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Cancer-associated fibroblasts: Origin, function, imaging, and therapeutic targeting

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ABSTRACT

The tumor microenvironment (TME) is emerging as one of the primary barriers in cancer therapy. Cancer-associated fibroblasts (CAF) are a common inhabitant of the TME in several tumor types and play a critical role in tumor progression and drug resistance via different mechanisms such as desmoplasia, angiogenesis, immune modulation, and cancer metabolism. Due to their abundance and significance in pro-tumorigenic mechanisms, CAF are gaining attention as a diagnostic target as well as to improve the efficacy of cancer therapy by their modulation. In this review, we highlight existing imaging techniques that

Abbreviations: ANG, Angiopoietin; ANXA1, Annexin A1; ATP, Adenosine triphosphate; ATRA, All-*trans* retinoic acid; BCC, Breast cancer cells; BDL, Bile duct ligation; BSA, Bovine serum albumin; BXPC-3, Pancreatic cancer cell line; CAF, Cancer associated fibroblast; CAP, Cleavable amphiphilic peptide; CD26, Dipeptidyl peptidase-4; CD, Cluster of differentiation; CLSM, Confocal laser scanning microscopy; CM-101, Collagen targeting probe; CPP, Cell-penetrating peptide; CSC, Cancer stem cells; CTC, Circulating tumor cluster(s); CXCR, Chemokine receptor; DCE, Dynamic contrast enhanced; DGL, Dendrigraft Poly-L-Lysine; DOTA, 2,2′,2″,2‴-(1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetic acid; DOX, Doxorubicin; DRP, Damage response program; DTPA, Diethylenetriamine pentaacetate; EA, Ellagic acid; ECM, Extracellular matrix; EGFR, Epidermal growth factor receptor; EMT, Epithelial to mesenchymal transition; EPR, Enhanced permeation and retention; ER, Estrogen receptor; FAK, Focal adhesion kinase; FAP, Fibroblast activation protein; FAPI, FAP inhibitor; FDA, Food and drug administration; FDG, Fluorodeoxyglucose; FITC, Fluorescein isothiocyanate; FOLFIRI, 5-fluorouracil, leucovorin, irinotecan; FOLFIRINOX, Combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin; FPR2, Formyl peptide receptor 2; FSP1, Fibroblast-specific protein 1; FU, 5-Fluoro uracil; GA, 18β-glycyrrhetinic acid; GBq, Gigabecquerel; *GEM*, Gemcitabine; GPER, G protein-coupled estrogen receptor; GSH, Glutathione; HA, Hyaluronic acid; HBSS, Hanks balanced salt solution; HER2, Human epidermal growth factor receptor 2; HGF, Hepatocyte growth hormone; HIF, Hypoxia-inducible factors; HRCT, High resolution computed tomography; HSA, Human serum albumin; HSP47+, Heat shock protein 47; HSPG2, Heparan sulphate proteoglycan 2; HSTS26T, Human soft tissue carcinoma; HSV, Herpes simplex virus; ID / g, Injected dose per gram; IFN, Interferon; IFP, Interstitial fluid pressure; IGF1, Insulin-like growth factor; IL, Interleukins; IPF, Idiopathic pulmonary fibrosis; IPI-926, Inhibitor of the Hedgehog Pathway; ITGA11, Integrin subunit alpha 11; ITGA5, Integrin subunit alpha 5; JAK, Janus kinases; JNK, Jun *N*-terminal Kinase; KPC, Clinically relevant model of pancreatic ductal adenocarcinoma; KRAS, Kirsten rat sarcoma virus; LCP, Lipid calcium phosphate nanoparticle; LOXL2, Lysyl Oxidase like 2; LPD, Lipid-coated protamine DNA complexes; LPP, Lipoma-preferred partner; LST-Lip, Losartan encapsulated liposomes; LXA4, Lipoxin A4; MAPK, Mitogen-activated protein kinase; MCT4, Monocarboxylate transporter 4; MET, Hepatocyte growth factor receptor; MHC, Major histocompatibility complex; MMP, Matrix metalloprotease; MPS, Mononuclear phagocyte system; MRI, Magnetic resonance imaging; MSC, Mesenchymal stem cells; mTOR, mammalian target of rapamycin; MU89, Human melanoma; NF, Normal fibroblast; NH_2, Amine group; NK, Natural killer cells; NO_2, Nitric oxide; NODAGA, 1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid; NP, Nanoparticle; NSCLC, Non-small cell lung cancer; PAMAM, Poly (amidoamine); PD-1, Programmed cell death protein 1; PDAC, Pancreatic ductal adenocarcinoma; PDGF, Platelet-derived growth factor; PDGFR, PDGF receptor; PDT, Photodynamic therapy; PDX, Patient-derived xenograft; PEG, Polyethylene glycol; PEGPH20, PEGylated form of recombinant human hyaluronidase PH20; PET, Positron emission tomography; PFT, Pericytes to fibroblast transition; PGE2, Prostaglandin E2; PP, Poly (ethylene glycol)-poly (Caprolactone); PSC, Pancreatic stellate cells; PSMA, Prostate-specific membrane antigen; PTC, Papillary thyroid carcinoma; PTX, Paclitaxel; QD, Quantum dots; QP, Quercetin phosphate; RGD, Tripeptide Arginine-Glycine-Aspartate; RNA, Ribonucleic acid; ROCK, Rho-associated protein kinase; ROS, Reactive oxygen species; RUNX3, Runt related transcription factor 3; SATB, Special AT-rich sequence-binding protein 1; SBRT, Stereotactic Body Radiation Therapy; SDF-1, Stromal-derived factor 1; α-SMA, Alpha smooth muscle; SMO, Smoothened receptor; SNAI1, Snail family transcriptional repressor 1; SPECT, Single photon emission computed tomography; SRBC, Stroma rich bladder cancer; STAT, Signal transducer and activator of transcription; SUV, Standardised uptake value; TAM, Tumor-associated macrophages; TGF-β, Transforming growth factor; TIE2, Angiopoietin receptor; TKI, Tyrosine kinase inhibitors; TME, Tumor microenvironment; TNC, Tenascin C; TNF, Tumor necrosis factor; TRAIL, Tumor Necrosis Factor Related Apoptosis-Inducing Ligand; TSL, Thermosensitive liposome; TSP-1, Thrombospondin-1; UMUC3, Stromal rich bladder cancer cell line; VCAM-1, Vascular cell adhesion molecule 1; VDR, Vitamin D receptor; VEGF, Vascular endothelial growth factor; VEGFR, VEGF receptor; YAP, Yes associated protein 1.

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are used for the visualization of CAF and CAF-induced fibrosis and provide an overview of compounds that are known to modulate CAF activity. Subsequently, we also discuss CAF-targeted and CAF-modulating nanocarriers. Finally, our review addresses ongoing challenges and provides a glimpse into the prospects that can spearhead the transition of CAF-targeted therapies from opportunity to reality.

Keywords:
Cancer
Tumor Microenvironment
Fibrosis
Imaging
Drug resistance
Nanocarriers

Contents

1. Introduction

Tumor malignancy and progression are associated with the intrinsically aggressive nature of cancer cells as well as alterations in the tumor microenvironment (TME) [1]. Activated fibroblasts, vascular endothelial cells, pericytes, adipose cells, lymphatic endothelial cells, and immune cells together with the abundant extracellular matrix (ECM) constitute the complex TME [1,2]. Cancer cells hijack the normal processes of the homeostatic tissue environment and orchestrate the interplay between numerous cell types [3]. One such cell type that is widely studied in the context of tumor progression is cancer-associated fibroblasts (CAF) [4,5]. Together, cancer cells and CAF can directly or indirectly activate/deactivate other cell types in the TME and regulate key processes, resulting in tumor progression and therapy resistance. This is attained primarily by the release of growth factors such as transforming growth factor-β (TGF-β), stromal-derived factor-1 (SDF-1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and interleukins [6]. Such paracrine signals lead to angiogenesis, immune suppression, and enhanced drug resistance [7]. Additionally, CAF modulate the composition and the abundance of the ECM proteins, which indirectly affects key processes in the TME [8].

Although chemotherapy overall has been quite efficient, the administration of chemotherapeutic drugs often results in several issues, which include chemo-driven toxicity, chemo-resistance, and relapse of cancer. Thus, as a complementary strategy, several studies and clinical trials are ongoing to target the TME together with cancer. CAF, due to their irrefutable involvement in cancer progression have particularly been in the limelight. There are two primary mechanisms to address CAF: inhibiting the interaction between CAF and cancer cells to attenuate angiogenesis, tumorigenesis, and drug resistance. The alternate strategy is to reduce the secretion of ECM moieties such as collagen and hyaluronic acid (HA), which alleviates the interstitial fluid pressure (IFP), thereby allowing deeper penetration of drugs in the tumor stroma. The mechanism, as mentioned above, cannot be achieved by targeting the cancer cells alone and calls for the need to co-target CAF and cancer cells. For this, researchers have studied different strategies utilizing nanocarriers, which will be discussed later in this review. These strategies include co-targeting CAF and cancer cells, entirely depleting CAF, re-educating CAF back to their

quiescence state, and sequential delivery to target CAF and cancer cells. Although numerous studies on therapeutic targeting of CAF are reported, it is necessary to detect CAF density and CAF-biomarkers at an early stage to achieve efficient treatment. Therefore, in this review, we will also discuss various molecular imaging probes and protocols that have been explored to visualize CAF, thereby highlighting the potential of combined CAF imaging and targeting in individualized and improved cancer therapy.

2. Definition and heterogeneity of CAF

First recognized by Virchow in 1858 [9], fibroblasts are mesenchyme-derived quiescent cells that preserve the structural integrity of tissues as well as maintain a homeostatic tissue environment. Fibroblasts deposit the required ECM proteins, and release proteinases and their inhibitors, thereby balancing ECM production and degradation [10,11]. Furthermore, they secrete and respond to essential growth factors and cytokines, and interact with numerous other cell types. Quiescent fibroblasts are distributed in healthy tissue as single cells, particularly residing in close proximity to the basement membrane that separates the epithelial and the stromal compartment. These tissue-residing fibroblasts provide epithelial differentiation cues and maintain basement membrane integrity [12].

Fibroblasts in wounded and inflammatory sites are activated. In wounds, activated fibroblasts or myofibroblasts enhance the deposition of ECM and increase contractility leading to faster wound healing [13,14]. Once the process of healing and remodeling is complete, the activated fibroblasts disappear via apoptosis or possibly by reverting to quiescence. The normal physiological processes are then re-established [15]. However, in case of constant inflammation and stress (e.g. cancer, auto immune diseases or chronic physical conditions), there is a persistent repair response of wound healing which leads to a pathological condition known as tissue fibrosis. Due to the persistent repair response, fibrotic tissues are also called as wounds that never heal. Maintenance of these kind of continuous wound healing mechanisms is partly promoted by epigenetic modifications in fibroblasts leading to the emergence of a hyper activated fibroblast subpopulation [10]. Such subpopulation of hyper-activated fibroblasts with enhanced anti-apoptotic pathways and proliferation, elongated morphology, that resides within or in the vicinity of the tumor mass, and lack mutations found within the cancer cells can be termed CAF [10,16,17,20]. CAF produce abundant ECM components and proteinases, contribute to change in matrix stiffness, induce angiogenesis, modulate immune cell response, and synthesize growth factors and interleukins that promote cancer malignancy [18,19]. Unlike wounds that heal, tumors constantly provide an inflammatory environment and the fibroblasts maintain their activated phenotype [20]. Post-activation, CAF modulate several key aspects within the TME, thereby facilitating cancer growth, malignancy, and therapy resistance.

As a consequence, there is an emerging interest in targeting CAF as a viable option for cancer therapy. The challenge however is that CAF are not a homogenous group of activated fibroblasts, but rather a group of cell subpopulations with spatial, phenotypic, and functional heterogeneity [20]. This provides additional challenges in CAF imaging and CAF-targeted therapy.

CAF heterogeneity could arise as a consequence of a) different cellular origins b) the differentiation stage of CAF c) proximity of CAF to the tumor cells, and d) the presence of diverse soluble cues in the TME. Although the precise origin of CAF is still unidentified, they are shown to be derived from various cell sources such as resident stromal fibroblasts, endothelial cells via endothelial to mesenchymal transition (EndMT) [21], pericytes via pericytes to fibroblast transition (PFT), [22] epithelial cells via epithelial to mesenchymal transition (EMT) [23], and differentiation of resident mesenchymal stem cells (MSC), stellate cells [24], and adipocytes. CAF are also derived from recruited cell sources such as bone marrow-derived MSC, fibrocytes, and hematopoietic stem cells (Fig. 1) [11,25]. CAF are commonly identified as spindle shaped cells expressing markers such as alpha-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), and fibroblast-specific protein-1 (FSP 1) in combination with the absence of epithelial, endothelial or leucocyte markers [20]. A list of several CAF markers and secretomes is provided in Table 1. These markers are however unspecific for CAF and could also vary with the heterogeneous subpopulations of CAF residing within the TME. The specificity of the markers/secretomes cannot be precisely defined due to lack of a comprehensive knowledge and the prevalent issue of heterogeneity associated with CAF. Nevertheless, investigations into the expression or absence of a pre-selected combination of markers and secretomes have been an effective method to distinguish distinct CAF subgroups [26].

Several studies have emphasized the diverse functions of CAF subpopulations within the TME. For instance, four distinct CAF subsets were identified in metastatic breast cancer tumors depending on their expression of six different CAF marker proteins [64]. These subsets differed in their abundance and metastasis-inducing properties, where two out of the four subsets were shown to be more aggressive. Moreover, the pro-invasive subsets of CAF promoted tumor propagation via distinct mechanisms and exhibited functional heterogeneity [65]. Similarly, Bartoschek et al. demonstrated the presence of three distinct CAF subpopulations within a breast cancer TME [66]. The three different subtypes were named vascular CAF (vCAF), matrix CAF (mCAF), and development CAF (dCAF) depending on their origin [66]. vCAF were shown to originate from perivascular locations and were more prevalent within the tumor core. mCAF on the other hand were shown to be derived from resident fibroblasts, whereas dCAF shared gene expression patterns with the tumor epithelium. The vCAF and mCAF gene signatures were associated with greater metastatic dissemination and poor prognosis [66].

Costa et al. identified four distinct CAF subsets (CAF-S1 to S4) in metastatic lymph nodes which showed varying functionalities. Subpopulations CAF-S1 and CAF-S4 supported cancer progression via distinct mechanisms whereas CAF-S2 and S3 acted as non-invading subsets [64,67]. CAF-S1 (close to CD34 + stromal cells) led to enhanced migration of cancer and induced EMT, whereas CAF-S4 (close to pericytes) was highly contractile and led to ECM remodeling and enhanced cancer invasion. Similarly, in pancreatic ductal adenocarcinoma (PDAC), two distinct pancreatic stellate cells (PSC)-derived CAF subtypes were recognized by Öhlund et al. [68] One subpopulation was inflammatory CAF (iCAF) that have low α-SMA expression levels and high expression levels of inflammatory cytokines such as IL-6. Another subtype was myofibroblasts (myCAF), which expressed high levels of α-SMA but low levels of inflammatory cytokines. These distinct subpopulations of CAF were not only different phenotypically and functionally but were also located in different regions of the tumor. MyCAF resided in the periglandular region near the tumor whereas iCAF were located further away from the tumor site [69]. Another distinct sub-type of CAF was recognized in the PDAC environment and was termed antigen-presenting CAF (apCAF) [70]. These CAF expressed major histocompatibility complex (MHC) class II genes and presented antigens to CD4 + *T*-cells. Newly discovered apCAF therefore could play an important role in immune regulation in the PDAC environment.

Recently, a biobank of CAF from patient-derived biopsies of non-small cell lung cancer (NSCLC) was established [71]. The biobank included patients with epidermal growth factor receptor

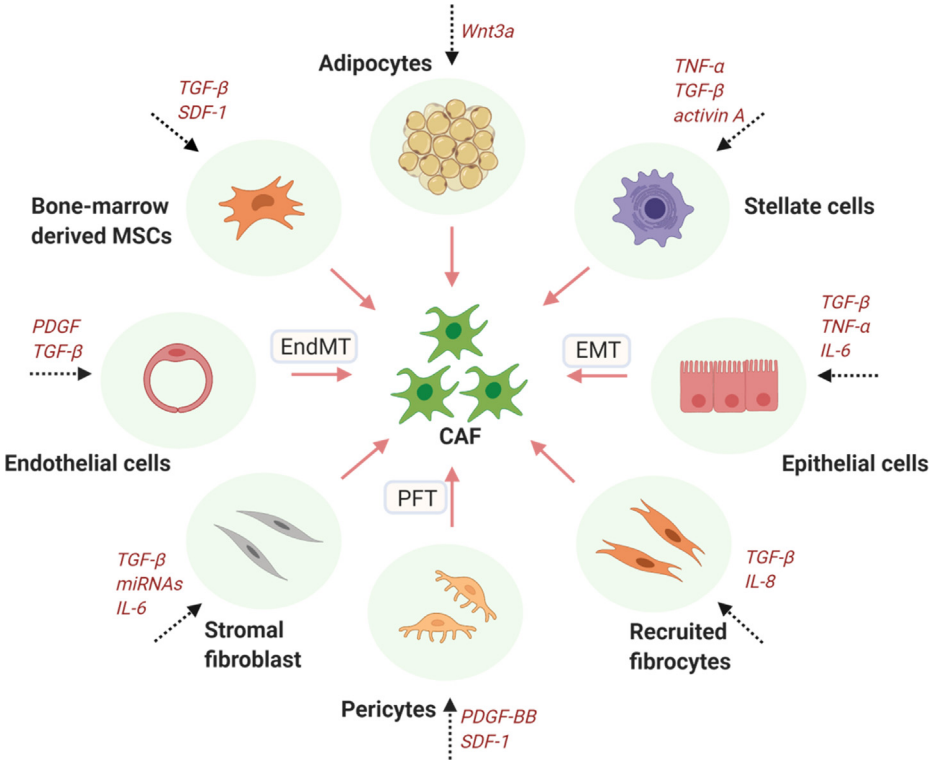

Fig. 1. Multi-cellular origin of CAF. Several cell types that reside within the TME such as normal fibroblasts, endothelial cells, epithelial cells, and pericytes, or recruited cell types such as bone-marrow-derived MSCs and fibrocytes can metamorphose into CAF. This transition occurs primarily due to the secretory factors released by the cancer cells such as TGF-β, PDGF, and IL-6. The diverse origin of CAF results in the manifestation of heterogeneous CAF subpopulations within the TME.

Table 1
List of CAF markers and secretomes.

CAF Markers		CAF Secretomes	
FAP [27,28]	Fibroblast activation protein	TGF-β1 [29–31]	Transforming growth factor β
FGF2 [32]	Fibroblast growth factor 2	IL-6 [33–35]	Interleukins
		IL-33 [36]	
		IL-17a [37]	
		IL-22 [38,39]	
		IL-32 [40]	
α-SMA [41]	α-Smooth muscle actin	CXCL12[42,43]	CXC Chemokine ligand
		CXCL1 [44]	
FSP 1 [48]	Fibroblast-specific protein	MMP-**1** [45,46]	Matrix metalloproteinases
COL11A1 [47,48]	Collagen type XI alpha 1 chain	LOX [49–51]	Lysyl oxidase (LOXL)
NAB2 [52]	NGFI-A-binding protein	VEGF [53]	Vascular endothelial growth factor
NG2 [54]	Neural/glial antigen 2	Gal-1 [55]	Galectin-1
MFAP5 [56]	Microfibril associated protein 5	Chi3L1 [57]	Chitinase-3-like protein 1
PDPN [58]	Podoplanin	HGF [59–61]	Hepatocyte growth factor
ASPN [62]	Asporin	ANXA1 [63]	Annexin A1

(EGFR) mutations and anaplastic large-cell lymphoma kinase (ALK) fusions. Three distinct subpopulations of CAF were identified. Subtype I highly expressed fibroblast growth factor 7 (FGF7) and protected the NSCLC from EGFR inhibitors (EGFR inhibitors are commonly prescribed for patients with EGFR-mutated NSCLC). Subtype II moderately rescued the cancer cells from EGFR inhibitors, while subtype III had minimal effect. The same rescue effect was observed in ALK^{+} NSCLC, where subtype III exhibited a better response to ALK inhibitors [71]. The heterogeneity can be an explanation of why patients with similar oncogene mutations have diverse therapy outcomes. By examining patient cohorts, it was established that patient tumors with Subtype III better responded to EGFR inhibitors as compared to subtypes I and II. The three distinct CAF subtypes required different treatment strategies to prevent CAF-mediated drug resistance. For instance, together with EGFR inhibitor, subtype I required the blockade of MET (HGF receptor) as well as fibroblast growth factor receptor (FGFR) pathway, while subtype II required only FGFR blockade and subtype III required no combination of therapy [71].

In conclusion, CAF show distinct spatial, phenotypic, and functional heterogeneity within the TME, and this heterogeneity has implications for therapy outcomes. A summary of several CAF subpopulations in different tumor types and their expression of selected biomarkers are listed in Table 2. In the future, further characterization of CAF, as well as the discovery of more CAF subpopulations, specific biomarkers, drug delivery systems, and novel imaging techniques, will contribute to new diagnostic and therapeutic opportunities for CAF-targeted interventions.

Table 2
List of CAF subpopulations in different tumor types and their associated markers.

Cancer type	Subpopulation	Biomarkers	Ref.
Pancreatic cancer	myCAF	• α-SMA ^{high,} HOPX, MYL9, COL12A1, THY1, IL-6 ^{low}	[68–70]
	iCAF	• IL-6 ^{high}, IL-8, CXCL12, α-SMA ^{low}	
	apCAF	• HLA-DRB1, CD74, HLA-DRA	
Lung cancer	Subtype I	• HGF ^{high}, FGF7 $^{High/low}$, p-SMAD2 ^{low}	[71]
	Subtype II	• FGF7 ^{High}, p-SMAD2 ^{low}, HGF ^{low}	
	Subtype III	• p-SMAD2, HGF ^{low}, FGF7 ^{low}	
Breast cancer	CAF-S1	• CD29 ^{Medium}, FAP ^{High}, FSP1$^{Low-High}$, α-SMA ^{High}, PDGFRβ $^{Medium-High}$, CAV1^{Low}	[64]
	CAF-S2	• CD29 ^{Low}, FAP ^{Negative}, FSP1$^{Negative-Low}$, α-SMA ^{Negative}, PDGFRβ ^{Negative}, CAV1^{Neg}	
	CAF-S3	• CD29 ^{Medium}, FAP ^{Negative}, FSP1 $^{Medium-High}$, α-SMA $^{Negative-Low}$, PDGFRβ ^{Medium}, CAV1 $^{Negative-Low}$	
	CAF-S4	CD29 ^{High}, FAP ^{Negative}, FSP1 $^{Low-Medium}$, α-SMA ^{High}, PDGFRβ $^{Low-Medium}$, CAV1 $^{Negative-Low}$	
Ovarian cancer	CAF- S1	• FAP ^{high}, α-SMA $^{medium-high}$, FSP1 $^{medium-high}$, CAV1 ^{low} CD29 $^{medium-high}$, PDGFRβ $^{medium-high}$	[72]
	CAF-S2	• CD29 ^{low}, FAP ^{negative}, α-SMA $^{negative/low}$, FSP1 $^{negative-low}$, PDGFRβ $^{negative-low}$, CAV1 ^{negative}	
	CAF-S3	• CD29 ^{medium}, FAP ^{low}, α-SMA ^{low}, FSP1 $^{medium-high}$ PDGFRβ ^{medium}, CAV1 $^{negative-low}$	
	CAF-S4	• FAP ^{low}, α-SMA ^{high}, FSP1 ^{high}, PDGFRβ $^{medium-high}$ CAV $^{negative/low}$	
Metastatic Lymph nodes	CAF-S1	• FAP ^{High}, CD29 $^{Medium-High}$, α-SMA ^{High}, PDPN ^{High}, PDGFRβ ^{High}	[65]
	CAF-S2	• FAP ^{Negative}, CD29 ^{Low}, α-SMA $^{Negative-Low}$, PDPN ^{Low}, PDGFRβ ^{Low}	
	CAF-S3	• FAP $^{Negative-Low}$, CD29 ^{Medium}, α-SMA $^{Negative-Low}$, PDPN ^{Low}, PDGFRβ $^{Low-Medium}$	
	CAF-S4	• FAP $^{Low- medium}$, CD29 ^{High}, α-SMA ^{High}, PDPN ^{Low} PDGFRβ ^{medium}	

3. Tumor-promoting functions of CAF

A plethora of evidence inclines towards the pro-tumorigenic capabilities of CAF. CAF can directly promote propagation and invasiveness of cancer cells and indirectly affect the surrounding ECM, vasculature, and modulate immune functions to drive cancer progression. Fig. 2 shows a brief overview of the role of CAF secretion in several key hallmarks of cancer.

3.1. CAF-derived pro-tumorigenic factors and exosomes promote cancer progression

The propagation of cancer cells from the primary tumor mass is promoted by CAF that alter the epithelial-like phenotype of cancer cells to a more mesenchymal-like phenotype. This phenomenon is termed EMT and is responsible for tumor growth, malignancy, and drug resistance [73]. Past studies have reported the effect of CAF-

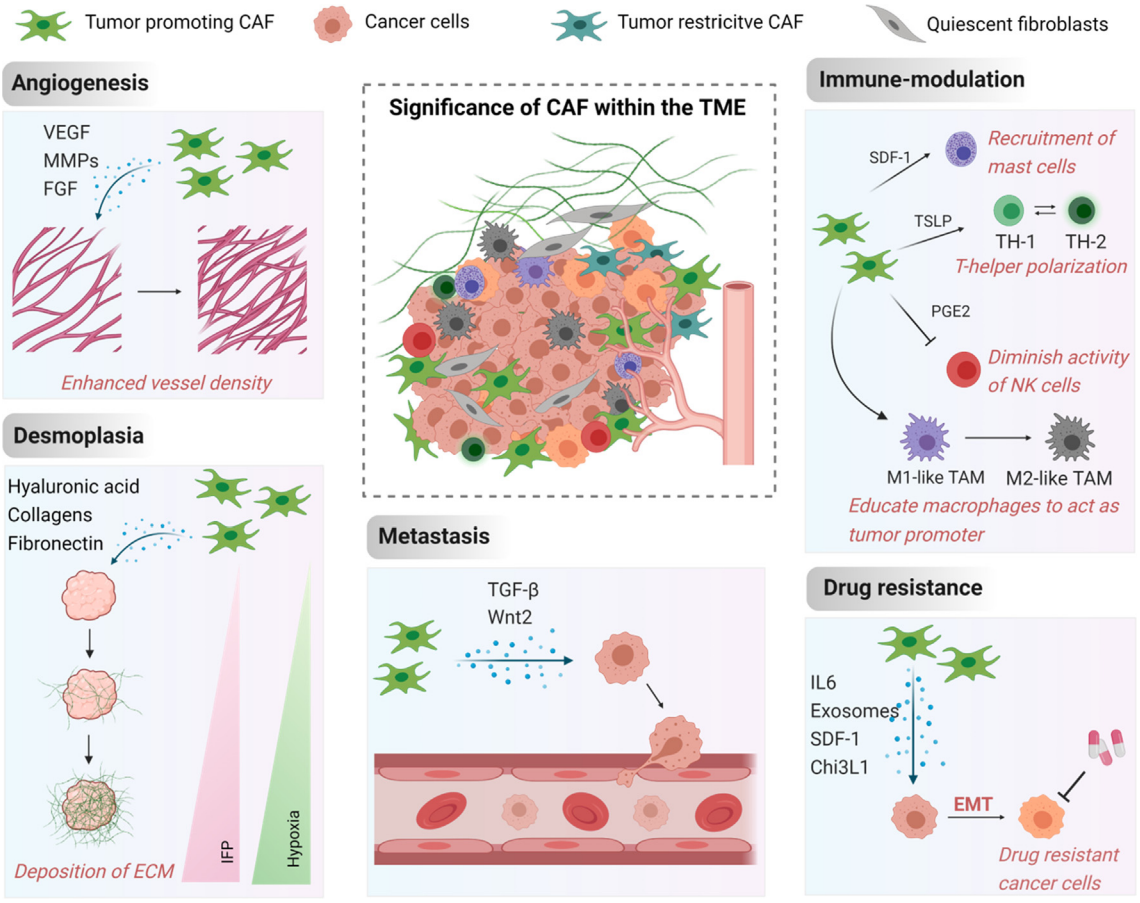

Fig. 2. Schematic overview of the role of factors secreted by CAF in several hallmarks of cancer. CAF influence several processes in the TME such as angiogenesis, desmoplasia, hypoxia, immune modulation, and EMT in cancer cells that result in tumor metastasis and high drug resistance. The illustration here depicts the complexity of the tumor microenvironment with CAF orchestrating the interaction of multiple cell types and processes.

secreted IL-6 in inducing EMT in several cancer types [34,74]. IL-6 *trans*-signaling activates the Janus kinases (JAK) family of tyrosine and activators of the transcription (STAT) family, particularly STAT3 [75]. The activation of the JAK1/STAT3 pathway leads to EMT in cancer cells. Additionally, other interleukins such as IL-33 released from CAF have been shown to induce EMT in cancer cells [76,77]. Table 3 lists several pro-tumorigenic paracrine activities of CAF on different tumor types.

Another mechanism by which CAF interact with cancer cells is via the secretion of exosomes. CAF-secreted exosomes comprise proteins, metabolites, and RNAs that have implications for cancer progression and therapy resistance. For instance, You et al. showed that CAF induce cancer cell EMT by releasing exosomes with increased levels of snail family transcriptional repressor 1 (SNAI1) expression [105,111]. SNAI1 is associated with tumor recurrence, increased cancer stem cell (CSC) activity, and control of tumor metabolism [112]. Another study showed that CAF secrete exosomes comprised miR-181d-5p leading to increased EMT in breast cancers [110]. Recently, Yang et al. showed that CAF-derived exosomes containing miR-210 led to lung cancer migration and proliferation. MiR-210 acted on the UPF1 via the PTEN/PI3K/AKT pathway activation [113]. CAF-derived exosomes also affect therapy. Gemcitabine (*GEM*), a standard chemotherapy drug, has been shown to induce the secretion of exosomes from CAF [108]. These exosomes are comprised of chemo-resistance stimulating factors and are taken up by cancer cells. It was observed that the exosomes consisted of miR-146 and SNAI1 mRNA. Once the exosome secretion from CAF was inhibited, there was a reduction in SNAI1 expression in cancer cells and a decrease in survival of the drug-resistant cancer cells [108].

3.2. Physical interaction of CAF with cancer cells and the ECM drives metastasis

Apart from the paracrine interactions, several studies have pointed toward the importance of direct cell–cell contact between CAF and cancer cells (Fig. 3) [114,115]. For instance, Labernadie et al. showed that CAF exerted a physical pulling force on the cancer cells that led to enhanced cancer invasion [116]. This pulling force was a consequence of the E-cadherin/*N*-cadherin junctions between the cancer cells and the CAF [116]. Impairment of the interaction between E-cadherin and *N*-cadherin decreased cancer cell invasion, which highlights a novel targeting strategy for the future [116]. CAF have also been shown to direct cancer cell migration and invasion by physically remodeling the surrounding ECM [117,118]. Gaggioli et al. showed that CAF generated tracks on the ECM that drive squamous cell carcinoma invasion [117]. The cancer cells retained their epithelial phenotype (absence of EMT) during the study thereby highlighting other mechanisms involved in cancer invasion. This study emphasized that cancer invasion requires a combination of secreted factors as well as physical remodeling. After the blockade of integrin α3, α5, or Rho–ROCK function in the CAF, the formation of tracks was reduced, thereby halting the invasion of the cancer cells [117].

It has been previously shown that CAF directly aid cancer cells in breaching the surrounding mesenteric basement membrane in localized tumors in the colon, thereby enhancing the propensity of colon cancer cells to metastasize [115]. This remodeling of the basement membrane was independent of the matrix metalloproteases (MMP) activity. The CAF within the TME not only utilized mechanical force to actively pull and stretch the basement membrane but also led to softening and remodeling of the basement membrane components. The alternate mechanism of CAF-mediated basement membrane breach could explain the failures of MMP inhibitors in clinical trials. These studies open an avenue for novel therapeutics focused on the ability of CAF to exert mechanical force on the surrounding ECM and basement membrane [115].

Recent studies have highlighted the contact-dependent intercellular exchange of genetic materials between the CAF and cancer cells via tunneling nanotubes [121–123]. Burt et al. showed that MSC-derived CAF can transfer mitochondria to acute lymphoblastic leukemia cells through tunneling nanotubes [119]. MSC exposed to reactive oxygen species transferred mitochondria to the cancer cells and prevented chemotherapy-induced cancer apoptosis. The reduction of mitochondrial transfer using microtubule inhibitors prevented this phenomenon. This study highlights how therapy-induced reactive oxygen species could trigger the activation of MSC into CAF and lead to therapy resistance in cancer cells via direct cellular interactions between CAF and cancer cells [119].

However, the direct cell–cell contact between CAF and cancer cells is not only limited within the TME. Previous results have indicated that circulating tumor clusters (CTCs) doṅt travel alone dur-

Table 3
The pro-tumorigenic activity of CAF in different cancer types.

Cancer type	Effect of CAF	Ref.
Ovarian cancer	• The release of FGF-1 by CAF promotes cancer invasiveness • CAF upregulates lipoma-preferred partner (LPP) in endothelial cells and increases vascular leakiness	[78–82]
Prostate cancer	• The release of lactate, enhance metabolic activity • Facilitate gland forming capability in cancer stem cells, Induce stemness of cancer cells	[18,83–88]
Colorectal cancer	• Release of IL-6 • Release of exosomes with high expression of miR-17-5p that leads to metastasis	[89–92]
Gastric cancer	• Promotes peritoneal dissemination • Involvement in cancer metastasis	[93–95]
Thyroid cancer	• Positive Expression of Notch1, TGF-β • Induces proliferation and invasion	[96,97]
Skin cancer	• Overexpression of collagen XI • Release of cytokines and chemokines that promote metastasis	[98]
Hepatocellular cancer	• Inhibit tumor apoptosis • Promote angiogenesis, immune suppression • Release of hepatocyte growth factor (HGF)	[99–103]
Lung cancer	• Increases cancer motility, Induce EMT • Regulate lung cancer stemness via IGFII/IGF1R	[104–106]
Pancreatic cancer	• Cancer cell proliferation via exosome signaling • Tumor growth via CXCL8 and FGF-2 signals	[107–109]
Breast cancer	• FGF-2 release by CAF promotes progression • Induction of EMT via exosomes containing miR-181d-5p	[32,110]

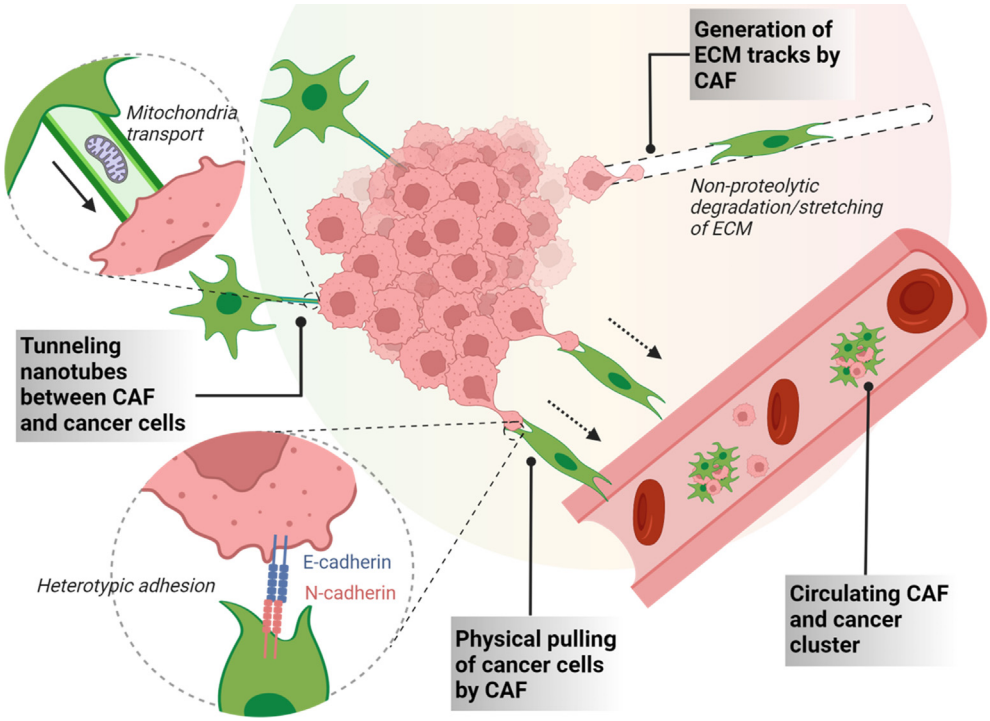

Fig. 3. Direct interactions between CAF and cancer cells and physical modulation of the ECM. The schematic depicts several mechanisms by which CAF directly drive metastasis. CAF directly interact with cancer cells via tunneling nanotubes or by E-cadherin/*N*-cadherin adhesion. CAF can remodel the basement membrane and ECM by physical stretching of the ECM strands. CAF also conjoin with cancer cells and physically protect cancer cells in the bloodstream. Image conceptualized from [116,117,119,120].

ing metastasis but together with passenger-CAF [124,125]. The passenger-CAF accompanies cancer cells to the secondary site where they support cancer cell survival and proliferation. Recently, Ortiz-Otero et al. showed that prostate cancer cells couple with CAF as cellular aggregates. CAF not only protect the cancer cells from the fluid shear stress but also preserve their proliferative capacity in the bloodstream [120]. These studies highlight the complex interplay between CAF and the cancer cells beyond paracrine signaling **(summarized in** Fig. 3**)**. It explains to an extent why targeting the cancer cells or CAF secretory factors alone does not result in efficient cancer therapy. Cancer cells have in their arsenal, various mechanisms to metastasize and evade therapy. Thus, targeting both the secretory factors as well as physical interactions between the CAF and cancer cells could be an alternative.

3.3. Interplay between CAF and cancer cell metabolism

Cancer cells are known to regulate their metabolism to survive and proliferate in nutrient-deficient conditions [126]. Cancer cells demand higher uptake of glucose and glutamine as compared to normal cells. Warburg observed that even in the presence of oxygen, tumor cells enhanced the uptake of glucose for ATP formation and enhanced the production of lactic acid [127]. Secretory factors from cancer cells shift CAF metabolism from oxidative phosphorylation to aerobic glycosylation (reverse Warburg effect), which in turn supports the metabolic demands of the cancer cells by providing lactate and pyruvate [128]. It has been shown that cancer cells initially secrete hydrogen peroxide in the surrounding microenvironment, thereby increasing oxidative stress in the CAF. As a result, the exposed fibroblasts shift their metabolic activity and display aerobic glycolysis [129]. In the same study, it was also observed that CAF exhibited increased glucose uptake, while cancer cells exhibited less dependence on glucose, possibly due to enhanced mitochondrial activity in cancer cells and reduced mitochondrial activity in CAF [129].

CAF also provide cancer cells with amino acids that are essential for tumor growth and progression. It was previously shown that activated PSC (fibroblast of the pancreas)-secreted non-essential amino acids that directly fuelled PDAC growth in nutrient-depleted conditions [130]. PSC was observed to secrete alanine, released via autophagy, which reduced cancer cellś dependency on glucose and serum nutrients. Recently, it was shown that CAF via the macropinocytosis of glutamine and the secretion of protein-derived amino acids facilitate PDAC survival [131]. In a glutamine limiting environment, CAF via CaMKK2-AMPK-RAC1 signaling enhanced macropinocytosis of glutamine from the surrounding environment. Moreover, suppressing aMKK2, ARHGEF2, or AMPK in CAF halted the macropinocytosis and reduced tumor growth [131].

CAF also undergo lipidomic modifications, which modulate cancer fate. It was shown in colorectal cancer patients that CAF enhance the secretion of exocrine lipids. There was a marked increase in fatty acids, phospholipids, and glycerides secretion by CAF, which were taken up by the colorectal cancer cells. This resulted in enhanced cancer cell migration. The cancer cell migration could be inhibited by targeting lipidomic interactions between CAF and cancer cells [132]. The metabolic perturbations in CAF as compared to normal fibroblasts can also be exploited for the imaging of the tumor site. For instance, the phenomenon of the reverse Warburg effect has been utilized in the past for the identification of high-risk breast cancer patients [133]. MCT4, a marker of hypoxia and aerobic glycolysis was immuno-stained and high MCT4 presence in CAF was correlated with decreased overall survival in breast cancer patients. This shows that the altered CAF metabolism can be utilized as a strategy for therapy and patient stratification [133].

3.4. Role of CAF in modulating angiogenesis

Increased angiogenesis, disorganized and leaky blood vessels are hallmarks of cancer [126]. Tumors require a continuous supply of nutrients and oxygen for their survival. Furthermore, cancer cells involve the vascular network to initiate the metastatic process [134]. Highly angiogenic TME directly correlates with a higher probability of metastasis and patient morbidity [135]. There is a growing number of studies that elucidate the influence of CAF on angiogenesis [136,137]. CAF can induce angiogenesis by secreting angiogenic factors, changing the surrounding ECM properties, and by downregulating angiogenic inhibitors [138,139]. CAF have shown to alter vascular fate in cancer microenvironment by the expression and secretion of factors such as VEGF [102,140,141], SDF-1 [42,142], IL6 [89], TGF-β [143–145], IGF [146], WNT2 [147], galectin-1 [55,148], HGF [149,150], HIF [151,152] and MMPs [153–155].

Apart from the release of soluble growth factors, CAF modulate the ECM stiffness and composition, which has implications in tumor angiogenesis. Bordeleau et al. elucidated the correlation between ECM stiffening and vascular phenotype [156]. Increased cross-linking of the matrix led to increased vascular outgrowth. Furthermore, increased matrix stiffness disrupted the VE-cadherin interaction between the endothelial cells which enabled vascular leakiness [156,157]. These studies, therefore, indicate that changes in ECM stiffness and composition can have a vast impact on vascular fate. Previous studies have indicated that CAF contribute to ECM deposition and reconfiguration leading to increased tumor progression and angiogenesis [158–160]. A study using *in-vitro* 3D models based on fibrin gel reported that CAF deform the surrounding ECM triggering vessel network formation [136]. CAF as compared to normal fibroblasts deformed the fibrin gel and resulted in vasculogenesis, which was attributed to the Rho/ROCK pathway and YAP signaling.

Many therapies in the past have attempted to halt cancer progression by inhibiting angiogenesis. In 2004, bevacizumab, a monoclonal antibody against VEGF-A was administered along with other chemotherapeutic agents in patients to treat metastatic colorectal cancer [161]. It was observed that the addition of bevacizumab significantly improved the survival rate of the patients. However, bevacizumab has failed to improve the survival of patients in several other cancer types [113,162,163]. Furthermore, targeting the ANG-TIE2 system (trebananib) along with VEGF (bevacizumab) as a combinatory therapy failed to improve the overall survival of patients with recurrent glioblastoma, leading to adverse effects [113]. Similarly, a phase III trial conducted with ramucirumab, a monoclonal antibody to VEGFR-2 along with a chemotherapeutic agent docetaxel failed to improve survivability in patients with metastatic breast cancer and led to increased toxicity [164].

One potential reason for the setback in anti-angiogenic therapies could be due to the interplay between CAF and the cancer cells. For instance, Crawford et al. showed that CAF can upregulate PDGF-C irrespective of the inhibition of VEGF, which can induce stability in blood vessels after VEGF inhibition[165]. Therefore, a future approach could be to inhibit angiogenic growth factors along with CAF as a combinatory therapy regimen.

3.5. Role of CAF in regulating the immune response

The hallmarks of cancer are chronic inflammation, immune cell infiltration, and the ability of cancer cells to evade immune response [126,166]. Immune cells such as natural killer cells (NK), T cells, tumor-associated macrophages (TAM), neutrophils, B cells, and dendritic cells (DC) are a vital part of the complex TME [167]. Both innate, as well as adaptive immune systems, are activated during the cancer pathogenesis and play a dual role of tumor suppressor as well as promoter. CAF play a pivotal role in supporting cancer cells to evade immune surveillance, recruit immune cells to TME and induce an immune-suppressive phenotype in immune cells [168].

It has been documented that there is an increased accumulation of TAM in the TME [138,139]. TAM has been shown to enhance tumor growth, angiogenesis, matrix remodeling, and immune-suppressive properties [169]. Growing evidence suggests that the crosstalk between CAF and macrophages tilts the macrophage behavior to function as a tumor promoter [88,199,200]. For instance, CAF educate M1-like TAM (anti-tumor immune response) to behave similarly to M2-like TAM (immune-suppressive response) with increased expression of CD163 and CD206, which are markers for M2 macrophages [170]. A recent study showed that CAF-secreted IL-6 promotes M2-like macrophage interaction with cancer cells as a consequence of the up-regulation of vascular cell adhesion molecule 1 (VCAM-1) expression in colorectal cancer cells [171].

Several studies have elucidated the potential of FAP-positive CAF in immune suppression [172–175]. For instance, Chen et al. showed that FAP-positive CAF induced immunosuppression and promoted immune checkpoint blockade resistance in a colorectal cancer mouse model [176]. The TME consisting of a high level of FAP positive CAF showed low resistance to anti PD-1 (programmed cell death protein 1) treatment. The study also showed that FAP-positive CAF restricted several anti-tumor immune response factors [176]. In a breast cancer mouse model, Cohen et al. showed that CAF secrete glycoprotein Chitinase-3-like-1 (Chi3Li) leading to immunosuppression [57]. CAF-secreted Chi3Li enhanced macrophage recruitment, induced M2-like phenotype, and inhibited T cell infiltration within the TME [57]. CAF also indirectly modulate the TAM infiltration to the tumor site by altering the surrounding ECM. For instance, Mazur et al. showed that CAF modify the surrounding collagen-1 by the secretion of FAP, which cleaves the collagen-1 to expose adhesion sites for macrophages [177]. Increased adherence of TAM on CAF-mediated cleaved collagen I substrate was observed as compared to untreated collagen I. This led to a higher accumulation of TAM in the TME.

NK cells have the proclivity to recognize tumor cells and eliminate them [178]. However, tumor cells can escape NK-mediated immune response and weaken the activity of NK cells with the support of TME [179]. Inoue et al. showed that the tumor-killing potential of NK cells was decreased due to the downregulation of poliovirus receptors in CAF [180]. In another study, Balsamo et al. showed that CAF inhibited NK activity in killing melanoma cells [181]. CAF through cell–cell contact as well as secretion of prostaglandin E2 (PGE_2) inhibited the expression of NKp44 and NKp30 activating receptors, thereby modulating NK behavior [181]. In another study, CAF-educated TAM prevented NK activation and recruitment [171]. Similar studies have shown that M2-like macrophages impair NK function [182,183].

CAF also manipulate *T*-cell function in the TME. For instance, Lakins et al. elucidated the effect of CAF on $CD8^+$ T cells [184]. CAF cross-presented tumor-derived antigens which led to the suppression of *T*-cell toxicity and resulted in *T*-cell death [184]. Similar studies have reported CAF-induced *T*-cell modulation and death in TME [35,185–187]. Apart from the immune-suppressive ability of CAF, they also partner up with several immune cells and gain reciprocal growth signaling. For instance, mast cells facilitated PSC proliferation via cell–cell communication and the release of soluble factors [188]. Mast cells were observed to secrete IL-13 and tryptase after tumor-mediated degranulation leading to PSC proliferation and pancreatic cancer growth [188]. In another study, it was observed that *T*-cells and CAF mutually supported one another by a reciprocal stimulatory feedback loop [189]. CAF

released IL-6 among other soluble factors, stimulated *T*-lymphocytes to release IFN-g and IL-17A, whereas *T*-cells released soluble factors that stimulated CAF to produce IL-6 [189].

Overall, the interplay between CAF, cancer cells, and immune cells is a significant hurdle in the treatment of malignant tumors. anti-CAF treatments in the future could be utilized to trigger a positive immune response against cancer cells. An overview of CAF targeting to elicit an immune response against the tumor is reviewed here [28].

3.6. CAF contribute to therapy resistance in tumors

One of the main hindrances in the treatment of cancer is the ability of cancer cells to develop resistance to therapy [74]. Cancer cells have an intrinsically built mechanism to evade chemo and radiotherapy [74]. On top of it, TME actively augments the drug resistance capability of cancer cells [55]. In this regard, the role of CAF has been extensively investigated. CAF regulate the cancer cells and other cell types in the TME through the exchange of soluble regulatory factors via paracrine signaling, by inducing EMT in cancer cells, maintaining the CSC stemness, and by modulating the ECM [114].

Herein, we discuss in more detail the influence of CAF on the progression and drug resistance of three major tumor types- lung, pancreatic, and breast tumors. In a 2018 statistical study, lung and breast cancers were found to be the most prominent forms of cancer with both cancer types having an incidence rate of 11.6 % worldwide [75]. Lung and breast cancer have a high cancer death percentage of 18.4 % and 6.6 % respectively. Pancreatic cancer, although not as common as lung and breast cancer, has a low survival rate of 9 % for 5 years. In the European Union, it is estimated for the year 2025 that pancreatic cancer will exceed breast cancer deaths by 25 % and will rank as the third leading cause of cancer deaths [190]. Taking these three main cancer types into consideration, we will discuss several mechanisms by which CAF contribute to drug resistance in cancer cells.

3.6.1. Therapy resistance in pancreatic cancer

The evidence of the involvement of CAF in pancreatic cancer progression and drug resistance is overwhelming [191]. PDAC, the most common type of pancreatic cancer, has a very high mortality rate and is characterized by strong desmoplasia [192]. CAF release several soluble factors that lead to drug resistance in PDAC. In a study, Wei et al. showed that pancreatic CAF secrete SDF-1, which leads to cancer malignancy and resistance to *GEM* [193]. SDF-1 secreted by CAF via the SDF1/CXCR4 axis upregulated Special AT-rich sequence-binding protein 1 (SATB-1) in the cancer cells resulting in their proliferation and drug resistance [193].

CAF also promotes the secretion of various ECM proteins that support pancreatic cancer progression. A recent study showed that pancreatic CAF deposit heparan sulfate proteoglycan 2 (HSPG2) or perlecan via NFκB signaling leading to increased pancreatic cancer invasiveness [194]. Interestingly, highly aggressive cancer cells with gain-of-function mutant P53 (GoF p53mut), educated the surrounding CAF to deposit perlecan providing an invasive milieu to the cancer cells. It was also observed that the educated-CAF elevated the invasiveness of less-aggressive P53-null cancer cells in the deposited matrix. This highlights that in a highly heterogeneous pancreatic cancer environment, cancer cells with less aggressive phenotypes can metamorphose to a metastatic phenotype in the presence of educated-CAF in their remodeled matrix [194]. The study further emphasized that mutant cancer-educated CAF assisted in *GEM*/Abraxane resistance via direct cell–cell contact and secretions of perlecan. The reduction of perlecan deposition reverted drug resistance in aggressive cancer cells [194].

A type of pancreatic CAF, PSC are quiescent pluripotent cells that constitute 4 to 7 % of the entire pancreatic tissue and activate into myofibroblast-like cells in the TME. Activated PSCs are highly migratory and upregulate the production of various sugars and ECM proteins such as collagens, laminins, fibronectin, and HA [195–198]. The pancreatic cancer environment may constitute up to 80 % of secreted ECM leads to a fibrotic and hypoxic environment and restricts immune cell and drug penetration to the tumor mass [199]. The growth of the tumor mass, its interaction with stromal components as well as the accumulation of ECM lead to a highly hypoxic microenvironment around the tumor vicinity that affects cancer progression and resistance to therapy [200]. The resulting hypoxic environment leads to EMT of pancreatic cancer cells and increases their ability to resist therapy [201]. In an *in-vivo* study, Bachem et al. injected pancreatic cancer cells (PC) alone or in conjunction with PSC in nude mice [202]. The combined PSC-PC injection led to faster tumor growth (3.2-fold higher tumor volume) as compared to the injection of PC alone. Immunofluorescent staining of collagen I, collagen III, and fibronectin showed a stronger presence of these ECM moieties when PC was injected together with PSC as compared to PC alone [202]. Hwang et al. showed that treatment of PC with conditioned media from human PSC led to higher invasiveness, proliferation, and resistance to radiation therapy as well as to *GEM* treatment [203].

An increase in ECM deposition also affects other cell types in the TME. In pancreatic cancer, the elevation of ECM and subsequent tissue pressure leads to the collapse of vasculature and provides a hindrance in drug penetration to the cancer cells. Provenzano et al. showed that the abundance of HA in the fibrotic pancreatic cancer ECM leads to collapse in vasculature due to elevation in the IFP [204].

3.6.2. Therapy resistance in lung cancer

Numerous studies have revealed the role of CAF in lung cancer growth and metastasis [33,106,205–207]. Furthermore, CAF are shown to induce drug resistance and lung tumor activation by the crosstalk of several inflammatory cytokines, exosomes, and growth factors [104,208,209]. One mechanism by which lung cancer cells acquire chemo-resistance is via CAF-mediated induction of EMT [208–211].

In the case of NSCLC, there is a prominent expression and mutation of the EGFR [212]. The activation of EGFR results in downstream pathways that are responsible for the proliferation, survival, and differentiation of cancer cells. EGFR-tyrosine kinase inhibitors (EGFR-TKI) such as Gefitinib and Erlotinib are clinically used to target the EGFR and show promising results in the initial application of the drug. However, as the treatment progresses, the NSCLC develops resistance to further EGFR-TKI treatment [213]**.** One possible mechanism by which the NSCLC develops resistance to TKI is via crosstalk with CAF. The secretion of soluble factors such as hepatocyte growth factors (HGF), Wnt16B, GLI1, and Annexin-A2 by CAF induce TKI resistance in the lung cancer cells [214–217]. Excess HGF triggers the overexpression of the hepatocyte growth factor receptor (MET) [218]. Bypass activation of MET allows cancer cells to depend on MET receptors rather than EGFR receptors for proliferation, survival, and differentiation [219] **(**Fig. 4). A previous study showed that NSCLC gains Erlotinib (an EGFR-TKI) resistance by upregulating GLI1 transcriptional activity and SMO-induced modulation of the actin cytoskeleton in CAF [220].

Previous research has indicated that CAF in the lungs can manipulate CSC plasticity [104]. CSC is linked with chemotherapeutic drug resistance [221]. It can be implied that CAF-mediated CSC plasticity could drive drug resistance and cancer metastasis [222,223]. In a previous report, it was shown that the presence of CAF maintained the stemness of the CSC and assisted in CSC

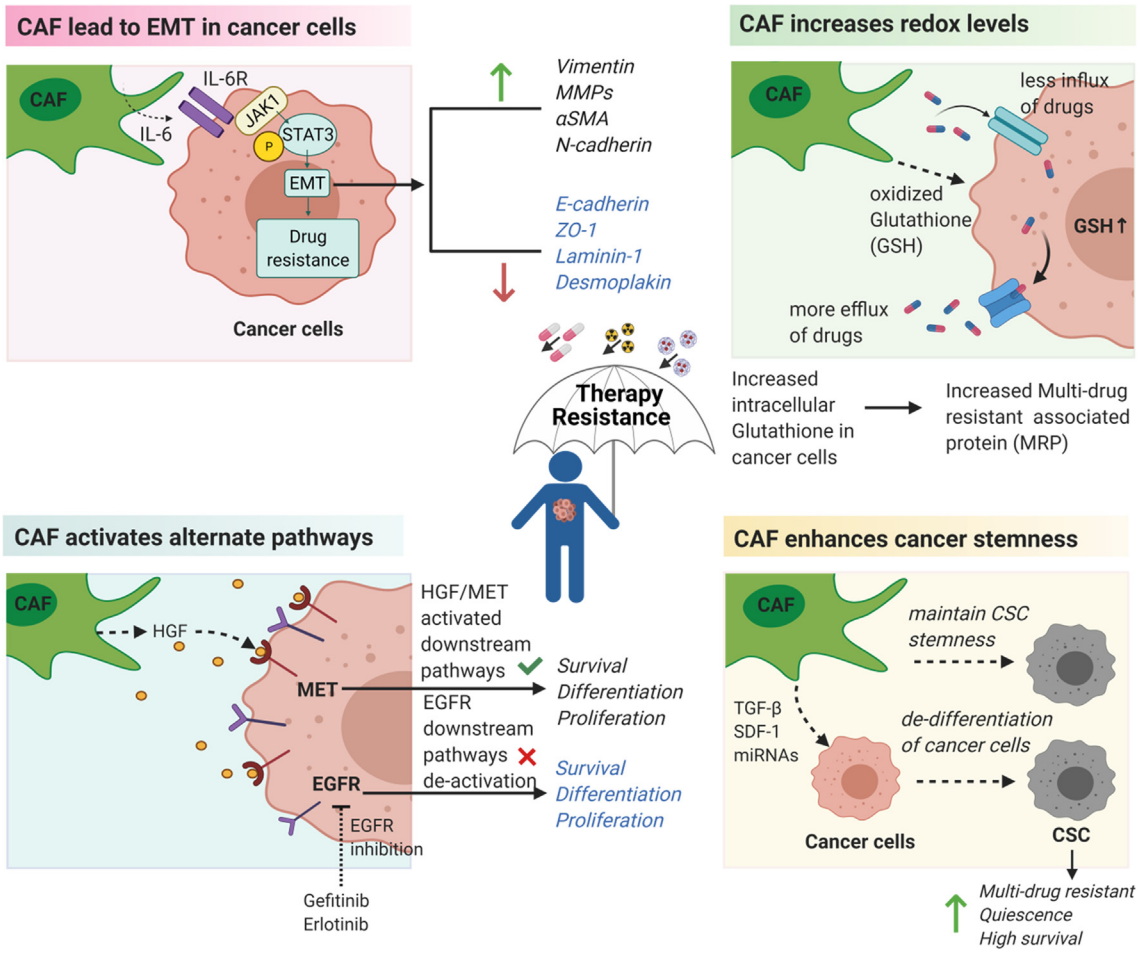

Fig. 4. CAF contribute to therapy resistance in cancer cells. CAF utilize several mechanisms to promote resistance to therapy. These include the release of paracrine signaling such as IL-6 that promotes EMT in cancer cells and the release of signals that trigger de-differentiation of cancer cells to CSC. CAF also promote the maintenance of CSC stemness. CAF in breast cancer release HGF that triggers overexpression of MET receptors thereby bypassing EGFR inhibition. CAF enhances intracellular glutathione levels in prostate cancer cells resulting in a high efflux of drugs from cancer cells.

growth via paracrine signaling, whereas the removal of CAF resulted in the differentiation of CSC into cancer cells (Fig. 4) [104]. CAF released factors such as IGF-II, HGF, and SDF-1, which facilitated the maintenance of CSC stemness. Interestingly, it was shown that lung cancer cells could de-differentiate to CSC in the presence of CAF [104].

3.6.3. Therapy resistance in breast cancer

CAF induce EMT in BCC, which eventually leads to therapy resistance [30,224–228]. For instance, Wen et al. showed that CAF released IL-32 that binds to integrin-β3 present on the BCC membrane. Interaction of IL-32 and integrin β3 resulted in the activation of P38 MAPK signaling followed by cancer cell EMT, and subsequent BCC invasion [40]. CAF also communicate with the tumor cells via exosomes [229,230]. Donnarumma et al. showed that CAF in a breast cancer environment delivered exosomes containing microRNAs (miRs −21, −378e, and −143) to the BCC which resulted in EMT of the cancer cells [231].

CAF increase the resistance of BCC to therapy [232]. Estrogen receptor (ER), a steroid receptor is a well-known BCC marker that is elevated in malignant breast cancers [233]. Tamoxifen has been used for the past 30 years in targeting ER-positive breast tumors but has a diminished rate of success due to BCC drug resistance [234]. Patients develop either immediate resistance to Tamoxifen or develop late-phase acquired resistance after successful initial therapy. Sun et al. observed that BCC showed Tamoxifen resistance due to the release of IL-6 by CAF. The secreted IL-6 induced EMT in BCC and decreased the ER-α expression by the degradation of the protein via the ubiquitin–proteasome pathway [51,235]. Yuan et al. showed that the interaction of CAF and integrin β1 on the BCC membrane results in G protein-coupled estrogen receptor (GPER)-mediated resistance of BCC to Tamoxifen [236].

Human epidermal growth factor receptor 2 (HER2) overexpression is prevalent in approximately 20 % of breast cancer cases [237]. Trastuzumab, a monoclonal antibody to HER2 has shown promising outcomes in treating HER2 expressing tumors. Recently, Sonnenblick et al. showed in phase III clinical trial studies that HER2-positive breast cancer patients with reactive stroma have a stronger resistance to therapy [238]. This increase in resistance to therapy could be attributed to a multitude of factors including but not limited to immune suppression by CAF, the release of soluble growth factors and cytokines, and the presence of integrin and ECM [238–241].

Apart from lung, breast, and pancreatic cancer, the majority of other cancer types also display CAF-mediated therapy resistance. One mechanism by which CAF promote drug resistance and cancer cell survival in prostate tumors is by increasing the intracellular glutathione (GSH) levels (Fig. 4). GSH is a known antioxidant against oxidative stress. Treatment with doxorubicin (DOX) is known to induce high levels of oxidative stress [242]. It is a possible mechanism wherein CAF-derived GSH plays a role in the efflux of DOX to counteract the induced oxidative stress. This process

Table 4
Examples of CAF-mediated drug resistance in different cancer types.

Cancer type	Mechanism	Ref.
Colorectal cancer	• CAF-derived interleukin-17A (IL-17A) maintains cancer-initiating cells • CAF induce the expression of hedgehog transcription factor *GLI2 in CSCs*	[245,246]
Bladder cancer	• Cisplatin resistance by enhancing ERβ/Bcl-2 signaling • Upregulation of anti-apoptotic genes	[247]
Ovarian cancer	• Cisplatin resistance in cancer cells by promoting STAT3 signaling	[248]
Esophageal squamous cell carcinoma	• CAF-secreted IL-6 leads to drug resistance	[249,250]
Gastric cancer	• IL-8 expression led to activation of NF-kβ and enhance cisplatin resistance	[251]
Melanoma	• Activation of MAPK and PI3K/Akt signaling pathways by hepatocyte growth factor via the MET receptor • Weakening of the BRAF inhibitor via increased integrin β1/focal adhesion kinase (FAK)/Src signaling	[61,252]

leads to poor accumulation of DOX, either due to decreased influx of DOX or increased efflux of DOX [243]. Drug resistance and poor accumulation caused by elevated GSH levels were also studied in ovarian cancer cell lines [244]. Table 4 provides some more examples of CAF-mediated drug resistance in different tumor types.

3.7. The tumor-suppressing function of CAF

The function of distinct CAF subpopulations as a tumor promoter or a suppressor is yet to be deliberated in its entirety. Although the evidence of the former outweighs the latter, the ability of certain subpopulations of CAF in resisting tumor malignancy should also be considered to fully understand cancer progression and prevent mishaps in clinical trials.

CAF build a desmoplastic environment around the tumor site to prevent cancer invasion. The activated fibroblasts simply respond to the unnatural stimulations generated by the cancer cells and present a defense mechanism to prevent further damage. Özdemir et al. showed that the depletion of α-SMA$^+$ CAF in PDAC led to increased tumor invasion in mouse models [253]. Depletion of CAF resulted in decreased tissue stiffness, total collagen content, and tumor vasculature. However, the intra-tumoral hypoxia and the number of CSC were enhanced. Strikingly, the reduction of CAF led to modulation of the immune system resulting in the decreased effector *T*-cells to regulatory *T*-cell (Teff/Tref) ratio. Furthermore, depletion of CAF in early-stage of the disease led to reduced infiltration of *T*-cells and B-cells within the TME [253].

It has also been shown that the stromal component prevents PDAC progression. The therapies that deplete the deposited ECM led to higher mortality outcomes for patients [254]. It has also been shown that a distinct subpopulation of CAF is restrictive to cancer in functionality [255]. For instance, Mizutani et al. showed that meflin-positive CAF in PDAC restricted tumor progression by depleting α-SMA positive subsets of CAF and ECM configuration [256]. It is therefore evident that CAF can be divided into two main subpopulations- one that is tumor-restrictive (rCAF) and the other that is tumor-promoting (pCAF). It is not fully understood whether the tumor-suppressing function of the CAF and the surrounding desmoplastic environment (secreted by the CAF) is limited only to specific cancer types such as pancreatic cancer or whether other cancer types too exhibit such outcomes. Single-cell RNA sequencing (scRNA–seq) experiments in the future could clarify the role of different subsets of CAF in different cancer types. A more focused discussion on the tumor-suppressing capabilities of CAF is provided in this review article [257].

In conclusion, therapies that target CAF should also consider the rCAF and pCAF subpopulations. It is yet to be elucidated whether selectively targeting distinct subpopulations of CAF while simultaneously preserving restrictive subpopulations has added benefits in therapy.

4. Imaging CAF

Imaging plays a crucial role in diagnosis and therapy monitoring. To image CAF, several probes are being developed that specifically target CAF and its processes. By doing so, researchers have an advantage to better understand the molecular processes and thus develop novel small molecule drugs and nanocarriers. The next half of this review will focus on a) how CAF and their associated processes can be imaged, and b) how CAF can be targeted using small molecule drugs and nanocarriers.

CAF are considered the main mediator of tissue fibrosis and possess unique expression profiles contributing to tumor-associated fibrosis [258,259]. Fibrosis is defined as the thickening or scarring of various tissues arising from tissue damage or inflammation that can lead to organ failure [260,261]. The excessive accumulation of collagen in fibrosis is very similar to the desmoplastic reaction or ECM production in different tumors [261–263]. Pancreatic and liver cancers are examples of characteristics of tumor-associated fibrosis, where 60–70 % of tumor tissue is composed of desmoplastic stroma. The role of CAF-mediated fibrosis to promote tumor proliferation and metastasis is well-known and is often associated with poor prognosis [264]. The dense collagen networks also restrict the diffusion and delivery of small molecular drugs and especially nano-based carrier systems. Proper and early diagnosis of fibrosis is crucial to develop a treatment plan or new therapies to suppress collagen networks, especially for desmoplastic tumors.

A biopsy is used as a gold standard to diagnose fibrosis. However, the limitations of biopsies such as invasiveness, sampling variability, and representing only a static snapshot of the total organ impede its use and highlight the need for better strategies [265]. Most of the studies now focus on targeting markers that are overexpressed in CAF as compared to normal fibroblasts. Some commonly expressed fibroblast markers such as α-SMA, FAP, vimentin, FSP1/S100A4 (S100 calcium binding protein A4), and PDGFR-α/β are used to target CAF, although these markers do not specifically define CAF [54,259,261], which represents a major challenge for the development of new therapies. Patient stratification either based on target expression or target engagement is currently lacking and is crucial owing to the vast heterogeneity of the fibroblasts population within and among patients.

Clinical non-invasive molecular imaging techniques, such as magnetic resonance imaging (MRI), positron emission tomography (PET), and single-photon emission computed tomography (SPECT) have been employed to detect tumor fibrosis. While molecular imaging has the potential to enable personalized care for different cancers, its application in understanding fibrotic tumors is limited. With the development of different contrast agents/peptides/antibodies and the revealing different markers expressed on fibrotic tumors, there is a growing demand for the use of molecular imaging techniques. An ideal probe should target molecular processes/receptors that are highly expressed in fibrotic tumors.

4.1. Fibroblast activation protein (FAP) imaging

Peptide-based/antibody-based endogenous radiotherapy has also been investigated to target the TME, particularly CAF. The most common marker expressed by CAF is the FAP, which acts as a potential target [266,267]. A method to image CAF using a FAP-specific enzyme inhibitor (FAPI) has been developed where FAPI-01 and FAPI-02 were synthesized for labeling with iodine-125 (^{125}I), lutetium-177 (^{177}Lu), and gallium-68 (^{68}Ga). Synthesis of FAPI-01 was achieved by an organotin stannylated precursor, and a bifunctional chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was chemically-linked for the incorporation of radiometals. FAPI-02 was obtained using DOTA as a precursor. [^{68}Ga]Ga-FAPI-02 showed better bio-distribution, tumor accumulation, and biochemical properties, which were further confirmed using [^{177}Lu]Lu-FAPI-02. Furthermore, [^{68}Ga]Ga-FAPI-02 also showed higher tumor accumulation in breast and pancreatic cancer metastases in humans ($SUV_{max\ avg}$ 1 h p.i. = 7.6 and 13.3 for pancreatic carcinoma and mammary carcinoma, respectively) as well as with locally advanced adenocarcinoma [268–270].

To further optimize tracer uptake and retention, derivatives of FAPI were developed [271]. Different FAPI derivatives (FAPI-02 - FAPI-15) were developed and tested *in vitro* for binding capacity using human embryonic kidney cells expressing murine FAP and dipeptidyl peptidase 4 (CD26). CD26 shows high homology to human FAP. Among these, FAPI-02 and FAPI-04 showed excellent serum stability, slower excretion, and specific affinity for FAP. Furthermore, FAPI-13 was also tested *in vivo* due to its resemblance to FAPI-04 and low EC_{50} [271]. *In vivo* studies performed with FAPI-02, FAPI-04, and FAPI-13 showed the highest tumor accumulation of FAPI-04 and FAPI-13 compared to FAPI-02, with a favorable tumor to blood ratio with FAPI-04. This was further confirmed by labeling FAPI-04 with ^{177}Lu. FAPI-04 was tested clinically in two patients with breast cancer, and in both the patients, the FAPI-04 accumulation in breast metastases was strong (SUV_{max}, 7–15.5 for patient 1 and 15.3–29.9 for patient 2) [271]. In addition to imaging, one patient also received 2.9 GBq of yttrium-90 ([^{90}Y]Y-FAPI-04) which showed accumulation of the tracer at 3 h and 24 h p.i. Clinically, this dose resulted in the reduction of pain medication, showed no toxicity, and uptake in healthy organs was low. Since FAPI-04 was developed by the incorporation of the 4,4-difluoroprolyl building block, this modification could have led to the prolongation of tumor retention time [271].

Chen et al. compared [^{68}Ga]Ga-DOTA-FAPI-04 with ^{18}F-FDG for the diagnosis of primary and metastatic lesions in patients with different types of cancers, mainly liver metastases and brain tumors using hybrid PET/CT (PET/Computed tomography). The results indicated that [^{68}Ga]Ga-DOTA-FAPI-04 had better sensitivity and diagnostic efficiency compared to ^{18}F-FDG [272]. Furthermore, the same group showed improved therapeutics by detecting malignancies in a patient with rectal cancer using [^{68}Ga]Ga-FAPI PET/CT [273]. Kratochwil et al. also showed better contrast and higher uptake of [^{68}Ga]Ga-FAPI-04 PET/CT in patients with 28 different types of cancers [274]. [^{68}Ga]Ga-FAPI-04 also accumulated in the injured myocardium, thereby representing a promising radiotracer for *in vivo* imaging of post-myocardial infarction fibroblast activation [275]. This compound also showed promising clinical results in patients with lower gastrointestinal malignancies [276]. Interestingly, Khreish et al. also observed that [^{68}Ga]Ga-FAPI-04 PET/CT showed intense tracer uptake in a 77-year-old patient with prostate cancer but developed prostate-specific membrane antigen (PSMA) negative phenotype due to high inter/intra lesion heterogeneity. Therefore, [^{68}Ga]Ga-FAPI-04 was tested and showed intense tracer uptake in all metastases, which demonstrated that [^{68}Ga]Ga-FAPI-04 could be used as a prognostic marker in prostate cancer [277]. An example of radiolabeled FAPI-04 is shown in Fig. 5**a**.

Watabe et al. radiolabeled FAPI-04 with a diagnostic β emitter copper-64 (^{64}Cu) and a therapeutic α emitter actinium-225 (^{225}Ac) and injected it in a pancreatic cancer xenograft mouse model. The results demonstrated that [^{225}Ac]Ac-FAPI-04 showed significant tumor growth suppression compared to control and therefore presented this data as a proof of concept to treat FAP-expressing pancreatic cancer. However, the study depicted only the Panc-1 mouse model, and further evaluation of different cancer models is essential to test its therapeutic effects [281]., The half-life of ^{225}Ac is much higher than the retention rate of the tracer. Thus, more research to fabricate FAP-targeting agents that can retain the therapeutic radionuclide longer in tumors is essential. Regardless of successful tumor accumulation and detection using radiolabeled DOTA FAPI-04, a further increase in retention time, and higher tumor-to-organ ratios were observed with FAPI-46 radiolabeled with ^{68}Ga and ^{177}Lu clinically **(**Fig. 5**b, 5c)** and pre-clinically, respectively. In FAPI-46, the linker region's modification between the quinolone moiety and the chelator might be the reason for higher retention and low normal tissue accumulation [280,282].

In general, [^{68}Ga]Ga-FAPI-PET/CT can detect both primary and metastatic tumors and shows promising results in uptake efficiency. This compound could be an ideal radiotracer to detect and target different kinds of FAP-positive tumors, thereby ensuring higher anti-tumor therapeutics. Different FAPI versions have also shown promising results clinically and pre-clinically in detecting prostate cancer and myocardial infarction fibroblast activation [283,284].

4.2. Imaging CAF-derived fibrosis

4.2.1. Collagen-based imaging probes

Excessive collagen deposition is another common hallmark for fibrosis and desmoplastic reaction in certain solid tumors making it a potential target. Several collagen targeting molecular probes have been developed and tested pre-clinically and clinically for fibrosis, in particular.

Using this imaging approach, Polasek et al. developed a peptide-based gadolinium-containing molecular MR probe (EP-3533) as an imaging biomarker for fibrosis. EP-3533 specifically targeted type I collagen in a pancreatic cancer mouse model and was successfully used to visualize and quantify fibrosis [285]. The same group previously showed the successful detection and staging of fibrosis using EP-3533 in liver fibrosis [286–288], pulmonary fibrosis [289], and cardiac fibrosis models [290]. However, this MR probe resulted in some retention of gadolinium in bone and other tissues [287,291], making it unsuitable for clinical translation. To enable clinical translation, the same group developed a collagen targeting probe (CM-101) that uses a stable macrocycle gadoterate meglumine (Gd-DOTA) chelate, which possesses a high kinetic and thermodynamic stability than the previously used linear gadopentetate dimeglumine chelate [292,293]. The study demonstrated in two different rat models (liver fibrosis induced by CCl_4) and bile duct ligation (BDL) showed fast blood clearance of CM-101, negligible accumulation of gadolinium in bone and kidneys, and accurate detection of fibrosis.

Furthermore, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) has been used to assess cancer-associated stroma, due to its ability to detect morphological and functional characteristics of tumor vasculature *in vivo*. Farace et al. used DCE-MRI with two different small molecules namely, the standard gadolinium chelate (Gd-DTPA) and an albumin binding MS-325 (Vasovist®) contrast agent to evaluate two different tumor models

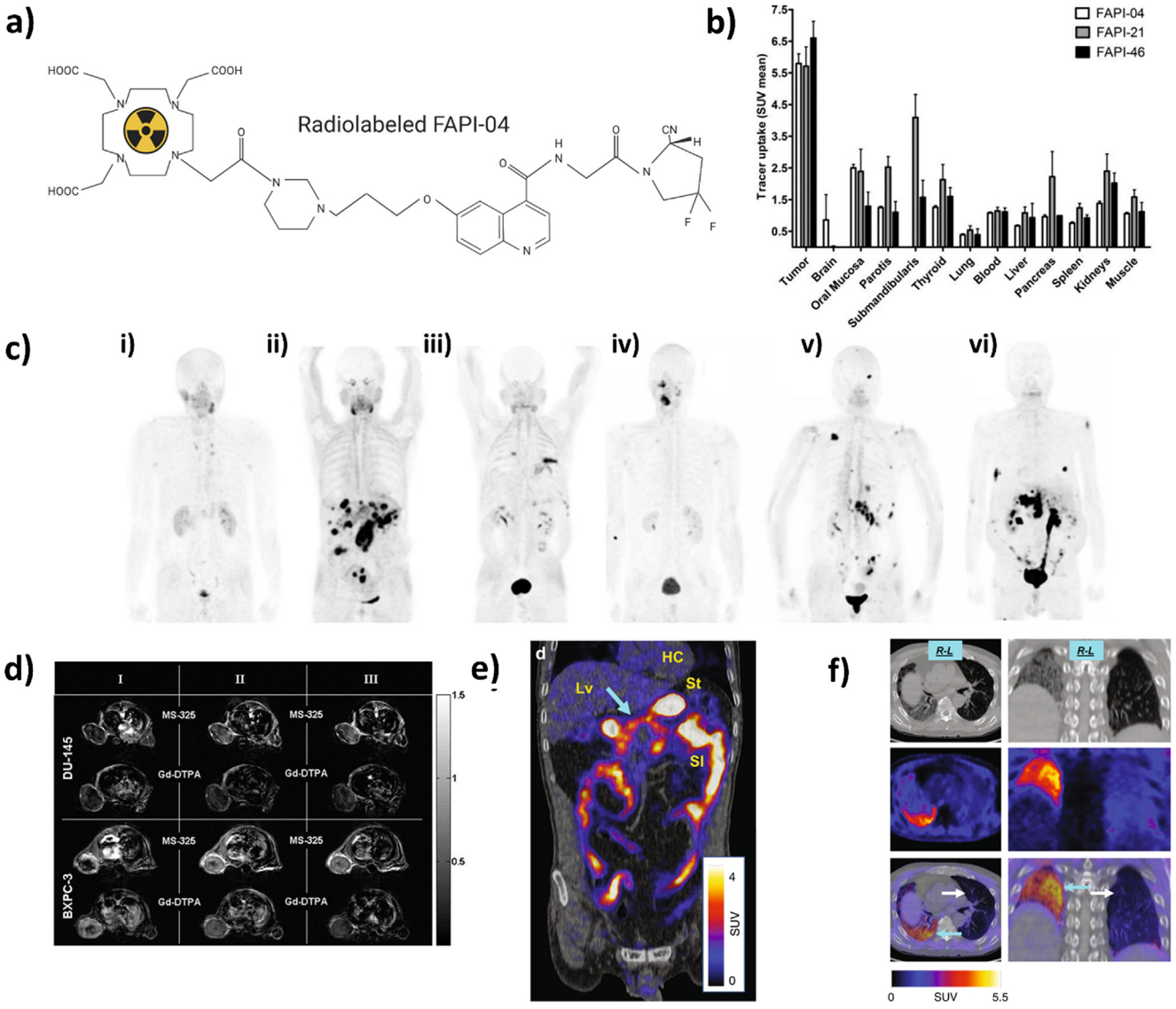

Fig. 5. Different probes used pre-clinically and clinically to image CAF. a) An example of radiolabeled FAPI-04 used clinically to image CAF. b) Tracer uptake (SUV_{mean}) of ^{68}Ga-labeled FAPI-04, FAPI-21, and FAPI-46 in tumor and healthy organs of patients suffering from metastasized mucoepidermoid, colorectal, ovarian, oropharyngeal, and pancreatic carcinoma. c) Whole-body PET/CT imaging of tumor patients with metastasized mucoepidermoid, oropharynx, ovarian, and colorectal carcinoma (i-vi) after 1 h intravenous administration of [^{68}Ga]Ga-FAPI-21 (i-iii) and [^{68}Ga]Ga-FAPI-46 (iv-vi) showing rapid accumulation of the radiotracer in primary tumors and metastases. d) Higher accumulations of an albumin-binding contrast agent (MS-325) compared to gadolinium-DTPA contrast agents in DU-145 and BxPC-3 tumors at early and late phases. e) Coronal PET/CT acquired from a patient diagnosed with pancreatic cancer. The light-blue arrow indicates tumor location with the highest accumulation of [^{68}Ga]Ga-NODAGA-$R_0$1-MG observed in the pancreas. f) PET/CT images of a 72-year-old male IPF patient. The right fibrotic lung shows the highest accumulation of [^{18}F]FP-$R_0$1-MG-F2. The white arrow indicates healthy lungs and the cyan arrow indicates fibrotic lungs. Images reproduced, with permission, from [278–280]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(prostatic DU-145 and pancreatic BxPC-3). The pancreatic BxPC-3 displayed a much higher stromal content compared to prostatic DU-145. The authors showed that MS-325 surpassed Gd-DTPA by producing enhanced MRI dynamic scans in BxPC-3 xenograft models, only during the early time points. At a later time point, the differences between the two models were unclear due to the washout observed specifically in BxPC-3 tumors. This was mainly related to the high stromal content in BxPC-3 tumors (Fig. 5**d**) [278].

The very first radiolabeled collagen-specific peptide was developed by Muzard et al. and was studied in a rat model of myocardial infarction and a mouse model of induced lung fibrosis. The peptide collagelin was radiolabeled with ^{99m}Tc (Technetium-99m) for SPECT imaging. They showed that ^{99m}Tc-collagelin accumulated to a greater extent in fibrotic scars in both the animal models compared to the peptide control (^{99m}Tc-*Pc*) [294]. Advancement was made with ^{99m}Tc-labeled collagen probes by Zheng et al., who developed ^{99m}Tc-CB1495. The peptide CB1496 (CPKESCNLFVLKD) showed a high affinity for type I collagen and an additional sequence Gly-(D)-Ala-Gly-Gly was introduced to couple ^{99m}Tc. In a fibrotic rat model, the authors showed a positive correlation of ^{99m}Tc-CB1495 uptake with hydroxyproline content in diseased areas. The imaging contrast obtained with ^{99m}Tc-CB1495 was better than ^{99m}Tc-collagelin. However, this study reports a very short circulation time of ^{99m}Tc-CB1495, and further studies are required to prolong its circulation time. Further, using bioinformatic models, the authors aim to improve the collagen affinity of CB1495 [295].

A similar collagen-binding peptide (CBP7) radiolabeled with ^{64}Cu was designed by Désogère et al. for PET imaging. The use of [^{64}Cu]Cu-CBP7 was not considered feasible for clinical translation

due to the high retention of CBP7 in the kidney (>60 % ID/g) of mice [296]. Thus, to improve on this front radionuclide with a short half-life such as ^{68}Ga or ^{18}F was preferred. The same group modified the peptide with a chelator to enable radiolabeling with ^{68}Ga and ^{18}F. ^{18}F-CBP8 demonstrated higher uptake in the lungs of control mice, suggesting aggregation [297]. [^{68}Ga]Ga-CBP8 was tested in a pulmonary fibrotic mouse model, where the probe demonstrated greater affinity with type I collagen in diseased sites, and the PET signal correlated with the collagen concentration. In addition, they tested the probe in the human lung tissues of three patients. Here, [^{68}Ga]Ga-CBP8 showed greater sensitivity to the early stages of fibrosis, during which, the collagen is new and less organized [298]. During the late stages of fibrosis, elastin is the major component and collagen becomes more organized and less sensitive to the peptide CBP8. [^{68}Ga]Ga-CBP8 shows great potential as a PET tracer to diagnose fibrosis at an early stage, which is a major challenge in developing potential therapy. Clinical trials have been organized to evaluate the potential of [^{68}Ga]Ga-CBP8 to detect radiation-induced fibrosis in lung and pancreatic cancer patients [299].

Besides targeting collagen to detect fibrosis, fibrin-fibronectin complexes have also been used as a specific molecular target for DCE-MRI. The specific binding of CLT1 peptide (CGLIIQKNEC) to such complexes is known to be observed in the ECM of different tumors and tissue lesions [300,301]. Chow et al., studied the possibility to detect and characterize liver fibrosis through molecular MRI using CLT1 peptide-targeted contrast agent (Gd-CP) and compared it with non-targeted control (Gd-C) [302]. The authors showed strong and prolonged contrast enhancement by dynamic liver imaging of Gd-CP than Gd-C. These results allowed for early detection of liver fibrosis by using an alternate molecular target. Although molecular MRI is also suitable for detecting tumor fibrosis, its use is limited [291]. In addition, the MRI contrast agents typically require micromolar concentration [291] to be visualized which is higher compared to the nanomolar concentration used in peptide-based radiation therapy.

4.2.2. Integrin-based imaging

Integrins are cell surface receptors that bind to the ECM proteins and are mainly involved in key processes such as cell survival, migration, proliferation, and differentiation. However, in some tumors, certain integrin receptors such as αvβ3, α5β1, and αvβ6 are overexpressed, which allows detection *in vivo* using PET tracers further paving the way to monitor and treat the disease. Similarly, integrin receptor overexpression was evaluated in the case of fibrotic diseases such as idiopathic pulmonary fibrosis (IPF), which is a chronic, fibrotic lung disease of unknown cause and is known to have similar links with cancer biology [303]. One such receptor αvβ6 overexpressed in both cancers and fibrosis is a potent activator of TGF-β which stimulates the production of ECM molecules such as collagen and fibronectin [304,305].

A cysteine-based peptide PET tracer was developed by Kimura et al. and evaluated pre-clinically and clinically to target αvβ6 overexpression in IPF and different tumors. $R_0$1-MG was recognized as a lead peptide based on lower kidney uptake in a BxPC3 xenograft model and was evaluated further as radio fluorinated PET tracer [^{18}F]fluoropropyl (FP)-$R_0$1-MG-F2 and [^{68}Ga]Ga-NODAGA-$R_0$1-MG. Clinical studies on patients suffering from pancreatic, cervical and lung cancer, and IPF demonstrated the highest uptake of the cysteine PET tracer with low background uptake in healthy organs (Fig. 5 **e,f**) [279]. This research highlights the broad potential of this novel PET tracer to detect and diagnose multiple diseases specifically overexpressing αvβ6 receptors. This study reports a very small number of diseased patients, and recruitment of additional cancer patients is ongoing to fully evaluate the potential of radiolabeled $R_0$1-MG peptide [306]. The use of αvβ6 receptors to detect fibrosis was successfully studied both pre-clinically and clinically using different peptide-based radiotracers [307,308].

In pre-clinical and clinical studies, IPF was also identified by overexpression of somatostatin receptors (SStR) which allowed the use of somatostatin analogs for imaging. Two different studies utilizing SPECT and PET tracers for IPF imaging in patients are studied by Lebtahi et al., and Ambrosini et al., respectively. Both the tracers, [^{111}In]In-octreotide ([^{111}In]In-NOC) and [^{68}Ga]Ga-DOTA-NOC showed a positive and linear correlation between the tracer uptake and fibrosis detected by high resolution computed tomography (HRCT) [309,310]. The role of SStR receptor expression to diagnose IPF is backed by several studies which show improved fibrotic mice survival, reduced collagen deposition, and fibrosis prevention by utilizing somatostatin analogs [311–313].

While collagen and integrin imaging is not as specific for CAF as compared to FAP, their abundant expression can be utilized for diagnostic purposes as described in the preceding paragraphs. Future research can shed light on the differences in expression patterns of collagens and integrins between the numerous stromal cell types residing in the TME.

5. Therapeutic targeting of CAF

5.1. Small molecules to target CAF

Several small molecule drugs and antibodies to target CAF are also investigated to enhance the efficiency of tumor therapy. In this regard, co-targeting FAP with small-molecule dipeptidyl peptidase inhibitor PT–100 and chemotherapy drug oxaliplatin reduced the accumulation of CAF in a xenograft tumor model thereby enhancing the chemotherapeutic response rate [314]. Similar studies were performed where both the tumor epithelia and surrounding CAF were targeted, which enhanced the chemotherapeutic efficacy [315–317]. The clinical efficiency of inhibiting FAP was tested using Val-boroPro (Talabostat) in a phase II study of patients with metastatic colorectal cancer. This phase II clinical trial demonstrated minimal clinical activity, probably due to incomplete inhibition of the targeted enzyme in CAF [318]. Similarly, minimal clinical outcome was observed when FAP was inhibited with Talabostat alone or in combination with chemotherapeutic drugs [319,320]. Targeting CAF using a monoclonal antibody (mAb) was one of the several approaches used. A humanized anti-FAP antibody (mAb F19; sibrotuzumab) was used in phase I clinical trials on patients with FAP-positive cancers to determine its safety, toxicity, and efficiency. This study resulted in positive clinical outcomes with no known adverse events and toxicities [321]. However, the antibody sibrotuzumab did not meet the minimum requirement of at least one partial or complete remission of 4 patients with stable disease to continue with the trial [322]. Ostermann et al. used a novel antibody-maytansinoid conjugate (mAb FAP5-DM1) to target FAP, which induced excellent therapeutic benefit and long-lasting tumor suppression in different xenograft mouse models [323]. Loeffler et al. showed enhanced tumor suppression after chemotherapy and anti-FAP Ab combinatory therapy [324]. However, clinical studies must be evaluated for these mAb to assess their efficiency. A recent phase I and II clinical trials is currently recruiting patients suffering from metastatic PDAC. The patients will either receive a combination of chemotherapeutic drugs or a combination of a FAP targeting antibody RO6874281 and a monoclonal antibody that targets PD-L1 [325]. The FAP targeting antibody RO6874281 has been shown to bind with FAP with high affinity and was studied in several different types of cancer [326–328]. Many clinical trials are ongoing that evaluate the potential of either monoclonal antibodies or a combination of monoclonal antibodies and chemotherapeutic drugs to modulate

CAF function. A hedgehog (Hh) signaling pathway inhibitor, sonidegib was used in combination with docetaxel in patients suffering from advanced TNBC in the phase Ib clinical trial. This combination treatment demonstrated anti-tumor activity in 3 out of 10 patients and the study also demonstrated that sonidegib could be safely administered with docetaxel [329]. More clinical trials using different targets to alternate CAF activity are still ongoing [330,331]. In addition, a combination of chemotherapeutic drugs and all-*trans*-retinoic acid (ATRA) was considered safe and tolerable in phase I clinical trials in PDAC patients [332]. ATRA reprograms pancreatic stroma to reduce the growth of PDAC tumors. After the encouraging results from phase I, this regime will be evaluated in phase II randomized clinical trials in locally advanced PDAC patients [333].

Losartan (Fig. 6**a**), an angiotensin II receptor antagonist, is approved to control hypertension in patients and is also known to possess anti-fibrotic effects. The anti-fibrotic effects are caused partly by the suppression of TGF signaling in CAF. As described previously, CAF are primarily activated by TGF-β, Hh or FAK signaling [334]. Furthermore, tumor blood vessel compression in desmoplastic tumors is mainly due to the collaborative interaction between hyaluronan and collagen, where the former's synthesis is mainly dependent on TGF-β. The effect of losartan on ECM production was demonstrated by Chauhan et al. in two different tumor models, i.e., E0771 breast cancer and AK4.4 pancreatic cancer, both known for their desmoplastic nature. The treatment reduced hyaluronan production, lowered solid stress in tumors, enhanced drug/oxygen delivery, and potentiated chemotherapy (Fig. 6**d-f**) [335]. This was also confirmed with different pre-clinical models [336–338]. The effect was attributed mainly to the high intratumoral distribution of losartan compared to other angiotensin inhibitors such as candesartan.

Based on these results, losartan was chosen for a clinical trial to study its efficacy combined with FOLFIRINOX (i.e., a combination of the drugs 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin)

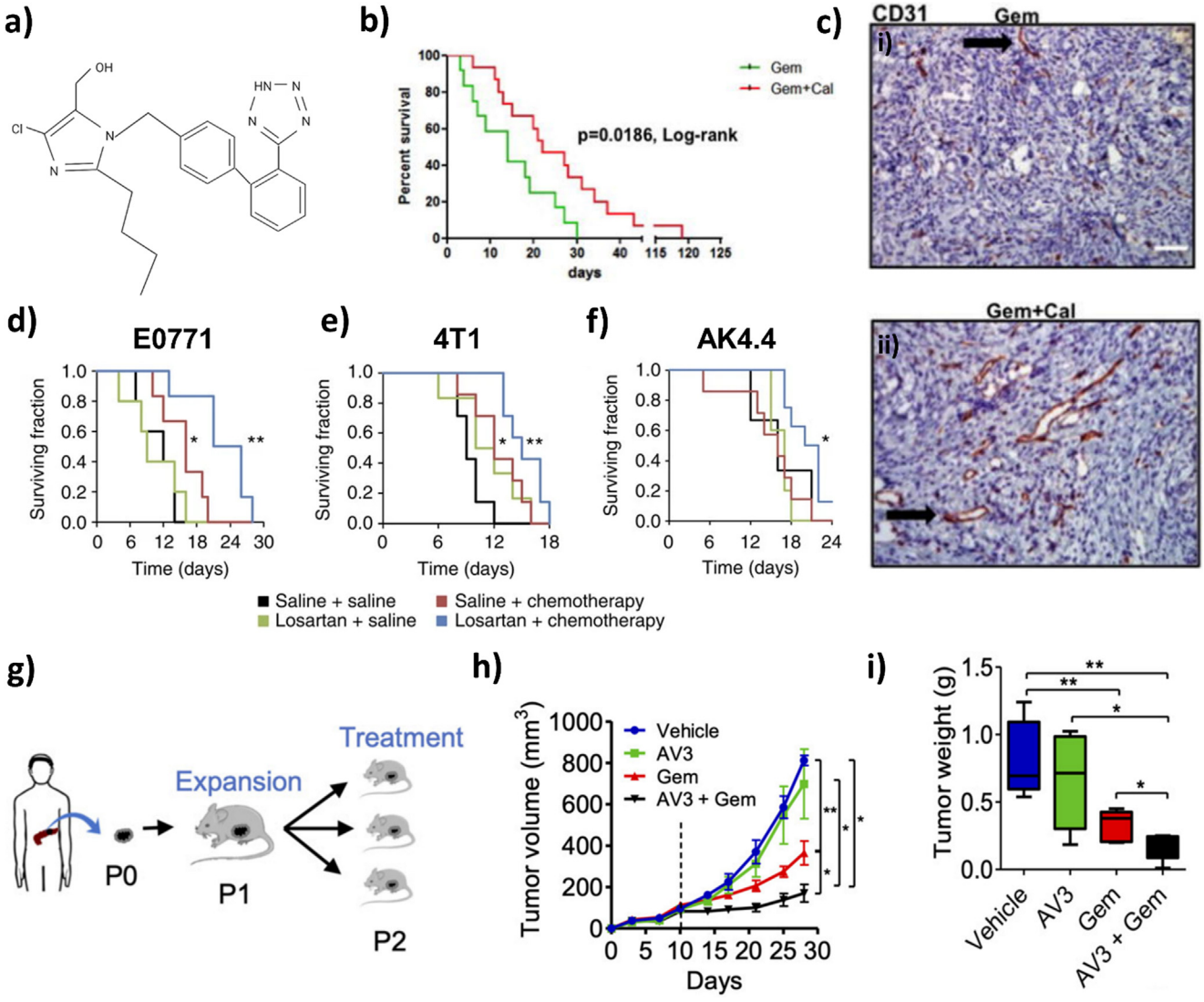

Fig. 6. Small molecule drugs to target CAF. a) An example of a small molecule anti-fibrotic drug, losartan. b) Kaplan-Meier survival analysis for KPC mice treated with i) gemcitabine (*GEM*) and ii) *Gem* + Calcipotriol (Cal). c) Representative CD31 images from *Gem* and *Gem* + Cal treated KPC xenograft mice. The black arrow in *gem*-treated mice indicates collapsed blood vessels. The black arrow in *Gem* + Cal treated mice indicates blood vessel with a lumen. d) Enhanced animal survival analysis with combination treatment of losartan and DOX for E0771 xenograft mice. e) Enhanced animal survival is obtained for 4TI xenograft mice treated with the combination of losartan and DOX. f) Enhanced animal survival obtained for AK4.4 xenograft mice treated with the combination of losartan and 5-fluoro uracil (5-FU). g) A schematic depicting the development of the human pancreatic PDX tumor model. h) Tumor volumes of the mice treated with vehicle alone, AV3, *Gem*, and AV3 + *Gem* highlighting the maximum reduction in tumor volume for the mice treated with the combination of AV3 and *Gem*. i) Lowest tumor weights of the Av3 + *Gem* treated mice at the end of the experiment, compared to other treatment groups. Images reproduced, with permission, from [335,339,340].

and radiotherapy in locally advanced PDAC. The preliminary results with 25 patients suggest that total neoadjuvant therapy resulted in prolonged survival rates and an R0 resection rate of 61 %, which means no cancer cells were seen microscopically at the resection margin [341]. This clinical trial supported the design of a second randomized phase II clinical trial using these drugs alone or in combination with nivolumab, an antibody that causes programmed cell death of cancer cells followed by chemo-radiotherapy. The status of this particular clinical trial is 'recruiting' with an aim to assess the R0 resection rate, progression-free survival, and overall patient survival either treated with or without nivolumab [342]. Another Phase 1/1b clinical trial is currently recruiting to study the efficacy of losartan combined with sunitinib for the treatment of pediatric and adult patients with relapsed or refractory osteosarcoma [343]. Furthermore, chronic use of angiotensin inhibitors independent of cancer stage and chemotherapy in resected PDAC patients showed longer overall survival rates. With a relatively small sample size of the tumor for RNA-seq analysis, the group confirmed the effects of angiotensin inhibitor drugs in non-metastasized PDAC patients [344]. Several ongoing clinical trials strengthen the use of losartan as an effective adjunct. Since losartan is already FDA-approved, it is considered safe and effective to be used along with other drugs to improve cancer treatment outcomes. Losartan has also been used with nano-drug delivery systems to further improve the nanocarrier's tumor penetration properties. This will be discussed later in **section 5.2.4**. An overview of the small molecules used to target CAF is listed in Table 5.

Sherman et al. showed that reprogramming tumor stroma and turning CAF back to quiescence using a vitamin D analog ligand (calcipotriol) assisted the chemotherapeutic response of *GEM* in PDAC mice **(**Fig. 6**b, c)**. Vitamin D receptor (VDR) is highly expressed in the stroma from human pancreatic tumors and acts as a genomic suppressor of the PSC activation state. The enhanced efficiency of *GEM* was achieved due to the ability of calcipotriol to reduce the inflammation and fibrosis markers in the pancreatic murine model. This study for the first time emphasizes CAF normalization [340].

Further, lipoxin A4 (LXA4), an endogenous bioactive lipid, was shown to reduce tumor growth *in vivo* by targeting LXA4 specific surface receptor formyl peptide receptor 2 (FPR2), which is highly expressed and upregulated in activated PSC. PSCs are considered the main source of CAF in pancreatic cancer; therefore, inhibiting the activation and migration of PSCs resulted in the reprogramming of tumor stroma and improving anti-cancer drugs' therapeutic efficiency [345]. The same group investigated the potential of targeting integrin α11 (ITGA11) highly expressed in human pancreatic cancer samples (activated PSCs). It was demonstrated that knocking down ITGA11 with short hairpin RNA (shRNA) inhibited the activation of PSCs by TGF-β both at gene and protein levels [346]. Additionally, the group also investigated the potential of integrin α5 (ITGA5) as a therapeutic target and studied the therapeutic potential of the novel peptidomimetic (AV3) for pancreatic cancer. This study was backed by several analyses of PDAC tissue samples obtained from 2001 to 2012 from 137 patients proving ITGA5 as a successful prognostic agent [339]. The therapeutic efficiency of AV3 was assessed with several *in vitro* assays and *in vivo* xenograft tumor models, including co-injecting tumor model and patient-derived xenograft (PDX) models **(**Fig. 6**g-i)**. It was found that the AV3 could reduce desmoplasia, which enhanced tumor perfusion and the efficacy of *GEM in vivo* [339].

5.2. Targeting CAF using nanocarriers

The use of nanocarriers such as nanoparticles (NP), nanogels (NG), liposomes, and micelles for the delivery of chemotherapeutic drugs is highly advantageous, mainly due to its size, tunable properties, high surface-to-volume ratio, and targeting abilities. The use of nanocarriers can overcome the limitation of conventional drug therapies by improving biodistribution, minimizing toxicity to healthy cells, and enabling tumor-specific targeting and retention. They can passively accumulate in the tumor interstitium due to the enhanced permeation and retention (EPR) effect. However, a *meta*-analysis by Wilhelm et al. showed that only 0.7 % (median) of administered NP are delivered to a solid tumor [347]. One of the potential causes of an inefficient NP delivery is the presence of tumor interstitium that consists of interstitial fluid, dense ECM deposition, and different cell types, including CAF. For instance, in desmoplastic tumors like pancreatic cancer, the development of dense, collagen-rich ECM secreted by CAF and increased IFP in the tumor hinders deeper NP penetration, resulting in lower NP concentration reaching the tumor [348]. This tumor desmoplasia progresses to such an extent that most tumor volume comprises activated stroma [349] while the pressure exerted by the vast number of CAF causes lymphatic vessel compression, thereby reducing the discharge of interstitial fluid [350]. An impaired drainage system and physiological changes in the stroma of the TME further add to the increased IFP [351]. Therefore, targeting CAF at an early stage can prevent the accumulation of dense ECM, elevated IFP, and improve nanocarrier delivery to treat cancer. Below, we will discuss the potential CAF targets utilized to design

Table 5
An overview of small-molecule drugs used to target CAF.

Key therapeutics	Outcome	Ref
PT 100 and Oxaliplatin	• PT 100 reduced the accumulation of CAF in xenograft colon cancer tumor models • PT 100 treatment led to enhanced oxaliplatin chemotherapeutic response	[314]
Val-boroPro (Talabostat)	• Val-boroPro resulted in an incomplete inhibition of FAP enzymatic activity in phase II clinical trial	[318]
Sibrotuzumab	• Safe administration of sibrotuzumab in patients with FAP-positive cancer in phase I clinical trial • Sibrotuzumab did not meet the minimum requirement of at least one partial or complete remission in the phase II clinical trial	[321,322]
Losartan and Doxorubicin	• Losartan educed stromal collagen and hyaluronan production in two different xenograft mouse models (pancreatic and breast) • Losartan enhanced drug/oxygen delivery that resulted in improved efficiency of doxorubicin	[335]
Losartan + FOLFIRINOX + Radiotherapy	• Losartan + FOLFIRINOX + Radiotherapy prolonged survival rates in PDAC patients in phase II clinical trial • An R0 resection rate of 61 %	[341]
Vitamin D analog ligand (Calcipotriol) + Gemcitabine	• Calcipotriol normalized the tumor stroma and returned CAF to quiescence • Calcipotriol enhanced gemcitabine efficiency	[340]
Lipoxin A4 (LXA4)	• LXA4 reprogrammed the tumor stroma by targeting FPR2, which is highly expressed in activated PSCs • Reduced tumor growth in-vivo	[345]
Short hairpin RNA (shRNA)	• PSC activation by TGF-β was inhibited by targeting Integrin α11β1 (ITGA11)	[346]
Peptidomimetic (AV3) + Gemcitabine	• AV3 reduced desmoplasia that led to decompression of blood vessels • AV3 Enhanced tumor perfusion and efficacy of gemcitabine *in vivo*	[339]

nanocarriers with improved tumor penetration and therapeutic efficiency.

5.2.1. Nanoparticles for depleting CAF

CAF-targeted nanocarriers are desired to provide a competitive advantage to achieve high therapeutic efficiency. Fig. 7**a** depicts an example of different molecules that play a role in eradicating CAF. To eradicate CAF with high specificity, it is essential to identify specific surface markers. Recent studies have shown that a targeted high-affinity molecule navitoclax (Nav) can specifically induce apoptosis of CAF at a low dose, indicating its potential to eradicate CAF in cancer [317]. On the other hand, tenascin-C (TNC) is an ECM-specific molecule that drives the progression of many types of human cancer and is mainly secreted by CAF [352,353].

To target CAF with high affinity, the FH peptide, which displays a high binding affinity to TNC was selected as a targeting moiety and, a nano-liposome containing both FH and Nav (FH-SSL-Nav) was developed to test the targeting ability and depletion of CAF. FH-SSL-Nav revealed better anti-tumor efficacy and CAF deleting effect than SSL-Nav (Fig. 7**b-d**), partly because some solid tumors are known to be resistant to Nav, and the dose regime in this study was much lower compared to the tumor dose therapy. Furthermore, the rapidly growing tumor cells treated with SSL-Nav may have overruled CAF eradicating effect leading to its low anti-tumor efficiency [354]. Further, Li et al. exploited ferritin nanocages to deliver $ZnF_{16}Pc$, a photosensitizer, to CAF (Fig. 7**e**). They achieved targeted delivery of photosensitizers by conjugating FAP-specific single chain variable fragment antibody (scFv). This treatment strategy, combined with localized photo-irradiation of the tumor, allowed the successful eradication of CAF (Fig. 7**g**) by depleting α-SMA levels, mainly expressed on the CAF surface. Furthermore, the collagen content, which is one of the main components of TME, was drastically reduced in photo-irradiated tumors. The scFv-Z@FRT mediated PDT enhanced tumor accumulation of BSA, 10 nm QDs, and 50 nm QDs (Fig. 7**f**) [355].

Ji et al. developed a novel cell-penetrating peptide (CPP)-assembled NP composed of FAP targeting mAb and chemotherapeutic drug DOX (PNP-D-mAb). The nanosystem resulted in reduced xenograft prostate cancer due to the combination of mAb targeting abilities, penetration by CPP, and DOX therapeutic effects [356]. The same group further developed a novel cleavable amphiphilic peptide (CAP) containing the sequence Ac-Ala-Thr-Lys (C18)-Asp-Ala-Thr-Gly-Pro-Ala-Lys(C18)-Thr-Ala-NH_2, which can be cleaved explicitly by FAP at Gly-Pro-Ala-X. The CAP monomer readily self-assembled into nanofibers in an aqueous solution, which later formed drug-loaded spherical NP (CAP-DOX-NP) after incorporating DOX. Once passively accumulated in the tumor

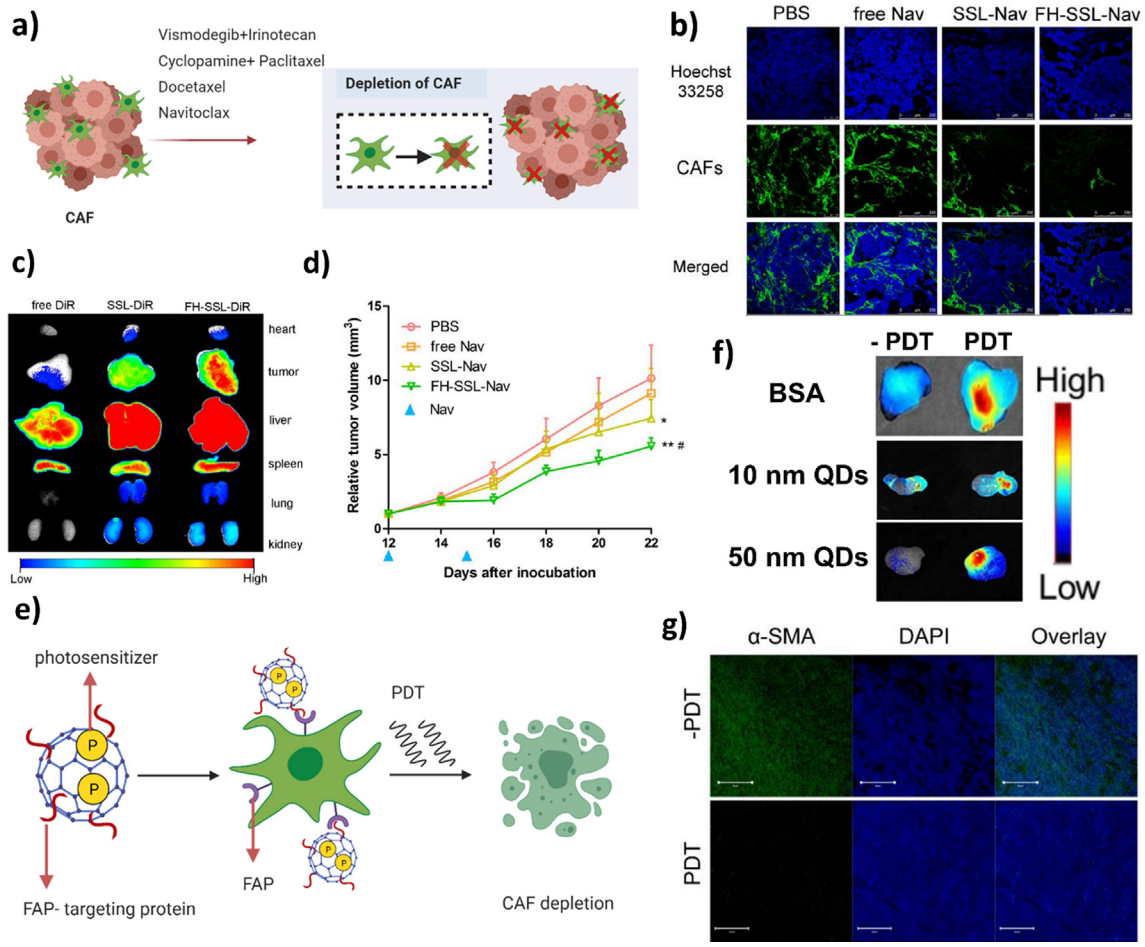

Fig. 7. Pharmacological strategies to deplete CAF for enhanced drug delivery and therapeutic efficacy. a) An example of factors that play a role in depleting CAF. b) Representative CLSM images of CAF from mice treated with PBS, free Nav, SSL-Nav, and FH-SSL-Nav, which depict CAF eradication after treatment with FH-SSL-Nav. c) Ex vivo image of tumors and main organs 48 h p.i. d) Relative tumor volume of mice treated with different formulations. e) A schematic of a FAP-targeted ferritin nanocage for PDT therapy to deplete CAF. f) Treatment with scFv-Z@FRT mediated PDT enhanced tumor accumulation (Ex-vivo) of BSA, 10 nm QDs and 50 nm QDs, comparatively to non-PDT treated groups. g) Confocal images of 4T1 tumor slices stained with FITC-conjugated anti-α-SMA antibody (Green), and DAPI (blue). Scale bar: 100 μm. Images reproduced, with permission, from [354,355]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 6
An overview of therapeutics used to deplete CAF using NP.

Delivery vehicle	Therapeutics	Outcome	Ref
Nano-liposome	• Nano-liposome comprising **peptide FH** and **Navitoclax** (Nav)	• Nav induced CAF apoptosis by targeting tenascin-C, which resulted in increased anti-tumor efficacy of the nano-liposome	[354]
Ferritin nanocage	• Ferritin nanocage comprising **photosensitizer** ($ZnF_{16}Pc$)**, a FAP-specific scFv targeting molecule** (scFv-Z@FRT) combined with photodynamic therapy	• scFv-Z@FRT mediated selective photodynamic therapy successfully eradicated CAF • Photodynamic therapy enhanced the accumulation of BSA, 10 nm QDs, and 50 nm QDs in the breast cancer tumor model	[355]
Cell-penetrating peptide (CPP)-assembled NP	• CPP-NP comprising **FAP-targeting mAb** and **Doxorubicin** (PNP-D-mAb)	• PNP-D-mAb achieved a maximum anti-tumor effect through CAF depletion in xenograft tumor model co-implanted with CAF and prostate cancer cells	[356]
Self-assembled cleavable amphiphilic peptide (CAP) NP	• CAP-NP comprising **FAP responsive peptide** (CAP) and **Doxorubicin** (CAP-DOX)	• CAP-DOX responded to FAP for efficient drug release and demonstrated promising anti-tumor efficacy of doxorubicin by depleting CAF	[357]

stroma, the NP disassembled upon cleavage of CAP and released the drug. This strategy was efficient in treating three tumor models by disrupting the stromal barrier, inducing CAF apoptosis, and enhancing the local drug accumulation [357]. An overview of different types of NP used to deplete CAF is listed in Table 6.

5.2.2. Re-educating CAF

Since it is known that fibroblasts can differentiate into CAF through TGF-β signaling [358], re-educating CAF to normal fibroblast is a novel approach to counter the pro-tumorigenic activity of CAF **(**Fig. 8**a)**. In their quiescent stage, PSCs are characterized by the presence of vitamin A-containing lipid droplets in their cytoplasm [359]. Once they are activated and characterized by the presence of α-SMA, they lose their vitamin A-lipid-containing droplets and increase the synthesis of ECM proteins, including collagen type I [359,360].

Patients suffering from PDAC are vitamin A deficient, and by treating the mouse model with All-*trans* retinoic acid (RA), activated PSC regained quiescence. This led to reduced Wnt16 expression resulting in slower tumor progression [363–365]. Taking this into account pH-responsive gold NP was developed, which utilized an active metabolite of vitamin A, RA, and heat shock protein 47 SiRNA (HSP47 siRNA, SiHSP47) to maintain PSCs quiescent and reduce collagen content, respectively (Au@PP/RA/SiHSP47) **(**Fig. 8**d)**. Treatment with this nanosystem combined with *GEM* increased accumulation, penetration, and decreased tumor progression in Panc-1/PSC tumor-bearing mice **(**Fig. 8**g)**. Furthermore, this system rendered PSC quiescent, induced lipid droplet formation, reduced expression of α-SMA, significantly reduced collagen content **(**Fig. 8**e, 8f)**, and restored homeostatic stromal function [361]. In an exciting study, Miao et al. developed lipid-coated protamine DNA complexes (LPD) for encapsulating sTRAIL (Engineered TNF-related apoptosis-inducing ligand) plasmids to target CAF and treat desmoplastic tumors (sTRAIL LPD). TRAIL is a transmembrane protein lacking a leader sequence for extracellular secretion and efficiently induces apoptosis in tumor cells while sparing healthy cells. Treatment with sTRAIL LPD induced apoptosis in adjacent tumor cells and improved the overall survival rate to 65 days. Interestingly, the residual CAF reverted to quiescence, as demonstrated by reduced expression of α-SMA and FAP by 90 % and 84 %, respectively [366].

Saha et al. showed that unmodified gold NP (AuNP) had an intrinsic property to reprogram the pancreatic TME by turning PSCs quiescent and reversing epithelial-mesenchymal crosstalk **(**Fig. 8**b, 8c)** [362]. A recent study showed that 20 nm AuNP reprogrammed CAF back to quiescence by enhancing lipid synthesis and lipid utilization [367]. These studies show that the intrinsic properties of the NP can be utilized to reprogram CAF [368]. An overview of different NP used to turn CAF back to quiescence is listed in Table 7.

5.2.3. Combinatory treatment to target CAF

Different types of NP targeting CAF have been studied [369–373]. An overview of molecules/drugs that diminish CAF activity is depicted in Fig. 9**a**. Miao et al. developed Anisamide coated lipid NP and encapsulated chemotherapeutic drug cisplatin (cisplatin NP). Cisplatin NP (1 mg/kg) significantly inhibited the tumor growth in a Stroma Rich Bladder Cancer (SRBC) model compared to free cisplatin [374,375]. However, this study reported relapse and resistance after repeated injections of cisplatin NP, and it was found that the protein Wnt16, a significant damage response program (DRP) molecule contributed to increased resistance of the drug cisplatin to the tumor [374,376]. The DRP molecules arise from non-specific uptake of the nanomedicines by CAF, causing tumor metastases and chemotherapeutic drug resistance [377]. Therefore, NP that can inhibit tumors and prevent DRP molecules simultaneously may improve treatment outcomes.

Taking this into account, Cun et al. developed multifunctional size switchable NP (denoted as DGL/*GEM*@PP/GA) that can co-deliver *GEM* and 18β-glycyrrhetinic acid (GA). GA is known to down-regulate the DRP molecule, Wnt16. Dendrigraft Poly-L-lysine (DGL) was attached to Poly (ethylene glycol)-poly (Caprolactone) (PP) using a Matrix Metalloproteinase-2 (MMP-2) substrate peptide and was self-assembled to form DGL@PP. *GEM* and GA were conjugated and encapsulated inside DGL@PP to form DGL/*GEM*@PP/GA [377]. Once passively accumulated in the tumor site, the release of a small *GEM*/DGL fraction was facilitated by a higher expression of MMP-2 in the TME. *GEM*/DGL penetrated deep inside the tumors owing to their small size of 28.7 nm while the larger GA/PP fraction remained in the TME and regulated the Wnt16 activity. This NP efficiency was studied in two different cancer mice models, pancreatic cancer (Pan02) and breast cancer (4T1). Owing to the two-step and size-specific delivery, DGL/*GEM*@GA/PP presented the best anti-tumor effect compared to DGL/*GEM*@PP, DGL@PP/GA, DGL/*GEM*, and *GEM* alone **(**Fig. 9**b, 9c)**. The study showed no inclination of tumor rebound eight days after the last injection with DGL/*GEM*@GA/PP. Additionally, the expression level of Wnt16 on the tumors was studied after different treatment injections, and reduced expression of Wnt16 and α-SMA was observed. This emphasizes the role of multi-target-based NP strategies to regulate the interplay between different aspects of TME [377].

Besides using GA as a naturally occurring molecule to downregulate Wnt16, Hu et al. developed a quercetin prodrug through

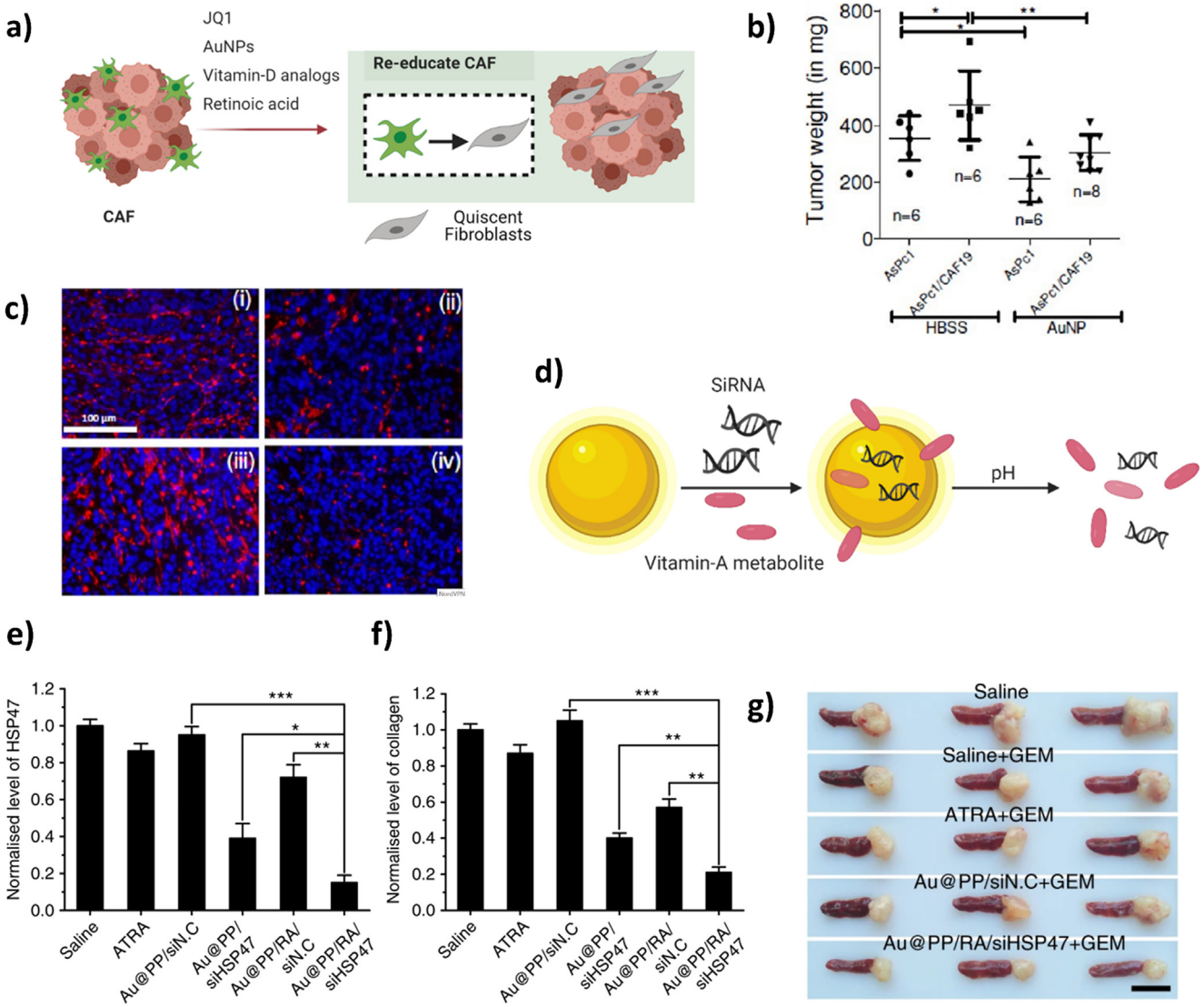

Fig. 8. Strategies to re-educate CAF to a quiescence state for enhanced therapeutic outcome. a) A schematic of factors that re-educate CAF. b) Tumor weight of AsPc1 and AsPc1/CAF19 xenograft mice model treated with HBSS (sham) or 20 nm AuNP 21 days post-treatment. c) Representative α-SMA stained sections of (i) AsPc1-HBSS, (ii) AsPc1-AuNP, (iii) AsPc1/CAF19-HBSS, and (iv) AsPc1/CAF19-AuNP groups after 21 days. d) A schematic of AuNP encapsulating SiRNA and vitamin A metabolite to modulate the TME and re-educate CAF [361]. e,f) Quantitative analysis of normalized HSP47 e) and collagen f) after treatment with Au@PP/RA/SiHSP47, implying a reduction in ECM deposition. g) Images of excised tumors with spleens demonstrating significant size reduction after treatment with Au@PP/RA/SiHSP47 + *GEM*. Scale bar: 1 cm. Images reproduced, with permission, from [361,362].

Table 7
An overview of different NP used to re-educate CAF.

Delivery vehicle	Therapeutics	Outcome	Ref
pH-responsive AuNP (Au@PP)	• Au@PP comprising **vitamin-A metabolite** (RA), **heat shock protein siRNA** (SiHSP47), (Au@PP/RA/SiHSP47)	• Au@PP/RA/SiHSP47 re-educated PSC to quiescence, which further led to reduced ECM and collagen production, and α-SMA levels • Au@PP/RA/SiHSP47 induced lipid droplet formation • Stromal modulation by Au@PP/RA/SiHSP47 increased gemcitabine accumulation	[361]
Lipid-coated protamine DNA complexes (LPD)	• LPD complexes encapsulated **sTRAIL** (Engineered TNF-related apoptosis-inducing ligand) (sTRAIL LPD)	• sTRAIL LPD induced tumor apoptosis and converted CAF back to its quiescence state • sTRAIL LPD reduced the expression of FAP and α-SMA	[366]
AuNP	• Unmodified AuNP	• Unmodified AuNP demonstrated its intrinsic property to revert PSC to quiescence	[362,367]

phosphorylation of the quercetin hydroxyl group (Quercetin Phosphate, QP) which showed superior downregulation of Wnt16 [379]. QP was encapsulated in lipid/calcium/phosphate NP (LCP-QP NP). Two systems were developed in this study: LCP-QP NP and LPC NP (encapsulating cisplatin without QP). The results demonstrated that the combination of LCP-QP and LPC NP (LCP-QP + LPC) significantly enhanced the tumor growth inhibition compared to the control, LCP-QP NP, and LPC NP in a stromal-rich UMUC3 bladder

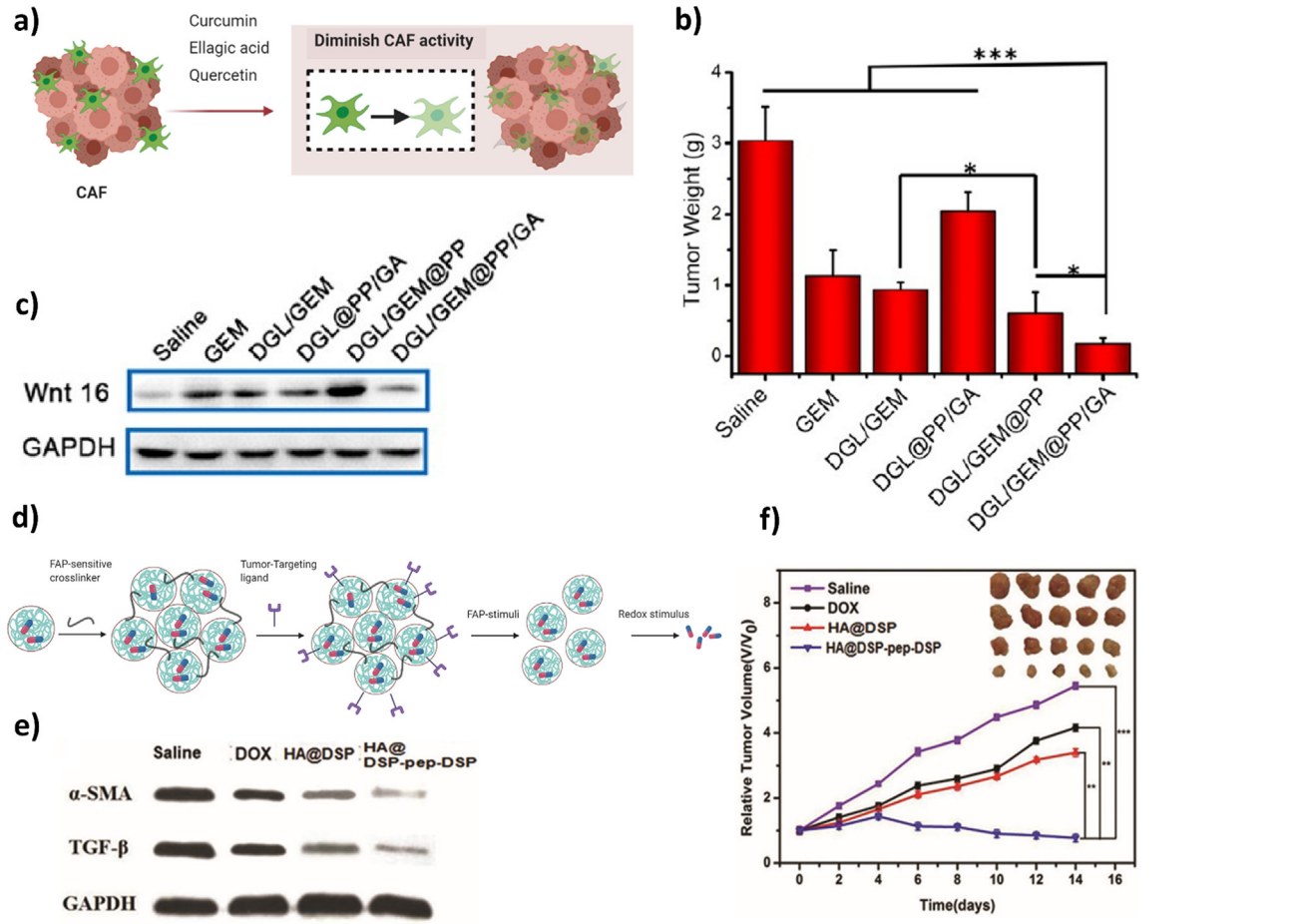

Fig. 9. Different types of nanocarriers used to diminish the activity of CAF. a) An example of certain factors that play a role to diminish CAF activity. b) Weight of Pan 02 tumor-bearing mice, which demonstrates the enhanced tumor weight reduction of mice treated with DGL/*GEM*@GA/PP. c) Reduced expression level of Wnt 16 after treatment with DGL/*GEM*@GA/PP. d) An example of disulfide-linked DOX NG crosslinked using a FAP degradable peptide. Upon accumulation of HA@DSP-pep-DSP via EPR effect and HA targeting abilities on the FAP α, the NG dissociate into smaller DOX-ss-DSP that further undergo degradation in the tumor due to redox sensitivity. This two-step strategy led to a reduction in CAF activity and enhanced the therapeutic efficiency of DOX [378]. e) Western blot of the tumor tissues treated with different formulations to investigate CAF-related expression level. The tumor of the mice treated with HA@DSP-pep-DSP demonstrates the lowest CAF-related expression (α-SMA and TGF-β). f) Relative tumor volume and tumor weight of tumor-bearing mice as a function of time using different treatment groups showing reduced tumor volume of the mice treated with HA@DSP-pep-DSP. Images reproduced, with permission, from [377,378].

cancer model. Furthermore, the expression of Wnt16 was significantly reduced in the TME, enabling better penetration and retention of LPC NP. [379].

In another such combination therapy by Wie et al. a thermosensitive liposome (TSL) was used to co-deliver human serum albumin (HSA) NP consisting of anti-tumor drug paclitaxel (PTX) and anti-PSC drug (Ellagic Acid, EA) [380]. PTX and EA were individually self-assembled with HSA. The formed complexes were further encapsulated in TSL by thin-film hydration to form TSL/HSA-PE. The TSL (176 nm) kept the drugs intact at 37 °C and only released (~9 nm) once the local tumor was heated to 40–42 °C. *In vivo* imaging of pancreatic cancer cell line (BxPC-3 and HPaSteC) injected mouse revealed higher tumor accumulation and penetration, tumor growth inhibition, and a significant reduction of α-SMA when treated with TSL/HSA-PE (heated). This led to a high tumor drug accumulation for PTX (4.86 μg/g tissue) and EA (4.75 μg/g tissue) due to its prolonged retention in the blood [380]. However, this study required the heating of tumor cells at 43 °C for one hour in a water bath, making it a tedious process and limiting its clinical efficiency.

In another study, CAF-responsive NP was developed for improving drug permeability and efficacy in desmoplastic TME. The trigger from CAF was obtained by crosslinking the NG using a peptide Asp-Ala-Thr-Gly-Pro-Ala. FAP, which is overexpressed in CAF, can cleave the polypeptide linkage between Pro and Ala [378]. First, the chemotherapeutic drug DOX was linked with the cationic poly (amidoamine) (PAMAM) dendrimer via a disulfide linkage (DSP). DSP was then cross-linked with the peptide (DSP-pep-DSP). Lastly, HA was modified on the surface (HA@DSP-pep-DSP) (Fig. 9**d**). Owing to the EPR effect and HA targeting ability, the NG could accumulate at the tumor site. In the presence of FAP, the NG were hypothesized to degrade and release smaller molecule DSPs to attack the tumor cells and penetrate further deeply. The nanosystem was tested *in vitro* (2D and 3D spheroids) and, lastly, in PC-3/CAF tumor-bearing mice. The results demonstrated that HA@DSP-pep-DSP showed enhanced tumor accumulation and penetration properties. Together with DOX and HA, synergistic effects on CAF and tumor volume reduction were observed (Fig. 9**f**). Expressions of TGF-β, α-SMA, and FAP also reduced significantly after the treatment (Fig. 9**e**), and the CAF morphology changed from dense ribbon to small dots [378]. These combination therapies, which include stimuli responsiveness to target CAF/proteins expressed on CAF and tumor, present a novel approach to improve anti-tumor efficiency, penetration, and retention.

These studies suggest that the depletion of desmoplasia and CAF improve the efficacy of the therapeutic drug pre-clinically.

Table 8
An overview of combinatory treatment to target CAF using NP.

Delivery vehicle	Therapeutics	Outcome	Ref
Multifunctional size-switchable NP	• Multifunctional size switchable NP synthesized from dendrigraft Poly-L-lysine (DGL) and poly (ethylene glycol)-poly (Caprolactone) (PP) comprising **Gemcitabine and 18β-Glycyrrhetinic acid** (GA) (DGL)/*GEM*@PP/GA)	• Upon cleavage by MMP-2 in the TME, the released DGL-*GEM* penetrated deep within the tumor to induce apoptosis • PP/GA accumulated in the TME and downregulated the activity of Wnt16	[377]
Lipid/calcium/phosphate NP (LCP-NP)	• LCP-NP encapsulated **Quercetin phosphate** (QP), (LCP-QP) LCP-NP encapsulated **Cisplatin** (LPC)	• LCP-QP downregulated Wnt16 expression, demonstrated by decreased collagen and α-SMA levels • LCP-QP improved the anti-tumor efficiency of LPC	[379]
Thermosensitive liposome (TSL)	• TSL to co-deliver human serum albumin (HSA) NP comprising **Paclitaxel** and an anti-PSC drug **Ellagic acid** (EA), (TSL/HSA-PE)	• TSL/HSA-PE rapidly released both HSA-PE and HSA-PTX in presence of heat stimuli • HSA-EA disrupted the PSCs-PDAC interaction, which resulted in superior anti-tumor effects of HSA-PTX	[380]
CAF-responsive peptide NP	• CAF-responsive NP comprising **Hyaluronic acid** and **Doxorubicin** (HA@DSP-pep-DSP)	• Release of smaller-sized HA@DSP in presence of FAP enhanced tumor accumulation and penetration of doxorubicin • CAF-responsive peptide NP reduced expressions of TGF-β, α-SMA, and FAP	[378]

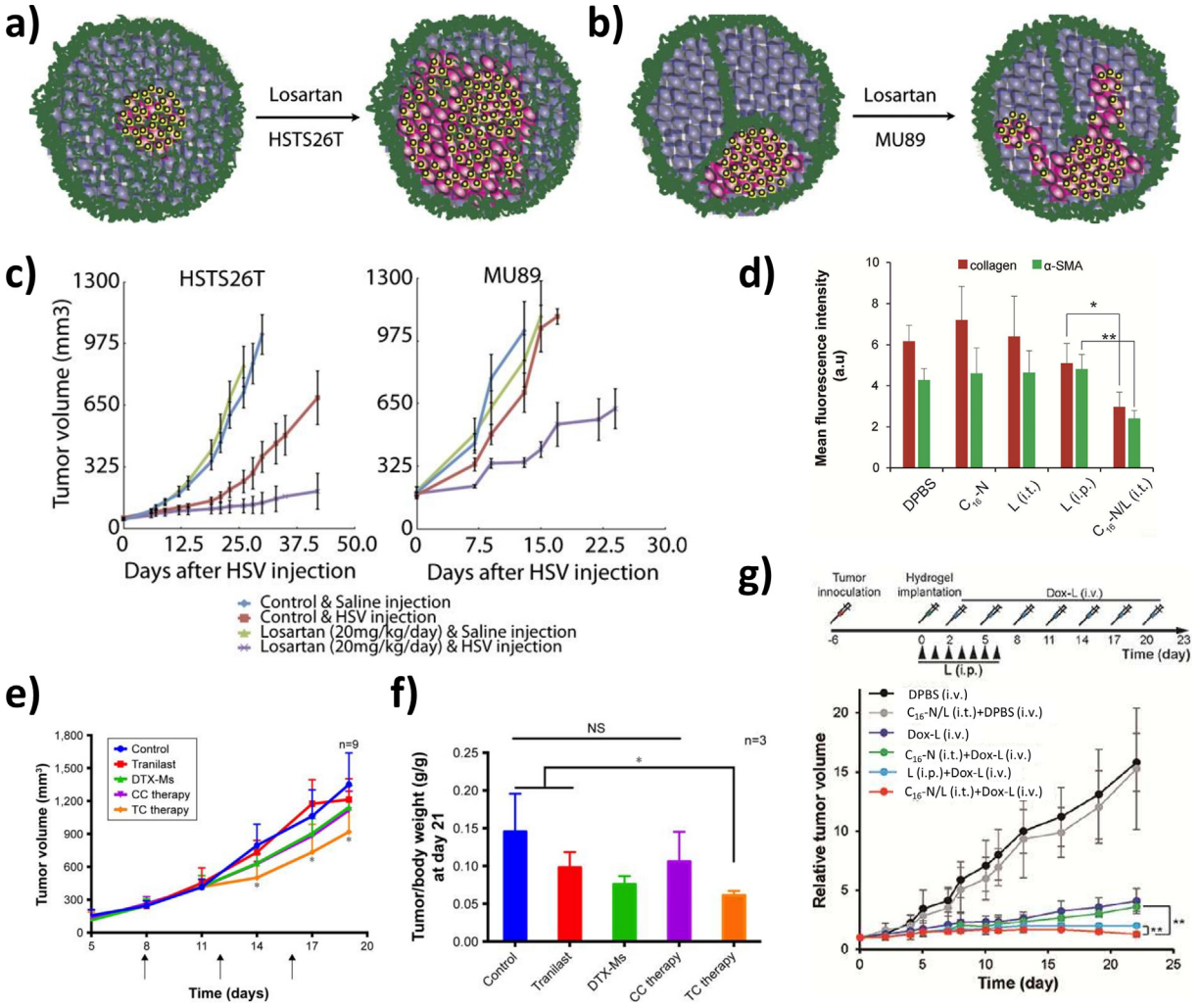

Fig. 10. Sequential delivery of small molecules and nanocarrier for enhanced therapeutic outcome. a,b) A schematic depicting the destabilization of collagen bundles (green) caused by pre-treatment with losartan in HSTS26T tumors a) and MU89 tumors. b) After treatment with losartan, the distribution of the HSV improves allowing it to infect (pink) a larger area. The purple color area indicates an area not infected by the HSV. c) Mice bearing HSTS26T tumors (Left) and MU89 tumors (Right) were treated with losartan for 2 weeks after which the mice were treated with HSV, which shows greater tumor volume reduction for the group treated with losartan and HSV. d) Reduced expression of collagen I and α-SMA in 4 T1 tumors treated with only C_{16}- N/L demonstrating modulation of TME. e) Indication of tumor volume for 20 days showing a relatively decreased tumor volume with the TC therapy group. TC therapy: Two-stage combination therapy, CC therapy: co-dose combination therapy, where tranilast and docetaxel micelles were administered together. DTX-Ms: docetaxel micelles f) Tumor coefficient at day 21 showing reduced tumor/body weight via TC therapy. g) Reduced tumor volume in the group treated sequentially with C_{16}- N/L and DOX. Images reproduced, with permission, from [391–393]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

However, clinical trials with *GEM* did not show satisfactory results [381,382]. Few preclinical studies reported that depleting CAF may not enhance tumor control and increase the risk of losing critical stromal elements required for tissue homeostasis, thereby leading to tumor progression [253,383,384]. This suggests that the role of desmoplasia is very context-dependent, and the patient benefit might require re-educating CAF to become more normal. Several vitamin A and vitamin D analogs are currently being tested to re-educate CAF in combination with chemotherapy or immunotherapy to treat metastatic pancreatic cancer [385–390]. An overview of combinatory treatments used to target CAF is listed in Table 8**.**

5.2.4. Sequential delivery of small molecule and nanocarrier for enhanced anti-tumor effect

The nano delivery systems developed till now utilize co-delivering drugs for CAF and cancer, which is advantageous if delivery to the same cell is the target. For delivery to two different target cells, the advantage can lie in administering two different doses separately, enhancing the synergic anti-tumor efficacy. Pang et al. developed a two-stage delivery system (TC: two-stage combination therapy) for breast cancer, wherein Tranilast, a clinical anti-fibrotic agent, is first delivered to downregulate CAF activity and cut off communication between CAF and cancer cells. In the second stage, 27 nm docetaxel-loaded micelles were administered, leading to a synergistic anti-tumor effect **(**Fig. 10**e, 10f)** [391].

Pei et al. developed NP in the size range of 20–30 nm for sequential delivery to target TGF-β signaling and KRAS mutation for pancreatic cancer. Pre-administration of antifibrotic Fraxinellone-loaded CGKRK peptide-modified NP (Frax-NP-CGKRK) recognized the overexpression of heparin sulfate proteoglycan in the tumor sites and rendered CAF quiescent, decreased collagen content, and increased blood perfusion in tumor vessels. This improvement allowed SiRNA-loaded lipid-coated calcium phosphate biomimetic high-density lipoprotein NP (SiKras-LCP-ApoE3) to silence oncogenic KRAS mutation and efficiently kill cancer cells [394].

The anti-fibrotic activity of losartan is also utilized in drug delivery to improve NP tumor penetration by suppressing the active TGF-β signals via thrombospondin-1 (TSP-1). A schematic depiction of collagen destabilization caused by losartan pre-injection is shown in Fig. 10**a,b**. Here, Frimpong et al. showed losartan improved efficacy of intratumorally injected oncolytic herpes simplex virus (HSV) and i.v. administered Doxil by retarding tumor growth in two xenograft mice models **(**Fig. 10**c)**. HSV is widely used in patients for gene therapy while Doxil is an FDA-approved liposomal formulation for encapsulation of the anti-cancer drug DOX [393]. To prevent the negative effect of losartan on blood pressure, it is important to enable its localized and prolonged delivery. Hu et al., showed in a 4T1 xenografted mice that pre-treatment with losartan-loaded peptide hydrogel (C_{16}-N/L) improved the intra-tumoral accumulation and penetration of PEGylated DOX liposomes by ~ 2-fold compared to treatment with losartan alone **(**Fig. 10**d, 10 g)** [392]. Xia et al. also demonstrated that pre-injection of losartan encapsulated liposomes (LST-Lip) improved anti-tumor efficiency of PTX-loaded liposomes modified with pH-sensitive peptide (PTX-TH-Lip) in 4 T1 xenografted mice. Additionally, they assessed the safety of LST-Lip on blood pressure [395]. Such delivery systems dramatically depend on the time window between the two doses delivery and with different types of nanocarriers in use, the dosing regime must be optimized for enhanced therapeutic effect. An overview of drugs and NP used for sequential delivery to enhance cancer therapy and target CAF is listed in Table 9**.**

6. Clinical translation, challenges, and future perspectives

CAF play a crucial role in modulating the TME and are responsible for stromagenesis within a tumor. The origin of CAF is incompletely understood and several precursor cell types are proposed for the generation of CAF. Furthermore, various subpopulations of CAF have been identified and could be associated with either tumor-promoting or tumor-inhibiting roles. CAF can trigger EMT and drug resistance in cancer cells, promote tumor growth and metastasis, and modulate angiogenesis and immune responses. Therefore, targeting CAF and/or the interaction between CAF and cancer cells to suppress tumor growth and drug resistance is a promising way forward for the development of improved anti-cancer (combination) therapies. However, our understanding of CAF behavior, expression profiles and markers, and associated signaling pathways are still expanding, and several challenges remain to exist. One of the major limitations in CAF biology is the absence of a specific marker to identify CAF, or even fibroblasts themselves. Usually, a combination of different markers is used to identify CAF. Some examples of commonly used CAF markers are α-SMA, FAP, FSP 1, PDGFRα/β, Vimentin, and Tenascin C [26]. However, the expression of these markers is not specific to fibroblasts and is also seen in other cell types, due to which the search for a CAF-specific marker still prevails. Further studies to understand the expression profiles of normal/quiescent fibroblasts are crucial to help in better distinguishing CAF from quiescent fibroblasts [396].

Table 9
An overview of sequential delivery to target CAF and enhance anticancer therapy.

First injection	Second injection	Outcome	Ref
Tranilast	**Docetaxel-loaded micelles**	• Synergistic anti-tumor effect compared to its respective controls was achieved by breaking down • CAF barriers using Tranilast (a clinical anti-fibrotic agent)and increasing micelles delivery efficiency	[391]
Fraxinellone-loaded CGKRK peptide-modified NP (Frax-NP-CGKRK)	**SiRNA-loaded lipid-coated calcium phosphate lipoprotein NP** (siKras-LCP-ApoE3)	• Frax-NP-CGKRK successfully targeted the tumor and regulated TGF-β signaling, reversed CAF to quiescence, and weakened the stromal barrier • A second injection of siKras-LCP-ApoE3 was efficiently internalized by tumor cells to silence KRAS mutation	[394]
Losartan	**Doxil**	• Primary injection of losartan improved the efficiency of Doxil by destabilizing the collagen	[393]
Losartan-loaded peptide hydrogel	**PEGylated Doxorubicin liposomes** (DOX-L)	• Losartan-loaded peptide hydrogel significantly inhibited the CAF and collagen synthesis • Losartan-loaded peptide hydrogel improved accumulation and penetration of DOX-L	[392]
Losartan encapsulated liposomes (LST-Lip)	**Paclitaxel-loaded liposomes** (PTX-LST-Lip)	• LST-Lip inhibited the collagen in tumors • PTX-LST-Lip exerted an enhanced anti-tumor efficiency due to the inhibition by collagen	[395]

Table 10
Current questions and challenges in CAF-targeted therapy.

Questions	Challenges
What are the biological challenges?	• No suitable specific markers of fibroblasts Several origins: Numerous cells that reside in the TME have been shown to metamorphose into CAF • CAF can also have functional and morphological heterogeneity based on their stage of differentiation • Little is known about the effect of cell culture practices on CAF modulation and its resemblance to *in vivo* conditions
What currently hinders the clinical translation of nanocarriers?	• Lack of suitable preclinical models that mimic the heterogeneity and complexity of TME to test the efficacy of nanocarriers • CAF and cancer cells develop resistance to therapies • Different CAF subtypes in tumors react differently to therapies due to their phenotypic and functional heterogeneities
What are the other challenges in targeting CAF?	• A limited number of studies show that subpopulations of CAF may have tumor suppressing ability as well. • Depleting such CAF subpopulations could result in tumor progression (although more evidence is needed to confirm this phenomenon).

Another key challenge in developing CAF-targeted diagnostics and therapeutics is the heterogeneity in CAF populations intertumorally, intratumorally, and across different patient populations. As mentioned in section 2, there are different subpopulations of CAF performing different tumor-promoting or tumor-restraining functions. Consequently, a too generic CAF targeting strategy may result in unfavorable outcomes. For instance, as discussed in section 3.7, depletion of certain kinds of tumor-restraining CAF can lead to enhanced invasion [253]. Conversely, reprogramming iCAF to myCAF can actually help restrain tumor growth [397]. This exemplifies the need to identify and classify different subpopulations of CAF and design subpopulation-specific strategies. However, given the current lack of knowledge on specific targetable markers and signaling pathways, CAF subpopulation targeting remains very difficult [396]. Table 10 summarizes some of the most pertinent challenges in developing CAF-targeted therapies.

Before targeting CAF or CAF-induced effects in the TME (such as desmoplasia) for cancer therapy, the shortcomings of previous clinical trials should be adequately assessed. Non-critical assessment of failures may not only result in developing further ineffective treatments but can also lead to conflicting outcomes in clinical trials. Table 11 gives an overview of the clinical trial outcomes related to CAF-targeting for cancer therapy.

In mouse models of pancreatic cancer, Olive *et al* showed that co-delivery of IPI-926, a drug that inhibits sonic hedgehog signaling (Shh) via targeting Smoothened (Smo), together with chemotherapy facilitated *GEM* accumulation at the pathological site [406]. As a result of depletion of desmoplasia and better *GEM* penetration, the treatment of PDAC models significantly improved. In subsequent clinical trials, however, poor outcomes were observed. This led to an in-depth study of the reasons for failure. In a follow-up study, by genetically deleting Shh and by long-term inhibition of Smo, it was observed that targeting these pathways enhanced tumor progression and metastasis, potentially due to increased vascularization [304].

In phase III clinical trial coined HALO-301, Pegvorhyaluronidase alfa (PEGPH20; a tumor stroma modulating agent that degrades HA) was expected to improve clinical outcomes of stage IV pancreatic cancer patients expressing high levels of HA. The concept behind using HALO was based on the fact that degrading HA would result in decreased tumor pressure and vascular compressing, thereby allowing the penetration of 'halo' in the surrounding tumor cells. The phase III trial was based largely on promising Phase I/II results [405]. However, the HALO-301 trial was halted prematurely due to failure in meeting the primary endpoint, which was the overall survival [405]. Although HALO-301 only included patients with a high expression of HA, it still failed in the trials which led to the understanding that the desmoplastic response alone does not cause chemoresistance, but additional intrinsic factors play a role [405]. In one study of 162 patients, the authors found that the dense stromal reaction was associated with improved disease-free survival, suggesting that stromal-depleting agents such as PEGPH20 may harm cancer treatment and enhance their metastatic capability [407]. In the future to target desmoplastic cancers, characterization of the complex interaction between TME and cancer is essential and for treatment, a combination of stromal modifying agents and other strategies must be considered [405].

From a biological and translational point of view, challenges for CAF targeting include the lack of specific markers, the intra-

Table 11
Overview of the clinical trial outcomes related to CAF-targeting for cancer therapy.

Target/ Inhibition	Drug/antibody	Outcome	Ref
FAP	**Sibrotuzumab** (anti-FAP antibody)	• Minimum clinical requirement of at least one complete/partial remission not met	[322]
LOXL2	**Simtuzumab** (IgG4 monoclonal antibody to LOXL2) + **FOLFIRINOX**	• Failed to show improved anti-tumor benefits in patients with metastatic KRAS mutant colorectal carcinoma.	[398,399]
LOXL2	**Simtuzumab + Gemcitabine**	• Failed to show improved anti-tumor benefits in patients with metastatic pancreatic adenocarcinoma.	[400,401]
Hh pathway	**Erivedge** (vismodegib)	• FDA-approved to treat basal cell carcinoma	[402]
Hh pathway	**Gemcitabine + Vismodegib**	• Adding Vismodegib to gemcitabine did not improve the overall response rate	[381,382]
Hh pathway	**Sonidegib**	• FDA-approved to treat basal cell carcinoma	[403]
FGFR	**Erdafitinib**	• FDA-approved to treat urothelial carcinoma in patients with susceptible FGFR genetic alteration	[404]
HA	**PEGPH20** (stroma modifying agent)	• Failed to show improvement in the duration of response for pancreatic tumors with high levels of HA.	[405]

tumoral and inter-tumoral heterogeneity of both cancer cells and CAF, and the lack of precise preclinical models. Moreover, little is understood about the origin of CAF and the inter-convertibility between the different subtypes. In a preclinical setting, it is of utmost importance that the tumor models used for testing recapitulate the complex interaction between the tumors and the surrounding environment. Genetically engineered mouse models (GEMM) have been shown to resemble human cancers with the promise of improved clinical outcomes of therapy when extrapolated from animal models to clinics [394]. But also GEMM are not perfect, as exemplified by the failure of IPI-926, which was evaluated in KPC mice (i.e. Kras and P53 double mutants) [406]. A detailed overview of the strengths and limitations of different pre-clinical cancer models is reviewed here [394]. While GEMM provide a better reflection of cancer development, including initiation, progression, and interaction with stromal and immune cells, it is challenging to work with GEMM, as they require experience, infrastructure, and continuous monitoring of disease development. Moreover, GEMM has certain biological limitations, such as differences between murine and human malignancies and the type of secondary responses occurring during tumor progression in humans *vs* mice.

On the material side, a prominent challenge includes the fabrication of delivery vehicles that can penetrate through desmoplastic tumors to reach CAF and tumors [394]. As described in more detail above, several nanocarriers have been studied for this purpose, showing that deeper penetration and improved therapeutic efficiency can be achieved by 1) downregulating CAF-induced ECM production; 2) depleting CAF; and/or 3) turning CAF into quiescent fibroblasts. In this context, the growing evidence of the presence of several subtypes of CAF within tumors raises the question of how distinct heterogeneities could be targeted. Related to this, there is still a vast impetus to develop personalized novel CAF-based nano-delivery systems depending upon patient-specific CAF heterogeneity. FAP-based CAF targeting of nanoparticles has already shown promising initial results in pre-clinical setups and its potential evaluation in clinical studies is eagerly awaited. Although the concept of antibody targeting cannot be compared directly with NP, the latter have certain advantages over the antibodies. They can e.g. be designed to deliver nucleic acids, can be made multifunctional to tackle more than one aspect of CAF targeting, and have a higher ability to load lower potency drugs.

Radionuclide imaging using FAPI has shown promising diagnostic applications in clinical trials for desmoplastic tumors. More research on finding the best therapeutic radionuclide to match the retention time of FAPI is required. Due to FAPIs lower retention time, shorter half-life radionuclides such as ^{213}Bi (Bismuth-213), and ^{188}Re (Rhenium-188) are more favorable to reduce unwanted toxicity. A further added advantage to this field can be achieved by using nanocarriers to increase the radiation dosage and also improve the retention time in the tumor. One of the challenges involved with nanocarriers is the high uptake of NP in the liver and spleen, which reduces the dose delivered to the tumor and also delivers unwanted radiation to the liver and spleen. In an aim to improve this, several strategies such as decreasing the NP size and NP pre-targeting were employed. In pre-targeting approaches, first, a nanomedicine is injected and allowed to accumulate at the target site for a sufficient time period. Following this, a second radiolabeled probe is administered that binds to the pre-administered nanomedicine. This approach involves intense optimization of two different agents and the time gap between their administration [408]. On this front, Lee et al. developed a radiolabeled liposomal formulation with high tracer uptake in the orthotopic pancreatic tumor mouse model, while preventing liposomal retention in the liver and spleen [409]. The authors achieved this by taking advantage of the different esterase activity in the tumor (very low) and mononuclear phagocyte system (MPS) (very high). In this study, the esterase-labile radiotracer-loaded liposomes upon accumulation in the liver and spleen were rapidly broken by the high esterase concentration, which resulted in the rapid clearance of the radiotracer. This strategy dramatically enhanced the tumor to-background ratio without affecting tumor uptake and also enabled early detection of an orthotopic pancreatic tumor model. Such strategies can also be employed for CAF targeting. Although promising, several questions remain unanswered such as: Is the clearance from MPS organs as effective as in humans? What is the differential esterase activity concentration in humans? Are there other endogenous enzymes present in the body which can be utilized? If these questions can be answered, more research on novel strategies/technologies to target CAF can be expected in the future [410].

Taking together, CAF have recently emerged as a key player in orchestrating multiple processes in the TME. While we did make good progress in recent years, there is still a lot to understand about their subtypes and roles. An improved understanding of CAF biology would shed light on important intratumoral events such as drug resistance, drug delivery issues, immunomodulation, and metastasis. Studies focused on understanding the different phenotypes of CAF subpopulation, their secretory profile, and their plasticity will be instrumental for unraveling the interplay between CAF, cancer cells, and other components of the TME. Identification of fibroblast-specific markers is crucial to classify and selectively target the CAF population. Techniques like scRNA-seq, cytometry by time-of-flight (CyTOF) combined with different *in vitro* and *in vivo* model systems can help decipher CAF subpopulation, their functional roles, and biomarkers [396]. Based on the target CAF subpopulation, effective anti-CAF therapies can be designed to either eliminate or reprogram CAF subpopulation to enhance therapeutic outcomes. To optimally do this, we need a deeper insight into the behavior of CAF intratumorally, intertumorally, and across different cancer patient populations. Understanding the molecular crosstalk and cellular signaling between cancer cells and CAF can help to get a deeper insight into CAF behavior. Identification of molecular pathways and signaling molecules can assist in the recognition of targets that can be potentially exploited for developing CAF targeted therapies. A summary of different signaling pathways involved in the crosstalk between CAF and cancer cells can be found in this review [411]. Bridging the gap by compiling and expanding known information, analyzing *meta*-data, using novel (e.g. single-cell) analysis techniques, and optimizing experimental conditions in CAF research will in the upcoming years advance our understanding and create new possibilities. Thus, there is still a lot to be explored and optimized when intending to generate CAF-based interventions. Ongoing and future studies will have to set out to systematically analyze the potential of CAF-directed diagnostics and therapeutics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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