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STATE-OF-THE-ART REVIEW

Phenotypic heterogeneity, stability and plasticity in tumor-promoting carcinoma-associated fibroblasts

Yoshihiro Mezawa and Akira Orimo

Department of Pathology and Oncology, Juntendo University School of Medicine, Tokyo, Japan

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Correspondence
A. Orimo, Department of Pathology and Oncology, Juntendo University School of Medicine, Tokyo 113-8412, Japan
Tel: 81 (0)3 5802 1039
E-mail: aorimo@juntendo.ac.jp

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Reciprocal interactions between cancer cells and stromal cells in the tumor microenvironment (TME) are essential for full-blown tumor development. Carcinoma-associated fibroblasts (CAFs) are a key component of the TME together with a wide variety of stromal cell types including vascular, inflammatory, and immune cells in the extracellular matrix. CAFs not only promote tumor growth, invasion, and metastasis, but also dampen the efficacy of various therapies including immune checkpoint inhibitors. CAFs are composed of distinct fibroblast populations presumably with diverse activated fibroblastic states and tumor-promoting phenotypes in a tumor, indicating intratumor heterogeneity in these fibroblasts. Given that CAFs have been implicated in both disease progression and therapeutic responses, elucidating the functional roles of each fibroblast population in CAFs and the molecular mechanisms mediating their phenotypic stability and plasticity in the TME would be crucial for understanding tumor biology. We herein discuss how distinct fibroblast populations comprising CAFs establish their cell identities, in terms of cells-of-origin, stimuli from the TME, and the phenotypes characteristic of activated states.

Introduction

A growing body of recent evidence indicates the tumor microenvironment (TME) to play key roles in determining disease progression and clinical outcomes [1–3]. Various stromal cell types, such as fibroblasts,

Abbreviations
ADT, androgen signaling deprivation therapy; apCAF, antigen-presenting CAF; ATRA, all-trans-retinoic acid; BET, bromodomain and extra-terminal; BMP, bone morphogenetic protein; CAFs, carcinoma-associated fibroblasts; CARs, chimeric antigen receptors; CAV1, caveolin-1; CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; ChIP, chromatin immunoprecipitation; CSF1, colony-stimulating factor 1; CyTOF, mass cytometry; DNMTs, DNA methyl transferases; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; EpCAM, epithelial cell adhesion molecule; FACS, fluorescence-activated cell sorting; FAP, fibroblast activation protein; FISH, fluorescent in situ hybridization; FSP-1, Fibroblast-specific protein 1; GEMM, genetically engineered murine model; H3K27me3, tri-methylation at histone H3 lysine 27; HDACis, histone deacetylase (HDAC) inhibitors; HIF-1α, hypoxia-inducible factor 1 alpha; HSF1, heat-shock factor 1; iCAFs, inflammatory CAFs; IDH3α, isocitrate dehydrogenase 3α; LRRC15, leucine-rich repeat-containing protein 15; MDSCs, myeloid-derived suppressor cells; MET, mesenchymal–epithelial transition; MHC, major histocompatibility complex; MMTV, mouse mammary tumor virus; MSCs, mesenchymal stem cells; mTORC1, target of rapamycin complex 1; myCAFs, myofibroblastic CAFs; NetG1, Netrin G1; NGL1, NetG1 ligand; NNMT, nicotinamide *N*-methyltransferase; NOX4, NADPH oxidase 4; PDGF, platelet-derived growth factor; PD-L1, programmed death-ligand 1; PDPN, podoplanin; pEMT, partial EMT; PGE2, prostaglandin E2; PHD2, prolyl hydroxylase domain protein 2; RAR, retinoic acid receptor; RASAL3, RAS protein activator-like 3; ROS, reactive oxygen species; SCCs, squamous cell carcinomas; SCNAs, somatic copy number alterations; scRNA-seq, single-cell RNA sequencing; SDF-1, stromal cell-derived factor 1; sFRP4, secreted frizzled-related protein 4; sst1, somatostatin receptors; TAMs, tumor-associated macrophages; TAZ, transcriptional co-activator with PDZ-binding motif; TEAD, transcriptional enhanced associate domain; TGF-β, transforming growth factor-β; TME, tumor microenvironment; YAP, yes-associated protein; ZEB1, zinc finger E-box binding homeobox 1; α-SMA, α-smooth muscle actin.

vascular endothelial cells, and inflammatory immune cells, constitute the TME [4]. These stromal cells associate with one another and coevolve with nearby carcinoma cells during the course of tumor progression.

Stromal fibroblasts are often abundant in human epithelial carcinomas, including those of the colon, breast, pancreas, and lung, as well as other tumor types such as melanoma and mesothelioma. These fibroblasts acquire different activated phenotypes, allowing their production of extracellular matrix (ECM) proteins, numerous growth factors, cytokines, metabolites, and lipid mediators [3,5–8]. α-Smooth muscle actin (α-SMA)-positive myofibroblasts, which are well-known activated fibroblasts present in wounds and fibrotic tissues, are frequently observed within desmoplastic stroma of most human tumors [9–11]. Activated fibroblasts barely expressing α-SMA, as exemplified by inflammatory cytokine production, are also present in the tumor-associated stroma of human carcinomas. Carcinoma-associated fibroblasts (CAFs) influence apposed epithelial cells not only to promote transformation of altered epithelial cells into tumorigenic cells, but also to accelerate the growth and progression of advanced tumors [3,5–7,12,13]. In addition, these fibroblasts exert crucial influences on tumor hallmarks, such as tumor cell invasion and metastasis, stemness, tumor heterogeneity and epithelial–mesenchymal transition (EMT), neoangiogenesis, ECM remodeling, metabolism, inflammatory responses, immune suppression, and therapeutic resistance [3,5,7,8].

Recent studies have demonstrated heterogeneous fibroblast populations present in various tumors [14–19]. These fibroblast populations exhibit potentially tumor-promoting abilities with diverse states of activation due to having originated from different progenitors, such as resident stromal cells and bone marrow-derived cells [20]. Exposure of the progenitors to distinct stimuli from the TME also promotes differentiation into various tumor-promoting CAF populations presumably maintaining their activated states with plasticity during tumor progression. However, it remains unclear how diverse activated fibroblast populations emerge and acquire their tumor-promoting abilities. In this review, we highlight the heterogeneity of CAFs and their diverse functions, focusing on stability and plasticity, in an effort to understand the precise influences of these fibroblasts on tumor progression.

What is the definition of CAFs?

Carcinoma-associated fibroblasts are activated fibroblasts present in large numbers in tumor-associated stroma. Different progenitors of CAFs are recruited into a tumor mass at a relatively early stage in cancer development. These cells then differentiate and polarize into activated, tumor-promoting fibroblasts presumably by adapting to various TME-derived stimuli including growth factors and cytokines, metabolites, exosome-derived factors, undernutrition, hypoxia, and acidification during tumor progression. However, a precise molecular definition of CAFs has yet to be established, mainly due to the lack of CAF-specific markers and the inter- and intratumor heterogeneity of these fibroblasts, as described in the following sections.

Carcinoma-associated fibroblasts showing a unique spindle morphology are usually detected in ECM immunohistochemically employing several tentative, but not exclusive, markers. These include α-SMA, fibroblast-specific protein 1 (FSP-1; also known as S100A4), fibroblast activation protein (FAP), podoplanin (PDPN), caveolin-1 (CAV1), platelet-derived growth factor (PDGF) receptors α and β, and the ECM components tenascin-C, periostin, and collagen type 1α2 [3,5–7], all of which are barely, if at all, detectable in quiescent fibroblasts. Vimentin is also used as a marker of mesenchymal cell lineages including both activated and quiescent fibroblasts. Epithelial cell adhesion molecule (EpCAM), CD45, and CD31, which are markers for epithelial, hematopoietic, and endothelial cells, respectively, are routinely employed to apply negative selection in order to eliminate these cells from mesenchymal cell populations. To date, combinations of positive and negative selection markers for CAFs have been used to characterize these fibroblasts.

Distinct fibroblast populations comprising CAFs in tumor-associated stroma

CAFs expressing the myofibroblastic and inflammatory traits

The ability of CAFs to modulate various aspects of tumor hallmarks has repeatedly been demonstrated by different research groups. Diverse soluble factors produced by different fibroblast populations in CAFs mediate their multi-functional propensities [3,5,7]. However, the functional roles of distinct fibroblast populations have yet to be fully elucidated.

Large numbers of α-SMA-positive myofibroblasts, the most well known of the activated fibroblasts comprising CAFs, are found predominantly in desmoplastic stroma rich in dense collagen fibers in various

tumors [10]. Myofibroblasts are also frequently detected in wound healing and fibrotic conditions, in accordance with the concept that 'tumors are wounds that do not heal' [21,22].

α-SMA-negative activated fibroblasts showing significant production of various inflammatory cytokines are also present in tumor-associated stroma of many cancer types. These fibroblasts are capable of impinging on recruitment of tumor-promoting inflammatory immune-suppressive cells, such as neutrophils and myeloid-derived suppressor cells (MDSCs), into tumors [23]. The α-SMA-negative CAFs resemble perivascular and sublining fibroblast-like synoviocytes, cells which are activated fibroblasts characteristic of rheumatoid arthritis. These fibroblasts produce numerous cytokines and proteases that exacerbate chronic inflammation and cartilage destruction [24].

Single-cell analyses assessing the CAF subtypes

Recent technically advanced single-cell analyses, including single-cell RNA sequencing (scRNA-seq), fluorescence-activated cell sorting (FACS), and mass cytometry (CyTOF), revealed various fibroblast populations to exist concomitantly in CAFs. The CAF subtypes often included two major fibroblast populations; one is a fibroblast population with the myofibroblastic trait, designated myofibroblastic CAFs (myCAFs) while the other exhibits the inflammatory trait and is thus termed inflammatory CAFs (iCAFs) in head and neck [19], pancreatic [15,16,25], and breast [26,27] cancers (Fig. 1).

Activation of transforming growth factor-β (TGF-β) canonical signaling does, in fact, induce the myCAF state, while two major inflammatory pathways including JAK-STAT and NF-κB signaling boost the iCAF state in fibroblasts. Since TGF-β signaling and IL-1-JAK-STAT signaling suppress each other in fibroblasts [28], these myCAF and iCAF states are in principle mutually exclusive. However, the two states are likely to be interconvertible in accordance with stimuli from the TME, resulting in generation of various degrees of transiently polarized activated states allowing an intermediate property with plasticity in CAFs (Fig. 1). If activated fibroblastic states vary significantly under conditions in which the TME changes dynamically, considerable numbers of fibroblasts with the intermediate phenotypes may result in a tumor. However, the mechanistic actions underlying the heterogeneity of CAFs and their phenotypic plasticity remain poorly understood.

The immunosuppressive role of CAFs in different human carcinomas is widely recognized [29], but the particular fibroblast populations in CAFs mediating immunosuppression have not as yet been identified. Costa *et al.* [17] described four distinct CAF subsets (S1-4) in single-cell suspensions, derived from 18 human breast cancer specimens, employing multicolor FACS analysis with antibodies for six CAF markers including integrin β1/CD29, FAP, α-SMA, FSP1, PDGFRβ, and CAV1. The CAF-S1 subset expressing $CD29^{med}FAP^{high}FSP1^{low\text{-}high}\alpha\text{-}SMA^{high}PDGFR\beta^{med\text{-}high}CAV1^{low}$ was markedly enriched in triple-negative breast cancers. Importantly, the CAF-S1 subset not only stimulated recruitment of effector T lymphocytes through production of stromal cell-derived factor 1 (SDF-1)/CXCL12, but also promoted their differentiation into $FOXP3^{+}$ regulatory T cells, resulting in promotion of immunosuppressive function (Fig. 1). The follow-up study showed the CAF-S1 subset to be abundant in a mesenchymal form of human high-grade serous ovarian cancer associated with poor outcomes. This CAF subset also functioned as an immunosuppressor by impinging on T-cell attraction via SDF-1, especially its SDF-1β isoform, in tumors [30]. This indicated myCAF-produced SDF-1 to act as an immunosuppressor in human breast and ovarian cancers.

In contrast, a few studies using scRNA-seq obtained contradictory results, indicating SDF-1 mRNA to be barely expressed in myCAFs, being instead highly expressed in iCAFs in human head and neck, breast, and pancreatic carcinomas [15,19,27]. These conflicting results obtained by scRNA-seq and FACS analyses might be attributable to the different gene sets employed to define the myCAF populations, in association with the substantial biological differences between measurements of RNA versus protein.

In human colorectal tumors, CAFs with the myCAF state produce TGF-β ligands including TGF-β1, 2, and 3 in significantly higher amounts than do epithelial cells, leukocytes, and endothelial cells. Such stromal TGF-β also attenuated the therapeutic efficacy of blocking programmed cell death 1 or programmed death-ligand 1 (PD-L1) by promoting immune evasion through T-cell exclusion, which restricts T cells to the stroma away from the tumor center, and suppression of T_H1-effector phenotypes in human colonic and urothelial tumors [31,32]. Leucine-rich repeat-containing protein 15 (LRRC15) is predominantly expressed in the myCAF population expressing PDPN in pancreatic ductal adenocarcinoma generated employing a genetically engineered murine model (GEMM) (Fig. 1). The $LRRC15^{+}$ myCAF subset was also detected in various human cancers and correlated with attenuated efficacy of PD-L1 blockade therapy [16].

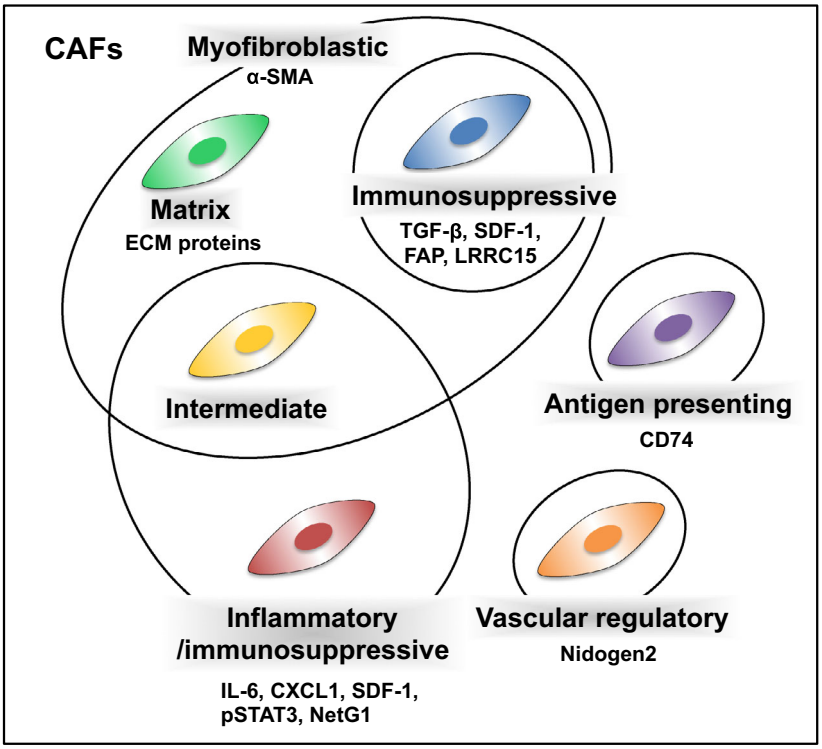

Fig. 1. Distinct CAF subtypes associated with potential functions. Various CAF populations with myofibroblastic, inflammatory, immunosuppressive, antigen presenting, matrix, or vascular regulatory states are characterized based on scRNA sequence using different human carcinomas including those of the pancreas, breast, head and neck, and colon. CAFs with the intermediate state between myofibroblastic and inflammatory phenotypes would also likely exist [28]. Several genes representing each CAF subset are indicated. Immunosuppressive CAFs expressing TGF-β, SDF-1, FAP, and LRRC15 indicate the myofibroblastic state [16,17], whereas those expressing IL-6, CXCL1, SDF-1, pSTAT3, and NetG1 correspond to the inflammatory state [23,28,37]. apCAFs express MHC class II molecules such as CD74 [15,16,26,27]. Matrix and vascular regulatory CAFs are characterized by expressions of ECM proteins and Nidogen 2, a basement membrane protein, respectively [20,40].

While the precise molecular mechanism(s) has received little research attention, TGF-β released from the $LRRC15^{+}$ myCAF subset may, at a minimum, contribute to the observed resistance to PD-L1 blockade therapy. Taken together, these findings indicate the myCAF subset-produced TGF-β to influence tumorigenesis via immunosuppressive mechanisms.

Carcinoma-associated fibroblasts also inherently represent iCAF populations constituting an immunosuppressive environment established by the production of pro-inflammatory molecules, such as CXCL1, CXCL2, CCL2, IL-1, IL-6, and prostaglandin E2 (PGE2) [33–36]. The glutamatergic presynaptic protein Netrin G1 (NetG1), which is highly expressed in pancreatic CAFs, is required to mediate the iCAF but not myCAF state in these fibroblasts (Fig. 1). NetG1 in CAFs has also been demonstrated to interact with its binding partner, NetG1 ligand (NGL1), on pancreatic cancer cells. CAFs then enhance direct transfer of glutamate, glutamine, and cytokines into cancer cells through macropinocytosis, resulting in promotion of survival of tumor cells under starvation conditions and attenuation of tumor cell death by natural killer cells [37].

The iCAF states could also be induced in CAFs through cancer treatment. Inhibition of a receptor for colony-stimulating factor 1 (CSF1) targets tumor-associated macrophages (TAMs), resulting in TAM depletion in tumors. However, in response to treatment with a CSF1 receptor inhibitor, CAFs further increase CXCL1 production recruiting MDSCs into tumors and thereby generating immunosuppressive TME and promoting tumor progression [38]. These findings, considered together, indicate different CAF subsets to play immunosuppressive roles via various mechanisms including adaptation to a range of therapies.

As another CAF subset possessing a putative immune-modulating trait, an antigen-presenting CAF (apCAF) population displaying major histocompatibility complex (MHC) class II molecules has been

characterized in pancreatic ductal adenocarcinoma and breast carcinomas (Fig. 1) [15,16,26,27]. This CAF subset can present antigens to T cells, but barely induces T-cell clonal proliferation due to a lack of the necessary costimulatory molecules, such as CD80, CD86, and CD40 [15]. The authors thus hypothesized that apCAFs act as a decoy receptor and suppress the immune response in the TME of pancreatic carcinomas. Antigen-presenting fibroblasts are also reportedly observed in patients suffering from rheumatoid arthritis. THY1^{positive}HLA-DRA (one of the MHC class II molecules) ^{high} synovial sublining fibroblasts are capable of marked productions of inflammatory cytokines, such as IL-6 and CXCL9 [39], indicating that apCAFs are not necessarily unique to carcinomas and also exist in inflamed tissues as part of the inflammatory response.

apCAFs were reported to be enriched in an FSP-1-positive fibroblast population in tumors raised by 4T1 murine breast cancer cells in immunocompetent BALB/c mice [27]. A PDPN-positive fibroblast population, which includes myCAFs and iCAFs, is also a constituent of CAFs in this murine breast cancer model. The low ratios of FSP-1-positive fibroblasts relative to those positive for PDPN in tumor-associated stroma were demonstrated to be associated with poor outcomes for breast cancer patients, indicating that the numerical balance between apCAFs and other CAF subtypes influences disease outcomes. However, whether apCAFs have an immune-promoting or an immune-suppressive function in human cancers remains unclear and the related mechanistic insights merit further investigation.

Carcinoma-associated fibroblast subtypes, in which genes enriched in ECM and vascular endothelial cells showed upregulation, have also been characterized in mouse mammary tumor virus (MMTV)-derived spontaneous breast tumors [40]. The authors demonstrated increased abilities of these CAF subtypes to stimulate invasion of cultured tumor cells and to predict poor outcomes in breast cancer patients. However, myCAF and iCAF subtypes were barely detected among their CAFs, indicating distinct activated fibroblastic states induced by interaction with the TME, which have diverse characteristics in different tumor models. The unified view of CAF subtypes presented in several previous reports also relies on various factors including differences between humans and mice, xenografts and spontaneous tumors, early vs. late tumor stages and technical variations in scRNA-seq.

Mechanisms underlying CAF emergence and diversity

Understanding the cell identity of CAFs

The intratumor heterogeneity in CAFs depends on the cells of origin of various CAF progenitors, ongoing activated fibroblastic states modulated by stimuli from the TME, and the stability and plasticity characteristics of the associated phenotypes (Fig. 2). However, the mechanism by which each CAF establishes its

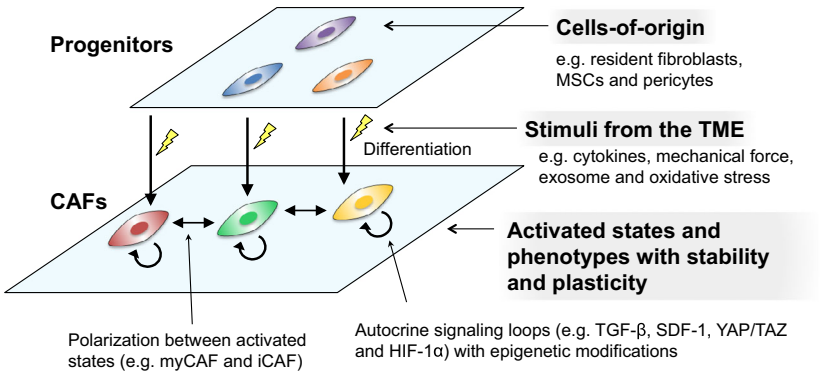

Fig. 2. Schematic representation of a model determining cell identity of CAFs. The cell identity of CAFs is influenced by cells of origin, ongoing activated states, and phenotypes with plasticity modulated by stimuli from the TME during tumor progression. Several progenitors, including resident fibroblasts, MSCs, and pericytes, differentiate into CAFs in response to various stimuli from the TME including growth factors, cytokines, exosomes, miRNA, mechanical force, oxidative stress, and therapy-induced DNA damage. Activated, tumor-promoting phenotypes in CAFs are mediated in a semi-stable fashion by establishment of self-stimulating autocrine signaling including the TGF-β, SDF-1, YAP/TAZ, and HIF-1α pathways, and activations of key transcription factors accompanied by chromatin remodeling through epigenetic modifications. Various activated fibroblast populations comprise CAFs in the TME, generating intratumor heterogeneity. The activated phenotype is modulated through interconvertible polarization associated with plasticity among fibroblasts. The responses are dependent on various stimuli from the TME during tumor progression.

individual cell type (cell identity) remains as yet poorly understood. Furthermore, little is known about the molecular mechanisms responsible for reprogramming the tumor-promoting ability of CAFs, a function generally maintained in a semi-stable fashion despite lack of continuous interaction with carcinoma cells.

Examination of the inherent cell lineage, the phenotype, and the cell state with plasticity becomes a classical and prospective approach to studying cell identity [41]. As an example, various subtypes of macrophages reside in different organs. The subtype specification is determined by series of different inputs of signals and transcription factors associated with the hard-wiring of the chromatin landscape. Macrophage lineage-differentiation signals control the transcriptional program common to all macrophage subtypes via PU.1 and C/EBPβ [42,43]. The tissue-identity signals are then induced by distinct environmental factors produced in different tissues, resulting in activation of the cognate transcription factors and thereby defining the cell identities of different macrophages, for example, red pulp macrophages in the spleen (through a Spi-c transcription factor induced by heme), peritoneal macrophages in the abdomen, and microglia in the brain. Functional-demand signals induced by factors such as hypoxia and bacterial molecules also cooperatively modulate the transcriptional program.

Macrophages are terminally differentiated via cooperation between macrophage lineage-differentiation factors and tissue-identity signals. The mature macrophages also reversibly polarize via functional-demand signals. The phagocytic M1-type macrophages, upon recruitment into a tumor, are in fact polarized into the immunosuppressive M2 type and thus contribute to tumor progression [44]. These observations allow us to speculate that macrophage subtypes determined by tissue-identity and functional-demand signals in different organs mirror the fibroblast heterogeneity which is generated by distinct TME-derived factors in a tumor. We also propose that fibroblast subsets with different activated states are modulated, through polarization, in collaboration with distinct TME-derived factors that depend on alterations inherent to carcinoma cells. Emergence, diversity, and plasticity of CAF subtypes might be determined by, in effect, a form of 'teamwork' comprising activation of various transcription factors associated with several epigenetic modifications. Multiple cells of origin for CAF progenitors would presumably play roles in additional further complexity. In the next section, we discuss the molecular mechanisms underlying the identities of activated, tumor-promoting CAFs accompanied by heterogeneity and stability.

Implications of cells-of-origin and TME-derived stimuli in CAF differentiation

Fibroblasts existing in normal healthy tissues have been demonstrated to be a highly heterogeneous group of mesenchymal cells showing distinct gene expression profiles and exerting different biological functions [45,46]. In tumors, CAFs are derived not only from resident fibroblasts in the affected tissues but also from various progenitors including pericytes [47], endothelial cells [48], adipocytes [49], and stellate cells of hepatic or pancreatic origin [50,51] (Fig. 2). Bone marrow-derived mesenchymal stem cells and fibrocytes also contribute to the generation of CAFs [20,52,53]. Collectively, these findings indicate that diverse cells of origin including distinct resident fibroblast populations contribute to generating various CAF subtypes.

Different progenitors are recruited into tumors and then differentiate into CAFs (Fig. 2). These fibroblasts reversibly acquire distinct activated states through polarization by stimuli from the TME. The differentiation/polarization program in CAFs relies on various TME-derived growth factors and cytokines as well as environmental stresses, which include TGF-β [54,55], PDGF-CC [56,57], sonic hedgehog [58], wingless type (WNT)7a [59], LIF [60], SDF-1 [55], IL-6 [61], IL-1α [28], IL-1β [23], oxidative stress [62], mechanical force [63], hypoxia [64], undernutrition, acidity, and therapy-induced DNA damage [65]. This broad range of TME-derived factors plays significant roles in generating different fibroblasts with distinct activated states. For example, TGF-β treatment conferred the myCAF trait on human dermal fibroblasts, while exposure to FGF2 induced the iCAF trait via activation of an ETV1 transcription factor in these fibroblasts [66], indicating different TME-derived paracrine factors to be responsible for CAF heterogeneity.

Autocrine signaling and transcription factors mediating stable CAF phenotypes

Activated, tumor-promoting traits are reportedly maintained in CAFs during propagation series in culture, despite lack of ongoing interaction with carcinoma cells [11,55]. Acquisition of self-stimulating autocrine signaling presumably plays significant roles in establishing such stable phenotypes. It would also allow continuous activation of particular transcription factors crucial for differentiation and polarization of progenitors into tumor-promoting CAFs. Polarization between myCAF and iCAF states might well be regulated in an interconvertible fashion during tumor progression. TME-derived signals would thus ostensibly

influence cellular states and phenotypes by acting cooperatively with the preexisting autocrine signaling, thereby allowing phenotypic conversion with plasticity. However, the mechanisms by which these activated phenotypes are maintained and modulated in CAFs during tumor progression remain poorly understood at the molecular level.

A study previously conducted in our laboratory demonstrated that establishment of autocrine signaling mediated by both TGF-β-Smad2/3 and SDF-1-CXCR4 signaling pathways is necessary for the induction and maintenance of myofibroblastic, tumor-promoting abilities in human breast CAFs [55]. Heat-shock factor 1 (HSF1), which is a heat-activated transcription factor, is required for tumorigenesis and reportedly mediates the TGF-β and SDF-1 signaling pathways in CAFs [67]. PU.1, which is a hematopoietic lineage-determining transcription factor, is also known to induce the profibroblastic gene signature in fibrotic states by acting through binding sites of the transcriptional enhanced associate domain (TEAD) which is a transcriptional output of the Hippo signaling pathway [68], highlighting the importance of PU.1-induced TEAD-Hippo signaling for induction of myofibroblastic properties in fibroses. However, the roles of PU.1 in CAFs have not yet been elucidated.

Constitutively activated inflammatory signaling pathways including JAK-STAT and NF-κB also mediate stable activated phenotypes of CAFs [28,60]. Activation of yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ) signaling in CAFs stimulates actomyosin contractility and ECM remodeling, thereby promoting invasion of apposed carcinoma cells [63]. HSF1-induced dickkopf-3 also activates β-catenin and YAP/TAZ signaling to further boost tumor-promoting ability in CAFs [69].

Epithelial–mesenchymal transition is regulated by several transcription factors including Snail1, Snail2, Twist, and zinc finger E-box binding homeobox 1 (ZEB1) as well as external signal cues, such as TGF-β, hepatocyte growth factor, and WNT, in tumor cells. The tumor cells acquire invasive, metastatic, stem cell-like, and therapy-resistant features through EMT [70]. The EMT-related transcription factors are also induced in CAFs, presumably via TGF-β derived from the TME, to promote tumor progression [71–76]. Forced Snail1 or Snail2 expressions were observed to boost TGF-β-induced myofibroblast differentiation in resident fibroblasts and to increase ECM stiffness. The ECM stiffness in turn mechanically activates YAP to produce profibrotic and ECM proteins which then promote tissue stiffness, thereby establishing a feed-forward loop of fibroblast activation. The IL-6 cytokine is known to imbue quiescent fibroblasts with activated inflammatory traits. Twist was indeed upregulated in IL-6-treated CAFs via STAT3 signaling to produce SDF-1 and remodel ECM, both of which promote tumor progression [74]. Twist1-induced CAFs also showed enhanced ECM stiffness via paladin and collagen α1(VI) [77]. Conditional knockout of the ZEB1 gene in $FSP1^{+}$ stromal cells in MMTV-PyMT-driven breast cancers resulted in decreased ECM deposition, neoangiogenesis, and tumor progression in mice [73], thereby revealing stromal ZEB1 to be required for creating protumorigenic CAFs.

Taken together, these observations indicate that different EMT-related transcription factors expressed in CAFs play major roles in promoting tumor progression through increased production of paracrine factors and greater ECM stiffness. However, the mechanisms by which each of the different EMT-related transcription factors impacts their target genes and modulates activated fibroblast states in CAFs remain elusive. Assessment of genome-wide DNA-binding sites corresponding to EMT-related transcription factors determined by the chromatin immunoprecipitation (ChIP)-sequence technique is anticipated to facilitate elucidation of the molecular roles of these factors in CAF activation.

Epigenetic alterations in CAFs

Previous reports indicated either genetic or epigenetic alterations that potentially mediate activated, tumor-promoting phenotypes in CAFs [78–80]. However, it has been classically believed that epigenetic alterations should explain a portion of the molecular basis of CAF phenotypes and that somatic genetic alterations would be minimal in these fibroblasts [81]. On the other hand, a recent study demonstrated enrichment of somatic copy number alterations (SCNAs) on chromosome 7 in $CD45^{-}CD90^{+}$ CAF populations, which had been extracted from all 9 human colon cancer patients examined using a single-cell genomics approach [82]. This finding clearly indicates potential clonal expansion of CAFs in tumors. However, whether these genetic alterations are functionally important in CAFs and whether the few genetic alterations detected in various other studies are attributable to intertumor heterogeneity of CAFs among patients, remain to be addressed in future studies.

In human squamous cell carcinomas (SCCs) of the skin, increased numbers of copies of the Notch 1 gene were also detected by fluorescent in situ hybridization (FISH) in CAFs extracted from nine patients [83]. Notch 1 silencing importantly impaired the tumor-

promoting ability of these fibroblasts, demonstrating Notch 1 gene amplification to mediate tumor-promoting CAFs in human skin SCCs. Downregulation of CSL/RBPJκ expression induced by ultraviolet radiation exposure was attributed to Notch1 gene amplification in CAFs via increased genomic instability and a decreased DNA damage response, indicating unique characteristics of skin CAFs susceptible to genetic alterations.

In terms of genome-wide DNA methylation, several analyses showed significant global hypomethylation with specific hypermethylated regions in CAFs derived from various human cancers including breast, lung, and gastric malignancies, as compared to their normal control counterparts [78,84,85]. In contrast, global hypomethylation was barely detectable in CAFs of prostate and gastric cancers according to other studies [86–88].

RAS protein activator-like 3 (RASAL3) is one of the GTPase-activating proteins that inactivate Ras through catalyzing GTP hydrolysis. Mishra *et al*. [89] demonstrated significantly downregulated RASAL3 expression via promoter hypermethylation in prostate CAFs in response to androgen signaling deprivation therapy (ADT). The attenuated RASAL3 expression activated Ras signaling in CAFs to increase the scavenging pathway for macromolecules, a process called micropinocytosis, which breaks down incorporated extracellular proteins and produces glutamine to fuel neighboring prostate cancer cells, resulting in the induction of ADT-resistant endocrine differentiation [89]. These findings indicate altered DNA methylation in the *RASAL3* gene in CAFs to be responsible for prostate cancer cells acquiring resistance to ADT.

Altered histone methylation status, for example global histone hypomethylation, reportedly contributes to the activated states in CAFs [90]. *S*-adenosyl methionine, which is the universal methyl donor for histones, transfers a reactive methyl group to nicotinamide via nicotinamide *N*-methyltransferase (NNMT), thus generating *S*-adenosyl homocysteine. Stromal NNMT expression was demonstrated to be upregulated in ovarian tumors by mass spectrometry-based proteomics using microdissection of formalin-fixed, paraffin-embedded specimens obtained from human ovarian carcinomas. The increased stromal NNMT staining was associated with poor outcomes for ovarian cancer patients. Inhibition of NNMT by shRNA increased global histone methylation while, conversely, decreasing activated fibroblastic states and the tumor-promoting ability of CAFs. Further treatment with an EZH2 histone methyltransferase inhibitor restored the activated states in these fibroblasts, indicating that global histone hypomethylation induced by NNMT promotes the protumorigenic phenotype of CAFs.

Tri-methylation at histone H3 lysine 27 (H3K27me3) is one of the markers of transcriptional repression. Maeda *et al*. [87] reported genomic regions with a significantly decreased H3K27me3 level in primary gastric CAFs as compared to their patient-matched counterparts. The altered H3K27me3 status in promoter regions of the *WNT5A* gene did indeed result in increased WNT5A mRNA expression in CAFs, thereby promoting invasion of apposed carcinoma cells [87].

Modulating the innate immune response in the TME by treatment with histone deacetylase (HDAC) inhibitors (HDACis) effectively attenuates tumor progression in murine tumor models [91,92]. Inhibition of class IIa HDAC by TMP195 suppressed primary tumor formation and metastasis of breast cancer cells by activating the phagocytotic ability of M1-type macrophages [91]. Treatment with the class I HDACi entinostat, with a DNA methyltransferase inhibitor, 5-azacytidine, also prevented metastasis of various tumor cells by disrupting the premetastatic niche through inhibited recruitment of MDSCs [92].

In addition to inflammatory cells, CAFs are also modulated by HDACis. HDAC6, which is one of the class IIb HDACs targeting non-histone proteins, activates STAT3 through phosphorylation in breast CAFs, producing the immunosuppressive molecule PGE2 [36]. CAFs treated with the HDAC6-selective inhibitor ACY1215 were demonstrated to have blunted tumor-promoting abilities via inhibition of STAT3 activation and PGE2 production [36], suggesting this HDACi to be a potential anti-CAF agent.

Treatment with a nonselective broad-spectrum HDACi, such as trichostatin A and vorinostat, which are class I and II HDACis, attenuated the myofibroblastic state in cultured CAFs and in murine models with fibrotic conditions and malignancies [93], indicating these HDACs to potentially mediate the myofibroblastic states in CAFs. Conversely, fibroblasts treated with the same HDACis boosted the productions of inflammatory cytokines including CXCL1, CXCL8, and IL-1 through the senescence-associated secretory phenotype to promote tumor progression [94,95]. However, the mechanism by which HDACi treatment attenuates the mCAF state and boosts the iCAF state, at the molecular level, remains largely unknown.

A hypoxic environment induces metabolic reprogramming in both carcinoma cells and stromal cells in the TME through epigenetic alterations. CAFs acquire glycolytic phenotypes under conditions of chronic hypoxia and produce metabolites including lactate and

pyruvate, which provide fuel to neighboring tumor cells [64]. Such metabolic changes are mediated by stable activation of hypoxia-inducible factor 1 alpha (HIF-1α) via promoter hypomethylation in the HIF-1α gene [64], reflecting epigenetic rewiring of HIF-1α stabilization in tumor-promoting CAFs. HIF-1α is also activated via posttranslational modification to increase glycolysis in colon CAFs and TGF-β-treated fibroblasts [96]. TGF-β-induced downregulation of isocitrate dehydrogenase 3α (IDH3α) expression decreases the level of effective α-KG by reducing the ratio of α-KG to fumarate and succinate, which in turn inhibits prolyl hydroxylase domain protein 2 (PHD2) [96]. PHD2 hydroxylates HIF-1α, leading to the binding of von Hippel–Lindau E3 ubiquitin ligase for proteasome-dependent degradation. Thus, the attenuation of PHD2 activity via downregulated IDH3α expression resulted in activated HIF-1α mediating glycolysis and the tumor-promoting phenotypes of CAFs.

Several studies have demonstrated CAF-derived metabolites, such as amino acids and lactate, to support tumor growth [97–99]. Many metabolic intermediates are substrates or co-factors of enzymes involved in epigenetic or post-transcriptional modifications [100–102], influencing the distinct metabolic features of diverse cell types including adipocytes, embryonic stem cells, and cancer stem cells [102–104]. Thus, establishment of activated tumor-promoting phenotypes in CAFs might depend largely on the crosstalk between hypoxic environments, metabolic features, and epigenetic alterations. However, the mechanism by which metabolic and epigenetic reprogramming governs the distinct activated states of CAFs in terms of phenotypic stability and plasticity remains an open question.

Tumor cell differentiation and plasticity controlled by CAFs

Cancer cells coevolve with CAFs, progressively acquiring their malignant phenotypes, including the capacities for invasion and metastasis. However, cellular and molecular insights underlying the invasion-metastasis cascade induced by CAFs remain as yet poorly understood.

Epithelial–mesenchymal transition is a transcriptional differentiation program enabling an epithelial cell to shed its epithelial characteristics, including cell–cell adhesion, and thereby acquire an array of mesenchymal traits induced by several EMT-related transcription factors including ZEB1. A single cancer cell in EMT thus moves away from the epithelium, which is a continuous epithelial sheet, facilitating local invasion and metastatic dissemination into distant organs.

E-cadherin, which is a component of a protein complex forming adherence junctions on epithelial cells, plays central roles in maintaining cell–cell adhesion and tissue integrity of the epithelium. Loss of E-cadherin expression thus promotes tumor cell invasion into the stroma-tumor interface through EMT. In contrast, membrane E-cadherin expression, which is partially maintained on tumor cells of human carcinomas, is required for collective invasion of tumor cell clusters [105]. E-cadherin thus plays dual roles, which are context-dependent, in modulating the invasion of either a single cancer cell or a multicellular tumor cluster.

Tumor cells, expressing both epithelial and mesenchymal markers through partial EMT (pEMT), have been observed in several human cancers. The tumor cells in pEMT are markedly tumorigenic and metastatic as compared to those with the complete EMT trait [106]. Our research group also observed tumor cells with pEMT, as exemplified by the weak membrane E-cadherin-positive cancer cells expressing nuclear ZEB1 in tumor budding (tumor clusters invading the stroma) of patient-derived colon tumor xenografts [107].

The pEMT is presumably induced by a signal(s) from the tumor-associated stroma, enabling tumor cell clusters to form, with each tumor cell partially maintaining cell–cell adherent ability. The tumor cell clusters efficiently colonize distant organs as compared to a single tumor cell. However, the precise cellular and molecular mechanisms underlying CAF-induced tumor cell cluster formation through pEMT remain poorly understood.

Our research demonstrated CAFs to endow apposed breast cancer cells with highly malignant phenotypes through pEMT *in vivo* [108] (Fig. 3). Co-inoculation of CAFs with otherwise minimally metastatic breast cancer cells into immunodeficient mice resulted in the generation of highly invasive and metastatic tumor cells, while control fibroblasts showed barely any induction of metastatic potential [108]. The breast cancer cells isolated from tumors containing CAFs showed an increased ability to form tumor cell clusters composed of two distinct cancer cell populations, one in a highly epithelial (E-cadherin^{high}ZEB1$^{low/negative}$: E^{high}) state and the other in a hybrid epithelial/mesenchymal (E-cadherin^{low}ZEB1^{high}: E/M) state. Oncogenic cell–cell adhesion molecules, such as carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) and CEACAM6, both of which associate with E-cadherin, were also upregulated in E^{high} tumor cells and thus enhanced the abilities of these cells to colonize and form metastatic nodules in distant organs. These observations are consistent with

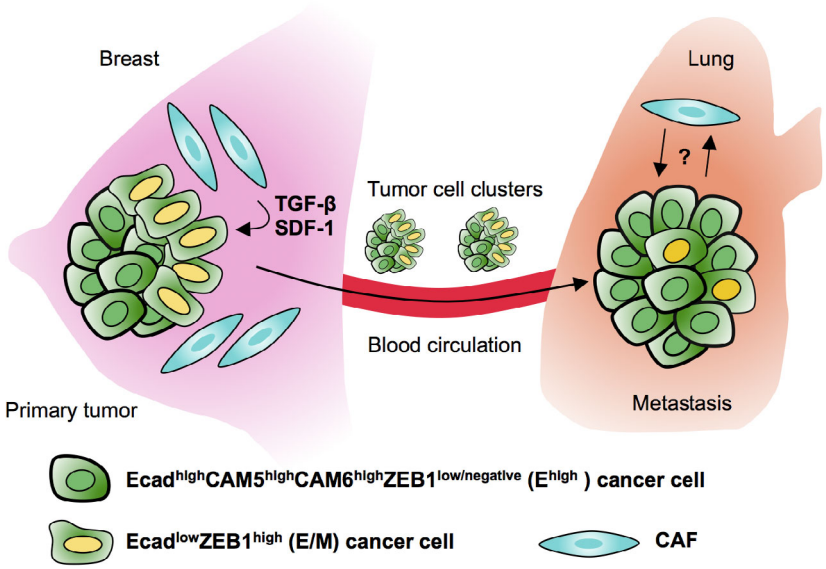

Fig. 3. Schematic illustration of CAF-induced metastatic dissemination. Through partial EMT, CAFs enable breast cancer cells to form a multicellular tumor cluster with highly invasive and metastatic properties [108]. The tumor cell cluster consists of two tumor cell populations: one with E-cadherin^{high}CAM5^{high}CAM6^{high}ZEB1$^{low/negative}$ (E^{high} state) and the other with E-cadherin^{low}ZEB1^{high} (E/M state). Acquisition of both E^{high} and E/M states mediates the increased invasion and metastatic abilities induced by CAFs. TGF-β and SDF-1 produced by CAFs are essential for the induction and maintenance of these aggressive phenotypes via activation of Src kinase in breast cancer cells. In the lung, the mesenchymal trait was significantly downregulated in tumor cell clusters with the E/M state through MET, resulting in the promotion of metastatic colonization.

the known oncogenic properties of E-cadherin mediating proliferation and anoikis resistance of disseminating cancer cells during metastatic spread [109]. In contrast, the E/M trait is likely to be related to the invasive propensity of tumor cells. It is noteworthy that the CAF-induced highly metastatic properties were maintained, when tumor cells were injected into secondary mice without addition of CAFs, implying that CAF had induced stable phenotypic switching in breast cancer cells. Mechanistically, CAF-produced TGF-β and SDF-1 mediated the formation of tumor cell clusters with the E^{high} and E/M states via Src kinase activation, which is crucial for the invasive and metastatic phenotypes.

Disseminating mesenchymal tumor cells usually revert to their original epithelial traits to colonize distant organs through mesenchymal–epithelial transition (MET), which is a crucial process for generation of micrometastasis. CAFs are capable of activating the bone morphogenetic protein (BMP) signal in disseminating mesenchymal cancer cells, thereby promoting the colonization of distant organs through Smad1/5 signaling by these cells [110]. BMP4 and BMP5 released from CAFs were also shown to induce epithelial differentiation with the luminal phenotype in primary bladder cancer cells, thereby suppressing local invasion by these cells [111,112]. The BMP signal pathway counteracts TGF-β signaling in various models including fibrosis [113–115]. Collectively, these findings indicate CAF-derived TGF-β and BMP paracrine signaling to delicately fine-tune epithelial–mesenchymal plasticity in cancer cells during the invasion-metastasis cascade.

Metastatic colonization by disseminating breast cancer cells is reportedly facilitated by the formation of a niche comprised of pulmonary CAF-produced tenascin-C, periostin, CXCL9, and CXCL10 in experimental mouse models [116–118]. Upon activation of p38α stress-activated protein kinase, pulmonary CAFs were also shown to form the premetastatic niche via the production of inflammatory cytokines, such as CXCL1, 3, and 5 that can recruit neutrophils into the affected lungs prior to the dissemination of melanoma cells in mice [119]. The CAF subset in axillary lymph nodes affected by metastases plays roles in promoting invasion and metastasis through activation of Notch signaling in breast cancer cells [120]. These findings indicate that unique CAF subtypes are engaged in the establishment of niches allowing metastatic outgrowth in distant organs. However, we do not as yet know whether CAFs in metastatic sites resemble those in a primary tumor site or whether metastasis-associated

CAFs play roles in facilitating metastatic spread into other distant organs.

CAF-targeted therapeutic approaches

Given the tumor-promoting ability of CAFs, these fibroblasts are increasingly being recognized as a potential therapeutic target. Possible therapeutic approaches have been examined in different experimental animal models, based on omics data and related functional assays using CAFs in bulk [3,8,51].

At present, CAF-targeted approaches are divided into three categories: first, CAFs were depleted pharmacologically or genetically. Numerous FAP-expressing stromal cells are present in human carcinomas and chronically inflamed tissues where they play immunesuppressive roles. Depletion of FAP-expressing CAFs by systemic administration of OMTX705, a humanized anti-FAP antibody linked to a novel cytolysin, TAM470, reportedly ameliorated intratumoral myofibroblast accumulation, attenuated tumor growth, and improved drug efficacy in several preclinical patient-derived colon tumor xenograft models [121,122]. However, depletion of FAP-positive fibroblasts caused unacceptably severe toxicities, including cachexia and anemia in GEMM for pancreatic ductal adenocarcinoma sensitive to diphtheria toxin treatment and in a tumor xenograft model with adaptive transfer of T cells genetically engineered with FAP-reactive chimeric antigen receptors (CARs) [123,124]. The unanticipated toxicities were due to FAP-expressing cells also being present in unaffected normal organs including skeletal muscle and bone marrow, highlighting the importance of targeting activated fibroblasts only present within a tumor.

The second approach is neutralization and/or degradation of molecules including growth factors, cytokines, and ECMs, which mediate communication between cancer cells and stromal cells including CAFs. Given the complex crosstalk among signaling pathways involving these cells which promote tumor progression, while inhibiting a single pathway may exert some efficacy, it is unlikely to eradicate tumors.

The third strategy is normalization of CAFs either transcriptionally or epigenetically (Fig. 4). NADPH oxidase 4 (NOX4), which is a reactive oxygen species (ROS)-producing enzyme, is one of the target genes of TGF-β signaling in fibroblasts. ROS are required for induction and maintenance of myCAFs [62,125]. The treatment of CAFs with a NOX4 inhibitor, GSK137831, clearly attenuated the myCAF state, resulting in the inhibited growth of carcinoma cells co-injected into mice

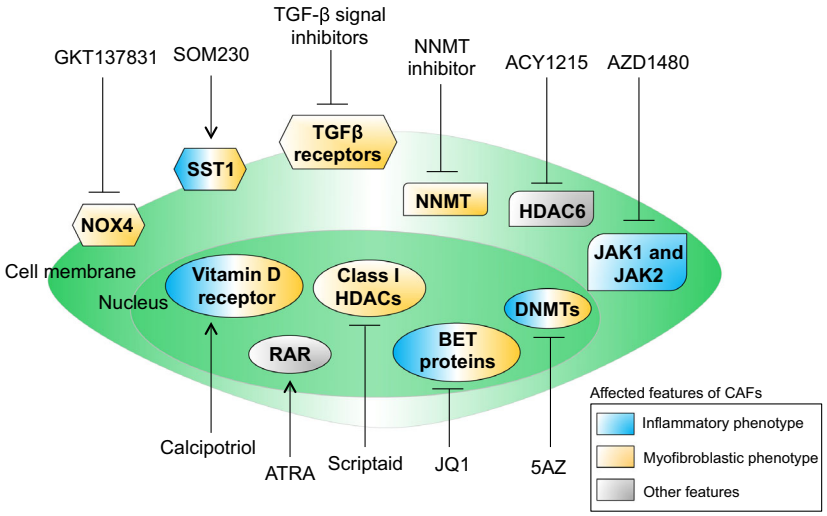

Fig. 4. CAF-targeting therapeutic approaches. Treatment with the various chemical and natural compounds depicted reportedly attenuates activated tumor-promoting traits in CAFs, as gauged by *in vitro* and *in vivo* assays. The indicated genes responsible for targeting CAFs and the resulting phenotypes for the myofibroblastic and inflammatory states, upon treatment with these compounds, are shown. Inhibition of NOX4 or TGF-β receptors normalizes the myofibroblastic state in CAFs, resulting in improved efficacy of immune checkpoint inhibitors [32,126]. Suppression of the class I HDAC or NNMT also suppresses the myofibroblastic state in CAFs [90,93]. Activation of sst1 and the vitamin D receptor or inhibition of BET proteins and DNMTs diminish both myofibroblastic and inflammatory phenotypes in CAFs [127,129–132]. Exposure of CAFs to JAK inhibitors downregulates the inflammatory phenotype. Inhibition of HDAC6 reduces STAT3 activation and COX2 expression, resulting in decreased production of PGE2 and relevant immune suppression in CAFs [36]. ATRA treatment increases production of sFRP4 acting through the RAR on CAFs and inhibits Wnt-β-catenin signaling in tumor cells, resulting in attenuation of tumor cell growth [128].

and reduced responsiveness to immunotherapy [126]. Inhibitors of the JAK-STAT pathway, such as ruxolitinib and AZD1480, targeted iCAFs through amelioration of tumor inflammation [28]. A vitamin D receptor agonist, calcipotriol, inhibited both the myCAF and the iCAF state, presumably via attenuation of TGF-β-Smad3 and STAT3 signaling pathways, respectively, indicating normalization of pancreatic CAFs into a quiescent fibroblast state [127]. Pancreatic stellate cells equivalent to CAFs, when treated with all-trans-retinoic acid (ATRA), increased production of secreted frizzled-related protein 4 (sFRP4) acting through the retinoic acid receptor (RAR) on the surface of these fibroblasts [128]. sFRP4 then attenuated canonical Wnt-β-catenin signaling in apposed cancer cells, resulting in attenuated tumor cell proliferation and increased apoptosis [128].

Activation of mammalian target of rapamycin complex 1 (mTORC1) stimulates production of IL-6 in pancreatic CAFs through high protein synthesis, which increases resistance to gemcitabine, in pancreatic tumor cells [129]. Treatment with the somatostatin analogue SOM230 attenuated IL-6 production through inhibition of mTORC1 by acting on somatostatin receptors (sst1) on the surfaces of myCAFs, thereby attenuating chemoresistance [129]. As mentioned earlier, tumor-promoting abilities of CAFs are likely maintained by different epigenetic regulations. Inhibitors of epigenetic modifiers, such as HDACs, bromodomain and extra-terminal (BET) proteins, DNA methyl transferases (DNMTs), and NNMT, have been demonstrated to attenuate the tumor-promoting ability of CAFs [36,90,93,130–132]. Given the various fibroblast populations of which CAFs are comprised, it may well be important to consider CAF subtype-specific targeting approaches in the future.

Perspectives and concluding remarks

Recent advances in genome-wide single-cell omics technologies have opened new avenues to understanding intratumor heterogeneity in tumor cells as well as stromal cells including CAFs [133]. Several CAF subsets were identified by scRNA-seq analysis. The subsequent clustering analyses allowed characterization of tentative CAF markers and assessment of their activated phenotypes in the TME. The differentiation trajectory of activated states in each CAF lineage was predicted by pseudo-time reconstruction analysis through identification of genes differentially expressed over time and retrospective inferences regarding sequential gene activation events [16].

Spatial interactions among CAF subtypes and other cell types are difficult to assess employing single-cell omics data. Epithelial carcinomas often consist of multifocal transformed glands with different cancer cell subtypes. Tissue localizations are an important parameter influencing the differentiation states of various cell types in the TME [100,134]. Spatial information obtained by transcriptomics and proteomics of tissues is therefore required to precisely illustrate the cell identities of different cells [135,136].

Conventional immunohistochemistry and *in situ* hybridization have been employed to assess spatial information of proteins and genes of interest in tissues. However, simultaneous detection of large numbers of different cell types is difficult. Multiplexed error-robust FISH, high content in situ hybridization with subcellular resolution [137], imaging CyTOF [134], and advanced serial immunofluorescence staining [138] have also been applied to demonstrate spatial interactions among various cells in the TME. For instance, multiplexed imaging CyTOF for breast cancer specimens revealed T cells to be spatially associated with CXCL10-producing stromal cells [134]. The multiplexed spatial imaging approaches also advance our understanding of how environmental and spatial parameters affect phenotypic functional diversity in cells. Notably, spatial transcriptomics technology, which allows *in situ* quantification of the mRNA on spatially barcoded tissue microarrays, has reportedly been applied to various disease models including human malignancies and the associated TME [139].

Various CAF subsets, which are known to express profibrotic genes, inflammatory molecules, ECM proteins, immune-suppressive molecules, and angiogenic factors, have been identified in different tumor models, though the extents to which they functionally resemble each other remain unclear. One of the future challenges is thus integration of CAF subsets, as reported in different studies within a single organization [8]. To this end, isolation and culturing the CAF subsets of interest should be pursued by performing *in vitro* and *in vivo* functional assays, even though several experimental biases must be considered. To compensate for such limitations, highly multiplex protein and mRNA evaluation, as described above, can serve as a powerful modality for inferring several of the functions of CAFs. These efforts are hoped to allow establishment of a relevant nomenclature for CAF subsets as well as the discovery of novel therapeutic opportunities.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

YM and AO wrote the manuscript and prepared all figures.

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