



2016 Colorectal Cancer: Global view

## Fibroblasts, an inconspicuous but essential player in colon cancer development and progression

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### Abstract

Tumor microenvironments have a crucial role in cancer initiation and progression, and share many

molecular and pathological features with wound healing process. Unless treated, tumors, however, do not heal in contrast to wounds that heal within a limited time framework. Wounds heal in coordination of a myriad of types of cells, particularly endothelial cells, leukocytes, and fibroblasts. Similar sets of cells also contribute to cancer initiation and progression, and as a consequence, anti-cancer treatment strategies have been proposed and tested by targeting endothelial cells and/or leukocytes. Compared with endothelial cells and leukocytes, less attention has been paid to the roles of cancer-associated fibroblasts (CAFs), fibroblasts present in tumor tissues, because their heterogeneity hinders the elucidation on them at cellular and molecular levels. Here, we will discuss the origin of CAFs and their crucial roles in cancer initiation and progression, and the possibility to develop a novel type of anti-cancer treatment by manipulating the migration and functions of CAFs.

**Key words:** Angiogenesis; Drug resistance; Extracellular matrix; Immune evasion; Transforming growth factor- $\beta$ ; Invasion; Metastasis

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**Core tip:** Tumor microenvironments have a crucial role in cancer initiation and progression, and consist of various types of cells, such as endothelial cells, leukocytes, and fibroblasts. Compared with endothelial cells and leukocytes, less attention has been paid to the roles of cancer-associated fibroblasts (CAFs), fibroblasts present in tumor tissues, because their heterogeneity hinders the elucidation on them at cellular and molecular levels. Here, we will discuss the origin of CAFs and their crucial roles in cancer initiation and progression, and the possibility to develop a novel type of anti-cancer treatment by manipulating the migration and functions of CAFs.

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## INTRODUCTION

Dvorak proposed that tumors are wounds that do not heal<sup>[1]</sup>, based on his discovery of vascular endothelial growth factor (VEGF), which is produced in wound healing sites as well as tumor sites<sup>[2]</sup> and can account for chronic hyperpermeability-mediated fibrin deposition in solid tumors and in early stages of wound healing<sup>[3]</sup>. Moreover, solid tumors and wound healing process share many pathological and molecular features.

Irrespective of the causes and the severities of wounds, healing proceeds to repair the structure and functions of injured organs and tissues, through a series of processes; hemostasis, humoral inflammation with microvascular permeability and extravascular clotting, cellular inflammation with inflammatory cell infiltration, angiogenesis, and generation of mature connective tissue stroma<sup>[4]</sup>. These steps proceed through the interaction between the parenchymal cells and the stroma, a complex mixture of inflammatory cells, matrix proteins, and tissue cells such as fibroblasts and endothelial cells. Injured organs and tissues are completely replaced by proliferating parenchymal cells in the case of acute and mild wound, but if not replenished completely, they are filled with connective tissue. Tumor cells, regardless of their site of origin, behave like parenchymal cells in normal tissues and proliferate by interacting with the stroma<sup>[4]</sup>.

Fibroblasts are a major cell type within the stroma and contribute to tissue remodeling in development and tissue homeostasis, by providing structural scaffolding and growth regulatory mediators. Moreover, following tissue injury, fibroblasts exhibit an activated and contractile phenotype with enhanced expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and are referred to as myofibroblasts<sup>[5]</sup>. Myofibroblasts synthesize increased amount of various types of collagens and extracellular matrix proteins (ECM) to provide scaffold and to eventually aid in wound repair<sup>[5]</sup>. Furthermore, the cells are important sources of many growth factors and cytokines that regulate wound healing processes<sup>[6]</sup>.

Primary normal fibroblasts isolated from various human tissues, can restrict the *in vitro* cell proliferation of various types of human cancer cell lines, under co-culture condition<sup>[7]</sup>. Indeed, if Nod-like receptor pyrin domain-containing protein 6 (NLRP6) is absent in fibroblasts within the stem cell niche in the colon, regeneration of the colonic mucosa and processes of epithelial proliferation and migration are impaired

and consequently, colitis-associated tumorigenesis is accelerated in mice<sup>[8]</sup>. Thus, under normal conditions, fibroblasts work as a sentinel cell to maintain epithelial tissue homeostasis and to prevent initiation of tumorigenesis in colon, in a NLRP6-dependent manner.

Like fibroblasts in wound healing process, fibroblasts present in tumor tissues exhibit an activated and a myofibroblast-like phenotype with  $\alpha$ -SMA expression and are referred as cancer-associated fibroblasts (CAFs)<sup>[9]</sup>. In contrast to fibroblasts in normal tissues, CAFs in most solid tumors are presumed to promote tumor development and progression by providing cancer cells with a myriad of growth factors<sup>[9,10]</sup>. However, in pancreatic ductal cancer, CAFs can deliver immune stimulating signals. As a result, depletion of CAFs induces immunosuppression with increased intra-tumoral regulatory T cells (Tregs) and eventually accelerates tumor progression with reduced survival<sup>[11]</sup>. Hence, it still remains elusive on the pathophysiological roles of CAFs in the development and progression of solid tumors.

We will herein discuss the pathophysiological roles of CAFs and their clinical relevance mainly in colorectal cancer (CRC), but will mention CAFs in other types of cancer if necessary.

## CAFS IN COLITIS-ASSOCIATED COLON CARCINOGENESIS MODEL

Accumulating evidence indicates the crucial contribution of chronic inflammation to tumor development and progression<sup>[12]</sup>. Colitis-associated colon carcinogenesis (CAC) is one typical example of this pathological process. CAC frequently ensues from chronic intestinal inflammatory changes observed in patients with inflammatory bowel diseases such as ulcerative colitis (UC), particularly those with a long duration, extensive involvement, and severe inflammation<sup>[13]</sup>. Pathological features of UC include mucosal damage and ulceration with prominent leukocyte infiltration, and these changes involve rectum at first and extend proximally.

Oral administration of dextran sulfate sodium (DSS) solution to rodents can cause acute inflammatory reaction and ulceration in the entire colon, similar to that observed in human UC patients, and therefore, is widely used to reproduce human UC<sup>[14]</sup>. Moreover, repeated DSS ingestion alone can cause a small number of colon carcinomas in about a half of mice<sup>[15]</sup>, suggesting that inflammatory response alone can cause colon carcinoma. The incidence of DSS-induced colon carcinogenesis is accelerated and increased by a prior administration of azoxymethane (AOM)<sup>[16]</sup>, which can alone cause colon cancer by inducing O<sup>6</sup>-methyl guanine formation and mutations of the  $\beta$  *catenin* gene<sup>[17]</sup>. Thus, the combined treatment with AOM and DSS is used frequently to recapitulate the molecular mechanisms underlying CAC.

A transcription factor, NF- $\kappa$ B, is a key player in

**Table 1** Pathological changes in various mice after azoxymethane/dextran sulfate sodium treatment

	Ingested DSS concentration	Body weight loss (> 20%)	Granulocyte infiltration (> 400/field)	Fibroblast accumulation (> 20% type I collagen <sup>+</sup> areas)	Tumor numbers
Wild-type mice	3.0%	+	+	+	> 20
CCR1-deficient mice	3.0%	-	-	-	< 5
	4.5%	+	+	+	> 20
CCR5-deficient mice	3.0%	-	-	-	< 5
	4.5%	+	+	-	< 7
CCL3-deficient mice	3.0%	-	-	-	< 5
	4.5%	+	+	-	< 5

Table is prepared according to Sasaki *et al.*<sup>[22]</sup>. DSS: Dextran sulfate sodium.

inflammation and its activity is triggered by I $\kappa$ B kinase (IKK) complex, in response to a wide variety of pro-inflammatory stimuli such as infectious agents and pro-inflammatory cytokines<sup>[18]</sup>. Greten *et al.*<sup>[19]</sup> demonstrated that IKK $\beta$  has crucial roles in AOM/DSS-induced CAC by two distinct pathways; prevention of apoptosis of epithelial cells and enhancement of growth factor expression by myeloid cells. Thus, these observations would indicate the crucial involvement of inflammatory cell infiltration in CAC development. Given the crucial roles of NF- $\kappa$ B in regulation of gene expression and biological functions of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$  and chemokines<sup>[18]</sup>, we examined the AOM/DSS-induced colon carcinogenesis process by using mice deficient in the tumor necrosis factor receptor (*TNF-R*)*p55* gene<sup>[20]</sup>. We revealed that genetic ablation of the *TNF-Rp55* gene in myeloid cells resulted in reduction in intracolonic infiltration of inflammatory cells, particularly macrophages, and neovascularization, and subsequent tumor formation. Moreover, a TNF inhibitor reduced tumor progression even when it was administered after multiple tumors developed in colon. Similar phenotypes were observed by genetic inactivation of a macrophage-tropic chemokine receptor, the *CCR2* gene, in myeloid cells or the administration of *CCR2* inhibitors after multiple colon tumors developed<sup>[21]</sup>.

These observations prompted us to further examine the roles of another macrophage-tropic chemokine, CCL3, and its receptors, *CCR1* and *CCR5*<sup>[22]</sup>. Mice deficient in *CCL3*, *CCR1*, or *CCR5* gene, did display marginal inflammatory reactions and subsequently develop few colon tumors when AOM was administered together with 3% DSS ingestion. All wild-type (WT) mice failed to survive 4.5% DSS ingestion, whereas CCL3-, *CCR1*-, or *CCR5*-deficient mice survived this high concentration of DSS solution with a prominent mucosal damage and leukocyte infiltration in colon. Interestingly, *CCR1*-deficient mice developed multiple colon tumors, whereas the same treatment caused only a small number of colon tumors in CCL3- or *CCR5*-deficient mice (Table 1)<sup>[22]</sup>. These observations would indicate that inflammatory cell infiltration is necessary but not sufficient for the development of

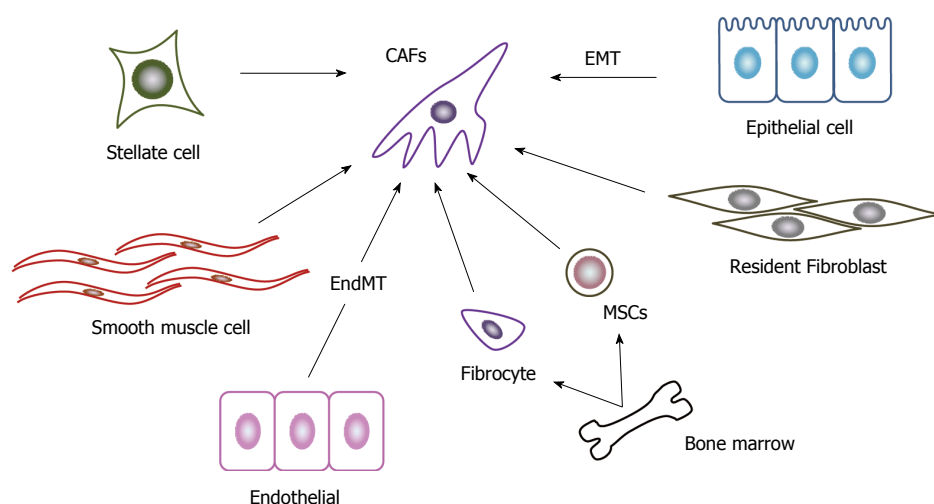
CAC in this model.

The most prominent difference in pathological change is that CCL3- or *CCR5*-deficient mice exhibited reduced accumulation of CAFs in colon, which was evident in the later phase of CAC in this model, compared with WT or *CCR1*-deficient mice (Table 1). Several groups including ours further demonstrated that CAFs expressed abundantly growth factors such as hepatocyte growth factor (HGF)<sup>[23]</sup>, epiregulin<sup>[24]</sup>, and heparin-binding epidermal growth factor-like growth factor (HB-EGF)<sup>[22]</sup> to promote tumor cell proliferation in the later phase of this CAC model. Moreover, the deficiency of the *CCR5* gene results in reduced growth of the tumors arising from either subcutaneous or orthotopic intracecum injection of a syngeneic mouse colon adenocarcinoma cell line, colon 26. Furthermore, this attenuated tumor growth was associated with a reduction in type I collagen-positive fibroblast numbers but not inflammatory cell infiltration<sup>[22]</sup>. These observations would indicate the crucial involvement of CAFs in rather progression of CAC than its development.

## ORIGINS OF CAFs

The lack of a specific marker to identify CAF<sup>[25]</sup> hampers the precise identification of the origin of CAF.  $\alpha$ -SMA, a robust CAF marker, is also expressed by normal colonic fibroblasts under *in vitro* culture conditions<sup>[26]</sup> and other cell types such as pericytes and smooth muscle cells surrounding vasculature, and cardiomyocytes<sup>[27]</sup>. There are several additional candidate CAF markers including fibroblast activation protein (FAP)- $\alpha$ <sup>[28,29]</sup>, S100A4/fibroblast specific protein-1<sup>[30,31]</sup>, neuronal antigen-2, platelet-derived growth factor (PDGF) receptor- $\beta$ , and prolyl 4-hydroxylase<sup>[32]</sup>. However, these molecules can be expressed by other cell types than CAFs and therefore, are not specific to CAFs. The lack of a definitive maker for CAFs, imply the phenotypical and functional heterogeneity of CAFs in CRC and this assumption is further strengthened by global gene expression profiles<sup>[33]</sup> (Figure 1).

The most possible cellular source of CAFs in CRC is resident fibroblasts in colon tissues. Supporting this notion, CAFs in liver metastatic foci of CRC exhibit the



**Figure 1 Origin of cancer associated fibroblasts.** A variety of cells can generate to CAFs. The most important cellular source of CAFs in CRC is presumed to be resident fibroblasts. Stellate cells in intestine may be able to transform into CAFs similarly as stellate cells in liver and pancreas do. Other potential sources include epithelial cells undergoing EMT, endothelial cells undergoing EndMT, smooth muscle cells, and bone marrow-derived cells including fibrocytes and MSCs. CAFs: Cancer associated fibroblasts; CRC: Colorectal cancer; EMT: Epithelial-mesenchymal transition; EndMT: Endothelial-mesenchymal transition; MSCs: Mesenchymal stem cells.

similar protein expression pattern as liver resident fibroblasts<sup>[34]</sup>. Kojima and colleagues demonstrated that resident human mammary fibroblasts progressively convert into CAF-like cells with enhanced  $\alpha$ -SMA expression and pro-tumorigenic capacity, during the course of tumor progression in a breast tumor xenograft model<sup>[35]</sup>. Moreover, these cells express transforming growth factor (TGF)- $\beta$  and a chemokine, stromal-derived factor (SDF)-1/CXCL12, the molecules which further initiate and maintain the differentiation of fibroblasts into CAF-like and the concurrent tumor-promoting phenotype in an autocrine and amplifying manner<sup>[35]</sup>.

Stellate cells are vitamin A-containing and lipid droplet-containing cells in their quiescence state and are present in various tissues including liver, pancreas, kidney, and intestine<sup>[36]</sup>. These cells activate  $\alpha$ -SMA expression under inflammatory and oncogenic conditions and acquire myofibroblast-like phenotypes as CAFs do. Most of hepatocellular carcinomas arise in a cirrhotic liver with prominent fibrosis. Activated hepatic stellate cells are the major source of extracellular proteins during fibrogenesis. Moreover, they can induce hepatocellular carcinoma cell growth, neovascularization, and immune evasion, to promote tumor progression<sup>[37]</sup>. Similar to hepatocellular carcinoma, pancreatic cancer is characterized by a prominent desmoplastic/stromal reaction. Like hepatic stellate cells, activated pancreatic stellate cells can produce abundantly the collagenous stroma of pancreatic cancer. Moreover, these cells can also interact closely with cancer cells to facilitate local tumor growth and distant metastasis, to mediate angiogenesis, and to induce immune evasion<sup>[38]</sup>. However, it has to be yet determined whether stellate cells in intestine, can also behave in a similar manner in the course of colon carcinogenesis, as those in liver and pancreas do.

Other types of cells are proposed to be a cellular

source of CAFs, based on the analyses on other types of cancers than CRC. Accumulating evidence indicates that under chronic inflammatory conditions, epithelial cells can undergo epithelial-mesenchymal transition (EMT) to acquire myofibroblast-like phenotypes and to participate in the synthesis of the fibrotic matrix<sup>[39]</sup>. A breast carcinoma biopsy provided evidence to indicate the occurrence of EMT and a coincidental  $\alpha$ -SMA-positive stromal reaction<sup>[40]</sup>. Moreover, when a mouse breast cancer cell line, MCF-7, was injected into nude mice, together with HBFL-1, a mammary gland epithelial cell line without tumorigenicity, HBFL-1 cells acquired myofibroblast-like phenotypes and conferred a significant 3.5- to 7-fold increase in MCF-7 tumor size in nude mice. Thus, breast cancer can transform its own nonmalignant stroma to facilitate its own growth<sup>[40]</sup>. Furthermore, when human epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (EGFR-TKI)-resistant lung cancer cells were used as a xenograft model, EMT-derived tumor cells give rise to about a quarter of CAFs, which provide cancer cells with resistance to EGFR-TKI<sup>[41]</sup>. Meanwhile, several independent groups argue against EMT as the origin of CAFs<sup>[42,43]</sup>.

Bone marrow-derived mesenchymal stem cells (MSCs) are proposed to be another origin of CAFs, because MSCs and CAFs exhibit similar immunophenotypes and share the potential to differentiate to various types of cell lineages such as adipocytes, chondrocytes, and osteoblasts<sup>[44]</sup>. Bone marrow-derived and circulating fibroblast progenitors, fibrocytes, also exhibit similar phenotypic and functional characteristics in chemically induced rat breast carcinogenesis model<sup>[45]</sup>, suggesting that fibrocytes can be an origin of CAFs.

TGF- $\beta$ 1 can induce proliferating endothelial cells to undergo a phenotypic conversion into fibroblast-like cells with the emergence of mesenchymal markers

and the reciprocal down-regulation of CD31<sup>[46]</sup>. Moreover, when endothelial cells were irreversibly tagged by crossing Tie2-Cre mice with R26Rosa-lox-Stop-lox-LacZ mice, endothelial-to-mesenchymal transition (EndMT) was evident at the invasive front of the tumors in the B16F10 melanoma model and the Rip-Tag2 spontaneous pancreatic carcinoma model<sup>[46]</sup>. Choi and colleagues further demonstrated that endothelial heat shock protein (a synonym of HSP27 in humans and HSP25 in rodents) has a crucial role in the maintenance of endothelial phenotypes and that its deficiency mediates the EndMT to accelerate fibrosis and eventually tumorigenesis in lungs<sup>[47]</sup>. Smooth muscle cells can be another source of CAFs in several cancers, particularly prostate cancer. Normal prostate stroma is enriched in smooth muscle cells, but during prostatic carcinogenesis in rats and humans, smooth muscle cells disappear with reciprocal appearance of CAFs, which can promote carcinogenesis in genetically abnormal but non-tumorigenic epithelial cells<sup>[48]</sup>. Thus, smooth muscle cells may be a source of CAFs in prostate cancer but it remains to be clarified whether CAFs originate from this population in other types of cancer, particularly CRC.

Evidence is accumulating to indicate functional heterogeneity of CAFs in various types of cancers including colon cancer<sup>[33]</sup>. This heterogeneity may arise from the differences in the origin of CAFs. Alternatively, CAFs are generated by the intricate interactions with tumor microenvironments consisting of cancer cells and other resident cells<sup>[10,25]</sup>. Thus, the heterogeneity of tumor microenvironments can induce a wide variation in CAFs. Nevertheless, this heterogeneity of CAFs can affect the clinical course of colon cancer patients<sup>[49]</sup>.

## RECRUITMENT AND ACTIVATION OF CAFs

Among chemokines, CCL2 and CCL3 can recruit fibroblasts<sup>[50]</sup>, while CXCL12<sup>[51]</sup>, CCL21<sup>[52]</sup>, and CCL3<sup>[53]</sup> can recruit fibrocytes in chronic inflammation. We proved that CCL3 is produced locally at tumor sites and is associated with CAF accumulation<sup>[22,54]</sup>. Moreover, in a mouse gastric cancer model, a substantial proportion of CAFs originate from bone marrow-derived mesenchymal stem cells, which are recruited to tumor site in a TGF- $\beta$  and CXCL12-dependent manner<sup>[55]</sup>. Thus, CAF accumulation may be regulated by the cooperation with chemokines and other fibroblast-tropic factors such as TGF- $\beta$ .

Normal fibroblasts can inhibit *in vitro* cell proliferation of cancer cells<sup>[7]</sup>. Moreover, normal intestinal fibroblasts can maintain epithelial homeostasis to prevent carcinogenesis<sup>[23]</sup>. Thus, malignant cells must reprogram normal fibroblasts into CAFs with protumorigenic activity. This process is mediated by

cancer cell-derived factors including TGF- $\beta$ , CXCL12<sup>[35]</sup>, PDGF<sup>[56]</sup>, and interleukin (IL)-6<sup>[57]</sup>. On the contrary, several lines of evidence indicate that CAF phenotype can persist in the absence of continued exposure to cancer cell-derived factors<sup>[58]</sup>. This may be explained by the observation that CAFs increasingly acquire the capacity to express TGF- $\beta$  and CXCL12, the cytokines which can act to initiate and maintain the differentiation into CAFs in auto-stimulatory and cross-communicating manner<sup>[35]</sup>. Alternatively, CAF may acquire irreversible genetic and/or epigenetic changes during the course of the differentiation, as similarly observed on tumor endothelial cells<sup>[59]</sup>.

## CAFS IN CARCINOGENESIS

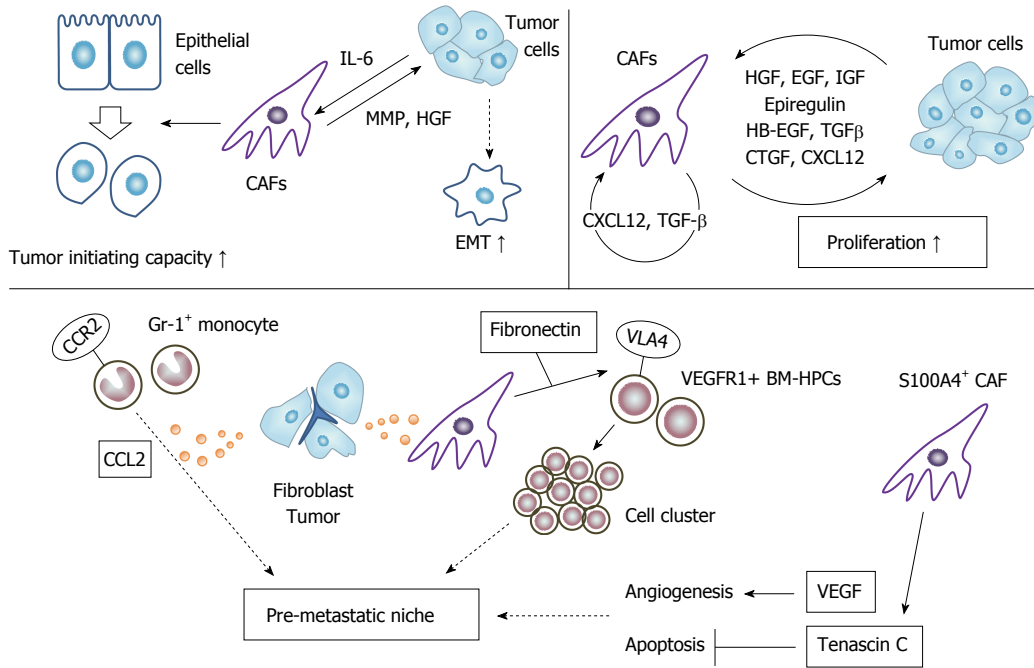
### *Cancer cell growth and stemness*

Human pre-malignant prostatic epithelial cells can transform to neoplastic cells when they are *in vitro* co-cultured with CAFs derived from human prostate cancer tissues<sup>[60]</sup>. Secreted factors from CAFs are believed to be responsible for this tumor-initiating capacity. Indeed, when human mammary epithelial cells are grafted into immunodeficient mice, together with fibroblasts overexpressing TGF- $\beta$  and/or HGF, the engrafted cells develop into a proliferating tissue that closely resembles human ductal carcinomas<sup>[61]</sup>. These observations raise the possibility that CAFs can initiate malignant transformation of epithelial cells by secreting these growth factors (Figure 2).

Under the influence of cancer cell-derived IL-6, CAFs can secrete metalloproteinases, to stimulate EMT and cancer stem cell phenotypes in prostate cancer<sup>[62]</sup>. CAFs in colon cancer secrete HGF, activate  $\beta$ -catenin-dependent transcription and subsequently induce cancer stem cell clonogenicity<sup>[63]</sup>. Moreover, CAF-derived HGF also restores the cancer stem cell phenotype in more differentiated tumor cells both *in vitro* and *in vivo*. Furthermore, human colon cancer-derived and chemotherapy-treated CAFs produce abundantly interleukin-17A (IL-17A), which increases chemotherapy-resistant cancer stem cells<sup>[64]</sup>.

CAF produce various growth factors and cytokines, which can promote cancer cell proliferation; HGF<sup>[23]</sup>, EGF<sup>[65]</sup>, epiregulin<sup>[24]</sup>, HB-EGF<sup>[22]</sup>, insulin-like growth factor (IGF)<sup>[66]</sup>, TGF- $\beta$ <sup>[35]</sup> connective tissue growth factor<sup>[67]</sup>, and CXCL12<sup>[58]</sup>. Actually, most of these growth factors can be produced by cancer cells and can enhance the growth of CAFs. Moreover, CAF-derived TGF- $\beta$  and CXCL12 affect CAF proliferation in an autocrine manner<sup>[35]</sup>. Thus, these growth factors form a vicious positive feedback loop between cancer cells and CAFs, and may eventually accelerate tumor progression.

Thus, CAFs can contribute to tumor development and progression by initiating malignant transformation, enhancing the proliferation of cancer cells, and inducing the cancer stem cell phenotype.



**Figure 2 Action of cancer associated fibroblasts on tumor cells.** CAF can induce tumor cells to enhance their tumor initiating capacity (stemness), and to undergo EMT. CAFs provide tumor cells with various growth factors to promote their growth. CAFs can also instigate pro-metastatic niche by inducing tumor cell cluster and angiogenesis, and suppressing tumor cell apoptosis. CAFs: Cancer associated fibroblasts; EMT: Epithelial-mesenchymal transition; VEGF: Vascular endothelial growth factor.

**Cancer cell migration, invasion, and metastasis**

Colon cancer cell lines exhibit enhanced migratory ability in a wound healing assay and increased clonogenic capacity in the presence of CAF-derived conditioned media, compared with normal fibroblast-derived conditioned media<sup>[26]</sup>. Moreover, co-injection of CAFs with a human colon cancer cell line into nude mice, significantly enhances tumor growth with increased tumor cell proliferation, compared with normal fibroblasts<sup>[26]</sup>. Gene ontology analysis further reveals that genes overexpressed in CAFs are associated with biological processes such as development (*TGFB2*, *PDGFC*, *cMET*, *CADM1*, *WNT1*) and cell-cell signaling (*TFAP2C*, *NTF-3*, *SEMA5A*, *EFNB2*, *INHBA*)<sup>[26]</sup>. These gene products can modulate the functions of cancer cells to promote their invasion and metastasis (Figure 2).

CAF expressed various matrix metalloproteinases (MMPs) and MMP-mediated ECM degradation results in proteolytic destruction of basement membrane and aids tumor cells to invade surrounding tissues<sup>[68]</sup>. Moreover, MMP-mediated enhanced invasiveness also involves proteinase-activated receptor 1 (PAR1) expressed on cancer cell surface. CAF-derived MMP-1 cleaves PAR1 at its proper site to deliver PAR1-mediated signaling pathway in cancer cells, which is associated with cancer cell migration and invasion<sup>[69]</sup>.

Fibroblasts as well as tumor cells produce abundantly CCL2, which recruits Gr-1-positive, CCR2-expressing inflammatory monocytes into lung<sup>[70]</sup>. Recruited inflammatory monocytes subsequently instigate a pre-metastatic niche, which favors lung metastasis of mouse mammary cancer. A pre-metastatic niche

can be formed by direct actions by CAFs<sup>[71]</sup>. S100A4-positive CAFs produce abundantly VEGF-A, which plays an important role in the establishment of an angiogenic microenvironment at the metastatic site to facilitate colonization. Moreover, S100A4-positive CAFs produce tenascin-C to provide cancer cells with protection from apoptosis.

Gene expression-based classification systems have identified an aggressive colon cancer subtype with mesenchymal features, possibly reflecting EMT of tumor cells. Comparative analysis of stroma<sup>high</sup> and stroma<sup>low</sup> CRC shows that the neoplastic cells in stroma<sup>high</sup> tumors express specific EMT drivers including ZEB2, TWIST1, and TWIST2<sup>[72]</sup>. Moreover, type I collagen dominates the extracellular matrix in these aggressive colon cancer with EMT markers. Mimicking the tumor microenvironment, Matrigel enriched with type I collagen can induce colon cancer cells to express tumor-specific mesenchymal gene, to suppress gene expression of hepatocyte nuclear factor 4, a transcriptional activator of epithelial differentiation, and its target genes, and to invade collectively patient-derived colon tumor organoids<sup>[72]</sup>. Thus, CAF-derived type I collagen can induce EMT in cancer cells to promote their invasion.

Bone marrow-derived hematopoietic progenitor cells expressing VEGF receptor 1 (VEGFR1) home to tumor-specific pre-metastatic sites<sup>[73]</sup>. Primary tumor-derived factors induce resident fibroblast in pre-metastatic sites to express fibronectin, which interacts with VLA-4 on VEGFR1-positive cells to induce cell clusters and to promote subsequently pre-metastatic

niche formation. Fibronectin expression in fibroblasts is regulated by sphingosine-1-phosphate (SIP)-SIP receptor-STAT3 pathway<sup>[74]</sup>.

CAFs can activate STAT3 pathway in cancer cells to promote malignant progression. CRCs frequently display mutational inactivation of the TGF- $\beta$  pathway with elevated TGF- $\beta$  production. Actually, cancer cell-derived TGF- $\beta$  stimulates CAFs to secrete IL-11, which triggers gp130/STAT3 signaling in tumor cells<sup>[75]</sup>. This cross-talk can provide metastatic cells with a survival advantage.

### Drug resistance

CAF-derived ECM has profound impact on cancer chemotherapy<sup>[76]</sup>. ECM forms a physical barrier and as a consequence, most anti-cancer drugs show limited penetration into solid tumors<sup>[77]</sup>. Moreover, cancer cells acquire chemoresistance through the activation of various pro-survival signal pathways including PI3K/Akt, Erk, Rho/Rock, and p53 after binding to ECM<sup>[76]</sup>. Adhesion of small cell lung cancer cells to ECM confers resistance to chemotherapeutic agents because the adhesion activates  $\beta$ 1 integrin-stimulated tyrosine kinase to eventually suppress chemotherapy-induced apoptosis<sup>[78]</sup>. Similar mechanisms may also work in the case of resistance to radiotherapy in glioma cells<sup>[79]</sup>.

In addition to ECM, CAF-derived soluble factors have been demonstrated to be involved in drug resistance. CAF-derived CXCL12 mediate drug resistance to conventional chemotherapeutics<sup>[80]</sup>. This resistance can arise from the ability of CXCL12 to promote cancer cell survival by activating focal adhesion kinase, Erk, and Akt,  $\beta$ -catenin and NF- $\kappa$ B, in CXCR4-expressing cancer cells<sup>[81]</sup>.

The presence of driver mutations in receptor tyrosine kinase (RTK) pathways positions RTKs for potential targets for cancer therapy and accordingly, many anti-cancer drugs have been developed by targeting these RTKs<sup>[82]</sup>. However, RTK-mediated signals converge on common critical downstream cell-survival effectors such as PI3K and Erk, and consequently, most cells can be rescued from drug sensitivity by simply exposing them to one or more other unrelated RTK ligands. Among these RTK ligands, HGF confers resistance to the BRAF inhibitor in BRAF-mutant melanoma cells<sup>[83]</sup>. Likewise, CAF-derived HGF can confer the resistance to EGF-receptor inhibitors to human non-small cell lung cancer cells which is otherwise sensitive to the inhibitors<sup>[84]</sup>.

CRC initiating cells (CICs) are resistant to conventional chemotherapy in cell-autonomous assays, but CIC chemoresistance is also increased by CAFs. Comparative analysis of matched CRC specimens from patients before and after cytotoxic treatment revealed a significant increase in CAFs after cytotoxic treatment<sup>[64]</sup>. Chemotherapy-treated human CAFs promoted CIC self-renewal and *in vivo* tumor growth associated with increased secretion of specific cytokines

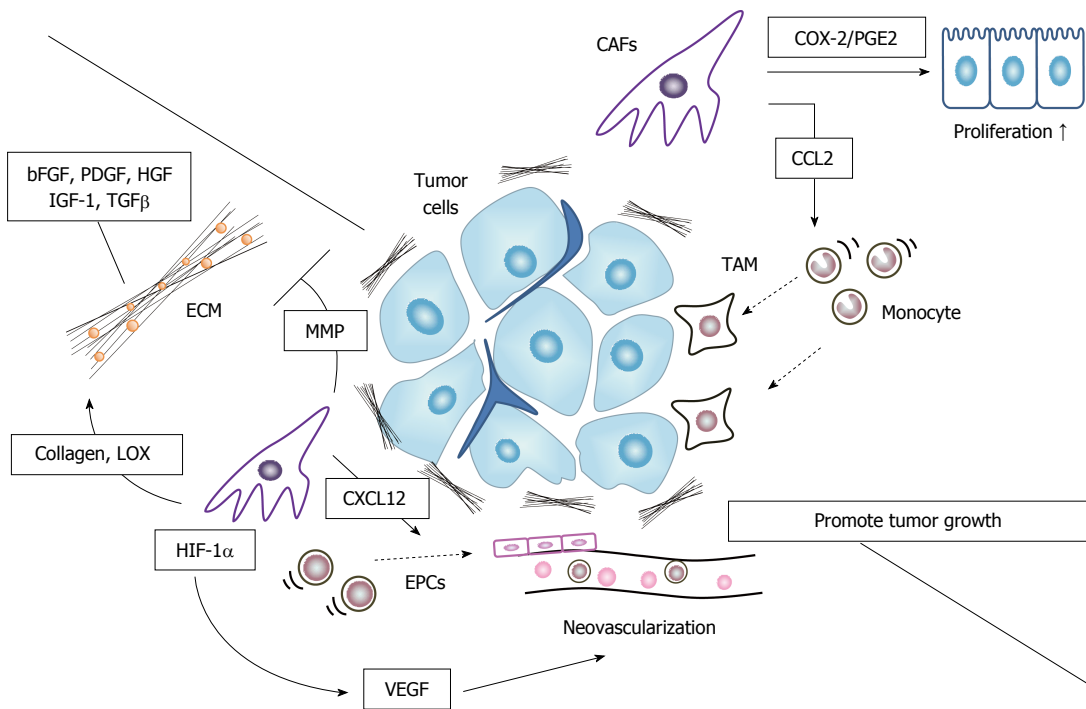
and chemokines, including IL-17A. Exogenous IL-17A increased CIC self-renewal and invasion, and targeting IL-17A signaling impaired CIC growth. Notably, IL-17A was overexpressed by colorectal CAFs in response to chemotherapy and this observation was validated directly in patient-derived specimens without culture<sup>[64]</sup>. These data suggest that chemotherapy induces remodeling of the tumor microenvironment through activating CAFs to secrete cytokines such as IL-17.

### Tumor microenvironments

Inflammation, particularly chronic one, is closely associated with tumorigenesis of various types of cancer<sup>[12,85]</sup>. CAFs, as a major cellular component of cancer-associated inflammation, mediate tumor-enhancing inflammation by expressing a proinflammatory gene signature in an NF- $\kappa$ B-dependent manner<sup>[86]</sup>. NF- $\kappa$ B activation enhanced the expression of several chemokines such as CCL2 and proinflammatory genes such as cyclooxygenase 2 (COX-2) in CAFs. CAF-derived CCL2 mediates the recruitment of blood monocytes to tumor sites<sup>[87]</sup>, favoring the generation of tumor-associated macrophages with a potent pro-tumorigenic activity. Simultaneously, COX-2 generates prostaglandin E2, which can promote both normal and malignant colonic epithelial cell proliferation<sup>[88,89]</sup> (Figure 3).

Another prominent feature of CAFs is their ability to synthesize ECM, which can serve as a reservoir for various growth factors such as TGF- $\beta$ , bFGF, PDGF, HGF, and IGF-1<sup>[90]</sup>. In response to mechanical stress present in tumor tissues, CAFs exhibit an increase in contractility, which can augment the production of collagen<sup>[91]</sup>. Moreover, CAFs synthesize the specific ECM including type I collagen, oncofetal nectin splice variants, periostin, and hyaluronan, and as a consequence, remodel ECM to promote tumor growth<sup>[90]</sup>. CAFs also express abundantly lysyl oxidase (LOX), an enzyme responsible for cross-linking type I collagen. LOX-mediated cross-linking and the resultant tumor tissue stiffness are associated with tumorigenesis<sup>[92]</sup>. Mechanical stress activates CAFs to express members of MMPs, which regulate the degradation of ECM<sup>[90]</sup>. MMP-mediated ECM degradation can also promote cancer cell invasion<sup>[68]</sup>.

Tumor microenvironment is characterized by abundant neovascularization, and this process can be induced by CAFs. CAF-induced MMP activation degrades ECM and eventually causes neovascularization<sup>[68]</sup>. Moreover, CAFs, particularly those in invasive margins, are a rich source of a CXC chemokine, SDF-1/CXCL12<sup>[58]</sup>, which mediates the recruitment of endothelial progenitor cells and subsequent tumor neovascularization. Furthermore, hypoxia can induce CAFs to express a transcription factor, hypoxia-inducible factor (HIF)-1 $\alpha$ , which in turn induces the expression of VEGF, a potent angiogenic factor<sup>[93]</sup>. VEGF production by CAFs can be further enhanced by CAF-derived IL-6, whose expression can



**Figure 3 Cancer associated fibroblasts in tumor microenvironment formation.** CAFs promote pro-tumorigenic microenvironment by producing extracellular matrix to provide tumor cells with a growth advantage, recruiting tumor-associated macrophages (TAMs) to foster tumor cell growth, and inducing neovascularization. CAFs: Cancer associated fibroblasts; EMT: Epithelial-mesenchymal transition; VEGF: Vascular endothelial growth factor; ECM: Extracellular matrix proteins; EPCs: Endothelial progenitor cells; MMP: Matrix metalloproteinase; HIF-1 $\alpha$ : Hypoxia-inducible factor 1 $\alpha$ .

be augmented in the presence of colon cancer cells<sup>[94]</sup>.

**Tumor immunity**

Direct evidence on the involvement of CAFs in suppressed tumor immunity, comes from the study, which revealed that deletion of FAP-positive stromal cells enhances tumor immunity<sup>[28]</sup>. This study, however, did not clarify the cellular and molecular mechanisms in detail, but several candidate mechanisms have been proposed. CAF-derived prostaglandin E2 and indoleamine 2,3-dioxygenase can inhibit natural killer cell functions, thereby contributing to immune escape and subsequent tumor progression<sup>[95]</sup>. CAF-derived tenascin also contributes to immune suppression at tumor sites. Soluble tenascin inhibits proliferation of human T cells induced by the combination of anti-CD3 antibody and fibronectin. Tenascin further attenuates T cell proliferation driven by IL-2, while it prevents high level induction of IL-2 receptor<sup>[96]</sup>. Prostate cancer stem-like cells present in the draining lymph nodes use tenascin-C to inhibit T-cell receptor-dependent T-cell activation, proliferation, and cytokine production, and as a consequence, cancer stem-like cells are protected from T cell-mediated immune surveillance<sup>[97]</sup> (Figure 4).

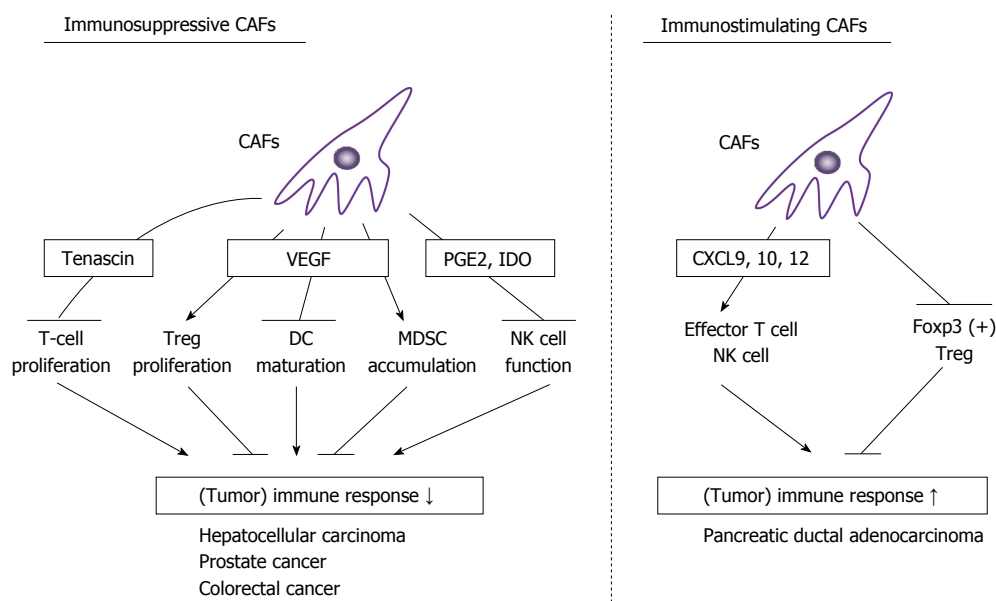
CAFs abundantly express TGF- $\beta$ 1, which can suppress the functions of various immune cells, particularly effector T cells and natural killer cells<sup>[98]</sup>. TGF- $\beta$  regulates Treg maturation and thereby suppresses immune responses. VEGF is also produced by CAFs and exhibits immunosuppressive effects<sup>[99]</sup>.

VEGF can suppress the maturation of dendritic cell precursors, promote the proliferation of Tregs, and the accumulation of myeloid-derived suppressor cells (MDSC) in peripheral immune organs, and thereby inhibits T-cell immune responses.

*In vivo* vaccination with a DNA vaccine against FAP eliminates CAFs and eventually causes a shift of the immune microenvironment from a Th2 to Th1 polarization. This shift is characterized by increased protein expression of IL-2 and IL-7, suppressed recruitment of tumor-associated macrophages (TAMs), MDSCs, and Tregs, and decreased tumor angiogenesis and lymphangiogenesis<sup>[100]</sup>. These observations suggest the roles of CAFs in intratumor Th2 polarization and subsequent depression of tumor immunity. This Th2 polarization is mediated by CAF-derived thymic stromal lymphopoietin (TSLP), which induces *in vitro* myeloid DCs to up-regulate the TSLP receptor, secrete Th2-attracting chemokines, and acquire TSLP-dependent Th2-polarizing capability *in vitro* and *in vivo*<sup>[101]</sup>. Moreover, CD90-positive CAFs in colon cancer produce IL-6, which induces the polarization of tumor promoting inflammatory T helper 17 cells in infiltrating lymphocytes as well as the expression of cancer stem cell markers in colon cancer cells<sup>[102]</sup>.

CAFs can produce a myriad of chemokines, which can attract and activate immunosuppressive cells, such as M2-polarized TAMs, MDSCs, and Tregs, thereby suppressing tumor immunity<sup>[103]</sup>. Simultaneously, CAFs can produce chemokines that promote the





**Figure 4 Double-edged actions of cancer associated fibroblasts in tumor immunity.** CAFs exhibit double-edged actions in tumor immunity. In most types of cancers, CAFs can dampen tumor immunity by suppressing T cell proliferation, NK cell activity, and DC maturation, and inducing Treg proliferation and MDSC accumulation. In some types of cancers such as pancreatic ductal adenocarcinoma, CAFs can enhance tumor immunity by enhancing effector T cell and NK cell functions and depressing Treg activities. CAFs: Cancer associated fibroblasts; VEGF: Vascular endothelial growth factor; MDSC: Myeloid-derived suppressor cells; NK: Natural killer.

recruitment of effector T cells and natural killer cells, including CXCL9, CXCL10, and CXCL12<sup>[103]</sup>. If the latter chemokines are predominant chemokines produced by CAFs, they can enhance specific tumor immunity instead of suppressing it. Indeed, depletion of CAFs induces immunosuppression and accelerates pancreas cancer with reduced survival in a mouse pancreatic ductal adenocarcinoma (PDAC) model<sup>[11]</sup>. The immunosuppression is associated with increased Foxp3-positive Treg numbers and can be reversed by the immune checkpoint therapy using anti-CTLA4 antibody. Thus, in this model, CAFs restrain Tregs from expansion to keep tumor cells under immune surveillance.

## CAFS AS A PROGNOSIS MARKER IN CRC

CAFs can be a useful marker to predict disease recurrence in patients with various type of cancer<sup>[10]</sup>. Similar observations are obtained on CAFs in CRC. Tumors with abundant  $\alpha$ -SMA-positive CAFs are associated with shorter disease-free survival for stage II and III CRC after curative CRC surgery<sup>[104]</sup>. Likewise, high intra-tumor stroma proportion was associated with shorter overall and disease-free survival in stage II and stage III CRC patients after curative surgery<sup>[105]</sup>. CAFs express abundantly FAP- $\alpha$  and SDF-1/CXCL12. Colon cancer patients with high intra-tumor stromal FAP- $\alpha$  expression tend to have more aggressive disease progression and experience metastasis or recurrence<sup>[106]</sup>. Similarly, intra-tumor FAP- $\alpha$  and SDF-1 expression is shown to be involved in tumor re-growth

and recurrence in rectal cancer patients treated with pre-operative chemo-radiation therapy<sup>[107]</sup>.

The analysis was conducted on CAFs established from primary human colon cancer and revealed that CAFs exhibit significant differences in their promigratory effects on cancer cells upon co-culture with cancer cells<sup>[33]</sup>. Moreover, CAFs' promigratory effects on cancer cells are associated with fibroblast activation and stemness markers. CAF signature is identified from the gene expression signature derived from the most protumorigenic CAFs and shows a remarkable prognostic value for the clinical outcome of patients with CRC. Berdiel-Acer and colleagues conducted a transcriptomic profile of normal colonic fibroblasts, CAFs at primary tumor and CAFs at liver metastasis sites, and they identified 19-gene classifier that can predict recurrence with high accuracy in patients with CRC and correlates with fibroblast migratory potential<sup>[108]</sup>. Moreover, this 19-gene classifier can identify low-risk patients very accurately, and this identification is of particular importance for stage II patients, especially T4N0 patients clinically classified as being at high risk, who would benefit from the omission of chemotherapy. The same group further developed a 5-gene classifier for relapse prediction in Stage II/III CRC by analyzing gene expression patterns in CAFs<sup>[109]</sup>. The 5-gene classifier in CAFs was significantly associated with increased relapse risk and death from CRC among stage II/III patients. These studies also proved the existence of heterogeneity in CAFs in terms of gene expression signatures.

Molecular classification of CRC based on global gene expression profiles have defined three subtypes;

chromosomal-unstable tumor (CCS1), microsatellite-unstable/CpG island methylator (CIMP)-positive tumor (CCS2), microsatellite-stable/CIMP-positive tumor (CCS3)<sup>[110]</sup>. CCS3 subtype exhibits upregulation of genes involved in matrix remodeling and EMT and has a very poor prognosis. However, a more detailed analysis revealed that their predictive power arises from genes expressed by stromal cells rather than epithelial tumor cells<sup>[111]</sup>. Moreover, functional studies indicate that CAFs can increase the frequency of tumor-initiating cells and that this enhancing effect is further augmented by TGF- $\beta$  signaling. Furthermore, poor-prognosis CRC displays a gene program induced by TGF- $\beta$  in tumor stromal cells. These observations would indicate that CAF-mediated gene expression profiles can be used to predict the prognosis of colon cancer patients.

## CAF AS A TARGET FOR CANCER TREATMENT

Kraman and colleagues demonstrated that genetic depletion of FAP-expressing cells causes rapid hypoxic necrosis of both cancer and stromal cells in Lewis lung carcinoma-bearing mice depending on interferon- $\gamma$  and TNF- $\alpha$ . They also demonstrated that depleting FAP-expressing cells allows immunological control of tumor<sup>[28]</sup>. However, the same group demonstrated that FAP-positive cells of skeletal muscle are the major local source of follistatin and that those in bone marrow express CXCL12 and kit ligand. As a consequence, experimental ablation of these cells causes loss of muscle mass and a reduction of B-lymphopoiesis and erythropoiesis<sup>[29]</sup>. Thus, it is probable that depletion of FAP-positive cells in tumor tissue can cause cachexia and anemia, and as a consequence, it may be difficult to target FAP to deplete CAFs.

We demonstrated the crucial involvement of the CCL3-CCR5 axis on AOM/DSS-induced colon carcinogenesis by recruiting and activating CAFs. Systemic delivery of a CCR5-antagonist-expressing vector is well tolerated by tumor-bearing mice and reduces significantly tumor mass together with decreased CAFs, when it is given even after multiple tumors develop<sup>[22]</sup>. An antagonist to another chemokine, CXCL12, inhibits CAF-mediated integrin  $\beta$ 1 clustering at the cell surface and eventually the invasive ability of gastric cancer cells, suggesting that the inhibition of CXCL12/CXCR4 signaling in gastric cancer cells may be a promising therapeutic strategy against gastric cell invasion<sup>[112]</sup>. Moreover, CAF-derived CXCL12 can provide prostate cancer cells with the chemoresistance to a cytotoxic drug, docetaxel, and a CXCR4 antagonist can sensitize cancer cells to this drug in a subcutaneous xenograft model of prostate cancer<sup>[80]</sup>. PDAC-bearing mice frequently does not respond to immune checkpoint therapy with anti-programmed cell death ligand 1 (PD-L1) antibody despite the presence of tumor-specific CD8-positive cells, but depletion of

FAP-positive CAFs uncovers the antitumor effects of anti-PD-L1 antibody and inhibits tumor growth<sup>[113]</sup>. Moreover, FAP-positive CAFs express CXCL12 and as a consequence, a CXCR4 antagonist also induces rapid T-cell accumulation among cancer cells and acts synergistically with anti-PD-L1 to greatly diminish cancer cells in pancreatic cancer model<sup>[113]</sup>.

Normalization of CAFs is proposed as the strategy targeting CAFs. CAFs in prostate cancer exhibit reduced miR-15 and miR-16 expression, which is associated with the reduced post-transcriptional repression of Fgf-2 and its receptor Fgfr1<sup>[114]</sup>. The Fgf-2-Fgfr1 axis acts on both stromal and tumor cells to enhance cancer cell survival, proliferation, and migration. Moreover, reconstitution of miR-15 and miR-16 impairs considerably the tumor-supportive capability of stromal cells *in vitro* and *in vivo*<sup>[114]</sup>. Downregulation of miR-31 and miR-214 is observed in CAFs in ovarian cancer and the expression of these miRNAs induces a functional conversion of CAFs into normal fibroblasts<sup>[115]</sup>. Similar observations were also obtained on miR-31 and miR-148a expression in CAFs<sup>[116,117]</sup>. Phosphatase and tensin homolog deleted on chromosome 10 (Pten) expression in stromal fibroblasts suppresses epithelial mammary tumors. Pten-deficient mammary fibroblasts exhibit reduced miR-320 expression and reciprocally enhanced ETS2 expression, and can accelerate tumorigenicity when co-injected into mice with mouse mammary cancer cells<sup>[118]</sup>. miR-320 overexpression in fibroblasts reduces their tumorigenic activity upon co-injection with cancer cells<sup>[118]</sup>. These observations would indicate that the modulation of miRNA expression can reduce the protumorigenic capacity of CAFs, by dedifferentiating CAFs into normal fibroblasts.

Nintedanib is a broad spectrum tyrosine kinase inhibitor, with the VEGF receptor, FGF receptor, and PDGF receptor as target by binding the ATP pocket in a competitively reversible manner and is used as monotherapy for the treatment of idiopathic lung fibrosis (IPF)<sup>[119]</sup>. Nintedanib reduces lung inflammation and fibrosis in IPF as evidenced by the reduced deposition of type I collagen and the inhibition of fibroblast activation. VEGF, FGF, and PDGF are secreted by CAFs as well as cancer cells and TAMs, and their receptors are expressed by CAFs, cancer cells, and endothelial cells<sup>[120]</sup>. Moreover, the mechanism of fibroblast activation in IPF closely resembles that in cancer<sup>[121]</sup>. Hence, nintedanib is proposed to be used as a second line therapy for non-small cell lung cancer in combination with docetaxel<sup>[122]</sup>. Another anti-fibrotic agent, pirfenidone, is used for the treatment of IPF although its exact molecular mechanisms remain enigmatic<sup>[123]</sup>. The combination of pirfenidone and cisplatin leads to increased CAF cell death and decreased tumor progression in a human non-small cell lung cancer xenografted model<sup>[124]</sup>. These observations would indicate that these anti-fibrotic

agents may be used for the treatment of cancer with abundant fibrotic changes.

Given the capacity of TGF- $\beta$  as a potent fibrotic molecule<sup>[98]</sup>, anti-TGF- $\beta$  monoclonal antibody was developed and tested in clinical trials for several types of cancer. It can induce anti-tumor effects but simultaneously cutaneous keratoacanthomas/squamous cell carcinomas<sup>[125]</sup>. This may arise from double-edged activities of TGF- $\beta$ ; a tumor suppressor for normal epithelial cells and a tumor driver in tumor microenvironments<sup>[98]</sup>.

## FUTURE PERSPECTIVES

Accumulating evidence indicates the crucial involvement of inflammation in cancer development and progression<sup>[12]</sup>. Inflammation is a dynamic host response, wherein various types of cells participate in a concerted manner<sup>[4]</sup>. However, until recently, much attention has been focused on two processes involved in inflammation; neovascularization and inflammatory cell infiltration. Various agents have been developed as anti-cancer drugs, by targeting neovascularization, but with limited success<sup>[126]</sup>. Despite the remarkable successes of immunotherapies that modulate the adaptive immune system consisting of lymphocytes and dendritic cells<sup>[127]</sup>, the plasticity and the heterogeneity of inflammatory leukocytes, monocytes/macrophages and granulocytes, have hindered the elucidation of the precise roles of these cells in carcinogenesis, and eventually the development of anti-cancer agents targeting inflammatory leukocytes<sup>[128]</sup>. Under these circumstances, fibroblasts, another type of cells in inflammation, have emerged as an important player in cancer-related inflammation<sup>[10,25,129]</sup>.

CAFs express a NF- $\kappa$ B-dependent gene signature in mouse tumor models of skin, pancreatic and breast cancer<sup>[86]</sup>. These observations incited two independent groups to conduct fibroblast-specific deletion of the gene of *IKK $\beta$* , which is indispensable to NF- $\kappa$ B activation and to examine the effects of the gene deletion on AOM/DSS-induced colon carcinogenesis<sup>[130,131]</sup>. However, the results are completely opposite to each other; Koliaraki *et al.*<sup>[130]</sup> demonstrated a pro-tumorigenic activity of *IKK $\beta$*  whereas Pallangyo identified *IKK $\beta$*  as a tumor suppressor. A remarkable difference between these two groups seems to arise from the use of the different gene promoters to delete the *IKK $\beta$*  gene. Koliaraki *et al.*<sup>[130]</sup> used type VI collagen gene promoter whereas Pallangyo and colleagues used type I collagen gene promoter. The *IKK $\beta$*  gene deletion in type VI collagen-positive CAFs, decreased IL-6 production associated with decreased inflammation and suppressed tumor formation<sup>[130]</sup>. On the contrary, the *IKK $\beta$*  gene deletion in type I collagen-positive CAFs, resulted in enhanced HGF production and subsequent tumor growth promotion<sup>[131]</sup>. Thus, CAFs as a whole may act to promote carcinogenesis but a part of them, particularly

type I collagen-positive ones, may still retain normal fibroblast-like phenotypes and functionality to suppress carcinogenesis, because normal intestinal fibroblasts can regulate intestinal homeostasis to suppress colitis-associated tumorigenesis<sup>[23]</sup>.

Targeting CAFs can be a novel strategy to treat cancer, particularly inflammation-related cancer. In order to advance this strategy, however, more detailed and precise understanding of phenotypical and functional heterogeneity in CAFs is required to identify the CAF subpopulation and/or the molecules, which have crucial roles in cancer development and progression.

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