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Mukaida N *et al.* Fibroblasts in colon carcinogenesis

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Abstract

Tumor microenvironments play crucial roles in cancer initiation and progression, and share many molecular and pathological features with the wound healing process. In contrast with wounds that heal within a limited timeframe, tumors do not heal in the absence of treatment. Wounds heal through the coordination of a myriad of cell types, including endothelial cells, leukocytes, and fibroblasts. Similar sets of cells also contribute to cancer initiation and progression; consequently, anti-cancer treatment strategies that target endothelial cells and/or leukocytes have been proposed and tested. Less attention, however, has been paid to the roles of cancer-associated fibroblasts (CAFs). The heterogeneity of fibroblasts present in tumor tissues hinders the elucidation of their roles in tumor initiation and progression at the cellular and molecular levels. In this review, we discuss the origin of CAFs and their crucial roles in cancer initiation and progression, as well as the possibility of developing a novel type of anti-cancer treatment through manipulating the migration and functions of CAFs.

INTRODUCTION

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

Irrespective of the cause and the severity 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^[4]. These steps proceed through interaction between parenchymal cells and the stroma, a complex mixture of inflammatory cells, matrix proteins, and tissue cells such as fibroblasts and endothelial cells. In the case of acute and mild wounds, injured organs and tissues are completely replaced by proliferating parenchymal cells, 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^[4].

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 α -smooth muscle actin (α -SMA) and are referred to as myofibroblasts^[5]. Myofibroblasts synthesize increased amount of various collagens and extracellular matrix proteins (ECM) to provide a scaffold and to eventually aid in wound repair^[5]. Furthermore, the cells are important sources of many growth factors and cytokines that regulate wound healing processes^[6].

Under co-culture conditions, primary normal fibroblasts isolated from various human tissues can restrict *in vitro* proliferation of various types of human cancer cell lines^[7]. Indeed, if Nod-like receptor pyrin domain-containing protein 6

(NLRP6) is absent in fibroblasts within the stem cell niche of the colon, regeneration of the colonic mucosa and the processes of epithelial proliferation and migration are impaired. Consequently, colitis-associated tumorigenesis is accelerated in mice lacking NLRP6^[8]. Thus, under normal conditions, fibroblasts work as a sentinel cell to maintain epithelial tissue homeostasis and to prevent the initiation of tumorigenesis in the colon, in an NLRP6-dependent manner.

Like fibroblasts in the wound healing process, fibroblasts present in tumor tissues exhibit an activated and myofibroblast-like phenotype with α -SMA expression and are referred to as cancer-associated fibroblasts (CAFs)^[9]. 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^[9,10]. 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^[11]. Hence, the pathophysiological roles of CAFs in the development and progression of solid tumors are yet to be elucidated.

We will herein discuss the pathophysiological roles of CAFs and their clinical relevance in cancer, and specifically in colorectal cancer (CRC).

CAFs IN COLITIS-ASSOCIATED COLON CARCINOGENESIS MODEL

Accumulating evidence highlights the crucial contribution of chronic inflammation to tumor development and progression^[12]. Colitis-associated colon carcinogenesis (CAC) is a 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^[13]. Pathological features of UC include mucosal damage and ulceration with prominent leukocyte infiltration, firstly involving the rectum and extending proximally.

Oral administration of dextran sulfate sodium (DSS) solution to rodents can cause acute inflammatory reactions and ulceration in the entire colon, similar to that observed in human patients with UC; therefore, it is widely used to reproduce human UC^[14]. Moreover, repeated DSS ingestion can lead to the development of a small number of colon carcinomas in approximately 50% of mice^[15], suggesting that the inflammatory response alone can cause colon carcinoma. The incidence of DSS-induced colon carcinogenesis is both increased and accelerated by a prior administration of azoxymethane (AOM)^[16], which can alone cause colon cancer by inducing O⁶-methyl guanine formation and mutations of the *β-catenin* gene^[17]. Thus, combined treatment with AOM and DSS is frequently used to recapitulate the molecular mechanisms underlying CAC.

The NF-κB transcription factor is a key player in inflammation. NF-κB activity is triggered by the IκB kinase (IKK) complex in response to a wide variety of pro-inflammatory stimuli such as infectious agents and pro-inflammatory cytokines^[18]. Greten and colleagues demonstrated that IKKβ has crucial roles in AOM/DSS-induced CAC through two distinct pathways: prevention of epithelial cell apoptosis, and enhancement of myeloid cell growth factor expression^[19]. These observations indicate the crucial involvement of inflammatory cell infiltration in CAC development. Given the crucial roles of NF-κB in the regulation of gene expression and the biological functions of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and chemokines^[18], we examined AOM/DSS-induced colon carcinogenesis process using mice deficient in the tumor necrosis factor receptor (*TNF-R*)*p55* gene^[20]. We revealed that genetic ablation of the *TNF-Rp55* gene in myeloid cells resulted in reduced intracolonic infiltration of inflammatory cells, particularly macrophages, neovascularization, and subsequent tumor formation. Moreover, a TNF-inhibitor reduced tumor progression even when administered after multiple tumors had developed in the colon. Similar phenotypes were observed by genetic inactivation of a macrophage-tropic chemokine receptor gene, *CCR2*, in myeloid cells or following the administration of *CCR2* inhibitors after multiple colon tumors developed^[21].

These observations prompted us to examine the roles of another macrophage-tropic chemokine, CCL3, and its receptors, CCR1 and CCR5^[22]. Mice deficient in *CCL3*, *CCR1*, or *CCR5*, displayed marginal inflammatory reactions and subsequently developed few colon tumors when AOM was administered together with 3% DSS ingestion. Wild-type (WT) mice failed to survive 4.5% DSS ingestion. Mice deficient in *CCL3*, *CCR1*, or *CCR5* mice survived 4.5% DSS ingestion and displayed prominent mucosal damage and leukocyte infiltration in the colon. *CCR1*-deficient mice developed multiple colon tumors; however, the same treatment resulted in a small number of colon tumors in *CCL3*- or *CCR5*-deficient mice (Table 1)^[22]. These observations indicate that inflammatory cell infiltration is necessary, but not sufficient, for the development of CAC in this model.

Compared with WT or *CCR1*-deficient mice, *CCL3*- or *CCR5*-deficient mice exhibited reduced accumulation of CAFs in the colon. This accumulation was predominantly evident in the later phase of CAC in this model (Table 1). Several groups, including ours, further demonstrated that growth factors such as hepatocyte growth factor (HGF)^[23], epiregulin^[24], and heparin-binding epidermal growth factor-like growth factor (HB-EGF)^[22] are highly expressed in CAFs. These growth factors promote tumor cell proliferation in the later phase of this CAC model. Moreover, deficiency of the *CCR5* gene results in reduced growth of tumors arising from either subcutaneous or orthotopic intracecum injection of a syngeneic mouse colon adenocarcinoma cell line, colon 26. This attenuated tumor growth was associated with a reduction in type I collagen-positive fibroblast numbers but not with inflammatory cell infiltration^[22]. These observations indicate that CAFs are involved in the progression, but not development, of CAC.

ORIGINS OF CAFs (FIGURE 1)

The lack of a specific marker to identify CAFs^[25] hampers the precise identification of their origin. α -SMA, a robust CAF marker, is also expressed by normal colonic fibroblasts under *in vitro* culture conditions^[26] as well as other cell types such as pericytes, smooth muscle cells surrounding vasculature, and cardiomyocytes^[27]. Additional candidate CAF markers including FAP- α ^[28,29], S100A4/fibroblast

specific protein (FSP)-1^[30,31], neuron-glia antigen-2, PDGF receptor- β , and prolyl 4-hydroxylase^[32]. However, these molecules are not specific to CAFs and can be expressed by other cell types. The lack of a definitive CAF marker implies that CAFs in CRC are phenotypically and functionally heterogeneous. This assumption is further strengthened by global gene expression profiles^[33].

The most likely cellular source of CAFs in CRC is resident fibroblasts in colon tissues. Supporting this notion, CAFs in CRC liver metastatic foci exhibit similar protein expression pattern as resident liver fibroblasts^[34]. Kojima and colleagues demonstrated that resident human mammary fibroblasts progressively convert into CAF-like cells with enhanced α -SMA expression and pro-tumorigenic capacity, during the course of tumor progression in a breast tumor xenograft model^[35]. Moreover, these cells express transforming growth factor (TGF)- β and a chemokine, stromal-derived factor (SDF)-1/CXCL12, which further initiate and maintain the differentiation of fibroblasts into CAF-like and the tumor-promoting phenotype in an autocrine and amplifying manner^[35].

In their quiescence state, stellate cells are vitamin A-containing and lipid droplet-containing cells and are present in various tissues including the liver, pancreas, kidney, and intestine^[36]. Similar to CAFs, these cells activate α -SMA expression under inflammatory and oncogenic conditions, and acquire myofibroblast-like phenotypes. Most 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^[37]. Similarly, pancreatic cancer is characterized by a prominent desmoplastic/stromal reaction. Like hepatic stellate cells, activated pancreatic stellate cells can abundantly produce the collagenous stroma of pancreatic cancer. Moreover, these cells can also interact closely with cancer cells to facilitate local tumor growth and distant metastasis, mediate angiogenesis, and induce immune evasion^[38]. However, it has yet to be determined if stellate cells in the intestine, can also behave in a similar manner during colon carcinogenesis, as they do in the liver and pancreas.

Other cell types are proposed to be a source of CAFs, based on the analyses of cancers other than CRC. Accumulating evidence indicates that, under chronic inflammatory conditions, epithelial cells can undergo epithelial-mesenchymal transition (EMT) to acquire myofibroblast-like phenotypes and participate in the synthesis of the fibrotic matrix^[39]. A breast carcinoma biopsy provided evidence of EMT and a coincidental α -SMA-positive stromal reaction^[40]. Moreover, upon injection of the MCF-7 mouse breast cancer cell line into nude mice with HBFL-1, a mammary gland epithelial cell line without tumorigenicity, HBFL-1 cells acquired myofibroblast-like phenotypes. In addition, a significant 3.5- to 7-fold increase in MCF-7 tumor size in nude mice was observed. Thus, breast cancer can transform its own non-malignant stroma to facilitate its growth^[40]. 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 gave rise to about a quarter of CAFs, which provided cancer cells resistance to EGFR-TKI^[41]. However, several independent groups argue against EMT as the origin of CAFs^[42,43].

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

TGF- β 1 can induce proliferating endothelial cells to undergo phenotypic conversion into fibroblast-like cells including the emergence of mesenchymal markers and reciprocal down-regulation of CD31^[46]. 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 tumors in the B16F10 melanoma model and the Rip-Tag2 spontaneous pancreatic carcinoma model^[46]. Choi and colleagues further demonstrated that endothelial heat shock protein (HSPB1, 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^[47]. 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 the reciprocal appearance of CAFs, which can promote carcinogenesis in genetically abnormal but non-tumorigenic epithelial cells^[48]. While smooth muscle cells may be a source of CAFs in prostate cancer, it is unclear whether CAFs originate from this population in other cancer types, particularly CRC.

There is considerable evidence that indicates functional heterogeneity of CAFs in various cancers, including colon cancer^[33]. Heterogeneity may arise from differences in the origin of CAFs. Alternatively, CAFs are generated by intricate interactions with tumor microenvironments consisting of cancer and other resident cells^[10,25]. Therefore, the heterogeneity of tumor microenvironments can induce wide variation in CAFs. Nevertheless, the heterogeneity of CAFs can affect the clinical course of colon cancer patients^[49].

RECRUITMENT AND ACTIVATION OF CAFs

In chronic inflammation the chemokines CCL2 and CCL3 can recruit fibroblasts^[50], while CXCL12^[51], CCL21^[52], and CCL3^[53] can recruit fibrocytes. We have shown that CCL3 is produced locally at tumor sites and is associated with CAF accumulation^[22,54]. 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- β and CXCL12-dependent manner^[55]. Therefore, CAF accumulation may be regulated by cooperation with chemokines and other fibroblast-tropic factors such as TGF- β .

Normal fibroblasts can inhibit proliferation of cancer cells *in vitro*^[7]. Moreover, normal intestinal fibroblasts can maintain epithelial homeostasis to prevent carcinogenesis^[23]. Thus, malignant cells must reprogram normal fibroblasts into CAFs with pro-tumorigenic activity. This process is mediated by cancer

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

CAFs IN CARCINOGENESIS

Cancer cell growth and stemness (Figure 2)

Human pre-malignant prostatic epithelial cells can transform to neoplastic cells when co-cultured with CAFs derived from human prostate cancer tissues *in vitro*^[60]. Factors secreted from CAFs are believed responsible for this tumor-initiating capacity. Indeed, when human mammary epithelial cells are grafted into immunodeficient mice, together with fibroblasts overexpressing TGF- β and/or HGF, the engrafted cells develop into a proliferating tissue closely resembling human ductal carcinomas^[61]. These observations raise the possibility that CAFs can initiate the malignant transformation of epithelial cells by secreting these growth factors.

Under the influence of cancer cell-derived IL-6, CAFs can secrete metalloproteinases, to stimulate EMT and cancer stem cell phenotypes in prostate cancer^[62]. CAFs in colon cancer secrete HGF, activate β -catenin-dependent transcription, and induce cancer stem cell clonogenicity^[63]. 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 abundantly produce IL-17A, which increases chemotherapy-resistant cancer stem cells^[64].

CAFs produce various growth factors and cytokines, which can promote cancer cell proliferation: HGF^[23], EGF^[65], epiregulin^[24], HB-EGF^[22], insulin-like growth

factor (IGF)^[66], TGF- β ^[35], connective tissue growth factor (CTGF)^[67], and CXCL12^[58]. Most of these growth factors can be produced by cancer cells and can enhance the growth of CAFs. Moreover, CAF-derived TGF- β and CXCL12 affect CAF proliferation in an autocrine dependent manner^[35]. Thus, these growth factors form a positive feedback loop between cancer cells and CAFs, and may accelerate tumor progression.

It is clear that 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.

Cancer cell migration, invasion, and metastasis (Figure 2)

Wound healing assays performed in the presence of CAF-derived conditioned media show that colon cancer cell lines exhibit enhanced migratory ability, and increased clonogenic capacity compared with normal fibroblast-derived conditioned media^[26]. Moreover, co-injection of CAFs with a human colon cancer cell line into nude mice, significantly increased tumor cell proliferation, compared with normal fibroblasts^[26]. 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*)^[26]. These gene products can modulate the functions of cancer cells to promote invasion and metastasis.

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^[68]. Moreover, MMP-mediated enhanced invasiveness also involves proteinase-activated receptor 1 (PAR1) expressed on the surface of cancer cells. CAF-derived MMP-1 cleaves PAR1 to activate PAR1-mediated signaling pathways, associated with cancer cell migration and invasion in cancer cells^[69].

In the lung, fibroblasts and tumor cells abundantly produce CCL2, which recruits Gr-1-positive, CCR2-expressing inflammatory monocytes^[70]. 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 the direct actions of CAFs^[71]. S100A4-positive CAFs abundantly produce 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^{high} and stroma^{low} CRC shows that neoplastic cells in stroma^{high} tumors express specific EMT drivers including ZEB2, TWIST1, and TWIST2^[72]. Moreover, type I collagen dominates the extracellular matrix in aggressive colon cancers with EMT markers. Mimicking the tumor microenvironment, Matrigel enriched with type I collagen can induce colon cancer cells to express tumor-specific mesenchymal genes; suppress expression of hepatocyte nuclear factor 4, a transcriptional activator of epithelial differentiation and its target genes; and invade patient-derived colon tumor organoids^[72]. 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^[73]. Primary tumor-derived factors induce fibroblasts resident in pre-metastatic sites to express fibronectin, which interacts with VLA-4 on VEGFR1-positive cells to induce cell clusters and promote pre-metastatic niche formation. In fibroblasts, fibronectin expression is regulated by the sphingosine-1-phosphate (SIP)-SIP receptor-STAT3 pathway^[74].

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

Drug resistance

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

In addition to ECM, CAF-derived soluble factors have been demonstrated to be involved in drug resistance. CAF-derived CXCL12 mediates drug resistance to conventional chemotherapeutics^[80]. This resistance can arise from the ability of CXCL12 to promote cancer cell survival by activating the focal adhesion kinases, Erk, and Akt, β -catenin and NF- κ B, in CXCR4-expressing cancer cells^[81].

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 targeting these RTKs^[82]. RTK-mediated signals converge on common critical downstream cell-survival effectors such as PI3K and Erk. Consequently, most cells can be rescued from drug sensitivity by exposure to one or more unrelated RTK ligands. Among these RTK ligands, HGF confers resistance to the BRAF inhibitor in BRAF-mutant melanoma cells^[83]. Likewise, CAF-derived HGF can confer the resistance to EGF-receptor inhibitors in human non-small cell lung cancer cells otherwise sensitive to inhibitors^[84].

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^[64]. Chemotherapy-treated human CAFs promoted CIC self-renewal and *in vivo* tumor growth associated with increased secretion of specific cytokines and chemokines, including interleukin-17A (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^[64]. These data suggest that chemotherapy induces remodeling of the tumor microenvironment through activating CAFs to secrete cytokines such as IL-17.

Tumor microenvironments (Figure 3)

Chronic inflammation is closely associated with tumorigenesis of various types of cancer^[12,85]. CAFs, a major cellular component of cancer-associated inflammation, mediate tumor-enhancing inflammation by expressing a pro-inflammatory gene signature in an NF- κ B-dependent manner^[86]. NF- κ 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^[87], 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^[88, 89].

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

Tumor microenvironment is characterized by abundant neovascularization, which can be induced by CAFs. CAF-induced MMP activation degrades ECM and

eventually causes neovascularization^[68]. CAFs, particularly those in invasive margins, are a rich source of the CXC chemokine, SDF-1/CXCL12^[58], which mediates the recruitment of endothelial progenitor cells (EPCs) and subsequent tumor neovascularization. Furthermore, hypoxia can induce CAFs to express the transcription factor, hypoxia-inducible factor (HIF)-1 α , which in turn induces the expression of VEGF, a potent angiogenic factor^[93]. VEGF production by CAFs can be further enhanced by CAF-derived IL-6, whose expression can be augmented in the presence of colon cancer cells^[94].

Tumor immunity (Figure 4)

Deletion of fibroblast activation protein (FAP)-positive stromal cells enhances tumor immunity^[28] and provides direct evidence of the involvement of CAFs in suppressed tumor immunity. While several candidate mechanisms have been proposed, this study did not clarify the cellular and molecular mechanisms of CAFs involvement in suppressed tumor immunity. CAF-derived prostaglandin E2 and indoleamine 2,3-dioxygenase (IDO) can inhibit natural killer (NK) cell functions, thereby contributing to immune escape and subsequent tumor progression^[95]. CAF-derived tenascin also contributes to immune suppression at tumor sites. Soluble tenascin inhibits the proliferation of human T cells induced by the combination of anti-CD3 antibody and fibronectin. Tenascin further attenuates IL-2 driven T cell proliferation, and prevents induction of the IL-2 receptor at high levels^[96]. 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. Consequently, cancer stem-like cells are protected from T cell-mediated immune surveillance^[97].

CAFs abundantly express TGF- β 1, which can suppress the functions of various immune cells, particularly effector T cells and natural killer cells^[98]. TGF- β regulates Treg maturation and thereby suppresses immune responses. VEGF is also produced by CAFs and exhibits immunosuppressive effects^[99]. 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, thereby inhibiting T-cell immune responses.

In vivo vaccination with a DNA vaccine against FAP eliminates CAFs and causes a shift of the immune microenvironment from a Th2 to Th1 polarization. This shift is characterized by increased expression of IL-2 and IL-7, suppressed recruitment of tumor-associated macrophages (TAMs), MDSCs, and Tregs, and decreased tumor angiogenesis and lymphangiogenesis^[100]. These observations suggest roles for CAFs in intratumor Th2 polarization and the subsequent depression of tumor immunity. Th2 polarization is mediated by CAF-derived thymic stromal lymphopoietin (TSLP), which induces *in vitro* myeloid DCs to up-regulate the TSLP receptor (TSLPR), secrete Th2-attracting chemokines, and acquire TSLP-dependent Th2-polarizing capability *in vitro* and *in vivo*^[101]. Moreover, CD90-positive CAFs in colon cancer produce IL-6, which induces the polarization of tumor promoting inflammatory T helper 17 cells (Th17) in infiltrating lymphocytes, as well as the expression of cancer stem cell markers in colon cancer cells^[102].

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^[103]. Simultaneously, CAFs can produce chemokines that promote the recruitment of effector T cells and natural killer cells, including CXCL9, CXCL10, and CXCL12^[103]. If the latter chemokines are the 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 progression with reduced survival in a mouse pancreatic ductal adenocarcinoma (PDAC) model^[11]. This immunosuppression is associated with increased Foxp3-positive Treg cells and can be reversed by immune checkpoint therapy using the anti-CTLA4 antibody. Thus, in this model, CAFs prevent 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 types of cancer^[10]. Tumors with abundant α -SMA-positive CAFs are associated with shorter disease-free survival for stage II and III CRC after curative CRC surgery^[104]. High intra-tumor stroma proportion was associated with shorter overall survival and disease-free survival in stage II and stage III CRC patients after curative surgery^[105]. CAFs abundantly express FAP- α and SDF-1/CXCL12. Colon cancer patients with high intra-tumor stromal FAP- α expression tend to have more aggressive disease progression and experience metastasis or recurrence^[106]. Similarly, intra-tumor FAP- α and SDF-1 expression is involved in tumor re-growth and recurrence in rectal cancer patients treated with pre-operative chemo-radiation therapy^[107].

Analysis of CAFs established from primary human colon cancer revealed that CAFs exhibit significant differences in their pro-migratory effects on cancer cells upon co-culture with cancer cells^[33]. Moreover, CAFs promigratory effects on cancer cells are associated with fibroblast activation and stemness markers. CAF signature is identified by the gene expression signature of the most pro-tumorigenic CAFs and shows remarkable prognostic value for patients with CRC. Berdiel-Acer and colleagues conducted a transcriptomic profile of normal colonic fibroblasts (NCFs), primary tumor CAFs, and liver metastasis site CAFs, and identified a 19-gene classifier. In patients with CRC, this 19-gene classifier can predict recurrence with high accuracy, and correlates with fibroblast migratory potential^[108]. Moreover, this 19-gene classifier can accurately identify low-risk patients, which is of particular importance for stage II patients. T4N0 patients, clinically classified as high risk, would particularly benefit from this prognostic tool and subsequent 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^[109]. The 5-gene classifier in CAFs was significantly associated with increased relapse risk and death from CRC among stage II/III patients. These studies proved the existence of heterogeneity in CAFs in terms of gene expression signatures.

Molecular classification of CRC based on global gene expression profiles has defined three subtypes: chromosomal-unstable tumor (CCS1), microsatellite-unstable (MSI)/CpG island methylator (CIMP)-positive tumor (CCS2), and microsatellite-stable/CIMP-positive tumor (CCS3)^[110]. The CCS3 subtype exhibits upregulation of genes involved in matrix remodeling and EMT and has a very poor prognosis. However, more detailed analysis of the subgroups revealed that the prognostic predictive power arises from genes expressed by stromal cells rather than epithelial tumor cells^[111]. Functional studies indicate that CAFs can increase the frequency of tumor-initiating cells and that this enhancing effect is further augmented by TGF- β signaling. Furthermore, poor-prognosis CRC displays a gene expression program induced by TGF- β in tumor stromal cells. These observations 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 (IFN)- γ and TNF- α . In addition, they demonstrated that depletion of FAP-expressing cells allows immunological tumors control^[28]. However, the same group demonstrated that the FAP-positive cells of skeletal muscle are the major local source of follistatin and those in bone marrow express CXCL12 and kit ligand. Consequently, experimental ablation of these cells causes loss of muscle mass and a reduction of B-lymphopoiesis and erythropoiesis^[29]. Thus, it is probable that depletion of FAP-positive cells in tumor tissue can cause cachexia and anemia, and therefore, 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 through recruiting and activating CAFs. Systemic delivery of a CCR5-antagonist-expressing vector is well tolerated by tumor-bearing mice and significantly reduces tumor mass together with decreased CAFs, even when it is given after multiple tumors develop^[22]. An antagonist to the

chemokine, CXCL12, inhibits CAF-mediated integrin β 1 clustering at the cell surface and 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^[112]. Moreover, CAF-derived CXCL12 can provide prostate cancer cells with chemoresistance to the cytotoxic drug, docetaxel, and a CXCR4 antagonist can sensitize cancer cells to this drug in a subcutaneous xenograft model of prostate cancer^[80]. PDAC-bearing mice frequently do 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. However, depletion of FAP-positive CAFs uncovers the antitumor effects of the anti-PD-L1 antibody and inhibits tumor growth^[113]. FAP-positive CAFs express CXCL12; consequently, a CXCR4 antagonist also induces rapid T-cell accumulation among cancer cells acting synergistically with anti-PD-L1 to greatly diminish cancer cells in pancreatic cancer model^[113].

Normalization of CAFs is proposed as the strategy targeting CAFs. In prostate cancer, CAFs exhibit reduced miR-15 and miR-16 expression associated with reduced post-transcriptional repression of Fgf-2 and its receptor Fgfr1^[114]. 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 considerably impairs the tumor-supportive capability of stromal cells *in vitro* and *in vivo*^[114]. In ovarian cancer CAFs downregulate miR-31 and miR-214 and the expression of these miRNAs induces a functional conversion of CAFs into normal fibroblasts^[115]. Similar observations were made of miR-31 and miR-148a expression in CAFs^[116,117]. 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, enhanced ETS2 expression, and can accelerate tumorigenicity when co-injected into mice with mouse mammary cancer cells^[118]. miR-320 overexpression in fibroblasts reduces their tumorigenic activity upon co-injection with cancer cells^[118]. These observations indicate that the modulation of miRNA

expression can reduce the pro-tumorigenic capacity of CAFs, by dedifferentiating CAFs into normal fibroblasts.

Nintedanib is a broad-spectrum tyrosine kinase inhibitor targets the VEGF, FGF, and PDGF receptors binding to the ATP pocket in a competitively reversible manner. Nintedanib is used as monotherapy for the treatment of idiopathic lung fibrosis (IPF)^[119] and reduces lung inflammation and fibrosis in IPF as seen by reduced deposition of type I collagen and inhibition of fibroblast activation. VEGF, FGF, and PDGF are secreted by CAFs, cancer cells, and TAMs and their receptors are expressed by CAFs, cancer cells, and endothelial cells^[120]. Moreover, the mechanism of fibroblast activation in IPF closely resembles that in cancer^[121]. Hence, nintedanib is proposed as a second line therapy for non-small cell lung cancer in combination with docetaxel^[122]. Another anti-fibrotic agent, pirfenidone, is used to treat IPF although its exact molecular mechanisms remain unknown^[123]. 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^[124]. These observations indicate that these anti-fibrotic agents may be used for the treatment of cancers with abundant fibrotic changes.

Given the potent fibrotic capacity of TGF- β ^[98], the anti-TGF- β monoclonal antibody was developed and tested in clinical trials for several cancer types. The anti-TGF- β antibody can simultaneously induce anti-tumor effects and cutaneous keratoacanthomas/squamous cell carcinomas^[125]. This duplicity may arise from the double-edged activities of TGF- β ; a tumor suppressor for normal epithelial cells and a tumor driver in tumor microenvironments^[98].

FUTURE PERSPECTIVES

Accumulating evidence highlights the crucial involvement of inflammation in cancer development and progression^[12]. Inflammation is a dynamic host response, wherein various cell types participate in a concerted manner^[4]. However, until recently, much attention has been focused on two processes involved in inflammation: neovascularization, and inflammatory cell infiltration. Various agents targeting neovascularization have been developed as anti-cancer drugs,

with limited success^[126]. Despite remarkable successes of immunotherapies that modulate the adaptive immune system consisting of lymphocytes and dendritic cells^[127], the plasticity and heterogeneity of inflammatory leukocytes, monocytes/macrophages, and granulocytes has hindered the elucidation of their roles these in carcinogenesis. Likewise the development of anti-cancer agents targeting inflammatory leukocytes^[128] has been equally hindered. Under these circumstances, fibroblasts, have emerged as an important player in cancer-related inflammation^[10,25,129].

CAFs express an NF- κ B-dependent gene signature in mouse skin, pancreatic and breast cancer models^[86]. These observations incited two independent groups to conduct fibroblast-specific deletion of the gene of *IKK β* , required for NF- κ B activation, and examine the effects on AOM/DSS-induced colon carcinogenesis^[130,131]. However, the results obtained from the two studies are completely opposite to each other. Koliaraki and colleagues demonstrated a pro-tumorigenic activity of IKK β whilst, Pallangyo identified IKK β as a tumor suppressor. The differences observed by the two groups appear to arise from the use of different gene promoters to delete the *IKK β* gene. Koliaraki and colleagues used the type VI collagen gene promoter whereas Pallangyo and colleagues used the type I collagen gene promoter. *IKK β* deletion in type VI collagen-positive CAFs, decreased IL-6 production associated with decreased inflammation and suppressed tumor formation^[130]. In contrast, *IKK β* deletion in type I collagen-positive CAFs, enhanced HGF production and subsequently promoted tumor growth^[131]. Therefore, in general, CAFs may act to promote carcinogenesis, but a type I collagen-positive subset may retain normal fibroblast-like phenotypes and functionality. This type I collagen-positive subset can act to suppress carcinogenesis, because normal intestinal fibroblasts can regulate intestinal homeostasis to suppress colitis-associated tumorigenesis^[23].

Targeting CAFs can be a novel strategy to treat cancer, and inflammation-related cancer in particular. In order to advance this strategy, however, a more detailed and precise understanding of phenotypical and functional heterogeneity in CAFs is

required to identify the CAF subpopulation and/or molecules, with crucial roles in cancer development and progression.

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

	Ingested DSS concentration (%)	Body weight loss (> 20%)	Granulocyte infiltration (> 400/field)	Fibroblast accumulation (> 20% type I Collagen ⁺ 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*^[22]. DSS: Dextran sulfate sodium.

Figure Legends

Figure 1 The origin of CAFs. A variety of cells can become CAFs. The most important cellular source of CAFs in CRC is presumed to be resident fibroblasts. Stellate cells in the intestine may be able to transform into CAFs in a manner similar to what occurs in the liver and pancreas. Other potential sources include epithelial cells undergoing EMT, endothelial cells undergoing EndoMT, smooth muscle cells, and bone marrow-derived cells including fibrocytes and MSCs. CAFs: Cancer associated fibroblasts; CRC: Colorectal cancer; EMT: Epithelial-mesenchymal transition; MSCs: mesenchymal stem cells.

Figure 2 The action of CAFs on tumor cells. CAFs 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 a 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.

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 by 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 α : Hypoxia-inducible factor 1 α .

Figure 4 Double-edged actions of cancer associated fibroblasts in tumor immunity. CAFs exhibit double-edged actions in tumor immunity. In most cancer types, CAFs can dampen tumor immunity by suppressing T cell proliferation, NK

cell activity, DC maturation, and by 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.

Figure 1. The origin of cancer-associated fibroblasts (CAFs)

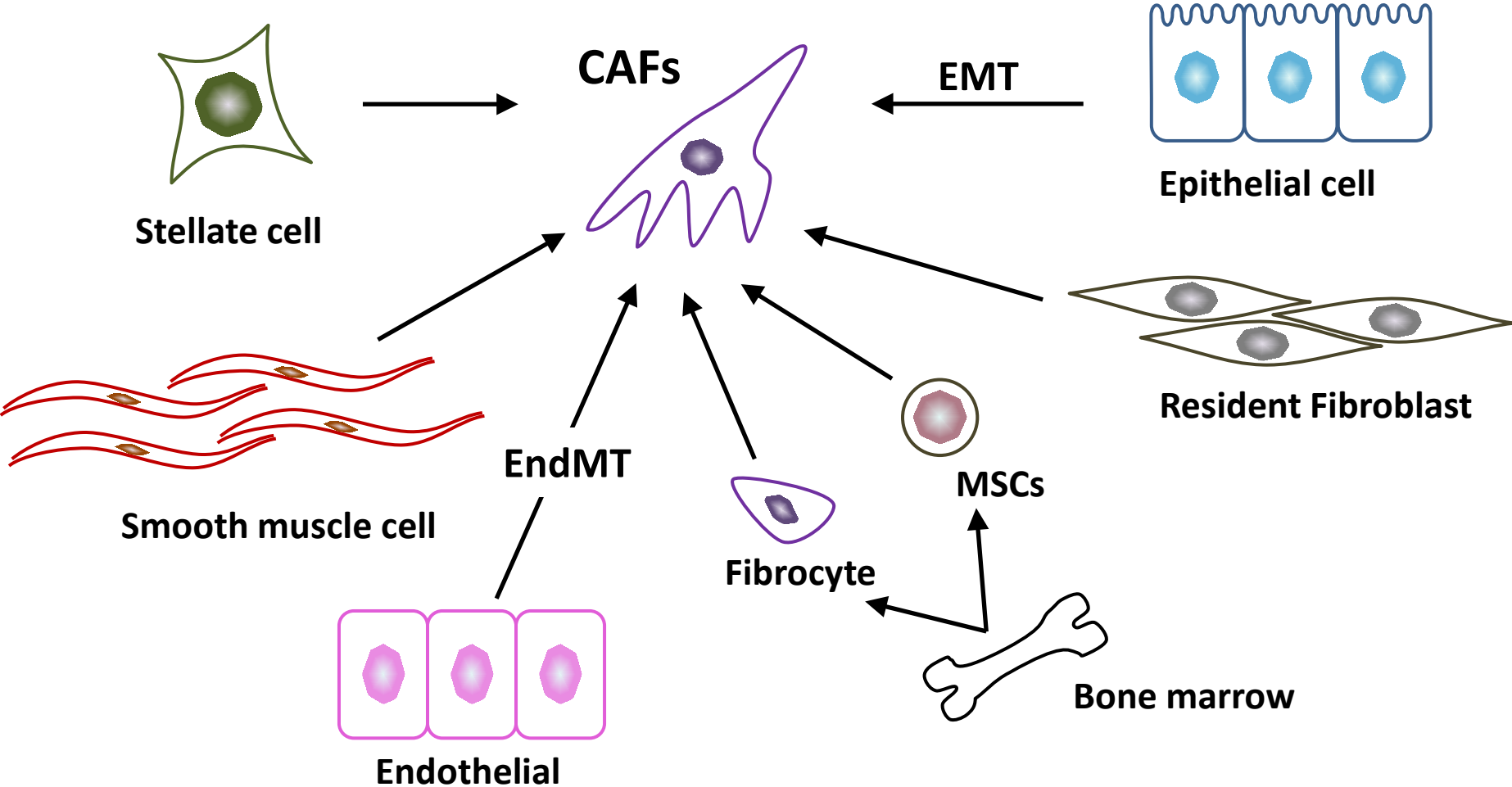


Figure 2. CAFs promote tumor proliferation, and maintain cancer cell stemness and migratory capacity

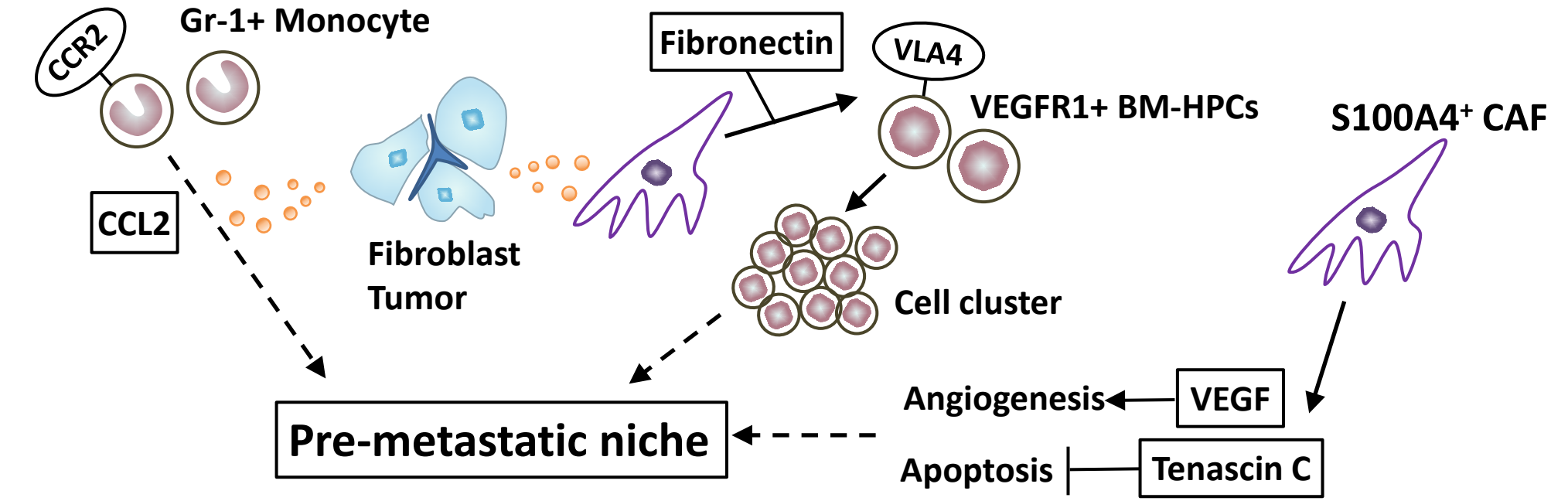
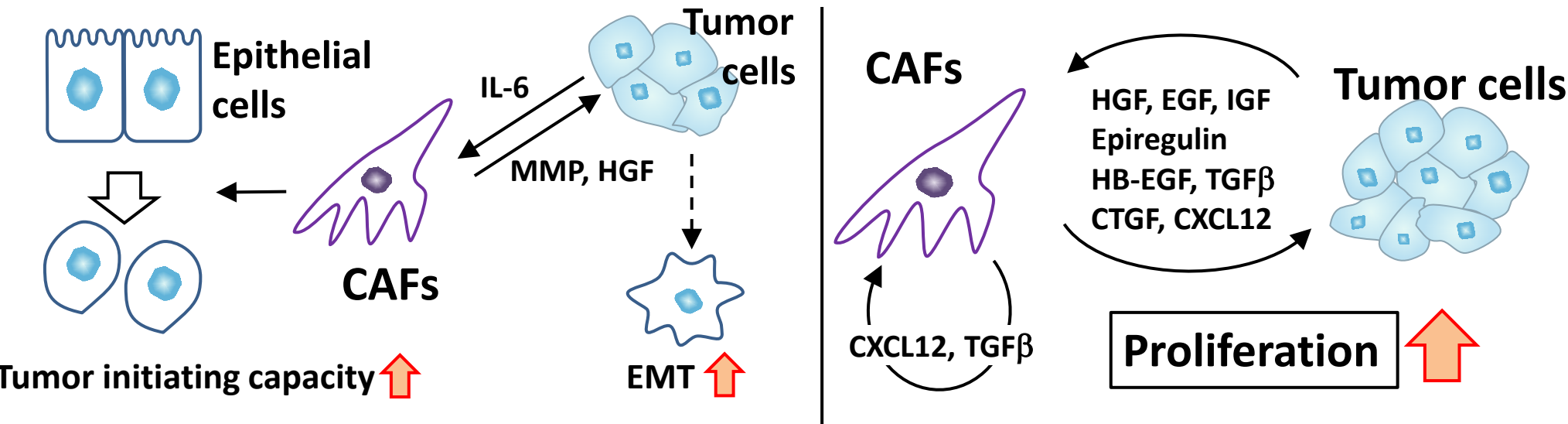


Figure 3. CAFs promote the formation of tumor microenvironments.

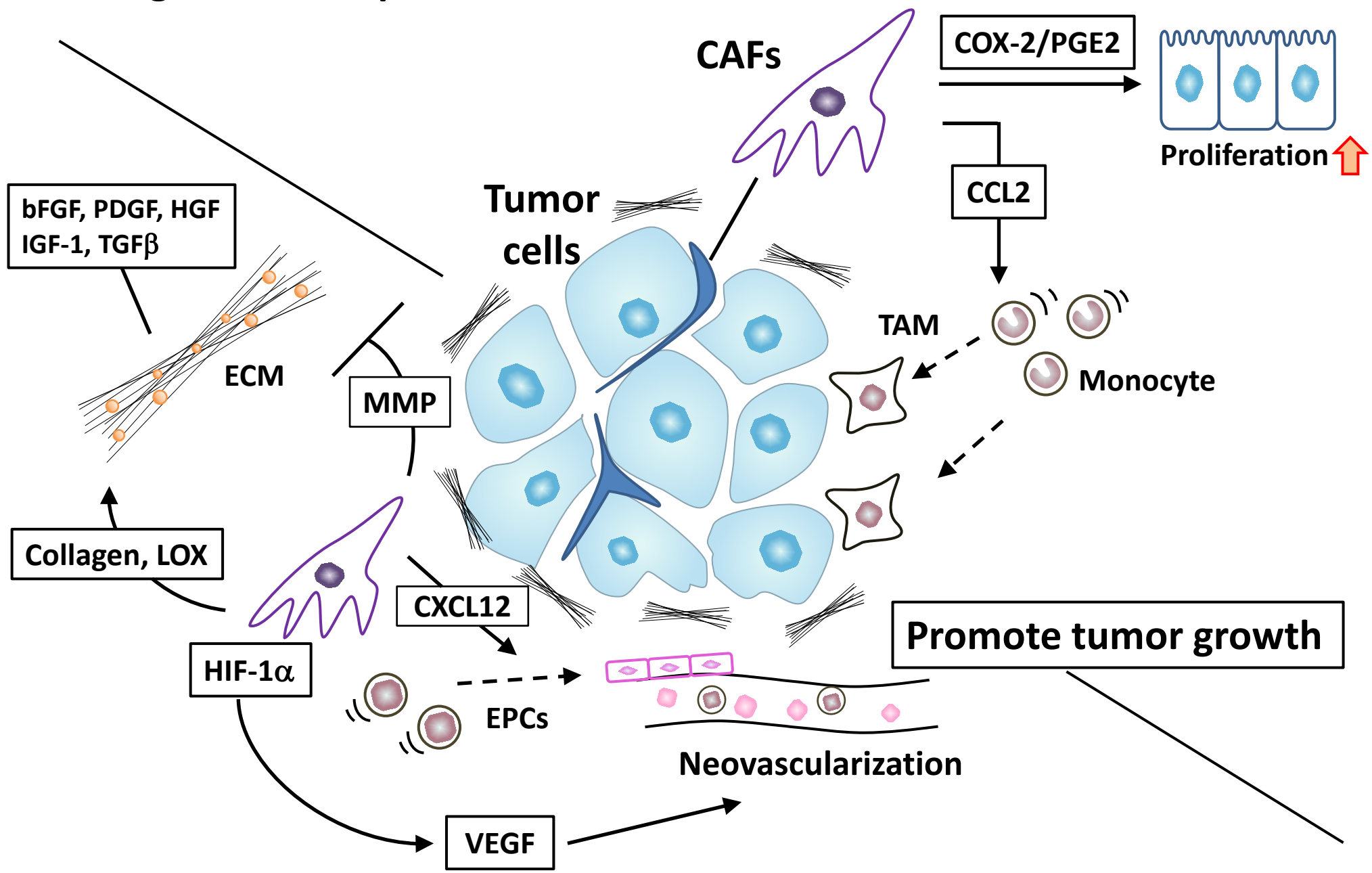
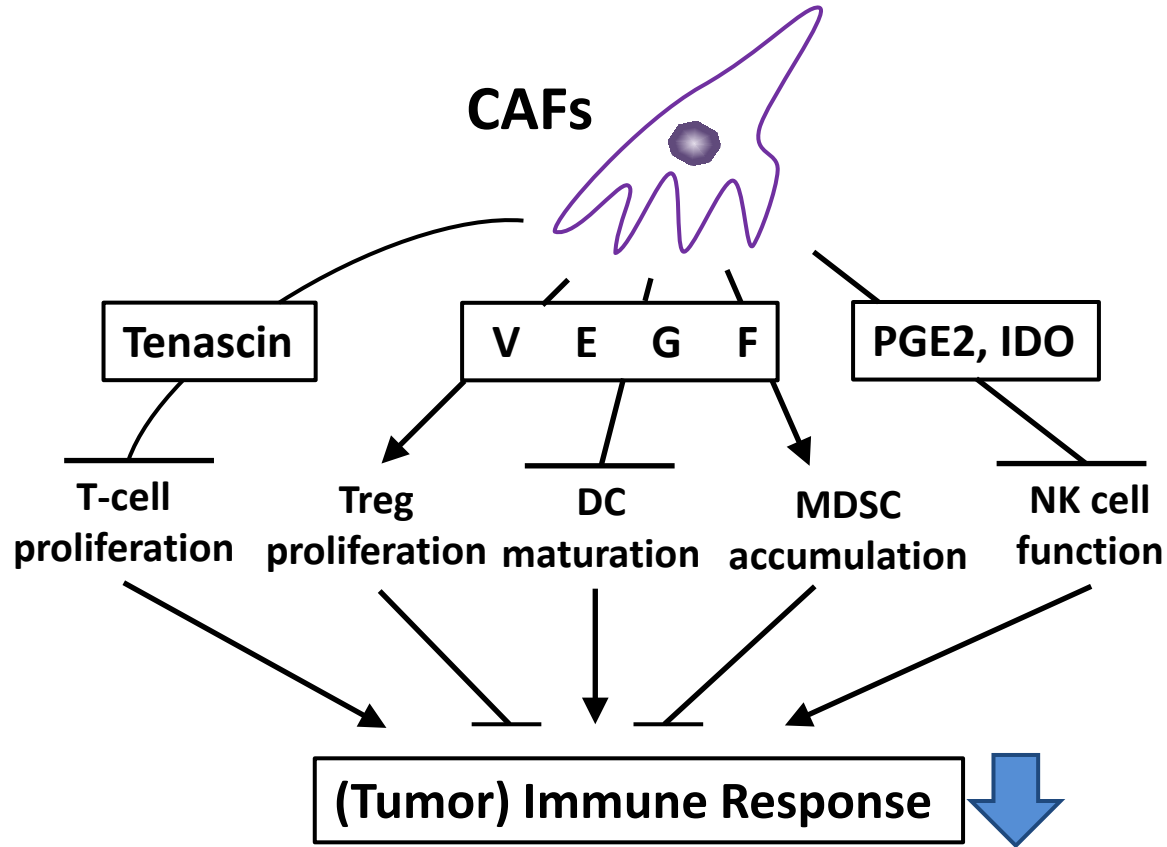


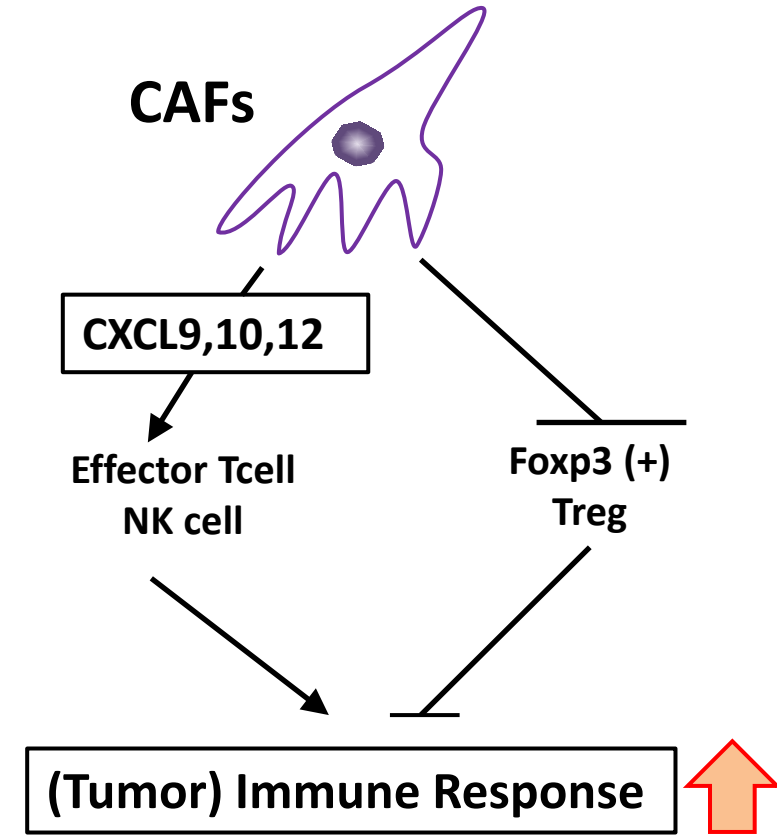
Figure 4. CAFs regulate tumor immunity

Immunosuppressive CAFs



- Hepatocellular carcinoma
- Prostate cancer
- Colorectal cancer

Immunostimulating CAFs



- Pancreatic ductal adenocarcinoma