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REVIEW
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Cancer-Associated Fibroblasts Build and Secure the Tumor Microenvironment

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Tumor cells reside in a highly complex and heterogeneous tumor microenvironment (TME), which is composed of a myriad of genetically stable non-cancer cells, including fibroblasts, immune cells, endothelial cells, and epithelial cells, and a tumor-specific extracellular matrix (ECM). Cancer-associated fibroblasts (CAFs), as an abundant and active stromal cell population in the TME, function as the signaling center and remodeling machine to aid the creation of a desmoplastic tumor niche. Although there is no denial that the TME and CAFs may have anti-tumor effects as well, a great deal of findings reported in recent years have convincingly revealed the tumor-promoting effects of CAFs and CAF-derived ECM proteins, enzymes, chemical factors and other downstream effectors. While there is growing enthusiasm for the development of CAF-targeting therapies, a better understanding of the complexities of CAF-ECM and CAF-cancer cell interactions is necessary before novel therapeutic strategies targeting the malignant tumor "soil" can be successfully implemented in the clinic.

Keywords: cancer-associated fibroblast, tumor microenvironment, extracellular matrix, therapy, mechanoreciprocity

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INTRODUCTION

In the last decades, despite considerable advances in the development of novel immunotherapies and targeted therapies, no significant improvements have been made in overall survival rates for patients with malignant solid tumors. One major reason for this lack of substantial improvement is the development of drug resistance in tumor cells, which usually reveals itself within a few months after patients are treated with anti-cancer drugs. An Achilles' heel of many current therapeutic approaches is that these therapies primarily target the fast-growing tumor "seeds" but largely ignore the fertilizing tumor "soil" – the tumor microenvironment (TME) (de Groot et al., 2017). The TME influences the penetration, distribution, and metabolism of therapeutic agents, and produces molecular factors and signals, which positively or negatively regulate how tumor cells grow, migrate and respond to therapeutic agents. As cancer-associated fibroblasts (CAFs) appear to be a major TME component in many tumors and are critical for shaping the "soil" within which the tumor cells thrive (LeBleu and Kalluri, 2018), they have become the prime target for the efforts to modify non-tumor cell behavior to suppress tumor growth. It is clear that the TME and CAFs are not always pro-tumorigenic due to the complexities of their interactions with tumor cells. However, in this review, we will mainly explore the tumor-promoting interactions between cancer cells and fibroblasts and how CAFs may be persuaded using novel therapeutic approaches to renounce their fealty to the tumor cells and even produce a tumor-suppressive "soil."

STROMAL FIBROBLASTS, MYOFIBROBLASTS, AND CAFs

Tumors are often referred to as "wounds that never heal" (Dvorak, 1986) because the stroma of a wound and a tumor share many similarities, such as fibroblast activation, increased extracellular matrix (ECM) protein production and intensive remodeling processes (Foster et al., 2018). Activated stroma is molecularly, biochemically and pathologically different from the normal stroma. In the stroma of normal human skin, fibrous proteins fill in the interstitial space between stromal fibroblasts while epithelial keratinocytes rest on the sheet-like basement membrane. Under normal physiological conditions, non-contractile fibroblasts are generally flat, spindle-shaped and recognized as quiescent and inert cells in the ECM (Valkenburg et al., 2018).

Myofibroblasts were first identified in the tissue wound repair process, during which fibroblasts or smooth muscle cells differentiate and gain a contractile phenotype (McAnulty, 2007). The major roles of myofibroblasts in wound healing are to contract the wounds and produce and organize the ECM (Darby et al., 2014). As the wound closes and heals, myofibroblasts become apoptotic and finally disappear as the scar is formed (Desmouliere et al., 1995). Myofibroblasts are different from normal fibroblasts in many aspects, including (1) ruffled membranes and a highly active endoplasmic reticulum (Baum and Duffy, 2011); (2) expression of alpha smooth muscle actin (α-SMA or ACTA2) and increased levels of vimentin (VIM) (Ronnov-Jessen and Petersen, 1993) and (3) formation of complex and organized stress fibers and fibronexus adhesion complexes (Rao et al., 2016). The bundles of microfilaments in myofibroblasts interact with the ECM proteins through fibronexus adhesion complexes, thereby allowing myofibroblasts to sense the tension in their surrounding microenvironment and maintain the cellular contractile force through the network of cytoskeletal proteins. As a feedback response, myofibroblasts increase matrix fibroplasia by producing ECM proteins, including collagen, elastin (ELN), fibronectin (FN1), tenascin (TNC), and remodeling enzymes, such as matrix metalloproteinases (MMPs).

Tumor growth recapitulates the basic wound healing program and shares many similarities, such as deposition and crosslinking of fibrin and FN1 and the recruitment of immune cells (Schafer and Werner, 2008). However, unlike a normal healing wound, which is restricted to a certain area and proceeds directionally through the steps of hemostasis, inflammation, proliferation, and maturation/remodeling, cancer cells distort the wound healing program and have the potential to migrate away or expand from the initiation site and invade adjacent tissues. CAFs are the fibroblasts found in the stroma of human cancers but differ from normal fibroblasts in their increased collagen and ECM protein production and up-regulated secretion of pro-tumor factors (Bauer et al., 2010; Xing et al., 2010; Pidsley et al., 2018). There are several important sources from which CAFs could be derived, including: (i) recruitment and activation of resident fibroblasts (Fukino et al., 2004); (ii) epithelial-mesenchymal transition (EMT) of resident epithelial cells (Petersen et al., 2001); (iii) endothelial to mesenchymal transition (EndMT) of resident endothelial cells (Zeisberg et al., 2007a,b) and (iv) differentiation of bone marrow mesenchymal cells (Quante et al., 2011). In a sense, CAFs or at least a subset of CAFs are wound-like myofibroblasts that mediate a deranged chronic wound healing program in tumors. For example, a large part of CAFs share similar features as α-SMA-positive (α-SMA+) myofibroblasts (Shiga et al., 2015). In addition, other than myofibroblastic CAFs, subpopulations of CAFs without α-SMA expression have also been reported, e.g., in pancreatic cancer (Ohlund et al., 2017).

HETEROGENEITY OF STROMAL FIBROBLASTS, MYOFIBROBLASTS, AND CAFs

Understanding the state, complexity and heterogeneity of normal fibroblasts may shed light on the origins of CAFs, how they form and how their transdifferentiation may be regulated in the early stages of tumorigenesis and at the tumor front. Two major populations of fibroblasts in the human dermis are papillary and reticular fibroblasts, which possess distinct morphology, molecular expression, and cellular functions (Harper and Grove, 1979). Janson et al. (2012) performed gene expression analysis on cultured papillary and reticular fibroblasts and identified 116 differentially expressed genes. However, except for matrix Gla protein (MGP), which is almost exclusively expressed in the reticular dermis, they did not discover any *in vivo* markers to separate the two fibroblast populations. Korosec et al. (2019) performed lineage identity and location studies of human dermis using two markers, fibroblast activation protein (FAP) and THY1 (Cluster of Differentiation 90 or CD90). They found that papillary fibroblasts are FAP^{+}; $THY1^{-}$, whereas FAP^{-}; $THY1^{+}$ fibroblasts are mainly of the reticular lineage. Their data showed papillary and reticular fibroblasts are not completely separated according to their spatial location.

However, recent studies have suggested that there exist more functionally distinct fibroblast subpopulations within the human dermis. A single-cell RNA sequencing (scRNA-seq) study by Philippeos et al. (2018) showed that there are five distinct fibroblast populations in adult human skin, which can be separated based on the expression of cell surface markers, including THY1, CD39, CD26 (DPP4), and regulator of G protein signaling 5 (RGS5), and are not spatially segregated. Tabib et al. (2018) performed single-cell transcriptomal analysis of cells obtained from whole skin without pre-purifying fibroblast populations. They identified two major fibroblast populations based on the expression of SFRP2/DPP4 and FMO1/LSP1 markers and five minor cell populations using CRABP1, COL11A1, PRG4, ANGPTL7, and SFRP4. In addition, there are several subpopulations in each major fibroblast population. These scRNA-seq data showed a complex and heterogeneous picture of fibroblast composition and functionality in the human dermis, which is simply beyond our original understanding of skin fibroblasts. Nevertheless, it remains to be understood how these subpopulations of fibroblasts react to either wounding or the tumorigenic process and evolve into myofibroblasts or CAFs.

Local fibroblasts are the most common origin of myofibroblasts (Hinz et al., 2007). However, several other cell types are able to differentiate into myofibroblasts, including smooth muscle cells or pericytes (Hinz et al., 2007). Fibrocytes, for example, can differentiate into myofibroblasts in skin, liver and lung tissues (Mori et al., 2005; Iwaisako et al., 2012; Ashley et al., 2017). In the liver, hepatic stellate cells are the source of myofibroblasts in liver fibrosis (Wells and Schwabe, 2015). Because of the nature of its diverse origins, myofibroblasts appear to be a heterogeneous group as well. α-SMA is the most commonly used marker to identify myofibroblasts (McAnulty, 2007). In addition, extra domain A fibronectin (EDA-FN), periostin (POSTN) and prolyl-4-hydroxylase (P4HB) have also been suggested as potential markers for myofibroblasts (Moore-Morris et al., 2014; Ngo et al., 2014; Kanisicak et al., 2016). A recent study proposed that amine oxidase, copper containing 3 (AOC3) and homeobox protein NKX2-3 are two biomarkers of pericryptal myofibroblasts in the colon and rectum (Hsia et al., 2016). Furthermore, markers that stain stromal fibroblasts can also be used to stain myofibroblasts, such as platelet derived growth factor receptor alpha (PDGFRA), THY1, and VIM, although they are not specific for myofibroblasts (Matthijs Blankesteijn, 2015).

Just like normal fibroblasts, CAFs appear to be a heterogeneous group of cells with different origins and different functions. This similarity was manifested by a study reported by Lambrechts et al. (2018). By performing scRNA-seq of 52,698 stromal cells isolated from human lung tumors and comparing with matching non-malignant lung samples, the authors identified five distinct types of fibroblasts in lung tumors, which all express their own unique set of collagens and ECM proteins that are different from non-malignant fibroblasts. Using a three-dimensional (3D) co-culture platform, Ohlund et al. (2017) identified two distinct populations of myofibroblasts and inflammatory fibroblasts in pancreatic ductal adenocarcinoma (PDA). More recently, the obscurity in CAF characterization has been further addressed by efforts to determine the exact composition of human tumor tissues using scRNA-seq. scRNA-seq data derived from head and neck squamous cell carcinoma (HNSCC) suggested that tumor myofibroblasts and CAFs may represent distinct fibroblast subpopulations (Puram et al., 2017). Overall, the authors were able to detect, in addition to normal fibroblasts and myofibroblast-like cells, two subsets of CAFs depending on the expression of FAP, THY1, connective tissue growth factor (CTGF) and podoplanin (PDPN). In their study, tumor myofibroblasts were identified based on the expression of α-SMA, melanoma cell adhesion molecule (MCAM), myosin light chain kinase (MYLK), and myosin light chain 9 (MYL9). Interestingly, scRNA-seq of colorectal cancer samples also revealed at least three fibroblast populations (Li et al., 2017). One population can be described as normal fibroblasts, the second one as myofibroblasts, which are positive for α-SMA, transgelin (TAGLN) and PDGFA, and the third one as a CAF population that is characterized by MMP2, decorin (DCN) and collagen type I alpha 2 (COL1A2). The authors determined that a key signaling pathway emanating from CAFs/myofibroblasts is transforming growth factor beta (TGF-β)/INHBA signaling, ascertaining that CAFs are not just ECM-producing factories. The scRNA-seq results of fibroblast populations are in good accordance with attempts to characterize CAFs using fluorescence activated cell sorting (FACS) (Costa et al., 2018). Such efforts in human breast cancer using six CAF markers, including FAP, integrin beta 1 (ITGB1), α-SMA, FSP1, platelet derived growth factor receptor beta (PDGFRB), and caveolin-1 (CAV1), allowed the authors to identify four distinct CAF populations, of which some were preferentially present in subsets of breast cancers. Two of the CAF populations expressed α-SMA and probably represent myofibroblast-like cells. However, a comparison of the two α-SMA+ populations revealed that one was similar to pericytes and expressed MCAM and a gene signature related to the regulation of actin cytoskeleton and muscle contraction. The second α-SMA+ population exhibited an immune-regulatory gene signature. These CAFs can function as immune-suppressors and regulators of T lymphocytes and create an immunosuppressive environment through a multi-step mechanism (Costa et al., 2018).

scRNA-seq studies of CAFs have suggested that CAF subtypes could be attributed to their origin in spatial subgroups of normal fibroblasts (Philippeos et al., 2018; Tabib et al., 2018). However, Biffi et al. (2019) reported that tumor-secreted TGF-β/ and IL1 can promote CAF heterogeneity. Subsets of CAFs can function to either support or suppress tumor cells. For example, it was reported that cancer cells undergo the EMT process and acquire invasive phenotypes through the activation of the TGF-β-SMAD signaling pathway induced by CAFs (Bellomo et al., 2016). In addition, by producing pro-angiogenic factors, such as fibroblast growth factor 2 (FGF2) and VEGFA (De Palma et al., 2017), CAFs regulate angiogenesis in the stroma, thereby providing essential nutrients for highly proliferative tumor cells. CAFs can also assist tumor cells in overcoming immune surveillance by recruiting immunosuppressive cells, such as M2 macrophages and myeloid-derived suppressor cells (MDSC) (Flavell et al., 2010; Yang et al., 2016). However, it was reported that ablation of subsets of α-SMA+ CAFs in PDA could result in a more aggressive cancer phenotype and reduced animal survival (Ozdemir et al., 2014). In summary, the heterogeneity of CAFs reflects the diversity and complexity of the TME, and more careful research is needed to fully comprehend the interactions among CAFs, tumor cells and the ECM.

CAF-DERIVED ECM PROTEINS

The tumor ECM is composed of a complex mixture of macromolecules, including fibrous proteins (collagen, ELN), proteoglycans (heparan sulfate, chondroitin sulfate), glycosaminoglycans (hyaluronic acid), and glycoproteins (FN1, laminins, TNC) (Botti et al., 2013). ECM proteins are not just bystanders of the tumorigenic process. Instead, they provide structural signals and support for tumor cells to grow and

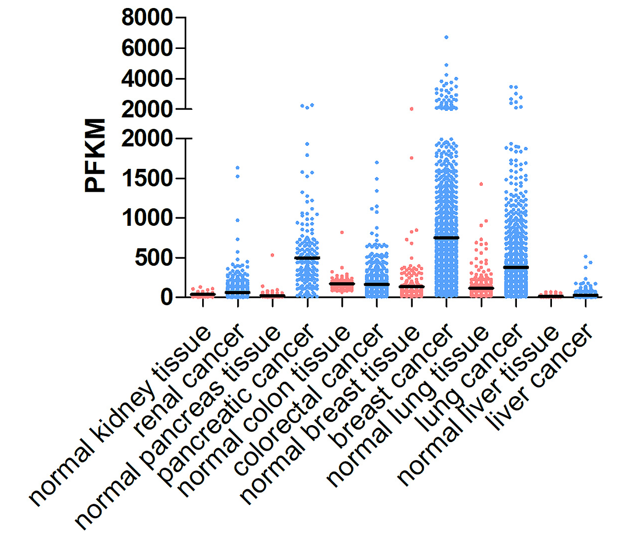

FIGURE 1 | COL1A1 expression levels in different cancer types and corresponding normal tissues. COL1A1 expression levels vary in different types of cancer, including renal cancer, pancreatic cancer, colorectal cancer, breast cancer, lung cancer, and liver cancer and generally are higher in tumor tissues than those in normal tissue. The data are obtained from https://www.proteinatlas.org/ENSG00000108821-COL1A1/tissue and https://www.proteinatlas.org/ENSG00000108821-COL1A1/pathology.

migrate. Although many other stromal cell types and tumor cells can also produce ECM proteins, CAFs appear to be the major player in the stroma that synthesizes, secrets, assembles and modifies the ECM composition and organization (Faouzi et al., 1999; Yoshimura et al., 2015; Erdogan et al., 2017). For example, elevated collagen production and crosslinking have been coupled with increased tumor stiffness and progression. It was estimated that fetal rat fibroblasts synthesize about 40 molecules of procollagen/cell per second (McAnulty et al., 1991). Many cancers are characterized by elevated levels of collagen production, e.g., COL1A1 (**Figure 1**). Faouzi et al. (1999) reported that myofibroblasts are the primary source of collagen (types I, IV, V and VI) in the stroma of human hepatocellular carcinoma. In addition, CAF-derived laminin was shown to induce cervical cancer cell migration via the interaction with integrin α6β4 (Fullar et al., 2015). In an *in vitro* ovarian cancer spheroid model, CAF-secreted versican promoted cancer invasion in a TGF-β-dependent manner (Yeung et al., 2013).

FN1 was first found to be overexpressed in human solid tumor specimens in 1981 (Stenman and Vaheri, 1981). Although tumor cells produce FN1 themselves, stromal cells, such as CAFs, are indispensable for bulk FN1 assembly (Attieh et al., 2017; Erdogan et al., 2017). Like collagen, the pro-tumorigenic role of FN1 is also well-acknowledged. In 1998, Menzin et al. (1998) proposed that FN1 may play an important role in regulating the invasive phenotype and poor patient prognosis in ovarian cancer. FN1 was also documented to promote cell cohesion, basement membrane invasion and tumor growth in glioblastoma (GBM). Depletion of FN1 in GBM cells resulted in weaker cell-cell contact and less collective migration in an *in vitro* spheroid model, highlighting the role of FN1 as a "biological glue" (Serres et al., 2014). The role of FN1 in cell cohesion has also been observed in fibroblast spheroids. FN1-depleted fibroblasts failed to form compact spheroids *in vitro*. Furthermore, the blockade of FN1-integrin interactions impeded fibroblast activation (Salmenpera et al., 2008).

Tenascin is another highly expressed ECM glycoprotein in the tumor stroma, such as the stroma of canine mammary carcinoma, pancreatic cancer and prostate cancer, and is associated with poor patient prognosis (Yoshimura et al., 2015; Cai et al., 2017; Ni et al., 2017). Mouse embryonic fibroblasts lacking TNC have robust overexpression of tissue plasminogen activator (tPA) and increased capacity to digest fibrin *in situ* (Brellier et al., 2011). Furthermore, they discovered that there was a correlation between *in vivo* TNC expression and fibrin accumulation in head and neck squamous cell carcinomas (SCC) and lung carcinomas, further confirming that TNC functions as a regulator of the fibrinolytic system.

CAF-DERIVED ECM ENZYMES

Tumor progression and metastasis require a distinct ECM biomechanical architecture, for which CAFs not only produce and secrete ECM proteins and also actively participate in the ECM proteolysis, crosslinking and assembly processes. In such a rigid and highly crosslinked tumor stroma, drug penetration is one potential reason for tumor cells to escape therapy. In addition, CAF-mediated ECM remodeling is a highly responsive process of receiving, processing and responding to the cellular, molecular and mechanical signals in the TME. The lysyl oxidase (LOX) family and MMPs represent two major types of remodeling enzymes produced by CAFs. As a highly adaptive and mechanically responsive stromal cell type, CAFs sense and respond to the ECM stiffness in a LOX/MMP-dependent manner and further fine-tune the CAF-ECM interactions.

The LOX family oxidases include five members: LOX and lysyl oxidase like (LOXL) 1, 2, 3, and 4 (Wang et al., 2016). They share similar structures and catalyze the cross-linking of collagen and ELN by oxidation, contributing to increased stiffness of the tumor stroma. In tissue fibrosis, it was demonstrated that fibroblast-derived LOX could be induced by different soluble factors, such as insulin-like growth factor-binding protein 5 (IGFBP5) (Nguyen et al., 2018) and POSTN (Kumar et al., 2018), and by the transcription factor hypoxia inducible factor 2 alpha (HIF2A) (Hikage et al., 2019). Elevated levels of LOX family oxidases are often observed in cancers and play a prominent role in cancer progression. Gene expression analysis of mouse mammary tumors revealed that activated fibroblasts are the major producers of LOX family oxidases (Pickup et al., 2013). When colon cancer patient-derived CAFs and normal fibroblasts were compared by proteomic analysis, LOXL2 was found to be overexpressed in CAFs and was identified as a predictive prognostic factor in stage II colon cancer patients (Torres et al., 2015). Similarly, LOXL2 expression in gastric CAFs was also demonstrated to be positively correlated with the invasive ability of gastric cancer cells (Kasashima et al., 2014).

TABLE 1 | Expression of MMP2 and MMP9 is correlated with cancer progression and metastasis.

MMP2	References	MMP9	References
Basal-cell carcinoma	Gozdzialska et al., 2016	Basal-cell carcinoma	Gozdzialska et al., 2016
Brain cancer	Wang M. et al., 2003; Tabouret et al., 2014	Brain cancer	Wang M. et al., 2003; Li et al., 2016
Breast cancer	Yari et al., 2014; Chen Y. et al., 2015; Ramos et al., 2016; Tabouret et al., 2016	Breast cancer	Wu et al., 2014; Yousef et al., 2014
Colorectal cancer	Groblewska et al., 2014	Gastric cancer	Wang et al., 2014; Chen S.Z. et al., 2015
Endometrial adenocarcinoma	Li et al., 2014	Liver cancer	Sun et al., 2014
Gastric cancer	Wang et al., 2014	Lung cancer	Lee et al., 2015; Zhang et al., 2015; Gong et al., 2016; Yu et al., 2016
Lingual and gingival cancers	Nishio et al., 2016	Pancreatic cancer	Jakubowska et al., 2016
Lung cancer	Zhang et al., 2015	Pituitary adenoma	Liu et al., 2016
Melanoma	Kamyab-Hesari et al., 2014	Prostate cancer	Oguic et al., 2014
Osteosarcoma	Zhang and Zhang, 2015	Squamous cell carcinoma	Stanciu et al., 2016
Ovarian cancer	Fu et al., 2015		

In breast cancer, LOXL2 inhibition showed anti-tumor effects in reducing tumor size and angiogenesis. Furthermore, a combination of LOX and LOXL2 inhibitors resulted in even smaller and less metastatic tumors (Chang et al., 2017). Interestingly, in mice bearing aggressive breast cancer, anti-LOXL2 monoclonal antibody AB0023 exhibited potent inhibitory effects in activated fibroblast as suggested by an 88% reduction of α-SMA+ cells by immunohistochemistry (IHC) after AB0023 treatment (Barry-Hamilton et al., 2010). The inhibitory effect was also shown to be closely associated with the reduction in cross-linked collagenous ECM matrix. Recently, an *in vitro* study using siRNA adenovirus vector to silence LOXL2 expression in mouse lung fibroblast also showed that the proliferation of lung fibroblasts was significantly decreased via the TGF-β/Smad signaling pathway (Wen et al., 2018). All these findings highlighted the role of CAF-derived LOX family oxidases in regulating tumor migration and invasion and potential beneficial outcomes of targeting CAF-synthesized LOX family oxidases.

The ability of cancer cells to digest surrounding ECM and localize to distal sites has long been attributed to MMPs, which are zinc-containing endopeptidases. MMPs play pivotal roles in creating the paths for tumor cells to leave the primary tumor niche and wade through the stiff matrix. There are 24 MMPs in mammals (Vandenbroucke and Libert, 2014), of which MMP2 and MMP9 are found to be overexpressed in many cancer types and promote tumor progression and metastasis (**Table 1**). CAFs were shown to be the major producer of MMP2 in mouse lung tumors as indicated by IHC staining results showing MMP2 primarily localizes to fibroblasts (Bates et al., 2015). Using the online database proteinatlas.org, we summarized the correlation between 13 MMPs and patient prognosis status in nine human cancers in **Table 2** based on the RNA-Seq data. Four MMPs (MMP10, MMP15, MMP24, MMP25) are shown to be favorable to patient prognosis as their expression levels are positively correlated with patient survival. However, the expression levels of eight MMPs (MMP1, MMP3, MMP7, MMP11, MMP12, MMP14, MMP19, and MMP28) are shown to be negatively correlated with patient survival. Interestingly, MMP9 seemed to have context-dependent roles in different cancer types. In conclusion, the roles of different MMPs in the TME need to be carefully examined based on cancer types and stages, and this should also be one major consideration when designing, dosing and scheduling MMP-targeting drugs for cancer patients (Iyer et al., 2012).

CAF-ECM INTERACTIONS

The interactions between CAFs and the ECM influence the stiffness of the tumor stroma and can be described using the term "mechanoreciprocity" (van Helvert et al., 2018), which consists of both "outside-in" and "inside-out" signaling modes (Shattil et al., 2010). The "outside-in" signaling mode is a well-established mechanism, by which ECM proteins can function as ligands and bind to integrin receptors on the cell membrane (**Table 3**). Integrins are transmembrane receptors composed of a heterodimer of α and β subunits. As shown in **Figure 2**, when the cells encounter a rigid ECM, the integrin molecules become dimerized to trigger the focal adhesion cascade and the activation of downstream signaling, including tyrosine protein kinase SRC and focal adhesion kinase FAK1, thereby converting external mechanical signals into cellular and biochemical signals inside the cells (Barczyk et al., 2010; Tucker and Chiquet-Ehrismann, 2015; Benito-Jardon et al., 2017). Integrin $\alpha 11\beta 1$ is a stromal cell-specific receptor for collagen and also known as an important regulator for fibroblast activation (Carracedo et al., 2010). Zhu et al. (2007) showed that the growth of the tumors formed by non-small-cell lung carcinoma (NSCLC) cell lines, A549, NCI-H460, and NCI-H520 mixed with integrin $\alpha 11$-deficient fibroblasts were significantly impeded as compared with the tumors derived from the mixture of either tumor cell line and wild-type fibroblasts. In this case, fibroblasts, originally good "listeners" and "responders" to the mechanical cues, lost their active ECM remodeling ability after the fibroblast-ECM interaction was blocked. In another example, cardiac fibroblasts cultured on a stiff matrix expressed increased amounts of LOX, further crosslinked collagen fibers and stiffened the ECM. To the contrary, the inhibition of the binding between $\alpha 2\beta 1$ integrin and collagen I ablated this effect and downregulated LOX expression (Gao et al., 2016). In the "outside-in" signaling mode, the mechanical cues can also activate other signaling pathways in fibroblasts, such as the mitogen-activated protein kinase (MAPK)

TABLE 2 | Correlations between MMP/LOX expression and the 5-year survival rates of cancer patients**.

MMP/LOX	Prognosis*	Cancer type	5-year survival		Sample size	*p*-value
			High expression	Low expression		
MMP1	Unfavorable	Renal cancer	60%	82%	877	9.90E-10
	Unfavorable	Liver cancer	36%	50%	365	0.0000042
	Unfavorable	Cervical cancer	59%	74%	291	0.00047
MMP3	Unfavorable	Pancreatic cancer	5%	34%	176	0.00041
	Unfavorable	Cervical cancer	45%	71%	291	0.00097
MMP7	Unfavorable	Liver cancer	38%	60%	365	0.00025
	Unfavorable	Lung cancer	34%	50%	994	0.00034
MMP9	Unfavorable	Renal cancer	64%	78%	877	0.000041
	Favorable	Endometrial cancer	81%	60%	541	0.00025
	Unfavorable	Liver cancer	37%	64%	365	0.00072
MMP10	Favorable	Urothelial cancer	48%	27%	406	0.00071
MMP11	Unfavorable	Renal cancer	65%	81%	877	0.00026
MMP12	Unfavorable	Liver cancer	33%	51%	365	0.00014
MMP14	Unfavorable	Renal cancer	58%	73%	873	0.00013
	Unfavorable	Ovarian cancer	20%	35%	373	0.00095
MMP15	Favorable	Renal cancer	86%	65%	877	8.50E-08
	Favorable	Urothelial cancer	49%	30%	406	0.00031
MMP19	Unfavorable	Renal cancer	62%	81%	877	8.60E-09
MMP24	Favorable	Renal cancer	74%	51%	877	8.20E-11
MMP25	Favorable	Head and neck cancer	51%	29%	499	0.000011
MMP28	Unfavorable	Pancreatic cancer	16%	40%	176	0.0000063
LOX	Unfavorable	Renal cancer	64%	87%	877	3.90E-08
	Unfavorable	Urothelial cancer	25%	51%	406	0.00033
	Unfavorable	Liver cancer	36%	52%	365	0.00074
LOXL1	Unfavorable	Glioma	0	10%	153	0.00013
LOXL2	Unfavorable	Lung cancer	31%	57%	994	1.50E-07
	Unfavorable	Renal cancer	54%	72%	877	2.90E-07
	Unfavorable	Cervical cancer	52%	82%	291	0.0000098
	Unfavorable	Glioma	0	10%	153	0.00018
	Unfavorable	Pancreatic cancer	6%	43%	176	0.00091
LOXL3	Unfavorable	Renal cancer	61%	77%	877	8E-08
LOXL4	Unfavorable	Glioma	2%	16%	153	0.00054
	Unfavorable	Ovarian cancer	23%	39%	373	0.00096

**The prognosis of each group of patients was examined by Kaplan–Meier survival estimators, and the survival outcomes of the two groups were compared by log-rank tests.*
***Data available from:*
MMP1: https://www.proteinatlas.org/ENSG00000196611-MMP1/pathology
MMP3: https://www.proteinatlas.org/ENSG00000149968-MMP3/pathology
MMP7: https://www.proteinatlas.org/ENSG00000137673-MMP7/pathology
MMP9: https://www.proteinatlas.org/ENSG00000100985-MMP9/pathology
MMP10: https://www.proteinatlas.org/ENSG00000166670-MMP10/pathology
MMP11: https://www.proteinatlas.org/ENSG00000099953-MMP11/pathology
MMP12: https://www.proteinatlas.org/ENSG00000262406-MMP12/pathology
MMP14: https://www.proteinatlas.org/ENSG00000157227-MMP14/pathology
MMP15: https://www.proteinatlas.org/ENSG00000102996-MMP15/pathology
MMP19: https://www.proteinatlas.org/ENSG00000123342-MMP19/pathology
MMP24: https://www.proteinatlas.org/ENSG00000125966-MMP24/pathology
MMP25: https://www.proteinatlas.org/ENSG00000008516-MMP25/pathology
MMP28: https://www.proteinatlas.org/ENSG00000271447-MMP28/pathology
LOX: https://www.proteinatlas.org/ENSG00000113083-LOX/pathology
LOXL1: https://www.proteinatlas.org/ENSG00000129038-LOXL1/pathology
LOXL2: https://www.proteinatlas.org/ENSG00000134013-LOXL2/pathology
LOXL3: https://www.proteinatlas.org/ENSG00000115318-LOXL3/pathology
LOXL4: https://www.proteinatlas.org/ENSG00000138131-LOXL4/pathology

TABLE 3 | Integrins and their ECM partners.

ECM Protein	Interacting Integrins	References
Collagen	α1β1, α2β1, α10β1, α11β1	Leitinger, 2011
Fibronectin	α4β1, α5β1, α8β1, αIIbβ1, αvβ3, αvβ6, αvβ8	Pankov and Yamada, 2002; Danen et al., 2005
Tenascin-C	α9β1, α8β1, αvβ1, αvβ6	Tucker and Chiquet-Ehrismann, 2015
Laminin	α3β1, α6β1, α7β1, α6β4	Belkin and Stepp, 2000; Yamada and Sekiguchi, 2015

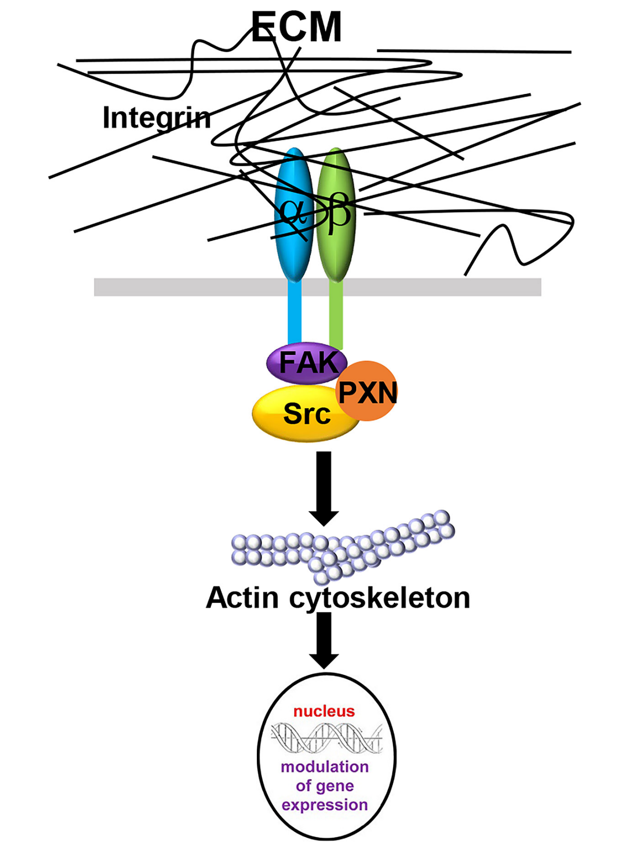

FIGURE 2 | The "outside-in" signaling mode in CAF-ECM interactions. When the membrane-bound integrin receptors interact with ECM proteins, integrin α- and β- subunits dimerize to activate downstream FAK1 and Src signaling and induce cytoskeleton remodeling, thereby converting external mechanical signals into cellular and biochemical signals inside the cells.

pathway (Wang J. et al., 2003; Paszek et al., 2005). In addition, it was reported that increased ECM stiffness could also activate the SRC-YAP-MYL9/MYL2 axis in CAFs to maintain the CAF phenotype. A positive feedback loop is established between CAF function and ECM stiffness, leading the stiff tumor stroma to become even stiffer and more favorable for cancer cell invasion (Calvo et al., 2013).

The "inside-out" signaling mode refers to the regulation of integrin-ECM interactions by intracellular signals. CAFs respond to tissue tension and exert their ECM remodeling and assembly abilities to further increase the stiffness of the stroma. The "inside–out" signaling mode is normally triggered by the binding of intracellular molecules, such as talin or kindlin, to the tails of integrins, leading to an increased affinity for extracellular ligands and enhanced ECM signaling (Shattil et al., 2010). For example, CAFs exert contractile forces and mediate extracellular FN1 assembly mainly via integrin αvβ3, leading to increased FN1 fibrillogenesis and ultimately contributing to tumor invasion (Attieh et al., 2017). Similarly, FN1 production and assembly were also observed in CAFs in prostate cancer. Erdogan et al. (2017) reported that CAFs produce an FN1-rich ECM with anisotropic fiber orientation as compared with normal fibroblasts and regulate cancer cell migration. In their study, CAFs remodel the FN1-rich ECM via the non-muscle myosin II (NMII)-α5β1 integrin axis.

DIRECT CAF-CANCER CELL CONTACT

Cancer-associated fibroblast-dependent tumor-promoting roles have long been attributed to the CAF secretome, but there is no denying that direct cell-cell contact also plays an important role in CAF-mediated cancer cell migration and invasion. Labernadie et al. (2017) discovered a heterotypic E-cadherin/N-cadherin adhesion complex between CAF and SCC cells. As shown in **Figure 3**, CAFs migrate through the ECM via integrin-mediated

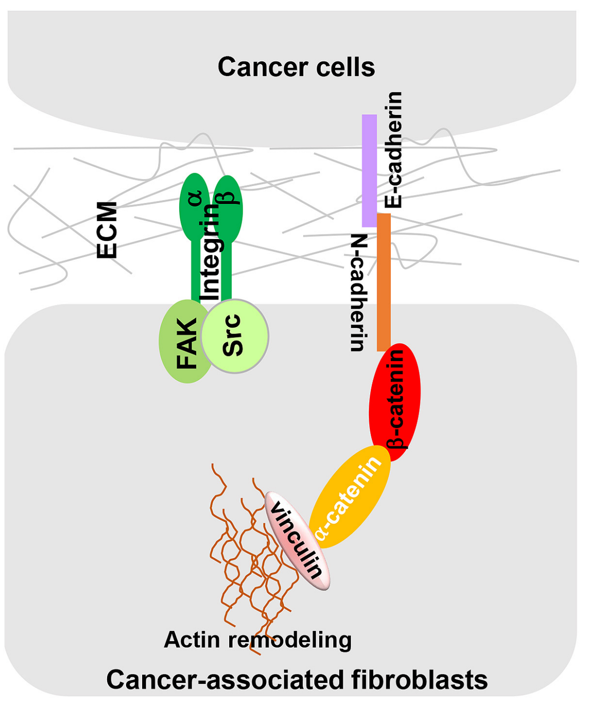

FIGURE 3 | A heterotypic E-cadherin/N-cadherin complex mediates CAF-SCC cell contact. As reported by Labernadie et al. (2017) intercellular physical force is transmitted between SCCs and CAFs by a heterophilic adhesion complex involving N-cadherin at the CAF membrane and E-cadherin at the cancer cell membrane. This heterotypic CAF-cancer cell interaction triggers β-catenin recruitment, α-catenin/vinculin interaction, and actin remodeling, allowing CAFs to exert an intercellular physical force on cancer cells and promote cooperative tumor invasion.

cytoskeleton remodeling and actomyosin reassembly while dragging tumor cells through CAF-cancer cell interaction via this heterotypic cadherin complex. The intercellular physical force between cancer cells and CAFs promote cooperative tumor invasion by triggering β-catenin recruitment, β-catenin/vinculin interaction and actin remodeling in both cell types.

In NSCLC, CAFs could potently enhance the motility of NSCLC cells through direct cell-cell contact via the hedgehog signaling pathway. Two co-culture systems (direct co-culture and indirect co-culture) were utilized to differentiate whether the motility-promoting effect is mediated by paracrine factors or cell-cell contact. Interestingly, increased tumor cell migration was only shown in a direct co-culture system, suggesting that CAF-promoted NSCLC cell migration is mediated by direct cell-cell contact (Choe et al., 2013). PDPN is a transmembrane glycoprotein that is known to be correlated with poor patient prognosis in lung adenocarcinoma (Ito et al., 2012). In an *in vitro* 3D collagen invasion model, PDPN-positive (PDPN+) CAFs accelerated lung tumor cell invasion into the collagen matrix. Ablation of PDPN reduced the invasive behavior of both CAFs and lung tumor cells. Because PDPN+ CAFs were observed to display high RHOA activity, RHO Kinase (ROCK) inhibitors were used to treat CAFs before co-culturing with lung tumor cells. ROCK inhibition suppressed PDPN-induced tumor cell migration, highlighting the role of the RHOA/ROCK axis in CAF-dependent tumor invasion (Neri et al., 2015).

INDIRECT CAF-CANCER CELL INTERACTIONS

Paracrine signaling between CAFs and cancer cells represents another well-studied mode of interaction between the two cell types that shapes the TME and promotes tumor growth. Hepatocyte growth factor (HGF) is a paracrine growth factor known to contribute to cancer progression. In cancer cells, HGF activates downstream RAS/MAPK and PI3K signaling pathways by binding to its receptor MET (Organ and Tsao, 2011). Cytokine antibody arrays suggested that HGF was the most significantly upregulated secreted factor in CAFs in breast cancer when compared to normal fibroblasts, which is positively correlated to their pro-tumorigenic ability to promote breast tumorigenesis in mice (Tyan et al., 2011). Similarly, the tumor-promoting functions of CAF-derived HGF were also observed in gastric cancer. By ablating HGF expression *in vivo*, CAFs failed to promote tumor growth in nude mice (Wu et al., 2013). Interestingly, CAF-derived HGF is also sufficient to induce RAF inhibitor resistance via the binding of its receptor MET and reactivation of the MAPK and PI3K/AKT signaling pathways in melanoma cells. 50 nM of recombinant HGF induced strong drug resistance to a BRAF inhibitor, vemurafenib, in several melanoma cell lines (Straussman et al., 2012).

CXCL12, also known as stromal cell-derived factor 1 (SDF1), is an important regulator in cancer initiation, angiogenesis, and metastasis (Orimo et al., 2005; Sugihara et al., 2015; Teng et al., 2016). In addition, CXCL12 was shown to induce angiogenesis by recruiting endothelial progenitor cells (EPCs) in breast cancer, thereby providing sufficient nutrients to fuel tumor growth and metastasis. Furthermore, after mice bearing breast cancer were treated with antibodies targeting CXCL12, reduced tumor volume and cell number were observed (Orimo et al., 2005). It was reported that CAF-derived CXCL12 activated TGF-β-regulated C-X-C chemokine receptor type 4 (CXCR4) expression in human prostatic epithelial BPH-1 cells to induce tumorigenesis. The CAF-conditioned medium was sufficient to induce CXCR4-AKT activation in BPH-1 cells *in vitro*. *In vivo* tumor grafting experiments also supported this claim. CXCR4-deficient prostate tumors were significantly smaller and less invasive as compared to control tumors, confirming the role of the CXCL12-CXCR4 axis in initiating tumor formation (Ao et al., 2007). The EMT process represents a pivotal mechanism used by cancer cells for migration and invasion. It was shown *in vitro* that CAF-derived CXCL12 functions as an important EMT inducer in breast cancer cells by regulating the Wnt/β-catenin signaling pathway (Shan et al., 2015). TGF-β is another multifunctional cytokine that is well-known for its role in inducing the EMT process. CAF-derived TGF-β1 promoted the aggressive phenotypes of breast cancer cells by inducing EMT through the activation of TGF-β/SMAD signaling. The EMT phenotype was reversed in the cells after the addition of TGF-β1 neutralizing antibody (Yu et al., 2014).

THERAPEUTIC PERSPECTIVES: TARGETING THE ECM MICROENVIRONMENT

Despite the growing enthusiasm for the development of CAF-targeting therapies, targeting CAFs has been challenging and lacks real and meaningful progress. One interesting example is FAP. Murine anti-FAP antibody F19 showed a significant tumor-inhibitory effect in xenograft models of lung, pancreas, and head and neck cancers with no obvious signs of toxicity (Ostermann et al., 2008). Because of promising pre-clinical data, a humanized version of murine anti-FAP antibody, sibrotuzumab, has been designed and tested in a phase I clinical trial and was determined to be safe and tolerable (Scott et al., 2003). However, in the phase II study in metastatic colorectal cancer, sibrotuzumab showed no therapeutic benefits (Hofheinz et al., 2003). Therefore, instead of targeting a specific subset of CAFs or CAFs in general, identifying the exact mechanisms that CAFs use to support cancer cells may help to develop better therapeutic strategies, e.g., based on CAF autophagy (Zhang et al., 2018), or based on the specific ECM proteins that are produced by CAFs.

TARGETING ECM PROTEINS

Humanized anti-collagen antibodies and ECM inhibitors have emerged as promising agents for cancer therapy (de Jonge et al., 2006; Koon et al., 2011). Halofuginone is an inhibitor of collagen I and was shown having anti-tumor activities in mouse models of prostate cancer (Gavish et al., 2002), pancreatic cancer (Spector et al., 2010) and lung cancer (Taras et al., 2006). D93/TRC093 is a humanized monoclonal antibody that specifically binds the HU177 cryptic collagen epitope within the tumor ECM with potential antiangiogenic and antitumor activities (Cretu et al., 2007; Caron et al., 2016). In the study conducted by Caron et al. (2016) D93/TRC093 was found to restrict the accumulation of α-SMA+ fibroblasts, which could be explained by the inhibition of integrin $\alpha_{10}\beta_1$-mediated fibroblast adhesion and migration on denatured collagen. In a phase I clinical study, TRC093 was shown to be well-tolerated and had tumor-inhibitory effects as monotherapy and in combination with bevacizumab in 19 patients carrying different types of solid tumors (Robert et al., 2010).

Conjugating a monoclonal antibody with a cell-killing agent is a new approach to develop novel targeted anti-cancer agents. In the past two decades, FN1-targeting antibodies have been designed and tested in different models. L19 is a monoclonal antibody known to target the ED-B domain of FN1. By attaching anti-angiogenesis drugs to L19, the fusion protein was demonstrated to exhibit strong anti-tumor effects in animal models carrying different tumors, including teratocarcinoma, colon adenocarcinoma and sarcoma (Birchler et al., 1999). Interleukin-2 (IL-2) is a cytokine factor and an important player in anticancer immunity. However, the cardiovascular toxicity of IL-2 remains a major clinical issue. To overcome this problem, a new strategy was designed by fusing IL-2 with L19 so that IL-2 can be precisely targeted to the tumor site, resulting in reduced side effects. This drug conjugate exerted strong immune-stimulatory effects and inhibited tumor growth in stage III melanoma patients (Danielli et al., 2015). Currently, L19-IL-2 in combination with L19-TNF is in a phase III clinical trial to evaluate its efficacy against advanced melanoma (ClinicalTrials.gov Identifier: NCT03567889). Similarly, TNC-targeting antibodies have also been conjugated with IL-2, and have shown some preliminary signs of anti-tumor activity in advanced solid tumors and metastatic breast cancer (Catania et al., 2015). Navitoclax (ABT-263) is a small molecule that was shown to have the ability to induce apoptosis in myofibroblasts (Lagares et al., 2017). Consequently, Navitoclax could be used to target CAFs in solid tumor. Navitoclax-loaded nanoliposome was modified with peptide FH (FH-SSL-Nav), which specifically binds to TNC, to precisely eradicate CAFs at the tumor site. Using a xenograft mouse model of hepatocellular cancer, FH-SSL-Nav was shown to have the ability to deplete CAFs and inhibit tumor growth (Chen et al., 2016). In January 2017, the National Cancer Institute (NCI) approved a phase Ib/II trial study to evaluate the side effects and best dose of the combination of MEK inhibitor Trametinib and Navitoclax in treating patients with advanced or metastatic solid tumors (ClinicalTrials.gov Identifier: NCT02079740).

TARGETING ECM REMODELING ENZYMES

Extracellular matrix remodeling plays an essential role in CAF-mediated desmoplastic reactions, which cannot be achieved without LOX-induced ECM crosslinking. LOX inhibitors have emerged as potential alternatives to target the desmoplastic TME and improve drug delivery efficacy. In an *in vitro* 3D spheroid model using four different mouse tumor cell lines, including Lewis lung carcinoma cell line (LLC), a fibrosarcoma cell line (MT6) and two breast carcinoma cell lines (4T1, EMT6), LOX inhibition significantly improved the diffusion of doxorubicin (Schutze et al., 2015). Blocking LOX family oxidases *in vitro* or *in vivo* has shown potent anti-tumor activities in breast and pancreatic cancer (Park et al., 2016; Chang et al., 2017). Nevertheless, caution should still be taken when considering using LOX inhibitors. In a rat model of prostate cancer, LOX inhibition seems to have context-dependent effects during different stages of tumor progression. Before tumor formation, LOX inhibitors showed strong tumor-inhibiting capacity. To the contrary, after prostate tumors were established, LOX inhibition did not affect or decrease tumor growth (Nilsson et al., 2016). In recent clinical trials, simtuzumab, a monoclonal antibody against LOXL2, failed to produce improved anti-tumor benefits when given in combination with other anti-cancer drugs, including 5-fluorouracil, leucovorin, irinotecan (FOLFIRI) and gemcitabine (Benson et al., 2017; Hecht et al., 2017).

Many MMPs have been known to be notorious for their roles in promoting cancer progression. As a result, more than 50 MMP inhibitors were investigated in clinical trials. In a pre-clinical study, an anti-MMP9 monoclonal antibody GS-5745 successfully inhibited tumor growth and reduced tumor metastasis in mice bearing colorectal tumors (Marshall et al., 2015). Nevertheless, despite exciting preclinical data, none of these MMP inhibitors displayed anti-tumor effects in clinical trials. Although there are many explanations for these failures, such as bad clinical trial design, poor oral bioavailability, and inadequate cancer stages (Vandenbroucke and Libert, 2014), one potential reason responsible for the failures of these MMP inhibitors might be the obscurity of the roles and functions of MMPs in the ECM microenvironment. In addition, the use of broad-spectrum MMP inhibitors also suppresses potential tumor-inhibiting MMPs. Therefore, although MMPs are attractive therapeutic targets, more research is needed to unravel the roles of different MMPs in different cancer types and/or during various cancer stages. Furthermore, more efforts are required to develop more specific and selective MMP inhibitors to avoid potential side effects.

TARGETING CAF-DERIVED MOLECULAR SIGNALS

Cancer-associated fibroblast-mediated paracrine signaling has also been envisioned as a potential target in cancer treatment. In a recent phase I–II study on myeloid leukemia, plerixafor, a CXCR4 inhibitor, resulted in improved recovery rate when given in combination with a FLAG-Ida regime (fludarabine, idarubicin, cytarabine, and G-CSF) (Martinez-Cuadron et al., 2018). To block TGF-β activity, TGF-β inhibitors and monoclonal antibodies have been designed and tested in clinical trials. Galunisertib, a TGF-β receptor kinase inhibitor, however, showed highly context-dependent tumor-inhibitory effects. While it showed promising clinical responses in neuroblastoma patients (Tran et al., 2017), galunisertib had no significant therapeutic effect in a phase II clinical study in recurrent glioblastoma patients (Brandes et al., 2016). The monoclonal antibody fresolimumab (GC1008), which is capable of neutralizing all human isoforms of TGF-β, has also been investigated in advanced malignant melanoma and renal cell carcinoma and showed early stage anti-tumor effects with no dose-limiting toxicity in a phase I clinical study (Morris et al., 2014). In 2017, several clinical trials investigating an anti-HGF antibody, rilotumumab, were published. In one clinical trial, improved antitumor activities of rilotumumab in combination with cisplatin and capecitabine were shown in patients with MET-positive advanced gastric or gastroesophageal junction cancer (Doi et al., 2017). The combined use of rilotumumab with erlotinib (an EGFR receptor inhibitor) also showed successes in treating advanced NSCLC (Tarhini et al., 2017). However, in another clinical trial on small-cell lung cancer patients, no significant clinical benefit of rilotumumab in combination with platinum-based chemotherapy was observed (Glisson et al., 2017). Similarly, in a phase III clinical study, the treatment utilizing rilotumumab plus epirubicin, cisplatin, and capecitabine as a first-line therapy on gastric or gastro-oesophageal junction cancer patients was unsuccessful (Catenacci et al., 2017). Taken together, targeting CAF-induced paracrine signaling appears to be spatial-temporal and case-dependent.

CONCLUSION

It is an astonishing feat of the tumor cells to abandon the basic rules of tissue homeostasis and to grow uncontrollably. Unfortunately, as we have learned from many modern targeted therapies, a simple approach to eliminate tumor "seed" is generally condemned to failure. It is becoming clear that the TME is actively involved in tumor initiation, progression, metastasis and the development of drug resistance. However, only after gaining enhanced knowledge about the TME, including the heterogeneous nature and complexity of CAF populations, a multiplex approach targeting CAFs and the ECM will naturally come by and provide desired clinical benefits.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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