to reap substantial benefits for cancer patients. An absolute pre-requisite for such an endeavor is a comprehensive understanding of the exact molecular basis of the complex signaling network in the TME, that controls the plasticity of both stromal and tumor cells, thereby modeling the complex cellular contexture, which ultimately forms a pro- and anti-tumorigenic milieu.

Cancer-associated fibroblasts (CAFs, also known as tumor-associated fibroblasts, TAFs) are a particularly abundant component of the tumor stroma and are known to influence pathology in multiple ways [16–18]. To understand the complex role of CAFs in tumor development, it is also important to consider their intrinsic heterogeneity and origins. Fibroblast populations from various body parts and within individual organs can have significantly different properties, including susceptibility to CAF phenotype acquisition and interaction with neighboring epithelial cells and immune cells [16,19]. Many studies have also reported different origins or predecessors of CAFs, including resident tissue fibroblasts, bone marrow-derived mesenchymal stem cells, hematopoietic stem cells, epithelial cells (epithelial-mesenchymal transition), and endothelial cells (endothelial-mesenchymal transition) [20–22]. Nonetheless, the majority of these studies have reported that CAFs express similar sets of markers as myofibroblasts, such as α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), and platelet-derived growth factor receptor (PDGFR)-α and β [21,23–25]. Importantly, these studies highlighted a pro-tumorigenic role for CAFs via secretion of various growth factors, cytokines, chemokines and the degradation of extracellular matrix (ECM) proteins [17,26]. In addition to inflammatory and mitogenic factors, CAFs also secrete $H_2O_2$ to effect stromal-mediated field cancerization, transform primary epithelial cells and aggravate cancer cell aggressiveness [27–29]. CAFs, or their conditioned medium, also contribute markedly to the development of drug resistance and to the expansion of cancer stem cells; thus, it seems that amelioration of their effects would not merely slow the growth of a tumor, but could, in fact, break the vicious cycle of cancer field expansion [28,30]. Hence, studying CAFs may hold the answer to some of the most persistent problems in clinical research today: recurring tumor, metastases, and drug resistance [31–33]. Despite the early enthusiasm, pharmaceutical inhibition of FAP-expressing CAFs has not, to date, proven remarkably successful [34–36] – although all such trials have involved advanced diseases, which is hardly an ideal indication for a therapy expected to interfere in the early stages of metastasis – but has at least demonstrated a lack of severe side-effects. Targeting CAFs will dictate a different therapeutic approach which is aimed at an entire field of interacting, dynamically evolving cells. The primary anti-tumor approach to target and eradicate the epithelial cancer cells is likely to remain as the mainstay treatment. Thus, the targeting of CAFs, or other stromal cells, and their communication networks will most likely be part of a multimodality approach or as adjunctive treatment to conventional tumor treatment.

## 2. Origin of CAFs

Emerging studies have reported that many kinds of cells could be recruited as CAFs predecessors: (1) resident tissue fibroblasts, (2) *peri*-tumoral adipocytes, (3) bone-marrow derived mesenchymal stem cells, (4) hematopoietic stem cells, (5) epithelial cells (through epithelial-mesenchymal transition; EMT), and (6) endothelial cells (through endothelial-mesenchymal transition; EndMT) [16,17,20–22]. Notably, a precise molecular definition of CAFs does not exist, and CAFs is rather a cellular state than a cell type [37]. Once recruited from the various

sources, a subset of these progenitors acquires CAFs phenotype (or status) through complicated activation processes which are still poorly studied and remain mostly enigmatic to scientists. Studies have demonstrated that unlike cancer epithelial cells, genetic alterations such as copy number changes and oncogene/tumor suppressor mutations are extremely rare in CAFs. Therefore, they do not appear to constitute the basis for the widespread tumor-promoting phenotypes exemplified by CAFs [38,39]. In stark contrast, epigenetic alterations (i.e. DNA methylation, histone modifications and nucleosome structure), changes in the expression of non-coding RNAs (i.e. miRNAs and long non-coding RNAs) [40,41] and the abnormal activation of several signal axis (i.e. NFκB, IL-6/STAT3, FGF-2/FGFR1 and TGF-β/SMAD) [42,43] are often observed upon acquisition of a CAF state. Thus, environmental factors play a huge part as well in determining if normal fibroblasts (NFs) transform to CAFs.

There are several markers characterizing CAFs and most of them are seen across different types of tumors. We show a non-exhaustive summary of the various markers characterizing CAFs (Table 1).

## 3. Molecular characteristics of CAFs

### 3.1. Transcriptomic changes in CAFs

CAFs have unique phenotypes and functions that are different from NFs. These characteristics of CAFs are governed in part by differences in their gene expression profile. Notably, these gene signatures are different among various tumor types in a subtype- and stage-specific manner, which underscores their prognostic value [83–86]. The analysis of these differentially expressed genes of CAFs in non-small cell lung [85], breast [87], colon [84,88] and pancreatic [89] cancers revealed that up-regulated genes are primarily associated with the special functions of CAFs in promoting cancer progression such as angiogenesis, EMT, cell adhesion and migration.

Despite the various studies highlighting the importance of CAFs in cancer malignancy and the prognostic potential of CAF gene signature, most of the identified genes were not amenable as therapeutic targets. Nuclear hormone receptors (NRs), one of the largest known classes of transcription factors, have been implicated in numerous types of cancers. In humans, the 48 known NRs play numerous roles in development, physiology, and pathology. Drugs that target NRs constitute one of the largest and most potent groups of pharmaceuticals currently in use and thus, hold great potential for use in improved anti-cancer treatment strategies. Most of the previous studies of the functional importance and relevance of NRs or their cognate ligands in tumor development and progression have been restricted to the epithelial cancer cells. A recent study identified a NR signature in CAFs of clinical cutaneous squamous cell carcinoma [30]. Among the 48 NR genes, 21 NR transcripts were differentially regulated, 18 were upregulated and 3 were downregulated in CAFs compared with NFs. Importantly, the study identified a cluster of driver NRs, consisting of peroxisome proliferator-activated receptor (PPAR) β/δ, androgen receptor (AR), glucocorticoid receptor (GR), retinoic acid receptor β (RARβ) and vitamin D receptor, as important modifiers of CAF function with profound influence on cancer cell invasiveness, proliferation, drug resistance, energy metabolism and oxidative stress status. Further investigation showed that RARβ and AR antagonists for concurrent therapy with cisplatin to inhibit acquired chemoresistance of transplanted xenografts. This work demonstrates that treatments targeting both the tumor epithelia and the surrounding CAFs can extend the efficacy of conventional chemotherapy.

### 3.2. Factors for activating CAFs

It was noticed that a subtle shift in the tumor milieu such as pH, lower glucose, and oxygen supply, accompanied by mixed “recruits” to a changed reservoir of growth factors, cytokines, or a stiff matrix could all trigger the activation of the CAF phenotype [10,16]. Regardless of the cellular origin of CAFs, many mechanistic studies have shown that signals that converged to the TGF-β are often employed and amplified for the transformation and activation processes of CAFs [90–93]. TGF-β1 expression has also been shown to positively correlate with CAFs formation and has a protective effect against apoptosis in breast cancer cells [94]. In addition, autophagy induced by TGF-β1 can promote tumor growth, and is involved in the protective effect of TGF-β1 and the formation of CAFs [94]. Clearly, TGF-β1 is required for normal fibroblast activation and transition to a CAF-like state. However, fibroblast-specific deletion of TGF-β1 Type II receptor (TGF-βRII) in mice led to spontaneous tumors in the prostate and forestomach, and promoted breast cancer progression as well [95–98]. The finding from a recent study has partly reconciled these paradoxical observations, with the underlying mechanism involving reactive oxygen species (ROS), specifically hydrogen peroxide ($H_2O_2$), in the transformation of CAFs [28]. NFs exposed to repeated low doses of $H_2O_2$ were initially responsive to TGF-β1, which facilitated their transformation to CAFs. Simultaneously, $H_2O_2$ triggered NFκB activation to suppress TGF-β signaling via the upregulation of the inhibitory Smad, Smad7. These combined characteristics synergistically create an ecosystem that encourages the conversion of NFs to CAFs, rendering CAFs refractory to TGF-β1 signaling and highly oxidative [28]. Indeed, in mature tumors, TGF-β1 is abundant and the TME is chronically subjected to low-grade inflammation and redox imbalance. ROS was also shown to be a potential driver to convert fibroblasts into highly migrating myofibroblasts through the accumulation of hypoxia-inducible factor-1α and C-X-C motif chemokine 12 (CXCL12) [99].

## 4. Stromal cell- stromal cell interactions

The concept and the definition of field cancerization were first introduced by Slaughter et al. in 1953 when he analyzed the tissues adjacent to squamous cell carcinoma [100]. The phenomenon was first observed in the organs and tissues of the respiratory tract and the upper part of the digestive tract, where multiple primary tumors and locally recurrent tumors originate from the anaplastic tendency of multiple cells. Presently, field cancerization refers to pre-malignant changes, in both epithelial cells and surrounding stromal cells, that occur in multiple and larger areas of the primary tumor [101,102]. It is a condition of major clinical significance because it is associated with increased frequency of multifocal and recurrent tumors surrounding the primary tumor, i.e. cancer fields. Clinical presentation of field cancerization has been reported among the commonest and deadliest cancer types in the world, including lung [103,104], breast [105,106], colorectal [107,108], prostate [109,110], stomach [111,112] and skin [113,114] cancers. Despite its prevalence and importance, field cancerization has been largely overlooked, in part, because very few studies have investigated the mechanisms of field cancerization, particularly the role of CAFs.

### 4.1. Field cancerization

According to the SMT, tumors are thought to be linked to genetic changes in apparently normal epithelial cells that can expand over time. Recent studies showed that genetic changes in stromal cells, like CAFs, can result in pro-tumorigenic changes to the adjacent epithelial cells. In a series of elegant experiments underscoring the importance of mesenchymal-epithelial communications, the lab of Paulo Dotto showed that a compromised Notch signaling in the underlying dermal fibroblasts results in stromal atrophy and inflammation that precede pre-malignant and malignant epithelial tumor development [115]. In the mouse, the deletion of a nuclear effector of Notch signaling, CSL (for CBF1/RBP-Jκ, Su(H), Lag-1) in dermal fibroblasts was sufficient for CAF activation and ensured multifocal keratinocyte tumors [115]. The silencing of CSL induced senescence of primary mouse and human

fibroblasts isolated from the dermis, oral mucosa, breast, and lung. CSL was down-modulated in stromal fibroblasts of premalignant skin actinic keratosis lesions and SCC, while tumor suppressor p53 expression and function was down-modulated only in the latter. Thus, the concomitant loss of CSL and p53 overcame fibroblast senescence, enhanced the expression of CAF effectors and promoted stromal and cancer cell expansion.

Of importance, a recent finding confirms the presence of stromal-mediated field cancerization, where the effects of stromal oxidative stress can be propagated and amplified, and effectively create a "mutagenic/oncogenic" field promoting multifocal tumor formations [28]. This finding highlights mesenchymal-mesenchymal and mesenchymal-epithelial communications in the propagation of field effect and the creation of a TME niche. An abundance of mutagenic ROS in the tumor stroma of patient biopsies indicates an extratumoral oxidative stress. CAFs secreted higher levels of $H_2O_2$ compared with normal cells, suggesting that extracellular $H_2O_2$ might act as a field effect carcinogen. Indeed, NFs treated with CAF-conditioned medium or exogenous $H_2O_2$ resulted in the acquisition of an oxidative, CAF-like state. Normal epithelial cells exposed to a chronic low dose of $H_2O_2$ also exhibited decreased phosphatase and tensin homolog (PTEN) and increased Src activities result from oxidative modification, leading to the pre-oncogenic transformation of these cells. The higher $H_2O_2$ production by CAFs was due to an impaired TGF-β signaling leading to the suppression of the antioxidant enzyme glutathione peroxidase 1 (GPx1). The expression of GPx1 is also regulated by p53 [116] and the ablation or mutation of p53 in CAFs increased growth and metastatic spread of prostate cancer cells because of the increased production of CXCL12 [117]. It is also interesting to note that the suppression of Notch/CSL and its associated gene expression in human dermal fibroblasts can be induced by stress/DNA damage caused by ROS, ultraviolet A ray, among others [115]. CSL expression is negatively regulated by p53, a key effector of the DNA damage response [118].

*In vivo*, the proliferative potential and invasiveness of composite tumor xenografts comprising cancerous or non-tumor-forming epithelia with CAFs and NFs could be attenuated by the presence of catalase. Importantly, the oxidatively transformed fibroblasts isolated from composite tumor xenografts retained their ability to promote tumor growth and aggressiveness when adoptively transferred into new xenografts [28]. Taken together, these findings indicated that CAFs engage redox signaling circuitries and mitogenic signallings, such as fibroblast growth factor and CXCL12, to reinforce their reciprocal relationship with the epithelial tumor.

### 4.2. CAFs interaction with tumor-associated immune cells

Cancer cells and CAFs secrete chemokines that facilitate the infiltration of tumor-associated immune cells, which include tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TIL) such as immunosuppressive regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Previous studies have shown that TILs do not exhibit anti-tumor functions, instead, they contribute to tumorigenesis and progression [119]. The Tregs are a subpopulation of TILs which modulate the immune system, maintain tolerance to self-antigens, and prevent autoimmune disease. Tregs secrete TGF-β and interleukin-10 (IL-10) to suppress or downregulate induction and proliferation of effector T cells at the tumor site [119]. In breast cancer, the presence of Tregs is associated with an invasive phenotype and poor prognosis [120,121]. In contrast to tumor stroma, Tregs are scarce in the normal stroma. Histochemical analysis of human breast cancer biopsies revealed that most of the TILs were distributed adjacent to CAFs in the cancer stroma [122]. This observation suggests a close interaction between CAFs and Tregs in the TME. Indeed, the depletion of CAFs in mammary tumors decreased Treg infiltration and pulmonary metastasis [123,124]. Reciprocally, Tregs-rich TILs and their secretory cytokines have a profound effect on the growth of CAFs, arresting CAFs at the G2/M phase [122].

Macrophages are the main component of inflammatory cells and are key players in the wound healing process. Drawing parallels between wound healing and tumor progression, the macrophages that infiltrate into the TME are known as TAMs. Circulating monocytes in the blood are the main sources of TAMs and the activities of tumor cell-derived cytokines drive the differentiation of tumor-infiltrating monocytes into mature pro-tumor macrophages [125–127]. Similar to TILs, TAMs are often detected in close proximity to CAFs in several tumor types, suggesting interactions between these two cell types [50]. Macrophages are activated by the cleavage of type I collagen by FAP derived from CAFs [128]. Hashimoto et al. reported that high density of either TAMs or CAFs was positively correlated with high malignancy of neuroblastoma. The study further demonstrated that the reciprocal communication between CAF-like mesenchymal stromal cells and TAM-like macrophages induces pro-tumor phenotypes *in vitro*, including increased angiogenesis for growth and tumor cell dissemination. Importantly, this CAF-TAM interaction accelerated the proliferation of neuroblastoma and prostate cancer [129,130].

CAFs recruit TAMs, MDSCs and Tregs to promote Th2 polarization of the TME. Th1 versus Th2 polarization is determined partly by the expression of Th1 and Th2 cytokines in the TME. Th1 polarization is associated with cell-mediated immunity and generally favors tumor rejection, while Th2 cytokine polarization generally prevents tumor rejection, promotes tumor growth and is commonly associated with solid tumors [131]. TAMs and MSDCs are the key producers of Th2 cytokines as well as other factors which suppress host anti-tumor immune responses and promote tumor growth [132]. This modulation of the TME promotes angiogenesis, lymphangiogenesis and suppresses anti-tumor responses to support tumor growth and metastasis [124].

Many studies have underscored the importance of communication between CAFs and TILs in chronic inflammation, cancer progression and metastasis [122,124,133–138]. Therefore, future research on anti-tumor treatments should include a viable strategy that disrupt CAF-TAM or CAF-TIL relationships [139].

## 5. Stromal cell – cancer cell interaction

CAFs can regulate cancer stem cells via cytokines and chemokines. These molecules can act on cancer stem cell directly to induce self-renewal [140]. CAFs can also reprogram and induce stemness in relatively undifferentiated tumor cells [141]. Chemokines secreted by CAFs are also involved in promoting cancer cell invasiveness. CAFs in gastric cancer have been shown to secrete CXCL12, which activates CXCL12/CXCR4 signaling, thereby promoting tumor growth and invasiveness [142]. This effect was also observed in breast [21] and non-small cell lung carcinomas [143]. Moreover, the subset of CAFs with a myofibroblast phenotype has been shown to create elongated collagen fibers which lead to a poor prognosis [144].

Gathering studies showed that CAFs can also interact with cancer cells through secreted miRNAs in extracellular vesicles. miR-1227 have been shown to transfer into CAFs by large oncosomes from tumorigenic prostate cells and enhance CAF migration [145]. MiR-409 is up-regulated in CAFs from prostate cancer and has been shown to induce EMT in cancer cells upon their secretion from CAFs [146]. Secreted miRNAs can also act in feedback loops between CAFs and cancer cells. MiR-133b is secreted from CAFs and act as paracrine stimulation of both fibroblasts and tumor cells to further increase their endogenous expression [147].

### 5.1. Chemoresistance

Cytotoxic chemotherapy remains as one of the mainstays of cancer treatment. It uses chemotherapeutic agents to eradicate rapidly dividing cancer cells by inducing apoptosis or inhibiting cell division. Despite being an important therapeutic option for most cancer patients,

drug resistance is typically associated with the cell autonomous processes, such as genetic and epigenetic changes in tumor epithelial cells influencing uptake, metabolism, and export of the drug. Furthermore, emergent evidence also points to the ever-evolving tumor stroma that creates a complex and intricate signaling network aimed at promoting drug resistance and survival of cancer cells after therapy. As the most prominent cell type in the tumor stroma, CAFs can contribute to drug resistance through the modulation of the physical, dimensional, and chemical aspects of the tumor tissues in an ongoing dynamic and co-evolutionary manner [37].

CAFs could impart adaptive drug resistance through their secretome that remodels the tumor stroma and directly reprogram cancer epithelial cells. CAFs can also modulate tumor cells in a paracrine manner including the exosomal transfer of mediators such as miRNAs and cytokines. For example, gemcitabine treatment increases the secretion of miR-146a and Snail by pancreatic CAFs in the form of exosomes, which are delivered to tumor epithelial cells, reinforcing their survival and proliferation power, contributing to poor prognosis [148]. However, treatment of gemcitabine-exposed CAFs with an inhibitor of exosome release, GW4869, significantly reduces survival in co-cultured epithelial cells, signifying the potential for exosome inhibitors as treatment options alongside chemotherapy for overcoming chemoresistance. Studies have also shown that exosomes secreted by CAFs can increase the number of cancer stem cells (CSCs), increase their growth rates and prime CSCs to promote tumor stemness [149].

CAFs-secreted TGF-β1 signaling often confers epithelial cancer cells resistance to chemotherapies, making it an attractive therapeutic target [150]. Platinum-based chemotherapy could be antagonized by glutathione and cysteine released by CAFs, which could be reversed by effector T cells in the TME boosted by a synergistic immunotherapy [151]. This suggests that the interplay between chemotherapy and immunotherapy holds high potential for cancer treatment. CAFs could dampen body's anti-tumor immune response through their secretion of pro-inflammatory and immunosuppressive factors in the TME. For instance, cisplatin treatment could stimulate CAFs to produce and secrete high levels of IL-11 into the TME, reigniting the p-STAT3/Bcl2 surviving signals, triggering anti-apoptosis and promoting colony formation in cancer cells in lung adenocarcinoma [152].

There are many other secreted factors from CAFs that have been proven to confer chemoresistance in several different types of cancer. In a pancreatic tumor environment, myofibroblasts in the tumor environment were shown to produce Insulin Growth Factor (IGF) which promotes the proliferation and survival of pancreatic cancer cells [153]. CAFs and TAMs could also work together to secrete IGF 1 and 2 into the TME, blocking cancer cell sensitivity to drugs causing chemoresistance [153]. In lung cancer, the expression of stromal cell-derived factor 1 (SDF-1) was increased, facilitating drug resistance via the CXCR4-mediated signaling pathway [32]. Of note, the increase in SDF-1 was caused by a down-regulation of miR-1, as previously shown to be required for transformation of NFs to CAFs [154]. Furthermore, it was shown in ovarian cancer that CAFs secrete soluble factors to endow chemoresistance not only against cisplatin and its family of chemodrugs, but also against platinum-based treatments [151]. Yan et al. managed to show that the resistance to cisplatin-induced apoptosis was due to STAT3 signaling with Survivin and Bcl-2 over-expression in gastric cancer [155]. In head and neck squamous cell carcinoma (HNSCC), CAFs have been shown to be able to confer resistance to cisplatin possibly by the release of paracrine factors to stimulate survival pathways such as the Akt pathway [156,157].

In addition, CAFs can enhance chemoresistance in pancreatic cancer via inhibiting the expression of caspases [158]. Ziani et al. show that CAFs commit themselves in the secretion of active MMPs to reduce the expression of NKG2D ligands, MICA/B, at the surface of tumor cells and consequently decreases the NKG2D-dependent cytotoxic activity of NK cells against melanoma tumor cells [159]. An overstressed TME, often represented by oxygen- and nutrient-deprivation and ROS stress, could also "educate" CAFs to metabolically reprogram themselves and undergo autophagy to provide epithelial cancer cell with high energy metabolites conferring them drug resistance [160]. Recently, pharmacological inhibition of the protein synthesis (mTOR/4E-BP1) pathway in CAFs has been shown to abolish the resistance of pancreatic cancer cells to gemcitabine [161]. The anti-malarial drug chloroquine, a potent inhibitor of autophagy, has been shown to significantly reduce tumor growth by selectively targeting CAFs [160]. However, in cancer, the autophagic process can serve as both a pro-death and a pro-survival process depending on cellular conditions [160]. Thus, one should exercise extra caution using strategies that aim to elicit autophagy in CAFs due to its paradoxical outcome.

In all, there are multiple ways in which CAFs can confer chemoresistance and they are different with regards to the type of cancer. Apparently, this list is not exhaustive, and it is possible that CAFs can utilize all the pathways to confer chemoresistance, regardless of the type of carcinoma. It is thus imperative that efforts to research on CAFs must continue to enhance our understanding of cancer and its treatments.

### 5.2. Metastasis and invasion

Metastasis is a multi-step process that culminates to the spread of cancer cells to tissues and organs beyond where the tumor originates, and the formation of new tumors (secondary and tertiary foci) [162,163]. CAFs in the tumor stroma play an important role in both cancer initiation and progression, and contribute significantly to the acquisition of hallmarks for cancer invasion and metastasis [164]. Based on the locations they are found, CAFs can be classified into 3 types, namely p-CAFs (CAFs from the primary tumor), c-CAFs (CAFs found in the blood circulation), and m-CAFs (CAFs from metastatic sites) (Fig. 1). Each CAF subtype may serve distinct function/s as well as have varying levels of potency in inducing cancer cell metastasis and invasion [31,47,165]. Currently, most experimental studies were focused on p-CAFs and m-CAFs, and the investigation of c-CAFs would provide a comprehensive understanding of the migration and/or transformation of CAFs. Taken together, CAFs stimulate invasion, ectopic survival, adhesion and colonization of a metastatic site by providing mechano-adhesive signals, track generation, soluble factors and packaged factors.

CAFs secrete many soluble factors that can be critical for cancer metastasis and invasion. Using three-dimensional mixed parental reduction mammary fibroblasts and human cancer cell lines co-cultures and mouse xenograft models of human breast cancer, decreasing CAFs Tiam1 expression induced invasion and migration in breast cancer cells. These long-lasting changes are both dependent on fibroblast secretion of osteopontin and affect the cancer cells even after their dissociation from the fibroblasts. This indicates a novel Tiam1-osteopontin pathway in breast CAFs. In a mouse model of breast cancer, the inhibition of fibroblast osteopontin prevented lung metastasis. Importantly, the expression patterns of Tiam1 and osteopontin in CAFs from human breast cancers were inversely correlated with invasiveness of the cancer cells. This supports that the Tiam1-osteopontin pathway in CAFs regulates breast cancer invasiveness and may be clinically relevant [166]. Consistent with a role for Notch signaling in field cancerization of skin squamous cell carcinoma [115], the level of Notch1 pathway activity in CAFs may determine either their tumor-promoting or tumor-suppressing effect on mouse melanoma. CAFs with elevated Notch1 activity inhibited melanoma growth and invasion significantly, while CAFs with no Notch1 signaling promoted melanoma invasion. These results revealed that the Notch1 pathway, as a molecular determinant, controls the regulatory role of CAFs in melanoma skin growth and invasion [167].

At the mechanobiological level, the onset of metastasis is considered to have occurred when cancer cells invade and breach the basement membrane (BM) which provides mechanical support to the epithelial

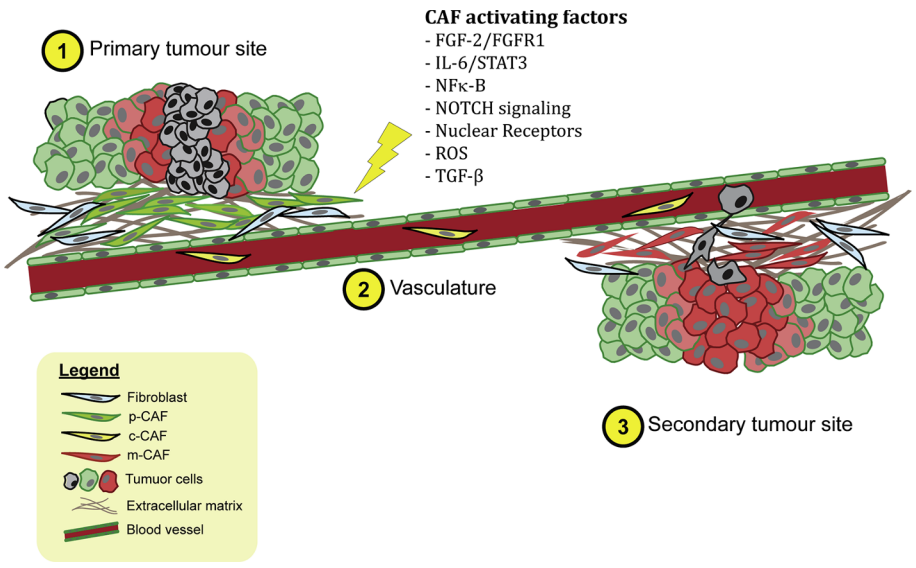


**Fig. 1.** CAFs and its activating factors. Upon activation by appropriate signals or mediators, such as TGF-β and NRs, NFs at the primary site are activated to CAFs (p-CAFs) and can contribute to chemoresistance, metastasis, and invasion. Circulating CAFs (c-CAFs) are detected in the vasculature. However, their origin remains unclear and it is still unknown if c-CAFs extravasate at the secondary tumor site. CAFs associated with secondary tumors are also known as m-CAFs. Similarly, their origin remains to be determined, i.e. they arise from fibroblasts at the secondary tumor site or have migrated from the primary tumor site.

tissues [168–170]. By expressing elevated amounts of matrix metalloproteinases, CAFs play a critical role in the degradation of the BM. The loss in integrity of the BM renders it permissive for invasion by cancer cells, allowing cancer cells to migrate freely through the gaps formed in BM [171–173]. Therefore, BM breaching can be achieved by the mechanical interactions and signal between CAFs and BM. Track generation by CAFs also contributes significantly to cancer metastasis and invasion. In a recent study, CAFs were isolated from two patients with salivary gland adenoid cystic carcinoma (ACC). Conditioned medium collected from the ACC-derived CAFs promoted ACC cell migration and invasion significantly. In addition, when the CAFs were co-cultured with ACC cells in a microfluidic device, the ACC cells followed the track, behind the CAFs that were localized at the invasion front. The inhibition of the ACC invasion promoted by CAFs can be achieved by both the matrix metalloproteinase and the CXCL12/CXCR4 pathway interference. This study demonstrated that apart from the secretion of soluble factors, CAFs also create an invasive track for ACC invasion in the ECM [174].

In a recent study, prostate cancer cells were found to migrate toward and along the axis of the CAFs, when co-culture with CAFs in a microfluidic device [175]. This was similarly observed in HNSCC. In contrast, cancer cells did not display migration directionality when co-cultured with NFs. These observations suggest a CAF-promoted directional cancer cell migration in different cancer types. Further investigation revealed that CAFs assemble a fibronectin (Fn)-rich and highly organized matrix to promote the migration of cancer cells. It has been shown that CAFs secrete high levels of Fn [176,177]. Fn is an abundant ECM protein which mediates either cell adhesion, migration, growth or differentiation in various biological processes [178]. Using traction-force microscopy, CAFs were observed to exert about 50% greater traction force on Fn than NFs did. CAFs also contracted collagen I gel to greater extent than NFs did in collagen gel contraction assays. The increased contractility and traction forces in CAFs were mediated by non-muscle myosin II and PDGFR-α. These forces were then transduced to Fn via α5β1 integrin. Thus, CAFs were able to organize Fn as parallel fibers to aid cancer cells in directional migration through an increased traction forces and contractility. Further examination of human prostate and pancreatic cancer tissues also provided evidence that aligned Fn fibers at the site of invasion are a prominent feature *in vivo*. Moreover, αv integrin was found to mediate the directionality and efficiency of prostate cancer cells migration in CAFs-derived matrices. In summary, CAF-dependent alignment of Fn confers directionality in the migration of cancer cells, and potentially contribute to cancer metastasis and progression [175].

Tumor cells with the appropriate profile of metastatic “driver” gene profile within the cancerized field become aggressive and gain the capacity to invade, intravasate, evade the immune system, and metastasize. However, in most cases, *in situ* epithelial cancer lesions do not progress into malignancy, even if they harbor many of the genetic changes found in invasive and metastatic tumors, indicating that tumor stroma can play an important role. In an epithelium to mesenchyme manner, it has been proposed that metastasized tumor cell may once again enact field cancerization, via the generation of m-CAFs, to corrupt its new microenvironment. Such early micrometastases will result in very different cancerized fields, which manifested as intertumor heterogeneity. Indeed, metastatic epithelial tumors produce an elevated level of ROS [27,28,179], in particularly $H_2O_2$. Diffusible $H_2O_2$ was elevated in the conditioned medium of cultured skin epithelia at various stages of oncogenic transformation, and $H_2O_2$ production increased with greater tumor-forming and metastatic capacity of the studied cell lines. Such exogenous $H_2O_2$, in combination with growth factors, could transform NFs at the new secondary site to CAF-like cells [27,28]. Thus, NFs act as a natural barrier of the establishment of micrometastases, whereas m-CAFs create a conducive amenable for the successful colonization of the metastasized cancer cells. In this context, clearly, other stromal cells like TAM and endothelial cells are also important determinants of productive engraftment of cancer cells at secondary sites.

Numerous studies have supported an important role for CAFs in promoting cancer metastasis and invasion. However, two recent studies indicate that the elimination of CAFs in cancer tumors might worsen the outcome. In a mouse model of pancreatic ductal adenocarcinoma, genetically induced deletion of α-SMA expressing CAFs led to an increase incidence of tumors and reduced animal survival [180]. Furthermore, the elimination of CAFs in this model also indicated the suppression of normal immune surveillance by promoting the formation of Tregs in the CAF-depleted tumors. In another study using human breast cancer xenograft mouse model, chemically-induced apoptosis of suicide-engineered CAFs at early time points after tumor implantation increased the presence of TAMs and the metastatic spread of breast cancer cells to the lung and bone [181]. Taken together, the apparent discrepancies from the various studies underscore the importance of phenotypic and functional heterogeneity of CAFs, and warrant further studies to understand the different roles of various subpopulations of CAFs before targeting them for tumor treatments [182].

## 6. Perspectives

In this review, various studies have established a role for TGF-β and Notch signallings in CAF activation and their subsequent effects on cancer cells. These two pathways have been known to synergistically work with each other [183,184], although the precise mechanism remains unclear. Thus, it will be interesting to investigate the functional synergism of these two pathways in CAFs. Several NRs such as PPAR, GR and RAR, have been shown to work either agonistically or antagonistically with TGF-β [185–187]. Understanding the roles of these various players in the overall signaling landscape of CAF activation would certainly aid in our better understanding the factors driving the cancer-promoting effects of CAFs.

The relationship between CAFs at different sites (i.e. primary, secondary and circulating CAFs) is also not clear. Currently, it is not known if CAFs at secondary tumor sites are CAFs that have migrated from primary sites or are mutated from NFs at the secondary site. This will also reveal to us if the circulating CAFs are removed by the host's immune system or they can make it to the secondary metastatic sites eventually.

In pre-clinical models, experimental immunotherapies, such as those against FAP-expressing CAFs, have shown some promising results in inhibiting tumor growth [188–190]. However, similar anti-FAP therapies demonstrated little or no clinical efficacy in human patients [191,192]. Off-target effects on FAP-expressing bone marrow stromal cells have also induced unintentional cachexia and bone toxicity in a T cell therapy against FAP-expressing cells [193]. The studies cited above are non-exhaustive and more clinical trials can be viewed online (https://clinicaltrials.gov). Therefore, more research is needed to design an optimum immunotherapy which has precise stromal cell elimination with little or no "off-target" side effects *in vivo*.

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