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Clinical and therapeutic relevance of cancer-associated fibroblasts

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Abstract

Cancer-associated fibroblasts (CAFs) found in primary and metastatic tumours are highly versatile, plastic and resilient cells that are actively involved in cancer progression through complex interactions with other cell types in the tumour microenvironment. As well as generating extracellular matrix components that contribute to the structure and function of the tumour stroma, CAFs undergo epigenetic changes to produce secreted factors, exosomes and metabolites that influence tumour angiogenesis, immunology and metabolism. Because of their putative pro-oncogenic functions, CAFs have long been considered an attractive therapeutic target; however, clinical trials of treatment strategies targeting CAFs have mostly ended in failure and, in some cases, accelerated cancer progression and resulted in inferior survival outcomes. Importantly, CAFs are heterogeneous cells and their characteristics and interactions with other cell types might change dynamically as cancers evolve. Studies involving single-cell RNA sequencing and novel mouse models have increased our understanding of CAF diversity, although the context-dependent roles of different CAF populations and their interchangeable plasticity remain largely unknown. Comprehensive characterization of the tumour-promoting and tumour-restraining activities of CAF subtypes, including how these complex bimodal functions evolve and are subjugated by neoplastic cells during cancer progression, might facilitate the development of novel diagnostic and therapeutic approaches. In this Review, the clinical relevance of CAFs is summarized with an emphasis on their value as prognosis factors and therapeutic targets.

Cancer initiation, progression and metastasis elicit a wide spectrum of dynamic alterations in host tissues, leading to the formation of a complex tumour stroma, also known as the tumour microenvironment (TME)[1–9]. In certain cancer types, such as pancreatic and breast cancers, the tumour stroma develops with a profound desmoplastic reaction resulting

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in an abundance of fibrous and/or connective tissue. In general, the tumour stroma is composed of extracellular matrix (ECM) components and a variety of cell populations, including immune cells, fibroblasts and vascular endothelial cells[7]. Essential elements of the tumour stroma, such as immune responses, inflammation, angiogenesis, metabolism, hypoxia, ECM remodelling and fibroblast heterogeneity, have been widely investigated as potential therapeutic targets[2,3,10].

Fibroblast populations found in primary and metastatic cancers, collectively referred to as cancer-associated fibroblasts (CAFs), have been widely studied and are implicated in tumour initiation, progression and metastasis[11–13]. CAFs are now known to be highly heterogeneous mesenchymal lineage cells with diverse putative functions as revealed in studies utilizing single-cell RNA sequencing (scRNA-seq) of different cancers[14–28]. Whether CAFs constitute a distinct class of mesenchymal cells with unique functions will remain an active area of research for years to come. In this Review, the proposed functional roles and clinical relevance of CAFs are discussed and summarized with particular emphasis on their heterogeneity, prognostic value and therapeutic potential. In addition, we provide insights and perspectives on future research and clinical studies involving CAFs.

The definition and origin of CAFs

Fibroblasts were first defined as cells that reside in connective tissues and synthesize collagens, especially type I collagen[29]. At present, fibroblasts are typically defined as interstitial cells of a mesenchymal lineage that are not epithelial, endothelial or immune cells[30]. However, the precise cellular origins and functions of fibroblasts remain ambiguous and challenging to determine owing to the substantial phenotypic and functional heterogeneity of these cells and, thus, a lack of definitive biomarkers[11,13]. Fibroblasts are arguably the most resilient and versatile cells contributing to the structural maintenance of tissues and participating in the wound healing processes of most organs. Quiescent fibroblasts become activated in response to tissue damage, during wound healing and as a consequence of neoplasia[31–34]. Such activation is usually defined based on ECM synthesis and remodelling abilities, secretory profiles, proliferative status, and/or the expression of certain markers such as α-smooth muscle actin (αSMA).

In the context of cancer, the definition of CAFs can be simply stated as fibroblasts (non-epithelial, non-cancerous, non-endothelial and non-immune cells) that are located within or adjacent to a tumour. The many putative origins of CAFs include quiescent tissue-resident fibroblasts and pancreatic or hepatic stellate cells[35–37] (FIG. 1). Other major cellular origins of CAFs identified in different studies include bone marrow-derived mesenchymal stem cells[23,38–41], endothelial cells[42,43] and adipocytes[44]. Nevertheless, the precise origins of CAFs and CAF subpopulations remain elusive, partially owing to the phenotypic and functional plasticity of these cells and the lack of well-defined lineage biomarkers. The identification of new biomarkers for non-malignant-tissue fibroblasts and CAFs through the interrogation of scRNA-seq datasets might facilitate subsequent lineage-tracing studies using specific Cre-recombinase drivers to precisely determine the cellular origins of CAFs in the context of spontaneous cancers in relevant genetically engineered mouse models (GEMMs).

CAFs share many basic characteristics, such as a secretory phenotype and capacity to synthesize ECM, with fibroblasts found in non-malignant tissues or in the setting of wound healing but can also have important alterations in epigenetic and transcriptional profiles[11,13,45]. The precise function of CAFs in cancer progression has remained elusive. Evidence from some preclinical studies suggests an initial role of fibroblast activation as part of the host response and defence mechanism against neoplasia[46,47]. Such defensive responses by CAFs can involve ECM-related physical barriers and encapsulation resulting from the desmoplasic reaction[48]. Findings from other studies suggest that, following tumour initiation, fibroblast activation and wound healing mechanisms are hijacked by cancer cells to support tumour growth[45,49]. Thus, fibroblasts probably have both tumour-supporting and tumour-suppressive functions that are context dependent and/or reflect the functional heterogeneity of CAFs.

CAF heterogeneity

A number of mesenchymal cell biomarkers have been used to identify CAFs, including but not limited to αSMA, fibroblast-specific protein 1 (FSP1, also known as S100A4), fibroblast activation protein (FAP), platelet-derived growth factor receptor-α (PDGFRα), PDGFRβ, desmin, discoidin domain-containing receptor 2 (DDR2) and vimentin. Heterogeneity in CAF biomarker expression was first demonstrated in co-staining studies[50]. More recently, the advent of scRNA-seq has enabled a deeper understanding of CAF heterogeneity across a wide range of tumour types, leading to the identification of a variety of biomarker genes defining different potential subpopulations of CAFs[14–17,19–28,51–54] (TABLE 1). Such studies involve the initial selection of CAFs via flow cytometry sorting and/or analyses based on the absence of exclusion markers (epithelial, immune and endothelial markers)[15,22,23,25] or the presence of putative cell-surface biomarkers, such as CD29, FAP, PDGFRα or CD90 (Thy1)[25,55]. Alternatively, CAFs have been identified based on their distinct transcriptional profile compared with epithelial and other stromal cells through scRNA-seq analysis[14,56,57]. Despite variability in CAF biomarkers used in different studies involving scRNA-seq, an αSMA-expressing myofibroblast subset has been consistently identified as one of the key subpopu lations of CAFs across multiple cancer types[15,16,19,21,25,58]. Other CAF subpopulations expressing biomarkers such as FAP (encoded by *FAP*), decorin (encoded by *DCN*) and/or podoplanin (encoded by *PDPN*) have also been identified (TABLE 1; Supplementary Table 1).

In the context of pancreatic cancer, scRNA-seq analyses of mouse and human tumours have been performed to identify CAF subpopulations[14–16]. Three distinct subsets of fibroblasts have been identified in the non-malignant mouse pancreas, two of which were also observed in several GEMMs of advanced-stage pancreatic cancer[16]. These CAF subsets overlapped with two major CAF subpopulations previously identified in both KPC (*LSL-Kras*$^{G12D/+}$;*Trp53*$^{R172H/+}$;*Pdx1-Cre*) mice and patients with pancreatic cancer[15,59]: ECM-producing CAFs, termed myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs)[59] (TABLE 1). Immunostaining of tumour organoids revealed differences in the spatial distribution of these two CAF subpopulations. Specifically, myCAFs were located in proximity to cancer cells whereas iCAFs were more distant, potentially indicating that myCAFs and iCAFs interact in a juxtacrine and paracrine manner, respectively, with cancer

cells[59]. A smaller subpopulation of antigen-presenting CAFs (apCAFs), characterized by MHC class II and CD74 expression but a lack of classical costimulatory molecules expressed by professional antigen-presenting cells, has also been identified in pancreatic tumours from GEMMs[15] (TABLE 1). The antigen-presenting function of fibroblasts in various physiological and pathological conditions was discovered decades ago and has received renewed interest in the past few years[60–64]. Nevertheless, precise identification of apCAFs, especially in human cancers, requires further in-depth investigation and functional validation of this CAF subset. Heterogeneous CAF subpopulations have also been observed in scRNA-seq analyses of human pancreatic tumour specimens, although whether apCAFs are present as a distinct subpopulation in patients with this disease remains unclear[14,15,17]. LRRC15 has been described as a novel biomarker of myofibroblasts in human pancreatic tumours and high expression of a gene signature of LRRC15$^+$ CAFs is correlated with a lack of response to immunotherapy[18]. LRRC15 is also expressed in a subset of CAFs identified in human breast tumours[24].

Distinct sets of biomarkers and different classification strategies have been used to define various CAF subpopulations in other cancer types, including melanoma, cholangiocarcinoma, and colorectal, head and neck, breast and lung cancers[19–26,53,54] (TABLE 1). Puram et al.[21] reported a differential prevalence of CAF subsets in lymph node metastases compared with primary human head and neck tumours, which suggests that CAF heterogeneity might be tissue-specific and/or altered by the metastatic microenvironment. Notably, ex vivo culturing of the CAFs led to loss of expression of activation markers and ligands such as complement component 3 (C3), IL-6 and hepatocyte growth factor (HGF)[21]. In the context of breast cancer and ovarian cancer, four CAF subsets, CAF-S1 to CAF-S4, have been defined[25,65,66]. In a subsequent study by Kieffer et al.[24], further analysis of the FAP$^+$ CAF-S1 subset using scRNA-seq revealed eight subclusters, three of which expressed genes related to ECM production and transforming growth factor-β (TGFβ) signalling and were correlated with the presence of CD4$^+$ T cells expressing PD-1 and/or CTLA4. Ex vivo coculturing of these subsets of CAF-S1, referred to as ECM-myCAFs and TGFβ-myCAFs, with T cells resulted in the upregulation of PD-1 and CTLA4 in the T cells and expansion of the TGFβ-myCAFs and ECM-myCAFs[24]. Similarly to Puram et al.[21], Kieffer et al.[24] also reported that CAF subtype identity ex vivo was dependent on the cell isolation and culture methodology used. Together, these studies highlight the need to study CAFs in physiologically relevant models.

Vascular CAFs, matrix CAFs, cycling (proliferative) CAFs and developmental CAFs have been described as being present in breast tumours[22] (TABLE 1). These CAF subsets are proposed to have disparate origins within the tumour, putatively including the perivascular niche, resident fibroblasts and transformed epithelium[22], potentially informing on their putative functions. Other studies have demonstrated that myCAFs (characterized by the expression of *PDPN*, *FAP*, *ACTA2*, *COL1A1* and *COL1A2*) are located at the invasive front of human breast tumours, whereas iCAFs (marked by the expression of *CXCL12*) are localized in distal areas of the tumour stroma and are associated with a higher number of lymphocytes[57]. Friedman et al.[23] profiled the dynamics of CAF subpopulations throughout breast tumour progression in mice and found that the transcriptional profiles of CAF subsets shift from a presumed immunoregulatory programme to wound healing and antigen-

presentation programmes, suggesting that CAF functions change during tumour progression. Data from a study using mouse models of melanoma similarly showed that the abundance of three CAF subclusters, denoted as Stromal 1 (S1; expressing high levels of *Pdpn*, *Pdgfra* and *Cd34*), S2 (expressing high levels of *Pdpn* and *Pdgfra* and low levels of *Cd34* and *Acta2*) and S3 (expressing high levels of *Acta2*), changed during tumour progression, with the S3 subpopulation dominating at later stages of tumour growth[27]. Together, these findings suggest that CAF subsets have distinct origins and functions, with the caveat that they are derived from correlative analyses of scRNA-seq and immunostaining data with extrapolation of CAF functions from transcriptional data and in vitro studies. Advances in imaging enabling highly multiplexed spatial analysis coupled with functional studies[54] might provide better insight into the precise origins and functional roles of CAF subsets.

The observation that the CAF subsets and their gene signatures can be very different across various cancer types is unsurprising considering their distinct cellular origins and microenvironments. One can therefore expect that the identification of a universal CAF biomarker system that is applicable to multiple cancer types will be difficult (BOX 1). Such differences will probably also necessitate rational biomarker-guided strategies for future CAF-targeted therapeutics, which will be discussed in later sections of this Review.

The observed plasticity and heterogeneity of CAFs could have several possible explanations: (1) dynamic and interchangeable shifts of individual classes of CAFs between either tumour-promoting or tumour-restraining phenotypes (as discussed below) (FIG. 2), depending on the complex context of the surrounding TME; (2) the existence of a wide spectrum of CAF subpopulations with distinct functions (FIG. 3); and (3) a diversity of CAF subpopulations that is likely to exceed the 3–4 major subpopulations defined in previous studies (TABLE 1; FIG. 3). Pseudotime mapping of scRNA-seq data suggests that CAFs from breast tumours are phenotypically plastic and can transition across different transcriptional states[57]. Similarly, Ohlund et al.[59] showed that pancreatic CAFs can transition between the myCAF and iCAF states in vitro. Notwithstanding, comprehensive in vivo lineage-tracing studies are needed to definitively prove the existence of transitions between CAF subsets during tumour progression. In addition, the transcriptome signatures and classifications of various CAF subpopulations based on scRNA-seq data require functional validation (BOX 2).

Despite the advances made with single-cell analysis techniques, measures should be taken in future studies to ensure correct interpretation of the functional roles of CAFs based on scRNA-seq datasets. Moreover, gene signatures of a given CAF subtype can vary across different cancer types, different stages of a given cancer type or between patients, thus underscoring the need to identify both universal and specific biomarkers for CAFs (or CAF subtypes) using samples from large cohorts of patients and multiple model systems with a unified and/or standardized classification system.

CAFs with tumour-promoting functions

Mouse model systems commonly utilized to investigate CAF functions include (1) transgenic models using thymidine kinase or diphtheria toxin receptor systems that ablate certain CAF lineages; (2) Cre-*loxP*-based transgenic models that enable the genetic deletion

of target genes in particular CAF lineages; and (3) conventional xenograft models using co-injection of cancer cells with CAFs to investigate the contributions of CAFs to tumour formation and progression[33,67–70]. The majority of studies demonstrating tumour-promoting roles of CAFs utilized co-implantation models that require ex vivo culturing of fibroblasts, which can induce transcriptional alterations and changes in CAF biomarker expression and, thus, loss of their in vivo identity[21,24]. Therefore, whether many of the proposed mechanisms by which CAFs promote tumour progression are actually conserved in autochthonous tumours and pathological contexts remains to be determined.

CAFs are the major producers of ECM components and various other secreted factors[15,59,71–75]. As CAFs dynamically evolve along with cancers, their secretome can modulate cancer progression and tumour immunity, both directly and indirectly, in a context-dependent manner (Fig. 2). Such regulatory functions can be exerted via growth factors, cytokines and chemokines, including CC-chemokine 2 (CCL2), CCL5, colony-stimulating factor 1 (CSF1), CXC-chemokine 5 (CXCL5), CXCL9, CXCL10, CXCL12 (also known as stromal cell-derived factor 1 (SDF1)), HGF, insulin-like growth factor 1 (IGF1), connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), IL-1, IL-4, IL-6, IL-6, IL-8, IL-10, leukaemia inhibitory factor (LIF), prostaglandin E_2 (PGE_2) and TGFβ[11,13,33,68,76–79]

Preclinical studies have revealed an immunosuppressive role of CAF-derived TGFβ, which can strongly influence the functions of various immune cell types, including T cells, macrophages and neutrophils[80–83]. In addition, CAFs have been reported to promote tumour progression via the secretion of CXCL12 (REF.[33]) and VEGFA[84], which promote angiogenesis. Other factors secreted by CAFs, such as LIF, IGF1, HGF, IL-6, WNT5α and bone morphogenic protein 4 (BMP4), have been shown to induce reciprocal signalling interactions from CAFs to cancer cells that promote tumour growth and progression[40,76,85,86].

CAFs are also important producers of ECM-degrading proteases, such as matrix metalloproteinases (MMPs), in addition to ECM-crosslinking enzymes and ECM components, which indicate their crucial roles in ECM remodelling in the TME[87,88]. The cytokines and chemokines, along with the remodelled ECM, produced by CAFs actively regulate immune responses and angiogenesis in tumours, thereby contributing to an immunosuppressive TME that is permissive for tumour progression[20,25,65,89,90] and therapy resistance mediated by various mechanisms, including via the regulation of interstitial fluid pressure, cell adhesion and cancer cell survival[67,91–95]. Genetic deletion of tissue inhibitor of metalloproteinase (TIMP) expression in fibroblasts is associated with increased ECM deposition and tumour growth as well as secretion of promigratory exosomes containing disintegrin and metalloproteinase domain-containing protein 10 (ADAM10)[96]. In mouse models of cholangiocarcinoma, depletion of αSMA^+ or *Lrat* lineage-traced CAFs is associated with a decreased tumour burden[54]. Genetic deletion of hyaluronan synthase 2 (*Has2*) or HGF (*Hgf*) in the putative majority of cholangiocarcinoma CAFs also reduced tumour growth[54]; however, whether survival is also affected remains to be determined. Expression of the deleted genes is largely restricted to specific CAF subpopulations, with myCAFs and iCAFs predominantly expressing *Has2* and *Hgf*, respectively. Nevertheless,

other CAF subtypes also have some expression of these genes and the functional roles of the various CAF subsets remain to be further elucidated.

Data from preclinical models and patient samples also suggest connections between certain CAF subsets, tumour immune responses and immunotherapeutic efficacy[18,20,24,97]. Specifically, FAP-expressing CAFs can have an immunosuppressive function, which might present an opportunity for improving immunotherapies via depletion of these CAFs or targeting CAF-derived factors such as CXCL12 or CCL2 (REFS[98–100]). In addition, CAFs can actively influence other cell types, including cancer cells and immune cells, through the production of various metabolites, including alanine, aspartate, lactate, deoxycytidine and lipids (such as lysophosphatidic acid)[101–107]. In particular, CAFs can provide various types of metabolic support to cancer cells through the release of alanine[101,102], deoxycytidine[103], proline[108] and lipid species[106], especially in the context of a nutrient-deprived TME. Exosomes derived from CAFs have also been shown to contain various surface proteins (such as WNT) and metabolites (such as TCA cycle intermediates) that can influence other cell populations in the TME, thus affecting cancer progression, stromal remodelling and drug resistance[109–113].

CAFs with tumour-restraining functions

Evidence from numerous studies underscores the fact that CAFs (or certain subtypes of CAFs) have context-dependent functions, imparting both tumour-promoting and tumour-restraining influences[11,12,47,50,58,59,75,114–120]. The mechanisms underlying the tumour-restraining functions of CAFs are probably dependent on promotion of anticancer immunity, a pro-inflammatory secretome, tumour-inhibitory signalling and the production of certain ECM components as barriers to tumour cell invasion and dissemination (FIG. 2).

In mouse models of pancreatic cancer, cell lineage-based depletion of αSMA$^+$ CAFs accelerates tumour progression, reduces fibrotic reactions and decreases survival[58]. In line with these findings, higher tumoural αSMA levels and stromal densities are associated with favourable overall survival outcomes in patients with pancreatic cancer[58,121]. Other studies have revealed that targeting the Sonic hedgehog (SHH)-Smoothened (SMO) signalling axis increases cancer cell proliferation and tumour formation owing to inhibition of SHH–SMO-mediated activation of the tumour-restraining phenotypes of myofibroblasts[117,120]. Tumour-restraining functions of SHH signalling and the fibrotic tumour stroma have also been identified in the context of bladder cancer[122]. Together, these findings indicate that αSMA$^+$ CAFs, namely myofibroblasts, possess tumour-restraining functions that are partially mediated through the SHH–SMO signalling pathway. However, definitive connections between transcriptional signatures of specific CAF subpopulations and these tumour-restraining functions remain to be demonstrated.

In preclinical models of pancreatic cancer, JAK inhibitors suppress IL-1-induced LIF signalling in inflammatory iCAFs, thereby skewing these cells towards an ECM-producing myofibroblast (myCAF) phenotype, increasing the myCAF to iCAF ratio and facilitating ECM deposition, which in turn results in a reduction in cancer cell proliferation and tumour growth[75]. We have shown that αSMA$^+$ myofibroblasts can exert tumour-restraining

functions through the deposition of type I collagen, the most abundant protein in the TME of pancreatic cancers. Specifically, mouse models with genetic deletion of collagen I (*Col1a1*) in αSMA$^+$ myofibroblasts using a system have markedly decreased collagen deposition, leading to accelerated pancreatic tumour development and progression[56]. This effect is associated with a more immunosuppressive stroma characterized by upregulation of CXCL5 in cancer cells, which results in increased recruitment of myeloid-derived suppressor cells and suppression of CD8$^+$ T cells[56]. Other studies have shown that deletion of *Col1a1* in CAFs promotes liver colonization of pancreatic and colorectal cancers[123] but does not affect cholangiocarcinoma growth[54]. These results, in combination with those of previous studies[56,58,75,117,120,122–124], underscore the specific tumour-restraining functions of myofibroblasts and the associated tumour stroma. By contrast, tumour-promoting functions of collagen in regulating cancer cell survival, proliferation, invasion and metabolism have also been reported[125–128]. Future studies are therefore required to further dissect the mechanisms underlying the context-dependent effects of stromal components such as collagen I.

The discovery of the tumour-restraining functions of CAFs provides a potential explanation for the unsuccessful clinical trials of therapeutic agents targeting CAFs or stromal components[129–134]. These observations suggest that future therapeutic strategies should avoid generic targeting of tumour-restraining CAF subpopulations in favour of precise reprogramming and normalization of tumour-promoting CAF subsets (preferably to non-malignant-tissue fibroblasts or tumour-restraining CAF subpopulations).

Value of CAFs as prognostic biomarkers

Many different biomarkers have been used to identify CAFs and their subtypes; however, none of these biomarkers are specific for CAFs (Supplementary Table 1). Owing to the expression of CAF biomarkers in other cell types (such as immune cells, endothelial cells, pericytes, smooth muscle cells and/or cancer cells), confusing conclusions might be drawn regarding the prognostic value of a given CAF biomarker in various cancer types. Indeed, many of the CAF biomarkers do not have any clear prognostic value when examined as single biomarkers in most cancer types included in analyses of the Human Protein Atlas based on The Cancer Genome Atlas datasets[135] (Supplementary Table 1). Nevertheless, numerous studies have investigated the prognostic value of commonly used CAF biomarkers in various cancer types (Supplementary Table 1). In addition, given the existence of distinct CAF subpopulations with both pro-tumour and antitumour functions, it is not surprising that contradictory phenotypes have been observed in different model systems using therapeutic or genetic strategies targeting a certain subset of CAFs harbouring a given biomarker set. Neither is it surprising to obtain contradictory results when attempting to generalize the prognostic value of the overall CAF population without differentiating the effects of heterogeneous CAF subpopulations.

Novel techniques, such as scRNA-seq and mass spectrometry-based cytometry by time of flight (CyTOF) as well as multiplex flow cytometry and multiplex immunostaining methods, have greatly facilitated the identification and validation of a variety of biomarkers for distinct CAF subpopulations at the transcriptional or protein level. Future studies

elucidating the functional roles of distinct biomarker-defined CAF subpopulations using clinical samples and clinically relevant model systems are necessary to further evaluate the prognostic values of CAFs and various CAF biomarkers. Current data indicate that certain subpopulations of CAFs have similar features and gene-expression signatures across different cancer types, while many other subpopulations are likely to vary substantially owing to the heterogeneous cellular origins of CAFs between cancer types (TABLE 1). Thus, additional investigations are needed to identify whether the prognostic value of specific biomarker-defined CAF subpopulations is either universal or cancer-type specific (BOX 1). Multiple biomarkers will probably need to be evaluated simultaneously in order to better inform on CAF function and, therefore, their prognostic effect. In addition, a better understanding of biomarkers for non-cancer-associated or quiescent fibroblasts will aid in identifying cancer-specific biomarkers.

Another intriguing issue relates to potential genetic alterations present within CAFs. Studies performed over the past two decades have revealed that CAFs can harbour genetic mutations[136–138], in contrast with the previous notion that CAFs are genetically stable. In 2020, a single-cell multiomics sequencing study demonstrated frequent somatic copy number alterations in CAFs from patients with colorectal cancer[55,139]. By contrast, data reported in 2021 demonstrate that CAFs from patients with breast cancer are copy number stable[140], highlighting potential cancer-specific dependencies. Other recent studies using single-cell whole-genome sequencing or scRNA-seq integrated with somatic mutation detection indicate the potential for detection of somatic mutations in CAFs (and other cell types of the TME) at the single-cell level[141–144]. Further advances in multiomics sequencing technology will provide additional insights into the genetic alterations of CAFs and their implications.

CAFs as potential therapeutic targets

Studies of the tumour-promoting functions of CAFs have identified various mechanisms, thus presenting multiple potential therapeutic targets (FIG. 4). These findings have led to numerous clinical trials testing strategies targeting CAFs and/or related signalling pathways (Supplementary Table 2). However, several therapeutic strategies, such as approaches targeting SHH–SMO signalling or hyaluronic acid, did not have sufficient therapeutic efficacy or, in some contexts, even shortened patient survival in clinical trials[129,130,133,134]. These unexpected results underscore the importance of considering both the tumour-promoting and tumour-restraining subtypes of CAFs.

Indeed, future therapeutic approaches targeting CAFs should differentiate the distinct subtypes and functions of CAFs, presumably based on a better-defined set of biomarkers (box 3). However, designing a therapeutic approach to specifically target tumour-promoting subtypes of CAFs is difficult owing to (1) the lack of definitive biomarkers and signalling pathways; (2) the dynamic interchangeability between CAF subtypes; and (3) the possibility that CAFs might have both tumour-promoting and tumour-restraining functions (FIG. 2). In other words, the spectrum of CAFs is typically nonbinary, with a lack of distinct cell polarization, and is dynamically regulated by a complex set of microenvironmental cues (FIG. 3).

Fine-tuning of specific CAF subpopulations might be important for therapeutic interventions targeting CAFs. For example, normalization and reprogramming of CAFs using vitamin D, calcipotriol or all-trans retinoic acid (ATRA) has been shown to restrain the tumour-promoting functions of CAFs and enhance the efficacy of pancreatic cancer therapy in preclinical models[145,146]. Combination therapy with paricalcitol, nivolumab and/or chemotherapy is currently being evaluated in a phase II clinical trial involving patients with pancreatic cancer (NCT02754726). As described previously, CAF reprogramming with JAK inhibitors can skew the iCAF subtype towards a myCAF subtype, which increases ECM deposition and restrains tumour growth[75]. A more comprehensive understanding of the tissue-specific gene-expression profiles and functions of physiological fibroblast populations as well as the precise mechanisms by which these characteristics are altered in cancer is urgently needed in order to design novel strategies for effective reprogramming of CAFs into a ‘normal’ or quiescent state.

The regulatory effects of CAFs and particular CAF subpopulations on tumour immunology and immunotherapy response have also been intensively investigated over the past few years. Several studies indicate connections between certain CAF subpopulations and immunosuppression, suggesting the potential for therapeutic interventions targeting these immunosuppressive CAF subpopulations in combination with immunotherapies[18,20,24,60,99]. For example, the CXCR4 antagonist motixafortide (BL-8040), which can inhibit the immunosuppressive CXCL12–CXCR4 axis driven by FAP-expressing CAFs, is currently being investigated in combination with pembrolizumab and/or chemotherapy in a phase II clinical trial involving patients with pancreatic cancer (NCT02826486). CAFs can directly or indirectly regulate, through complex interactions, the functions of other cell types within the TME and potentially even the microbiota, although more experimental evidence is needed regarding the latter effect (FIG. 4). Some of these interactions could present potential therapeutic targets following additional adequate and appropriate preclinical assessments (BOX 3).

Perspectives and future considerations

As we expand our understanding of CAF heterogeneity using the latest techniques and models, the clinical relevance of CAFs, including the prognostic indications and therapeutic strategies, needs to be reevaluated and redefined based on the distinct roles of specific CAF subpopulations.

Use of single-cell analysis techniques.

We anticipate that modern technologies, such as scRNA-seq and other single-cell analysis techniques (such as single-cell whole-genome sequencing, single-cell genome and transcriptome sequencing, and single-cell methylome and transcriptome sequencing), will be utilized widely and routinely as new standards for the detailed taxonomy of distinct CAF subpopulations at the single-cell level. Numerous marker genes of CAFs, as previously identified using conventional methods, such as flow cytometry, immunohistochemistry and immunostaining, have been confirmed using contemporary single-cell analysis techniques, including αSMA, FAP, LOX and PDGFRα (Supplementary Table 1). Despite the

unprecedented potential of scRNA-seq to decipher cellular heterogeneity, this technique has several limitations: (1) genes with low levels of expression are difficult to measure accurately owing to the limited sequencing depth; (2) transcript levels of given genes might not always reflect the actual protein levels owing to additional translational and posttranslational regulatory mechanisms; and (3) the cell-clustering algorithms, including the most commonly used non-linear dimensionality projections, namely t-distributed stochastic neighbour embedding (t-SNE) and uniform manifold approximation and projection (UMAP), will always yield subclusters according to artificially selected clustering parameters, even when no functionally or biologically meaningful subclusters exist[147]. Therefore, additional validation using complementary methodologies, including CyTOF, multiplex flow cytometry and/or multiplex immunostaining, is necessary for further investigations of the proposed CAF subpopulations. Functional validation using various in vitro and in vivo model systems is also required to establish whether the proposed CAF subpopulations are biologically relevant.

Unified CAF classification and taxonomy.

The current classification and taxonomy of CAF subpopulations lacks a unifying standardized system to reconcile the different (or even contradictory) observations made by various research groups, often in the same cancer type and using similar scRNA-seq or CyTOF techniques. Indeed, the subclustering and definition of CAF subpopulations is somewhat subjective and can therefore vary between observers. In this regard, a consensus on the major biomarkers and hierarchical clustering maps of CAF subpopulations based on scRNA-seq or CyTOF analysis needs to be developed in the coming years.

Subsequently, a standardized experimental system with specific, dedicated and reliable reagents and protocols needs to be developed for the identification, staining and/or labelling, sorting, cell culture, and functional assessment of CAF subpopulations both in vitro and in vivo, similar to those developed for research on immune cell populations[148]. This standardization will then enable precise definition and characterization of CAF subpopulations with specified functional roles and prognostic values, thereby facilitating the development of specific therapeutic interventions targeting certain CAF subpopulations and/or pathways in the future.

Functional validation of CAF subsets.

Current techniques, such as scRNA-seq and CyTOF, are focused on the identification of heterogeneous CAF subpopulations based on the expression profile of signature genes and proteins, respectively. These observations need to be complemented by functional studies in order to connect the gene and/or protein signatures of distinct CAF subpopulations with their precise functional roles in cancers. Further investigations of the functional roles of distinct CAF subpopulations require the utilization of sophisticated model systems, including new transgenic mouse models, organoids and patient-derived xenografts[13,52]. We expect such functional validation to be one of the key focus areas in the field of CAF research. The identification of novel biomarkers for fibroblasts and CAFs based on the new scRNA-seq datasets would facilitate subsequent investigations using specific Cre-recombinase drivers and lineage-tracing techniques to further elucidate the origins and

functions of CAFs in the context of GEMMs. For example, using novel dual-recombinase GEMMs[149,150], we were able to lineage trace CAFs and cancer cells, respectively, or genetically delete type I collagen specifically in αSMA$^+$ myofibroblasts[56], both in the context of autochthonous pancreatic cancer. In addition, a suite of Dre recombinase-based models has been developed for marking a range of cell types, including CAFs[151], which provides additional methods to track and target CAFs. In this regard, we have generated *Pdpn-tk* and *Pdpn-Cre* mouse models (enabling cell depletion and genetic manipulation of *Pdpn*-lineage cells) to investigate tumour lymphangiogenesis[152] and these GEMMs might also be useful in evaluating CAFs given that multiple studies have implicated PDPN as a CAF biomarker[15,21,23,153].

Owing to the highly dynamic heterogeneity of CAFs and the lack of specific biomarkers, establishing clinically relevant experimental models that enable real-time tracking of CAFs remains challenging. Future functional studies will undoubtedly benefit from novel cell lineage-tracing and genetic manipulation (Cre-*loxP*) model systems, although these models are still limited by the paucity of CAF-specific biomarker genes that can serve as driver promoters for Cre-recombinase and fluorescence reporter genes. Specifically, commonly used types of lineage-tracing models include (1) a conventional Cre driver line (such as αSMA-Cre[154]) integrated with a fluorescence reporter allele; (2) an inducible Cre driver line (such as αSMA-CreERT2 (REF.[155]), Gli1-CreERT2 (REF.[156]), Grem1-CreERT[157], Hoxb6-CreERT[157] or Islr-CreERT2 (REFS[157,158])) with a fluorescence reporter allele; (3) a fluorescence reporter gene driven by a promoter derived from a putative CAF biomarker gene, which enables real-time transcriptional tracking of the putative biomarker (such as αSMA-RFP[40,58,159,160], Col1a1-DsRed/YFP/eGFP[40,41], Gli1-eGFP[156] or FSP1-GFP[84,160]); and (4) cell lineage-depleting models using a thymidine kinase[54,58,123,160] or diphtheria toxin receptor[54,99,123,161] system (such as αSMA-tk[58] or FAP-DTR[98,99]). We expect that new biomarkers of CAFs identified using scRNA-seq and CyTOF techniques will facilitate the generation of new lineage-tracing models in the future. Intravital imaging has also been used to dynamically image CAFs in tumours[94,162]. Implementation of the aforementioned labelling systems combined with further development of high-throughput and non-invasive dynamic imaging techniques will elucidate how CAFs evolve during tumour progression.

Furthermore, connecting CAF heterogeneity with the clinical relevance and functional roles of the various CAF subpopulations in human cancers is also challenging, even with the utilization of transgenic mouse models, organoids and patient-derived xenografts. Currently, the speculated functional roles of the CAF subpopulations in human cancers are largely based on the expression profiles of gene clusters associated with certain cellular pathways, processes and/or activities. Discrepancies between observations made in experimental model systems and human cancers remain an obstacle to the clinical development of precise therapeutic strategies targeting certain CAF subtypes, although future research promises to be exciting and will probably help overcome such hurdles.

Conclusions

CAFs have long been investigated as an attractive therapeutic target; however, many therapeutic strategies targeting CAFs or associated stromal components have failed to

improve clinical outcomes, underscoring the importance of dissecting the heterogeneous subpopulations and diverse functions of CAFs in a context-dependent manner. Modern techniques, such as scRNA-seq and CyTOF, have provided new opportunities to decipher the heterogeneity of CAFs on the basis of signature gene and protein expression profiles. Nevertheless, measures should be taken in future studies to systemically define the functional roles of CAFs and CAF subpopulations in relation to the transcriptional signatures of CAFs. Comprehensive characterization and functional validation of CAF subtypes in both preclinical and clinical studies might inform the development of novel diagnostic and therapeutic approaches predicated on CAFs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- Cancer-associated fibroblasts (CAFs) are found in both primary and metastatic tumours; studies using modern cell sorting and sequencing technologies have provided exciting new insights into the potential therapeutic and prognostic value of CAFs.
- In particular, studies using single-cell RNA sequencing and genetically engineered mouse models have begun to reveal the heterogeneity and functional roles of CAFs, which are dynamic and context dependent.
- The precise functional roles of various CAF subtypes remain largely undefined, which requires future investigations integrating functional studies using multiple model systems with transcriptomic and/or proteomic data at single-cell resolution.
- The identification and precise characterization of the tumour-promoting and tumour-restraining functions of different CAF populations might provide opportunities to develop novel diagnostic and therapeutic approaches.

Box 1 |

Does a pan-CAF biomarker exist?

The question of whether a single biomarker can be used to identify all cancer-associated fibroblasts (CAFs) really starts with the question of whether a specific biomarker can exclusively define fibroblasts. The simple answer is no and this also extends to CAFs. For a long time, α-smooth muscle actin (αSMA) was considered an ideal biomarker to identify 'activated' fibroblasts[32,163]. This paradigm has now been demonstrated to be oversimplified[14–16,18,19,21–27,53,54] and many other biomarkers have been proposed (TABLE 1; Supplementary Table 1), clearly illustrating the heterogeneity of CAFs. Despite numerous different unbiased analyses of CAF populations, including proteomics, single-cell RNA sequencing, flow cytometry and multiplex staining techniques, a single biomarker that can identify all CAFs in a given tumour has not been identified. What we are left with are some biomarkers that might identify a percentage of CAFs and combinations of two or more biomarkers that could potentially identify all CAFs. Therefore, the search for an absolute biomarker of CAFs continues.

Box 2 |

A cautious approach to phenotypic classification of CAFs

The possibility that cancer-associated fibroblasts (CAFs) constitute a heterogeneous population of cells was first described in 2006 (REF.[50]). More recently, single-cell RNA sequencing has been used to identify multiple distinct subsets of CAFs in different tumour types. Using basic bioinformatic tools, such CAF clusters were classified using a presumptive functional nomenclature, for example, as myofibroblastic, inflammatory, matrix, cycling or developmental CAFs. The concern with this approach is that transcriptional patterns might not actually define cell phenotypes and their purported functions. For example, naming a CAF cluster ‘inflammatory’ implies that all CAFs in this subset have inflammatory properties. Moreover, thorough analyses suggest that CAFs in other clusters also have gene-expression profiles reflecting a putative inflammatory phenotype. Certain cytokines that have been used to define inflammatory CAFs (iCAFs), such as IL-6, can be expressed not only by iCAFs but also by myofibroblastic CAFs. In addition, extracellular matrix components can be expressed robustly not only by matrix-producing myofibroblastic CAFs but also by iCAFs. Thus, the nomenclatures of CAF subpopulations based on their transcriptomic profiles without functional validation might lead to invalid presumptions in this field of research. Such over-interpretation is analogous to endowing a functional nomenclature to an immune cell cluster without confirming their definitive functional biomarkers or precise functions. Moreover, this type of analysis assumes that function is solely dictated by transcript abundance and does not account for post-transcriptional regulation or spatial proximity of cell types for signalling. Unless careful functional analyses are performed, ascribing phenotypic names to CAF subsets can be misleading.

Box 3 |

Is targeting of CAFs a feasible approach to cancer therapy?

Many different strategies to target cancer-associated fibroblasts (CAFs) or associated factors that mediate cancer progression have failed clinically. This lack of success primarily reflects a lack of stringent preclinical studies and an inadequate understanding of the functions of CAFs in cancer. Clear evidence indicates that CAFs can possess both tumour-restraining and tumour-promoting functions. Therefore, it is important to determine if targeting a putative CAF functional mediator affects different pathways and results in cancer acceleration in an unexpected manner owing to inadequate preclinical assessment of the drug target. Additionally, standard approaches for cancer drug screening are unlikely to be applicable to CAFs given that CAFs demonstrate a greater propensity to alter their phenotypes in cell culture as compared with cancer cells. Nevertheless, with careful analysis of the putative CAF-related drug target in appropriate and relevant in vivo models of cancer (preferably autochthonous genetic models of cancer with the natural evolution of CAFs) and detailed validation of the target using genetic gain-of-function and loss-of-function tools, the development of a CAF-targeted therapy with potential benefits in controlling cancer is possible. The following mechanistic and strategic guidelines could be considered before targeting CAFs in the clinic:

1. Identify the relevant CAFs with a specific biomarker.
2. Establish their precise function in cancer progression and metastasis using genetic mouse models, and establish whether a given CAF subset is in totality tumour restraining or tumour promoting.
3. Perform in-depth analyses of the transcriptome and proteome of the relevant CAF subset to identify the potential mediators that positively influence cancer progression and metastasis.
4. Perform genetic experiments to determine the precise function of the identified CAF mediators in cancer progression and metastasis.
5. Conduct preclinical efficacy studies in models relevant to the human disease in order to establish the CAF mediator as a specific experimental drug candidate.
6. Perform drug validation experiments using genetic mouse models to confirm target engagement in CAFs.
7. Establish a clinical development programme in conjunction with a CAF biomarker assessment.

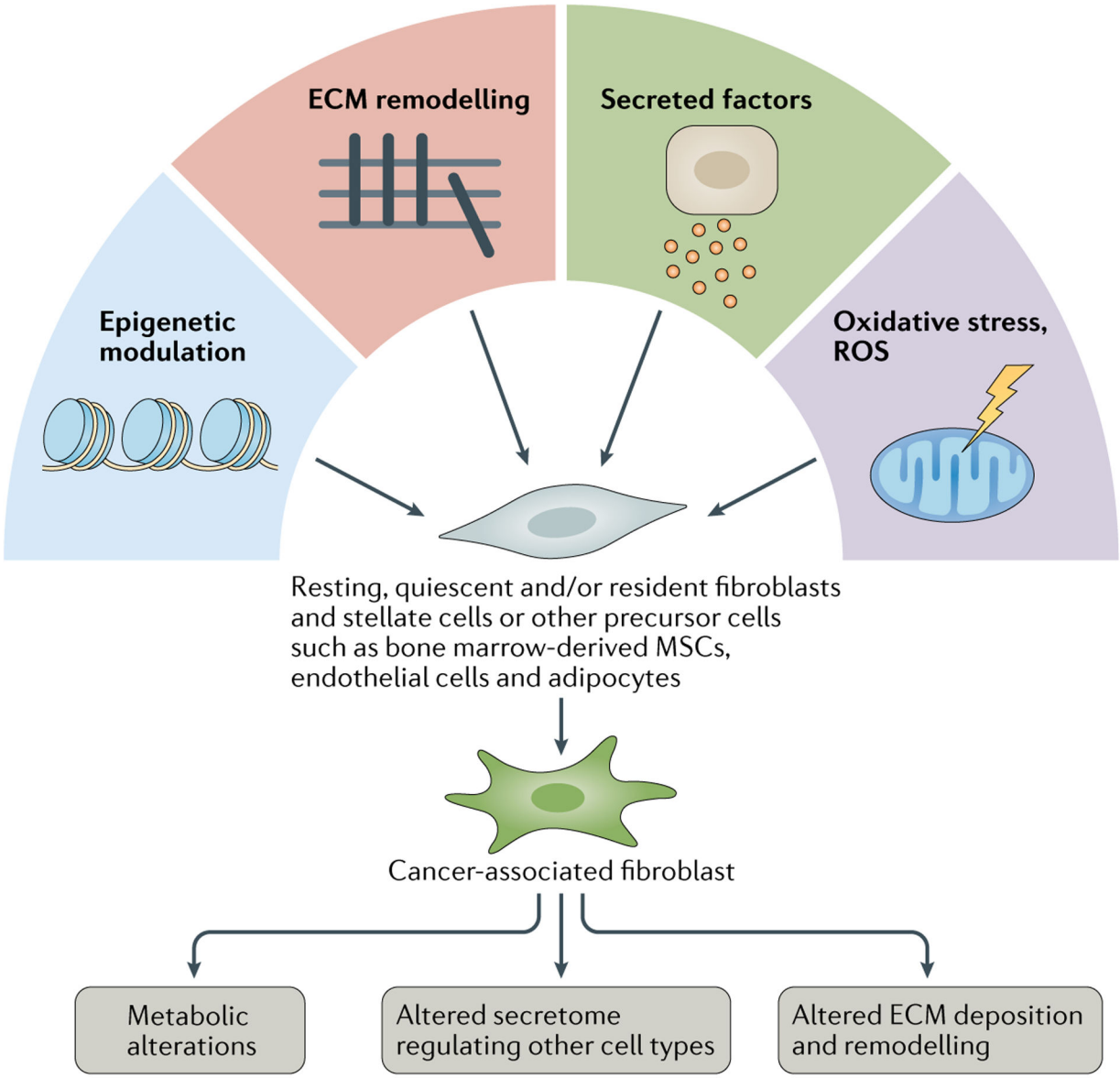

Fig. 1 |. Activation of CAFs.

Schematic illustration of various mechanisms involved in cancer-associated fibroblast (CAF) activation. Potential cellular origins of CAFs include quiescent, resting or specific tissue-resident fibroblasts (stellate cells), bone marrow-derived mesenchymal stem cells (MSCs), endothelial cells and other cell types. ECM, extracellular matrix; ROS, reactive oxygen species.

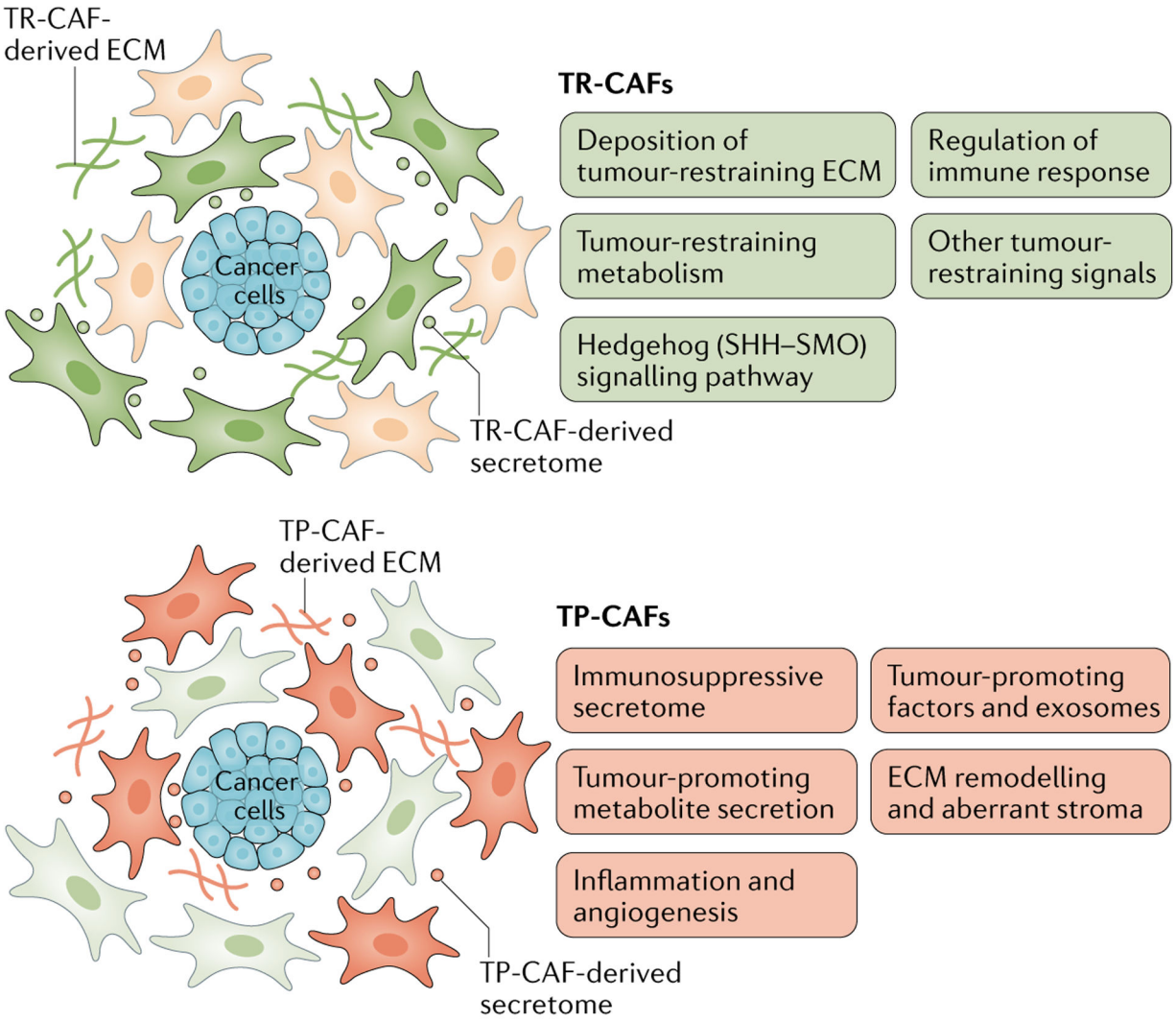

Fig. 2 |. The heterogeneity and plasticity of CAFs with both tumour-restraining and tumour-promoting functions.

Schematic illustration of heterogeneous cancer-associated fibroblast (CAF) subpopulations with potential tumour-restraining (TR) functions and tumour-promoting (TP) functions through various indicated mechanisms. However, the spectrum of CAF heterogeneity is likely to be non-binary, without distinct polarization, and therefore should not be oversimplified as either tumour restraining or tumour promoting, and might exhibit context-dependent plasticity that needs to be further elucidated. ECM, extracellular matrix; SHH–SMO, sonic hedgehog–smoothened.

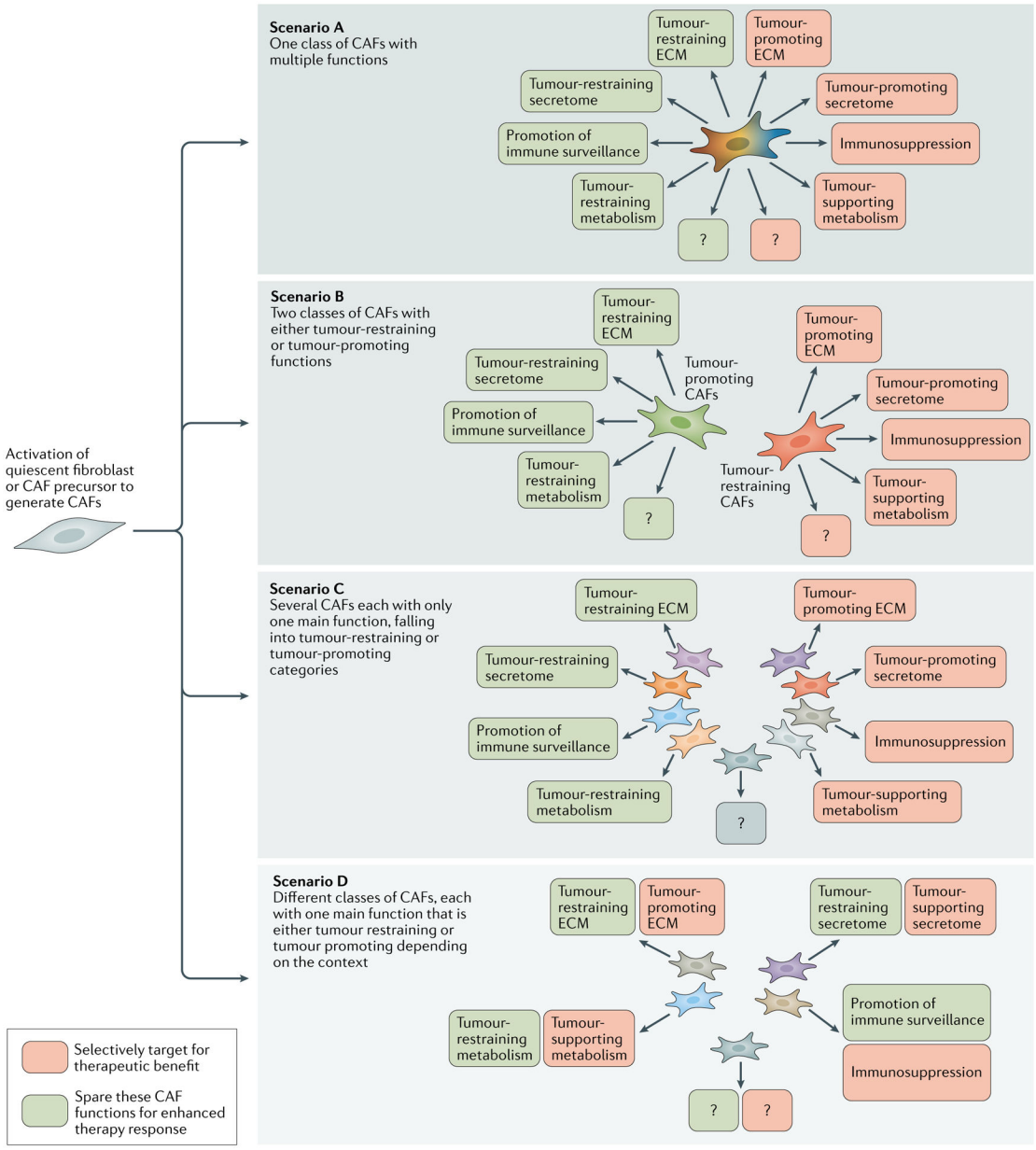

Fig. 3 |. Proposed models explaining the diverse functions and phenotypes of CAFs in cancer. Activation of cancer-associated fibroblasts (CAFs) might lead to heterogeneous CAF compositions according to various possible scenarios (A–D). These distinct models of CAF subset differentiation can be interchangeable and dynamically regulated by the tumour microenvironment. ECM, extracellular matrix.

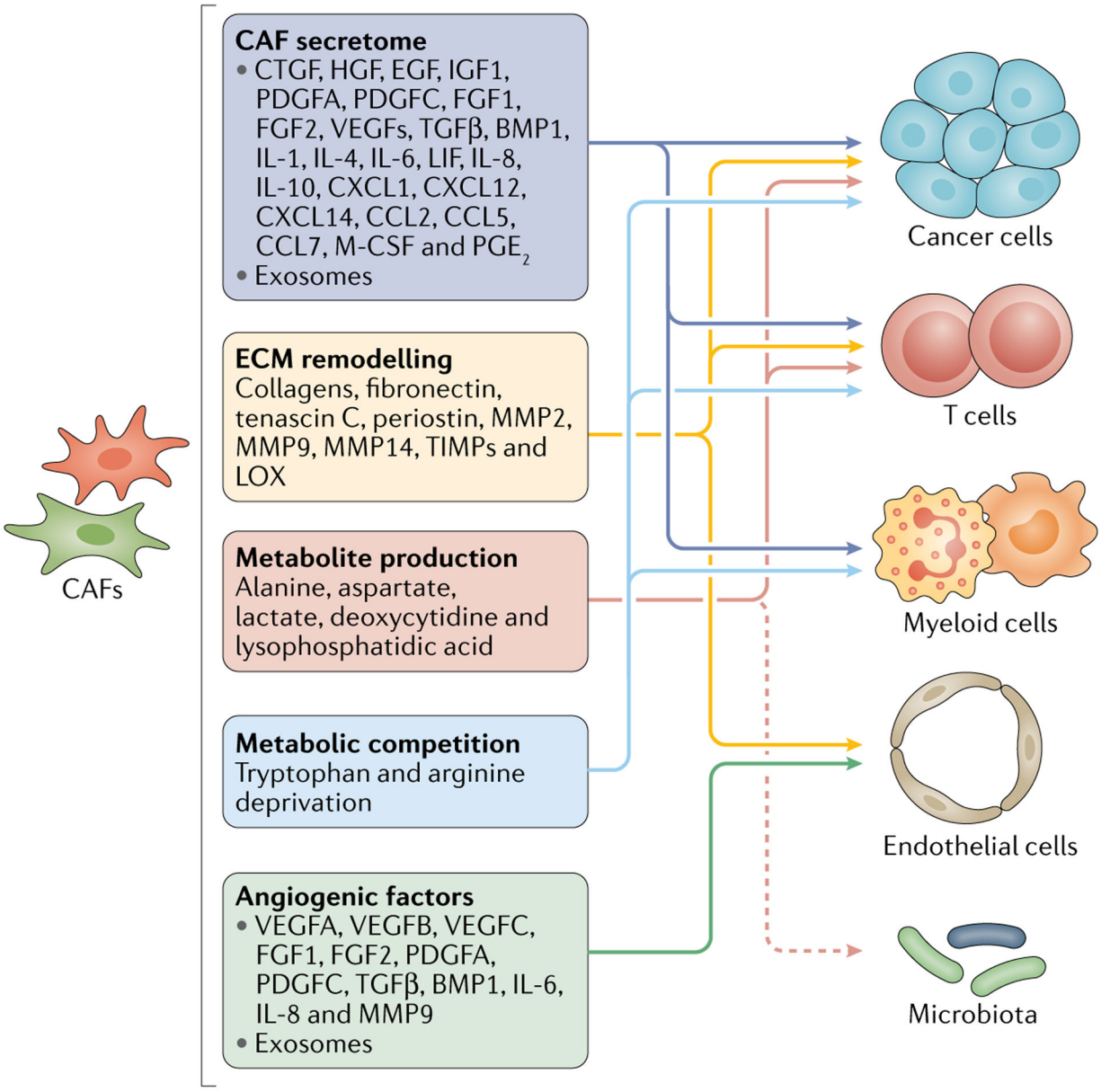

Fig. 4 |. Interactions between CAFs and other cell types in the tumour microenvironment.
Schematic illustration of the potential regulatory effects of cancer-associated fibroblasts (CAFs) on other cell populations (including cancer cells, lymphocytes, myeloid cells and endothelial cells) and the microbiota within the tumour microenvironment. BMP, bone morphogenetic protein; CCL, CC motif chemokine; CTGF, connective tissue growth factor; CXCL, CXC motif chemokine; ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; LOX, lysyl oxidase; M-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PGE_2, prostaglandin E_2; TGFβ, transforming growth factor-β ; TIMPs, tissue inhibitors of metalloproteinases; VEGF, vascular endothelial growth factor.

Table 1 |

CAF subpopulations identified in indicated cancer types

Tumour type	Sample type	CAF subpopulation	Biomarkers or characteristics of CAF subpopulation
Pancreatic cancer[14–18,56,59,75]	Patient samples; KPC, KIC and KPfloxC mouse tumours	Myofibroblast (myCAF)	αSMA, THY1, TAGLN, CTGF, IGFBP3, COL12A1, THBS2 and LRRC15
		Inflammatory CAF (iCAF)	CLEC3B, COL14A1 and LY6C
		Antigen-presenting CAF	CD74, SLPI, SAA3, MHCII and FSP1
Colorectal cancer[19,20]	Patient samples	CAF-A (FAP-CAF)	FAP, MMP2 and DCN
		CAF-B (αSMA-CAF)	αSMA, TAGLN and PDGFA
Head and neck cancer[21]	Patient samples	Myofibroblast	αSMA, MYLK and MYL9
		CAF1	FAP, PDPN, COL1A2, THY1, VIM, CAV1 and MMP11
		CAF2	FAP, PDPN, FOS, JUN, FGF7 and TGFB2
Lung cancer[26]	Patient samples	Cluster-1	Expression of ECM-related genes
		Cluster-2 (myofibroblast)	αSMA
		Cluster-4	Enriched at the leading edge of tumours
		Cluster-5	High mTOR gene signature; enriched in the tumour core
		Cluster-7	High mTOR signature; enriched at the leading edge of tumours
Melanoma[27]	B16-F10 mouse tumours	S1	Immune CAFs; CD34
		S2	Desmoplastic CAFs; TNC
		S3	Contractile CAFs; αSMA
Breast cancer and ovarian cancer[24,25,65,66]	Patient samples	CAF-S1	FAP-high,αSMA-high; contains 8 subclusters of CAFs; enriched in TNBC
		CAF-S2	Low expression of most detected markers; enriched in luminal A tumours
		CAF-S3	αSMA-low, $FSP1^+$ and $PDGFR\beta^+$
		CAF-S4	CD29-high,αSMA-high and FAP-low; enriched in TNBC
Breast cancer[57]	Patient samples	iCAF	CXCL12
		myCAF	ACTA2, FAP, PDPN, COL1A1 and COL1A2
Breast cancer[22]	MMTV-PyMT mouse tumours	Vascular CAF	αSMA and PDGFRβ
		Matrix CAF	Fibulin 1 and PDGFRα
		Cycling CAF	PDGFRβ
		Developmental CAF (dCAF)	SCRG1andSOX9

Tumour type	Sample type	CAF subpopulation	Biomarkers or characteristics of CAF subpopulation
Breast cancer[23]	4T1 mouse tumours	PDPN-CAF	Contains 6 subclusters: immune reg E (CXCL12), immune reg L (SAA3), ECM (fibrillin 1), wound healing (αSMA), inflammatory A (CXCL1) and inflammatory B (IL-6)
		S100A4-CAF	Includes 2 subclusters: protein folding (HSPD1) and antigen presenting (SPP1)
Bladder cancer[28]	Patient samples	myo-CAF	RGS5, MYL9 and MYH11
		iCAF	PDGFRα, CXCL12, IL-6, CXCL14, CXCL1 and CXCL2
Prostate cancer[53]	Patient samples	CAF-S1	αSMA and PDGFRβ
		CAF-S2	PDGFRα, CREB3L1 and PLAGL1
		CAF-S3	αSMA, HOXB2 and MAFB
Cholangiocarcinoma[54]	Patient samples; KRAS/p19 and YaP/AKT mouse tumours	myCAF	COL1A1, αSMA, COL8A1, COL15A1 and SERPINF1
		iCAF	CXCL12, HGF and RGS5
		mesCAF	Mesothelin

αSMA, α-smooth muscle actin; CAF, cancer-associated fibroblast; ECM, extracellular matrix; TNBC, triple-negative breast cancer.