

# Fibroblast heterogeneity in the cancer wound

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**Fibroblasts regulate the structure and function of healthy tissues, participate transiently in tissue repair after acute inflammation, and assume an aberrant stimulatory role during chronic inflammatory states including cancer. Such cancer-associated fibroblasts (CAFs) modulate the tumor microenvironment and influence the behavior of neoplastic cells in either a tumor-promoting or tumor-inhibiting manner. These pleiotropic functions highlight the inherent plasticity of fibroblasts and may provide new avenues to understand and therapeutically intervene in malignancies. We discuss the emerging themes of CAF biology in the context of tumorigenesis and therapy.**

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Although the concept of “seed and soil” was proposed over 100 years ago (Paget, 1889), investigators have only recently revealed the fundamental importance of the tumor-associated stroma in cancer development. Fibroblasts are essential in tissue homeostasis and wound healing, and consistent with the hypothesis that “a tumor is a wound that never heals” (Dvorak, 1986), an emerging body of evidence demonstrates that the fibroblasts in tumors, often referred to as cancer-associated fibroblasts (CAFs), are key players in the process of tumorigenesis (Hanahan and Weinberg, 2011). Indeed, recent studies have shown that many of the cancer-promoting and therapy-resisting properties of the stroma can be attributed to the activity of fibroblasts. It is necessary to acknowledge that CAFs can derive from multiple origins and make up a heterogeneous population of cells but nevertheless are united by their capacity to alter the tumor microenvironment and to change the fate of neoplastic cells.

To be able to fully understand the role of fibroblasts in cancer, it is important to consider the function of this cell type in normal tissues. Fibroblasts are elongated cells of mesodermal origin, showing a fusiform or spindle-like shape, and express fibroblast-specific protein 1 (FSP-1; Strutz et al., 1995). Beyond FSP-1, they show a complex expression pattern of protein markers, reflecting an inherent diversity within a population of fibroblasts (Anderberg and Pietras, 2009).

Fibroblasts are found embedded within the extracellular matrix (ECM) and are the most abundant cell type in connective tissue. The ECM is composed of fibrillar collagens, fibronectins,

hyaluronic acid, and proteoglycans, providing a structural framework for all tissues. The ECM also acts as a reservoir for cytokines and growth factors, and as a scaffold for cell migration. In fact, fibroblasts are the major producers of the ECM and thereby participate in tissue homeostasis, and the regulation of interstitial fluid volume and pressure. Fibroblasts are also highly involved in regulating tissue remodeling and repair. Upon tissue damage, fibroblasts proliferate and differentiate into myofibroblasts, a process characterized by de novo expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), contractile stress fibers, and splice variants of fibronectin (Serini et al., 1998; Tomasek et al., 2002). The synthesis of ECM and ECM remodeling proteases is up-regulated, resulting in deposition of a reactive stroma, often referred to as a desmoplastic reaction or desmoplastic stroma. The induced expression of  $\alpha$ -SMA alters cytoskeletal organization, which increases the contractile ability of myofibroblasts (Rønnov-Jessen and Petersen, 1996; Hinz et al., 2001). Myofibroblasts contract the ECM to bring the borders of the wound together, and secrete matrix proteins that repair the remaining tissue defects and attract epithelial cells to complete the healing process. Upon completion of wound healing, activated fibroblasts undergo apoptosis (Desmoulière et al., 1995) or a particular type of programmed cell death termed nemosis (programmed necrosis; Bizik et al., 2004).

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### Definition and markers of CAFs

CAFs are found in almost all solid tumors; however, their abundance varies between different types of cancers. For example, breast, prostate, and pancreatic cancers contain high numbers of CAFs, whereas brain, renal, and ovarian cancers demonstrate fewer (Neesse et al., 2011; Smith et al., 2013). They are defined as all the fibroblastic, nonneoplastic, non-vascular, nonepithelial, and noninflammatory cells found in a tumor (Fig. 1). However, there is no consensus on their molecular definition (Kalluri and Zeisberg, 2006; Orimo and Weinberg, 2007; Pietras and Ostman, 2010; Xing et al., 2010). CAFs can be distinguished from neoplastic cells that have undergone epithelial-mesenchymal transition and show a fibroblast-like morphology by their stable karyotype and the lack of genetic alterations. Although p53 mutations in CAFs have been reported (Kurose et al., 2002; Hill et al., 2005; Patocs et al., 2007), these studies have been criticized for using methods highly prone to generating experimental artifacts (Campbell et al., 2009). Moreover, recent studies have confirmed the lack of frequent mutations in CAFs (Qiu et al., 2008; Walter et al., 2008; Hosein et al., 2010).

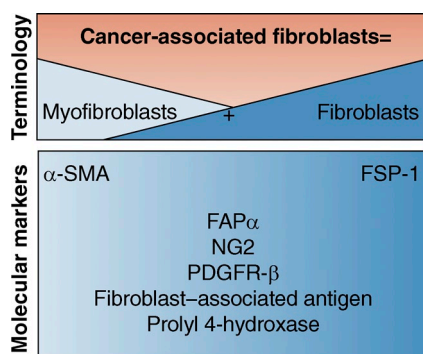
The molecular definition of CAFs is still a debated issue, and emerging data demonstrate that CAFs make up a complex and heterogeneous population of cells. Several markers have been suggested in the past to define CAFs, but it is now being appreciated that these markers do not mark all CAFs and that most of them are not even unique to CAFs or to the fibroblasts lineage.  $\alpha$ -SMA is a robust CAF marker, which usually identifies CAFs with myofibroblast morphology (Desmoulière et al., 2004). Nonetheless,  $\alpha$ -SMA is also expressed by normal fibroblasts (Hawinkels et al., 2014), and in some cases, normal fibroblasts show comparable or even more  $\alpha$ -SMA expression compared with CAFs (Madar et al., 2009; Berdiel-Acer et al., 2014).  $\alpha$ -SMA expression is detected in other cell types as well, such as pericytes and smooth muscle cells surrounding vasculature, visceral smooth muscle cells, and cardiomyocytes (Wendling et al., 2009). The cell-surface serine

protease fibroblast-activation protein  $\alpha$  (FAP $\alpha$ ) is another common marker for CAFs in many carcinomas including breast, lung, ovarian, and pancreatic (Garin-Chesa et al., 1990). However, FAP $\alpha$  is also not specific to CAFs, as it is highly expressed in quiescent mesodermal cells in multiple tissue types (Roberts et al., 2013), and activated fibroblasts during wound healing and collagen vascular disease (Wang et al., 2005). Another well-studied marker is FSP-1 (also known as S100A4), which seems to play a distinctive and somewhat dual role in cancer, as evident by a protective role of FSP-1-positive cells against carcinogen-mediated malignancy (Zhang et al., 2013a). Yet FSP-1 is also not restricted to CAFs and is also expressed by epithelial cells undergoing EMT (Okada et al., 1997) and bone marrow-derived cells, including macrophages, that respond to tissue injury (Österreicher et al., 2011; Cheng et al., 2012). Additional proteins expressed in some CAFs include NG2 (Neuronal Antigen-2, also known as Chondroitin sulfate proteoglycan), platelet-derived growth factor receptor- $\beta$  (PDGFR- $\beta$ ), fibroblast-associated antigen, and Prolyl 4-hydroxylase (Orimo and Weinberg, 2007). However, these markers are also not unique to CAFs. For example, NG2 is a neural marker that is expressed in the nervous system (Polito and Reynolds, 2005), and PDGFR- $\beta$  expression is also common to vascular smooth muscles, skeletal muscles, and myocardium (Cuttler et al., 2011). PDGFR- $\beta$  was recently reported to be expressed by invasive pancreatic cancer cells (Weissmueller et al., 2014) and thus may also represent a new EMT marker.

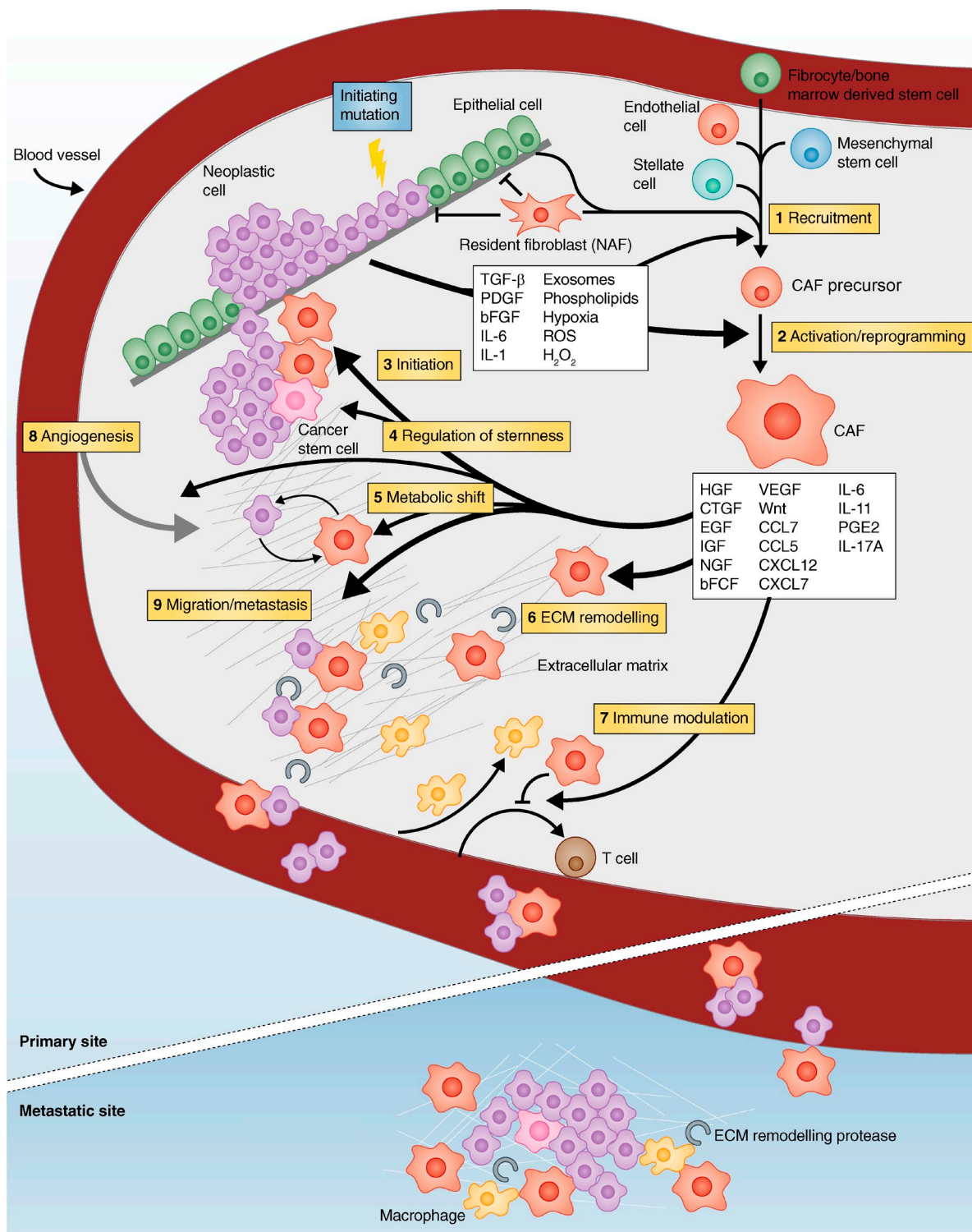
It is therefore important to keep in mind that there is currently no marker that completely and exclusively defines CAFs, and this should be taken into account when interpreting results from different models. Moreover, apart from FSP-1, which identifies a unique CAF population, these proteins are often expressed or coexpressed heterogeneously among CAFs, possibly reflecting different stages of activation and sources of CAFs (Fig. 1; Sugimoto et al., 2006).

### The origin of CAFs

Several tissues contribute to the population of CAFs found in an individual tumor (Fig. 2; Xing et al., 2010; Cirri and Chiarugi, 2011). Indeed, numerous cell types have been reported to transdifferentiate to CAFs. The most direct source of CAFs derives from resident tissue fibroblasts and mesenchymal stem cells (MSCs; Rønnov-Jessen and Petersen, 1993; Kojima et al., 2010; Paunescu et al., 2011; Quante et al., 2011; Vicent et al., 2012). The stellate cell is a distinct cell type that shares many of the same functions as fibroblasts. In its quiescent state it is a vitamin A-storing and lipid droplet-containing cell that can be found in the liver, pancreas, kidney, intestine, lung, spleen, uterus, and skin (Kordes et al., 2009). Similar to fibroblasts, stellate cells activate  $\alpha$ -SMA expression upon stimulation and acquire a myofibroblast-like phenotype. Stellate cells are responsible for the majority of the desmoplastic reaction seen in chronic pancreatitis and pancreatic cancer (Bachem et al., 1998), as



**Figure 1. Molecular definition of cancer-associated fibroblasts.** CAFs are composed of two morphologically distinctive populations: fibroblasts and myofibroblasts. Indicated are common molecular markers that define CAFs.



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**Figure 2. CAFs promote tumorigenesis: a schematic illustration representing all the fronts in which CAFs boost cancer.** By secreting soluble factors and lipid-based particles, transformed epithelium drives recruitment (1) and reprogramming (2) of several types of cells into CAFs, including resident fibroblasts (NAFs) that are naturally tumor-suppressive. CAFs are then activated to promote tumor initiation (3), cancer cell stemness (4), and to change tumor metabolism (5) by intensive cross talk of ligands/receptors, cell-cell contact, and remodeling the ECM (6). Concomitantly, CAFs prevent immune surveillance of tumor cells (7) while balancing inflammation and angiogenesis (8). Finally, CAFs stimulate invasion and metastasis by facilitating tumor cell dissemination, intra- and extravasation, and metastatic colonization (9).

well as in liver fibrosis (Yin et al., 2013), and are categorized as CAFs when seen in cancer tissue. In addition to these local sources of CAFs, bone marrow–derived cells termed fibrocytes can be recruited to the tumor and differentiate into myofibroblasts and fibroblasts (Ishii et al., 2003; Direkze et al., 2004; Mishra et al., 2008; Kidd et al., 2012).  $\alpha$ -SMA–expressing myofibroblasts have also been shown to originate from neighboring adipose tissue (Kidd et al., 2012). Endothelial–mesenchymal transition (EndMT), a process where endothelial cells down-regulate their CD31 expression and induce expression of FSP-1, is another potential source of CAFs (Zeisberg et al., 2007). FSP-1–positive fibroblasts can also be derived from local epithelial–mesenchymal transition (EMT), indicating that nontransformed epithelial cells may be an additional source of CAFs by undergoing EMT in response to stimuli from surrounding cells (Iwano et al., 2002). Therefore, CAFs may be derived from multiple tissue types, reflecting local and distant cues that are sensed during tumorigenesis, and thus cannot be referred to as one population of cells. The relative contribution of each source of CAFs is highly dependent on the experimental conditions used and less certain in actual human cancers.

#### Neoplastic cells recruit and activate CAFs

Early work of Stoker et al. (1966) demonstrated that normal quiescent fibroblasts inhibit the growth of polyoma-transformed cells by direct contact between the two cell types. It has later been shown that normal tissue-associated fibroblasts (NAFs) isolated from different organs can inhibit the growth of neoplastic cells (Flaberg et al., 2011), presumably due to the ability of fibroblasts to maintain epithelial homeostasis and proliferative quiescence (Trimboli et al., 2009). Indeed, this has been proposed as one reason why certain tumors found in autopsy studies appear dormant and nonprogressing (Bissell and Hines, 2011). Given these properties of NAFs, neoplastic cells must evolve to both recruit fibroblasts (from the different potential sources mentioned above) and to reprogram them into CAFs, thereby converting the fibroblast from being tumor suppressive to tumor supportive. Interestingly, once reprogrammed, the CAF phenotype can persist in cell culture even in the absence of continued exposure to intratumoral stimuli (Orimo et al., 2005). Several studies have shown that NAFs are functionally distinct from CAFs. Prostate CAFs are more proliferative and less prone to contact inhibition than their NAF counterparts (Madar et al., 2009). Furthermore, colonic CAFs more potently induce the proliferation and migration of neoplastic cells compared with NAFs (Berdial-Acer et al., 2014).

Oncogenic signaling within neoplastic cells frequently drives the recruitment and activation of fibroblasts by secreting fibroblast-activating factors (Yang et al., 2006), thereby mimicking the normal wound healing process where these factors are normally secreted from injured epithelial cells and macrophages (Fig. 2). TGF- $\beta$  is a major regulator of fibrosis and is one of the most important and well-studied cancer cell–derived factors affecting CAF activation (Lieubeau et al., 1994;

Löhr et al., 2001). TGF- $\beta$  binds to the type-2 TGF- $\beta$  receptor (TGFBR2) on the cell surface of fibroblasts, inducing recruitment and phosphorylation of TGFBR1 and expression of factors involved in paracrine signaling and ECM production and remodeling (Pickup et al., 2013a). Platelet-derived growth factor (PDGF) is also highly expressed in most neoplastic cells, which rarely express PDGF receptors (PDGFRs) themselves. Instead, other cell types, such as CAFs and endothelial cells, express PDGFR (Bronzert et al., 1987; Forsberg et al., 1993), and PDGF has been shown to stimulate proliferation of CAFs and to be an important initiator of the desmoplastic reaction in tumors (Shao et al., 2000). Basic fibroblast growth factor (bFGF) is another mitogen secreted by neoplastic cells that stimulates the proliferation and differentiation of fibroblasts to CAFs (Armelin, 1973; Strutz et al., 2000; Kwabi-Addo et al., 2004). Cytokines produced by neoplastic cells, including IL-6, also stimulate the production of CAFs (Giannoni et al., 2010). In addition, phospholipid derivatives, such as lysophosphatidic acid (LPA), can be secreted from neoplastic cells to stimulate differentiation of human MSCs into myofibroblast-like CAFs (Jeon et al., 2008).

Besides growth factors and cytokines, other mechanisms of CAF activation have been reported. For example, exosomes can transfer protein, RNA, and microRNA (miR) from one cell to another, and cancer-derived exosomes have been shown to contribute to the recruitment and activation of fibroblasts and MSCs within tumors (Webber et al., 2010; Cho et al., 2012; Gu et al., 2012; Kahlert and Kalluri, 2013). In addition, hypoxia and reactive oxygen species (ROS) promote activation of CAFs. ROS have been shown to support conversion of fibroblasts into myofibroblasts through the accumulation of the hypoxia-inducible factor (HIF)-1 $\alpha$ , whereas antioxidants have been found to reduce HIF-1 $\alpha$  levels, inhibiting numerous myofibroblast features (Toullec et al., 2010).

#### CAF modulates the tumor microenvironment

Upon reprogramming, many transcriptional changes occur that distinguish CAFs from their normal counterparts (Allinen et al., 2004; Nakagawa et al., 2004; Hu et al., 2005). Reprogramming cells into CAFs also involves changes in miR expression, affecting target genes posttranscriptionally (Aprelikova et al., 2010, 2013; Musumeci et al., 2011; Bronisz et al., 2012; Mitra et al., 2012; Masamune et al., 2014). Furthermore, epigenetic changes, such as global DNA hypomethylation frequently seen in neoplastic cells, have also been observed in CAFs (Jiang et al., 2008). Together, all these gene regulatory changes induce a shift in the metabolome and secretome, which maintain the differentiated state of myofibroblasts in an autocrine manner (Kojima et al., 2010) but also stimulate neoplastic cells in a paracrine manner (Fig. 2). In the following sections, we will discuss principle strategies used by activated CAFs to communicate these protumorigenic effects.

**CAF secretes soluble factors.** CAFs orchestrate key pathophysiological processes in cancer development through paracrine interactions involving the secretion of multiple soluble

factors. Growth factors such as hepatocyte growth factor (HGF), connective tissue growth factor (CTGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), nerve growth factor (NGF), basic fibroblast growth factor (bFGF), and members of the Wnt family (Orimo et al., 2001, 2005; Allinen et al., 2004; Bhowmick et al., 2004; Kalluri and Zeisberg, 2006; Erez et al., 2010; Räsänen and Vaheri, 2010; Chaffer and Weinberg, 2011; Cirri and Chiarugi, 2011), as well as many cytokines, such as CCL7 and CXCL12 (Orimo et al., 2005; Jung et al., 2010), have all been reported to be involved in this paracrine cross talk. The CAF secretome also regulates angiogenesis. Vascular endothelial growth factor A (VEGF-A) is induced in CAFs by neoplastic cells (Guo et al., 2008), as well as CXCL12, CXCL14, and CTGF (Orimo et al., 2005; Yang et al., 2005; Augsten et al., 2009). The three most studied CAF-secreted factors will hereafter be discussed in detail.

CXCL12 is highly overexpressed in CAFs (Allinen et al., 2004) and several *in vitro* and *in vivo* models have revealed a key role of CXCL12 and its receptor CXCR4 in tumor–stroma interactions. CXCL12 provides stimulatory input to CXCR4-expressing neoplastic cells, resulting in proliferation and migration (Domanska et al., 2012). In pancreatic cancer cells, CXCL12/CXCR4 signaling triggers invasion and EMT through noncanonical Hedgehog pathway (Li et al., 2012c) and further contributes to cancer progression by activation of the canonical Wnt pathway (Wang et al., 2008). CTGF has a key role in fibrosis (Gao and Brigstock, 2005) and is also highly up-regulated in numerous cancers (Jacobson and Cunningham, 2012), including pancreatic ductal adenocarcinoma (Charrier and Brigstock, 2013). CTGF expression is induced by TGF- $\beta$  (Pickup et al., 2013a) and hypoxia (Eguchi et al., 2013) in both activated pancreatic stellate cells and neoplastic cells (Hartel et al., 2004) and causes increased invasiveness of neoplastic cells, which can be inhibited through CTGF depletion (Eguchi et al., 2013). In head and neck squamous cancer cells, CTGF enhanced stem-like properties and increased expression of multiple pluripotency genes (Chang et al., 2013). HGF was originally identified as a mitogen for hepatocytes but also plays an important role in wound healing and tissue repair. Normally, HGF is sequestered in the ECM in a latent form. Proteolysis of the latent form of HGF enables binding to and signaling by the c-Met tyrosine kinase (Benvenuti and Comoglio, 2007). The c-Met signaling pathway is often affected in cancer, either by mutations in neoplastic cells leading to constant oncogenic c-Met signaling (Peruzzi and Bottaro, 2006) or by overexpression of HGF in CAFs, leading to increased proliferation of neoplastic cells. Furthermore, in a three-dimensional model of ductal carcinoma *in situ*, co-cultures with HGF-secreting fibroblasts were found to increase the invasive outgrowth by enhancing the ability of these preneoplastic cells to degrade the ECM (Jedezsko et al., 2009).

**CAFs modify tumor metabolism.** Metabolic alterations are not only restricted to cancer cells; CAFs also show important metabolic changes that promote tumorigenesis. Fibroblasts are metabolically active already in their quiescent state

(Lemons et al., 2010), but when transformed into CAFs they change their metabolic profile and show increased autophagy (Chaudhri et al., 2013). As an example, phosphoglycerate kinase-1 (PGK1), a part of the glycolytic pathway, is highly up-regulated in CAFs compared with normal fibroblasts. Upon overexpression of PGK1, normal fibroblasts gain a myofibroblastic phenotype, which promotes tumor cell growth when coimplanted with neoplastic cells *in vivo* (Wang et al., 2010).

Interestingly, CAFs have been reported to use aerobic glycolysis as a source of energy. In some tumors, CAFs express glycolytic enzymes related to the Warburg effect, such as the M2 isoform of pyruvate kinase and lactate dehydrogenase, whereas adjacent neoplastic cells do not (Pavlidis et al., 2009). A switch to aerobic glycolysis in CAFs is hypothesized to generate lactate and ketones, which are secreted into the intracellular space and act as paracrine onco-metabolites that fuel oxidative mitochondrial metabolism in neoplastic cells. This phenomenon is referred to as the “reverse Warburg effect” (Pavlidis et al., 2009). To further support the significance of this phenomenon, high stromal MTC4 expression (a functional marker of hypoxia, oxidative stress, aerobic glycolysis, and lactate efflux) was shown to be correlated with decreased overall survival in triple-negative breast cancer, whereas expression of MTC4 in neoplastic cells had no prognostic value (Witkiewicz et al., 2012). In *ex vivo* co-culture systems containing breast cancer cells and fibroblasts, glucose uptake was increased and mitochondria activity was decreased in fibroblasts and the opposite effect was observed in neoplastic cells (Martinez-Outschoorn et al., 2011). Mechanistically, neoplastic cells have been shown to secrete H<sub>2</sub>O<sub>2</sub>, which initiates an oxidative stress in the tumor stroma, switching CAFs to aerobic glycolysis (Martinez-Outschoorn et al., 2011). Furthermore, the TGF- $\beta$  pathway in CAFs induces a metabolic reprogramming that promotes mitochondrial activity in adjacent neoplastic cells (Guido et al., 2012). In contrast, other tumors are characterized by the conventional Warburg phenotype in which the neoplastic cells are driven by anaerobic metabolism, and CAFs have been reported to express lactate transporters (MCT1/MCT2) while reducing the expression of glucose transporters (GLUT1; Koukourakis et al., 2006), suggesting a role of CAFs in buffering and recycling the products derived from anaerobic metabolism in neoplastic cell.

There is a great diversity in metabolic phenotype among different tumors. In an attempt to map the metabolic heterogeneity in breast cancer, tumors were classified as having Warburg type (glycolysis in neoplastic cells, nonglycolysis in stroma), reverse Warburg type (nonglycolysis in neoplastic cells, glycolysis in stroma), mixed type (glycolysis in both stroma and neoplastic cells), and null type (nonglycolysis in both stroma and neoplastic cells; Choi et al., 2013). The results indicate a correlation between different metabolic phenotypes and different tumor subtypes (Luminal A, B, triple-negative, etc.).

Collectively, CAFs participate in a dynamic metabolic interplay with the neoplastic cells and can adapt to the metabolic needs of the tumor. This is important to keep in mind when

targeting metabolic pathways, and it also indicates that PET imaging may be specifically detecting glucose uptake in the tumor stroma rather than in the neoplastic cells in certain tumors.

**CAFs remodel the ECM.** One characteristic feature of activated CAFs is their ability to synthesize and remodel the ECM in the desmoplastic stroma. Severe desmoplasia is correlated with poor prognosis in lung, pancreas, breast, and colorectal tumors (Cardone et al., 1997; Maeshima et al., 2002; Tsujino et al., 2007; Erkan et al., 2008; Kawase et al., 2008). Changes in the composition and cross-linking of the ECM influence the stiffness of the tissue, which has a pivotal role in tumorigenesis (Erler and Weaver, 2009; Cukierman and Bassi, 2010). By regulating the remodeling of the ECM, CAFs drive the recruitment of other cells into the tumor, promote migration, and facilitate a metastatic phenotype of neoplastic cells.

Hyaluronic acid is a major component of the ECM and CAFs contribute to its production. Apart from serving as a structural component, hyaluronic acid possesses biological functions, such as macrophage recruitment (Kobayashi et al., 2010). Furthermore, CAFs express Lysyl oxidase (LOX), an enzyme responsible for cross-linking collagen I (Pickup et al., 2013b). In breast cancer, tumorigenesis is accompanied by changes in collagen cross-linking, and reduced LOX-mediated collagen cross-linking diminished tumor tissue stiffness and decreased tumor incidence in a xenograft mouse model (Levental et al., 2009).

In contrast, CAFs also regulate the degradation of the ECM. By expressing members of the matrix metalloproteinase (MMP) family, CAFs can facilitate tumor growth, invasion, and metastasis (Poulsom et al., 1992; Stetler-Stevenson et al., 1993; Sternlicht et al., 1999; Vosseler et al., 2009; Zigrino et al., 2009). MMP-mediated ECM remodeling is essential for cancer angiogenesis, and MMP13 expressed from CAFs plays a key role in promoting angiogenesis by releasing VEGF that is bound and sequestered in the ECM (Lederle et al., 2010). In addition, MMPs stimulate protease-activated receptors (PARs) on the surface of neoplastic cells, thereby promoting growth and invasion (Boire et al., 2005). Other proteases are also involved in ECM degradation. For instance, urokinase-type plasminogen activator (uPA) activates the serine protease Plasmin and has thereby been shown to increase proliferation, migration, and invasion (Danø et al., 2005; Noskova et al., 2009). Finally, the CAF marker FAP $\alpha$  is a membrane-bound glycoprotein with both collagenase and dipeptidyl peptidase (DPP) activities that are important for degrading the ECM (Scanlan et al., 1994) and stimulating tumor growth (Cheng et al., 2005).

### CAFs trigger tumor initiation and progression

Many studies have highlighted the importance of CAFs in initiation and progression of cancer mediated by the ability of the CAFs to alter important functions in neoplastic cells, such as cell cycle regulation, migration, and death (Shimoda et al., 2010). Interestingly, neoplastic cells of different origins differ in their responses upon stimulation from CAFs (Kadaba et al., 2013), illustrating a unique relationship with CAFs across tumor types.

By using *in vitro* co-culture and *in vivo* transplantation experiments, Olumi et al. (1999) showed that human prostatic CAFs induced the proliferation and the ability to initiate tumors from immortalized nontumorigenic human prostatic epithelial cells. This effect was not manifested by normal fibroblasts and CAFs did not affect growth of normal human prostatic epithelial cells under identical conditions (Olumi et al., 1999). In a similar study, senescent human fibroblasts were shown to stimulate premalignant and malignant, but not normal, epithelial cells to proliferate in culture and develop tumors when engrafted in mice (Krtolica et al., 2001). This elegantly demonstrates how fibroblasts can initiate tumor formation in premalignant cells that have not acquired sufficient genetic alterations for complete transformation. Interestingly, when regrafted into secondary mice in the absence of CAFs, the epithelial cells sustain their tumorigenic potential, showing that the CAF-mediated transformation of epithelial cells is long-lasting (Hayward et al., 2001).

Secreted factors from CAFs are believed to be responsible for this tumor-initiating potential. Although TGF- $\beta$  signaling helps maintain the CAF phenotype, ablating TGFBR in fibroblasts resulted in spontaneous neoplasia in the prostate, illustrating a tumor-suppressive function of TGF- $\beta$  signaling in early tumor development (Bhowmick et al., 2004). Notably, HGF can compensate for TGF- $\beta$  loss, making HGF a candidate for mediating the tumor-initiating effect of CAFs. Indeed, overexpression of HGF in fibroblasts induced hyperproliferation of normal epithelium in a xenograft model (Kuperwasser et al., 2004), which further supports an initiating role of HGF. In addition to tumor initiation, CAFs also participate in tumor maintenance and progression. For example, co-implantation of breast cancer cells with CAFs was shown to increase xenograft tumor growth (Orimo et al., 2005).

Further evidence for the role of CAFs in tumor progression comes from studies in which activated CAFs were inactivated or in which key proteins were reduced in CAFs through genetic ablation. Activated stellate cells in pancreatic cancer could be driven back into a quiescent state by retinoic acid, resulting in reduced paracrine cross talk, such as Wnt/ $\beta$ -catenin signaling. In a three-dimensional co-culture system, these quiescent stellate cells slowed tumor progression by reducing neoplastic cell proliferation and migration (Froeling et al., 2011). Mice deficient for the CAF marker FSP-1 also showed reduced engraftment of neoplastic cells and delayed tumor growth (Grum-Schwensen et al., 2005).

### CAFs regulate cancer stemness

There is accumulating evidence for the acquisition of stem-like plasticity in a subset of the cancer cells, usually referred to as cancer stem cells (CSCs). Inflammatory mediators and cytokines are the most common cues reported to induce stemness of neoplastic cells. Many factors secreted by CAFs, such as IL-6, IL-17A, CXCL7, PGE2, and HGF, were shown to trigger the Wnt- $\beta$ -catenin pathway in neoplastic cells and augment the CSC population (Giannoni et al., 2010, 2011; Vermeulen et al.,

2010; Liu et al., 2011; Li et al., 2012a; Malanchi et al., 2012; Lotti et al., 2013). CD44, a prominent CSC marker and receptor for hyaluronic acid, is up-regulated in breast, prostate, and colorectal cancers by different cytokines produced by CAFs and contributes to maintaining CSC self-renewal features and general stemness capacity (Giannoni et al., 2010; Liu et al., 2011; Lotti et al., 2013). Interestingly, CD44 is also expressed on CAFs, where it is indispensable for the existence of the neighboring CSC population. In response to hypoxia or reduced nutrient conditions, CAFs elevate CD44 and maintain stem cell properties of neoplastic cells (Kinugasa et al., 2014).

CAF also induce expression of other CSC markers, such as ALDH1 and Nestin, in many tumor types (Liu et al., 2011; Hamada et al., 2012; Matsuda et al., 2012). Expression of these stem cell markers and the elevation of the CSC population are correlated with aggressive tumors, which exhibit enhanced invasiveness and metastatic capacities (Giannoni et al., 2011; Li et al., 2012a; Malanchi et al., 2012). Together, these data show that CAFs maintain stemness in neoplastic cells to support further tumor progression.

### CAFs modulate the immune response

An inflammatory environment is known to promote error-prone, high-rate proliferation, thereby facilitating tumorigenesis (Grivennikov et al., 2010). Indeed, chronic inflammation is a prominent risk factor for many cancers, including hepatocellular carcinoma, gastric cancer, and pancreatic ductal adenocarcinoma (Pikarsky et al., 2004; Peek and Crabtree, 2006; Guerra et al., 2007). CAFs mediate tumor-enhancing inflammation by expressing a proinflammatory gene signature, which creates a microenvironment that attracts myeloid cells and supports tumor growth and angiogenesis (Erez et al., 2010; Torres et al., 2013). This signature, identified early in dysplastic lesions and maintained throughout tumorigenesis, is mediated via NF- $\kappa$ B-dependent signaling and initiated by IL-1 $\beta$  in skin cancer. Most importantly, normal fibroblasts can be induced by carcinoma cells to turn on this proinflammatory gene signature (Erez et al., 2010). A similar NF- $\kappa$ B-dependent inflammatory signature was activated by CAFs to support prostate cancer stemness and invasion (Giannoni et al., 2011).

Cyclooxygenase 2 (COX2), an NF- $\kappa$ B target involved in mediating inflammation and a part of the proinflammatory gene signature, is expressed by CAFs and neoplastic cells (Nakagawa et al., 2004; Erez et al., 2010), and has been shown to function as a mediator of tumor progression. Indeed, the implantation of lung carcinoma cells in COX2-deficient mice or treatment with COX2 inhibitors reduced tumor growth and vascular density (Williams et al., 2000). In a xenograft model of ductal carcinoma in situ, fibroblasts were shown to induce the expression of COX2 in neoplastic cells, affecting tumor growth and progression. As a consequence of increased COX2, MMP14 and VEGF expression were increased, causing a more invasive phenotype. Administration of a selective COX2 inhibitor, Celecoxib, impeded this invasion (Hu et al., 2009). Likewise, *in vitro* coculturing of pancreatic cancer cells with

CAFs increased expression of COX2, and a selective COX2 inhibitor abrogated migration of these cells (Sato et al., 2004).

Genetically altered neoplastic cells often generate neoantigens that can potentially be recognized and destroyed by the immune system. However, most tumors evade immune surveillance despite the abundance of an inflammatory milieu. Accumulating evidence shows that the tumor stroma and CAFs actively participate in modulating the immune response to help neoplastic cells escape detection, thereby supporting tumor progression (Singh et al., 1992; Liao et al., 2009; Iijima et al., 2011). CAFs have been shown to protect pancreatic tumors and lung carcinoma cells from the cytotoxic effect of T cells via different mechanisms. FAP $\alpha$ -positive CAFs were shown to produce CXCL12, which mediated the exclusion of T cells from neoplastic cells (Feig et al., 2013). CAFs also opposed the TNF- and IFN- $\gamma$ -mediated necrosis of neoplastic cells by T cells (Kraman et al., 2010). Depletion of FAP $\alpha$ -positive cells restored immunological detection and destruction of the tumor, indicating that FAP $\alpha$ -positive cells are immune-suppressive cells in the tumor microenvironment (Kraman et al., 2010; Feig et al., 2013). Additionally, pancreatic stellate cells induce the differentiation of peripheral blood mononuclear cells into immune-suppressive myeloid-derived suppressor cells, contributing to T cell inhibition (Mace et al., 2013). Finally, CAFs derived from hepatocellular carcinoma impair NK cell cytotoxic activity by reducing cytotoxic receptors on their cell surface and interfering with their cytokine production (Li et al., 2012b). This was mediated through the secretion of prostaglandin E2 (PGE2) from CAFs, which also interfered with NK cell functions in melanoma (Balsamo et al., 2009). In summary, CAFs possess the ability to manipulate the immune system, both by excluding and opposing T and NK cell functions and by maintaining an aberrant inflammatory pro-tumorigenic environment.

### CAFs promote cancer cell migration and metastasis

Neoplastic cell metastasis leads to the demise of most patients, and CAFs participate in this process. Neoplastic cells undergo epigenetic and genetic changes resulting in a metastatic phenotype, and CAFs coevolve with the neoplastic cells during tumor progression by acquiring a proinflammatory gene expression pattern (Saadi et al., 2010). Furthermore, gene signatures characterizing active CAFs are often correlated with poor clinical outcomes (Herrera et al., 2013).

The close proximity between neoplastic cells and CAFs yields an effective cross talk, which ultimately supports invasion and metastasis. TGF- $\beta$  response in CAFs led to secretion of IL-11, which in turn can induce GP130/STAT3 signaling in neoplastic cells to support tumor initiation and accelerate metastasis (Calon et al., 2012). Breast cancer cells also create a paracrine loop by reprogramming their surrounding MSCs into CAFs that secrete CCL5. The cancer cells then responded to CCL5 through the CCR5 receptor, resulting in augmented metastatic capacity (Karnoub et al., 2007). The noncanonical Wnt-planar cell polarity (PCP) pathway also promotes the dissemination of breast cancer to distant sites in the body. The

PCP pathway controls convergent–extension movements during embryogenesis and regulates cell motility by changing cell morphology and inducing protrusions. Interestingly, exosomes secreted from CAFs triggered Wnt-11 autocrine induction of the PCP pathway in neoplastic cells, enhancing breast cancer metastatic potential (Luga et al., 2012).

Another important consequence of CAF-mediated signaling on neoplastic cells is EMT, which is considered a critical process in the extravasation of tumors (Guarino et al., 2007). For example, pancreatic stellate cells in pancreatic tumors were shown to trigger EMT in co-cultured neoplastic cells, correlating with increased metastasis (Kikuta et al., 2010). Notch and COX2/NF- $\kappa$ B signaling have also been shown to be involved in acquiring and maintaining the EMT phenotype (Wang et al., 2009b; Li et al., 2012a). Indeed, CAF-secreted MMPs triggered the COX2–NF- $\kappa$ B cascade in prostate cancer cells to promote ROS accumulation and prostate cancer invasion (Giannoni et al., 2011).

Interestingly, CAFs can promote metastasis in the absence of EMT. CAFs use Rho-dependent signaling pathways to create force-dependent movements by which they migrate and form tracks in the matrix for the neighboring neoplastic cells to follow (Gaggioli et al., 2007; Goetz et al., 2011; Sanz-Moreno et al., 2011). Rho-dependent cytoskeletal remodeling in CAFs was shown to be activated by Palladin, Cav-1, or JAK signaling cascades, and led to Actomyosin contractility and enhanced invasion (Hooper et al., 2010; Goetz et al., 2011; Sanz-Moreno et al., 2011; Brentnall et al., 2012). Mesenchymal-derived Cav-1 also conferred microenvironment remodeling to favor the migration of neoplastic cells. CAFs further facilitated intra- and extravasation and were shown to accompany neoplastic cells to their metastatic site (Duda et al., 2010; Xu et al., 2010). Clusters of cells composed of neoplastic cells and fibroblasts have been shown to be more viable than single neoplastic cells in the blood stream and have a survival advantage when seeding in metastatic sites (Duda et al., 2010). By these means, neoplastic cells can sustain their epithelial features while relying on CAFs for invasion.

CAFs have also been reported to increase the rate of successful colonization by selecting for neoplastic clones bearing a specific metastatic advantage while still residing in the primary lesion. This effect is mediated through the secretion of CXCL12 and IGF (Zhang et al., 2013b). Successful colonization has also been shown to be dependent on proangiogenic factors secreted from FSP-1-positive cells (O’Connell et al., 2011).

### CAFs alter therapeutic responses

The protumorigenic effects of CAFs can also promote therapeutic resistance. Evidence for the clinical relevance of CAFs came from a study by Farmer et al. (2009), who found that breast cancer patients responded poorly to neoadjuvant chemotherapy if their tumors had increased expression of stromal genes. The stroma can interfere with therapeutic agents by two major mechanisms. First, it can simply function as an interstitial barrier that prevents efficient drug delivery (Jain, 1990). The second mechanism involves the activation of signaling

pathways that change the therapeutic outcome, driving the neoplastic cell to a more drug-resistant phenotype.

Conversely, a limited number of studies report that CAFs can also increase the anti-neoplastic potency of certain drugs. The mechanism for this unexpected sensitization may include the alteration of signaling pathways in neoplastic cells that are induced by CAFs (McMillin et al., 2013). Although we will not discuss this topic further, it highlights the need to consider the tumor microenvironment during therapeutic development.

**Environment-mediated drug resistance.** Although acquired drug resistance of neoplastic cells is considered to be a cell autonomous process that leads to adaptive biochemical, epigenetic, or genetic changes, the microenvironment can also participate in this process by providing extrinsic cues that promote cell survival and thereby impede drug toxicity. The impact of the tumor microenvironment on drug resistance was shown previously when mammary cancer cells that were refractory *in vivo* to cytotoxic drugs became sensitive upon transfer to an *in vitro* setting where the microenvironment was excluded (Teicher et al., 1990). The importance of the “soil” was further demonstrated in an experiment where colon carcinoma cells were injected at different sites, and showed varying responses to chemotherapy merely based on their location (Fidler et al., 1994). Compared with cell autonomous drug resistance, which develops over a long period of time, environment-mediated resistance can develop rapidly. Thus, environment-mediated resistance may protect neoplastic cells from apoptosis while they acquire the mutations needed for cell-intrinsic protection of chemotherapy (Meads et al., 2009).

Environment-mediated drug resistance can be further categorized into two major groups: soluble factor-mediated drug resistance and cell adhesion-mediated drug resistance (Meads et al., 2009). Soluble factor-mediated drug resistance is induced by cytokines and growth factors secreted from CAFs and other cell types in the stroma. By binding to neoplastic cell receptors, these factors cause transcriptional changes and also activate nontranscriptional mechanisms including degradation or redistribution of activators of apoptosis, and increased stability of suppressors of apoptosis and cell cycle regulators. Resistance due to secreted factors from stromal cells is common, particularly to targeted agents (Straussman et al., 2012). Many cancers have been shown to harbor driving mutations in receptor tyrosine kinase (RTK) pathways, constitutively activating these pathways and making the neoplastic cell “kinase-addicted.” Inhibiting these pathways has shown promising results *in vitro* but many times failed *in vivo*, where neoplastic cells soon develop resistance to such drugs. Wilson et al. (2012) made it clear that because of redundancy and overlap in many RTK signaling pathways, otherwise kinase-addicted neoplastic cells that respond to certain RTK inhibitors can be rescued from drug sensitivity simply by adding one or more RTK ligands. As CAFs are a major source of secreted RTK ligands, this gives a mechanistic explanation to how CAFs mediate resistance toward targeted therapy. Additional evidence was provided by Wang et al.



(2009a), who showed that CAFs recruited to lung tumors induced resistance to tyrosine kinase inhibitors against the EGF receptor (EGFR-TKIs). Interestingly, if HGF was inhibited, the resistance to EGFR-TKIs was eliminated, both *in vivo* and *in vitro*. This shows the striking impact that a single CAF-secreted growth factor can have on drug resistance.

Moreover, CAFs also play a role in targeted anti-angiogenesis treatments. Crawford et al. (2009) found that the expression of PDGF is up-regulated in CAFs from tumors that are resistant to anti-VEGF therapy and that a PDGF-neutralizing antibody impaired the observed anti-VEGF resistance, suggesting a crucial role for CAFs in mediating resistance to anti-angiogenesis therapy.

Soluble factor-mediated drug resistance is also directed against conventional chemotherapies. The CXCL12–CXCR4 signaling axis has been shown to mediate chemotherapy resistance (Singh et al., 2010; Domanska et al., 2012), and furthermore, pancreatic stellate cells drive resistance to chemotherapy-induced apoptosis and radiation-induced apoptosis via a mechanism involving activation of the AKT pathway (Hwang et al., 2008). As mentioned earlier, CAFs also secrete factors that stimulate neoplastic cells to undergo EMT and this process has also been shown to augment resistance to chemotherapy (Wang et al., 2009b).

Cell adhesion-mediated drug resistance occurs when neoplastic cells either adhere to the ECM or make direct cell-to-cell contact with stromal cells. Early observations revealed enhanced tumorigenicity and drug resistance when small cell lung cancer (SCLC) cells were grown on a basement membrane (Fridman et al., 1990). Furthermore, if the integrin-mediated contact with the ECM was blocked, the tumor load decreased *in vivo* (Fridman et al., 1990). Adhesion of SCLC cells to the ECM was later shown to enhance tumorigenicity and confer resistance to chemotherapeutic agents as a result of  $\beta 1$  integrin-stimulated tyrosine kinase activation, suppressing chemotherapy-induced apoptosis (Sethi et al., 1999). These results provide a mechanistic explanation behind cell adhesion-mediated drug resistance. Similar effects have been reported in ovarian cancer (Sherman-Baust et al., 2003) and pancreatic ductal adenocarcinoma cells (Miyamoto et al., 2004). Furthermore, resistance to radiotherapy can also be induced by cell adhesion, and  $\beta 1$ -integrin-mediated adhesion to fibronectin and collagens has been shown to be essential for cell survival after radiation-induced injury (Cordes et al., 2006).

Finally, cell adhesion can mediate resistance to targeted therapies using the same principles as described for soluble factor-mediated resistance. For example, PI3K/mTOR inhibitors caused cell death in matrix-deprived ovarian cancer spheroids, whereas matrix-attached cells were resistant (Muranen et al., 2012).

**Therapy-induced responses in CAFs.** Cancer therapies target all cells *in vivo*, including CAFs, which have been shown to undergo a DNA damage response that paradoxically results in the secretion of proteins that drive tumorigenesis. For example, after exposure to therapeutic agents, CAFs activate

canonical Wnt signaling in prostate cancer cells, promoting cancer cell survival and disease progression (Sun et al., 2012). Moreover, when breast cancer cells were injected into the irradiated fat pads of mice, increased neoplastic potential was observed compared with the same cells injected into non-irradiated fat pads (Barcellos-Hoff and Ravani, 2000). Along these lines, irradiated fibroblasts increased invasiveness in pancreatic cancer cells, an effect which could be inhibited with an HGF antagonist (Ohuchida et al., 2004). In addition, Lotti et al. (2013) compared colorectal tumors before and after cytotoxic treatment and found more CAFs in the post-treated tissue. These chemotherapy-treated CAFs, when isolated and co-cultured with CSCs, increased self-renewal, chemoresistance, and tumor growth of CSCs via IL-17A secretion. Collectively, these data illustrate how CAFs can gain new features upon exposure to chemo- or radiotherapy, which not only enables them to reverse or attenuate the effect of anti-cancer drugs but also contributes to a more aggressive cancer phenotype (Barcellos-Hoff et al., 2005).

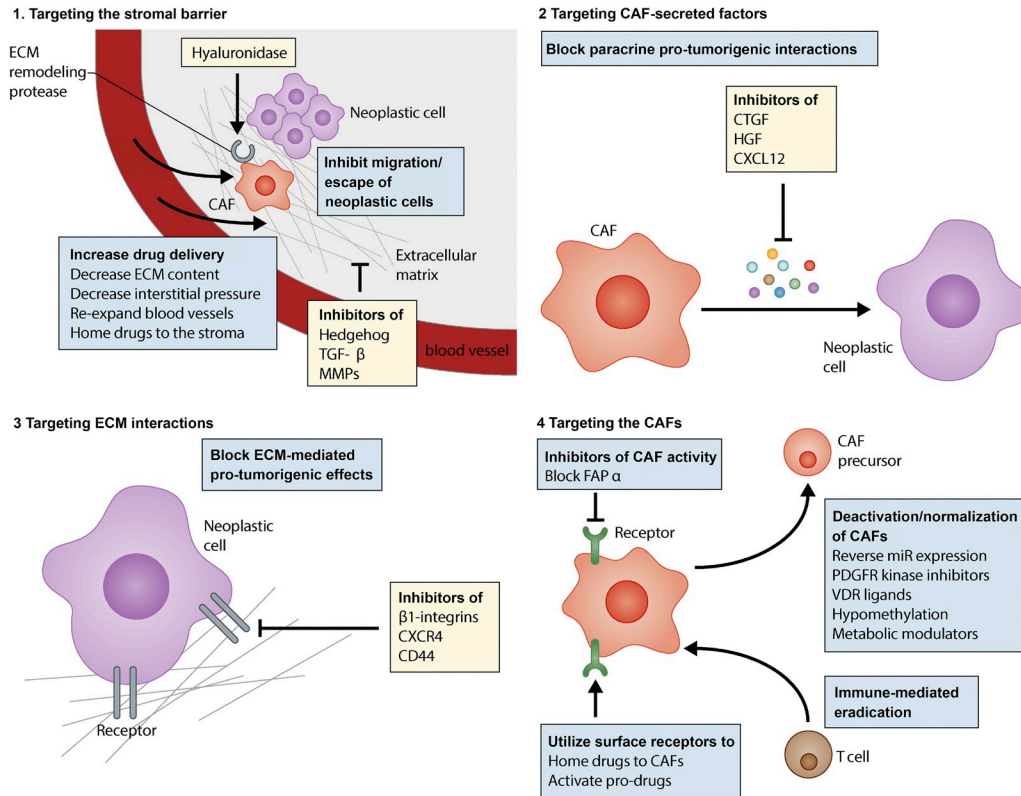
### Targeting the CAF-induced protumorigenic effects

The broad range of mechanisms used by CAFs to stimulate tumorigenesis and drug resistance (Fig. 2) provides multiple avenues to pursue therapeutic development. Indeed, four general strategies have been suggested to target CAFs and thereby achieve therapeutic benefits: (1) targeting the biophysical stromal barrier to increase drug delivery; (2) sequestering or inhibiting CAF-secreted factors that stimulate neoplastic cell behavior and drug resistance; (3) depleting or blocking the ECM components to reduce adhesion-mediated signaling; and (4) targeting the CAFs themselves to disable their downstream effects (Fig. 3).

Many informative experiments testing these strategies have been performed in combination with conventional chemotherapeutic or targeted drugs. The following section will briefly summarize some of the experiments that have provided additional insights to CAF/ECM targeting.

**1. Targeting the stromal barrier.** The tumor stroma can be considered a barrier that needs to be lowered so that drugs can be optimally delivered. A variety of strategies have been tested to increase drug delivery by overcoming the stromal barrier. Targeting the production of the ECM or degrading the ECM are both feasible strategies to loosen the stroma, resulting in a change in the interstitial pressure of the tumor and the consequent reexpansion of blood vessels. Hyaluronic acid, TGF- $\beta$ , and the Hedgehog pathway have all been tested as targets in this context.

High interstitial fluid pressure is evident in solid tumors, especially those rich in ECM, resulting in vascular collapse and effectively limiting perfusion and diffusion (Provenzano et al., 2012). Hyaluronic acid compresses vessels in collagen-rich tumors, and both collagen and hyaluronic acid are critical targets for decompressing tumor vessels. Enzymatic degradation of hyaluronic acid using hyaluronidase normalized the interstitial fluid pressure and expanded the microvasculature,



**Figure 3. Principle strategies for targeting CAF-induced pro-tumorigenic effects.** Presented are four general approaches to control and diminish CAFs and their activity toward tumorigenesis and drug resistance. Examples of targets that have been hypothesized and/or empirically tested are indicated.

leading to increased intratumoral delivery of chemotherapeutic agents (Provenzano et al., 2012; Jacobetz et al., 2013). Accordingly, the combination of hyaluronidase and conventional chemotherapy inhibited tumor growth and prolonged survival relative to monotherapy in mouse models of pancreatic cancer (Provenzano et al., 2012; Jacobetz et al., 2013). This concept is now being tested in clinical trials.

Inhibitors of TGF- $\beta$  were shown to decrease collagen I content and increase the fraction of perfused vessels in a mouse breast cancer orthotopic model, leading to preclinical benefit. Furthermore, the normalization of the tumor stroma improved intratumoral delivery of conventional chemotherapeutic drugs, leading to better drug response (Liu et al., 2012). TGF- $\beta$  inhibitors are also capable of inhibiting cancer progression in animal models (Akhurst and Hata, 2012), but this pathway is highly context-dependent and complicated to target. Indeed, TGF- $\beta$  signaling exhibits a seemingly biphasic action during tumorigenesis, being tumor-suppressive early in tumorigenesis and becoming tumor-promoting in later stages. Indirectly targeting MMPs by inhibiting the TGF- $\beta$  pathway is an alternative mechanism by which the stromal barrier may be attenuated. Low doses of the TGF-type I receptor inhibitor have been shown to increase the delivery of anti-cancer nanocarriers, thereby potentiating their tumor-inhibitory effect without altering either TGF signaling in neoplastic cells or the amount of fibrosis (Kano et al., 2007). Given that TGF- $\beta$  and MMP14 are

both involved in a pathway that regulates vessel stability, inhibiting this pathway may increase delivery efficacy by increasing perfusion of the tumor (Soussi et al., 2010).

Angiotensin receptor blockers (ARBs) were originally developed for the treatment of hypertension but also inhibit TGF- $\beta$  signaling (Diop-Frimpong et al., 2011), although the exact mechanism for this is unclear. Nevertheless, the Angiotensin signaling pathway is activated in stellate cells and contributes to the desmoplastic reaction (Sakurai et al., 2011). ARBs have been shown to inhibit the production of collagen I by CAFs (Diop-Frimpong et al., 2011), and in a subcutaneous xenograft mouse model, ARB reduced expression of  $\alpha$ -SMA in the tumor, resulting in reduced ECM and tumor inhibition (Masamune et al., 2013). By reducing collagen and hyaluronic acid production, ARBs can increase vascular perfusion and drug delivery (Chauhan et al., 2013). A retrospective study further suggested the role of the Angiotensin pathway in cancer by showing that pancreatic cancer patients that underwent gemcitabine monotherapy and at the same time received hypertension treatment with ARB had a significantly better survival than patients without ARB treatment (Nakai et al., 2010).

Hedgehog (Hh) signaling mediates tumorigenesis in many types of cancer (Thayer et al., 2003; Bailey et al., 2009). Interestingly, despite ligand production in neoplastic cells, Hh signaling is not activated in these cells. Instead, CAFs have been

demonstrated to respond to Hh stimulation by increasing the production of ECM (Yauch et al., 2008), indirectly supporting tumor growth. An inhibitor of Hh signaling, IPI-926, resulted in stromal depletion in a mouse model of pancreatic cancer. In combination with gemcitabine, IPI-926 increased the intratumoral vascular density, perfusion, and intratumoral concentration of chemotherapy, correlating with transient growth inhibition (Olive et al., 2009). Importantly, this study and the aforementioned studies targeting hyaluronan showed that by depleting the stroma barrier, drug delivery is increased, resulting in transiently improved survival. Although the combination of IPI-926 and chemotherapy was promising in a Phase I trial, this approach failed in a randomized phase II clinical trial. To address these conflicting data, a follow-up study was recently conducted, where Hh was genetically ablated in the epithelial compartment of a pancreatic cancer mouse model. Interestingly, despite a decreased number of stromal cells and an increased vascularization, these tumors were more aggressive and tended to be much less differentiated, subsequently leading to reduced survival of these mice (Rhim et al., 2014). Similar results were obtained in another study, where depletion of cancer-associated myofibroblasts in a pancreatic cancer model led to more aggressive and less differentiated tumors (Özdemir et al., 2014). Thus, the protective role of certain stromal elements should be kept in mind, although further interrogation of this phenomenon in other settings is required to evaluate the physiological role of the stroma in restraining cancer.

MMP inhibitors are another example that illustrates the complexity of the tumor stroma. These inhibitors were developed to block the degradation of the ECM, a process which was predicted to facilitate tumor progression. Early preclinical studies with MMP inhibitors were promising; however, randomized clinical trials were not positive (Dufour and Overall, 2013). An explanation for this may lie in the fact that MMPs have many different substrates, and besides ECM structures, they also affect chemokines and cell surface receptors that might have tumor suppressive effects (Dufour and Overall, 2013). Unfortunately, side effects limited the dosing of the MMP inhibitor, and therefore the results of this trial may be misleading.

It is also possible to use the tumor ECM as a homing signal for drugs, limiting reactivity to normal tissues. By conjugating drugs to antibodies or small molecules that recognize stromal epitopes, drugs can be accumulated in the tumor, limiting the area of cytotoxicity. For instance, the plasminogen activator inhibitor type 2 (PAI2) has high affinity to plasminogen activator (uPA), which is highly expressed in the stroma of pancreatic cancer. By conjugating a radioisotope onto PAI2, radioactivity was concentrated in the tumor and caused complete inhibition of tumor growth in a xenograft model (Qu et al., 2005). Additionally, nAb-Paclitaxel, an albumin-formulated Paclitaxel, was hypothesized to bind to the matricellular protein Osteonectin/SPARC that is present in many tumor types and thereby cause stromal depletion. This hypothesis was supported by data from patient-derived pancreatic cancer xenografts that demonstrated stromal loss after treatment (Von Hoff et al., 2011), and from resected

specimens of patients who were treated with nAb-Paclitaxel and gemcitabine that demonstrated loss of neoplastic cells and a deficient stroma (Alvarez et al., 2013). However, direct experiments in genetic mouse models did not corroborate stromal loss as the primary mode of action for nAb-paclitaxel but rather implicated drug–drug interactions leading to increased neoplastic cell death as an important aspect of its activity (Frese et al., 2012). Furthermore, mice harboring pancreatic tumors and lacking SPARC responded to nAb-Paclitaxel in an indistinguishable manner compared with mice that expressed SPARC (Neesse et al., 2014). To reconcile these seemingly contradictory findings, it is logical that the loss of neoplastic cells will lead to secondary decreases in stromal abundance.

**2. Targeting CAF-secreted factors.** CAFs secrete a variety of mitogens, chemokines, and matricellular proteins that promote tumor progression and drug resistance. Neesse et al. (2013) showed that targeting the stroma in a mouse pancreatic cancer model with a CTGF antibody yielded a synergistic effect with gemcitabine without increasing the intratumoral gemcitabine concentration. Mechanistic analysis revealed that CTGF deregulates the expression of the apoptosis modulating protein XIAP, highlighting the impact that secreted factors from CAFs have on neoplastic cells in terms of response to therapy. This modality is currently undergoing clinical investigation.

The HGF–Met pathway has been interrogated in many preclinical studies, either as monotherapy or in combination with other drugs. For example, a competitive antagonist of c-Met (NK4) has been shown to reduce liver metastasis and prolong survival in a mouse model of colon cancer (Wen et al., 2004). Furthermore, stromal fibroblast-secreted HGF is involved in mediating therapy resistance in melanoma. BRAF mutant melanomas are highly dependent on constitutive BRAF kinase activity, and inhibition of BRAF improves survival in patients (Bollag et al., 2010; Flaherty et al., 2010; Chapman et al., 2011; Sosman et al., 2012). However, CAF-derived HGF and subsequent activation of the HGF receptor c-Met have been shown to mediate resistance to targeted RAF inhibition. Interestingly, targeting both RAF and c-Met reversed the observed resistance (Straussman et al., 2012), illustrating how microenvironment-derived mediators of drug resistance can serve as promising targets to overcome the resistance problem with targeted therapies seen in the clinic.

The CXCR4 antagonist AMD3100 was shown to chemosensitize prostate cancer cells to the chemotherapeutic agent docetaxel in a synergistic manner (Domanska et al., 2012). As discussed previously, CXCL12 secreted from FAP $\alpha$ -positive cells was shown to be important for immune suppression in pancreatic cancer, providing a conceivable explanation as to why immunological checkpoint antagonists, such as anti-PDL1, have failed in pancreatic cancer (Royal et al., 2010). Notably, in a mouse model of pancreas cancer, CXCR4 inhibition promoted T cell accumulation in the tumor site and synergized potently with anti-PDL1 (Feig et al., 2013).

**3. Targeting ECM interactions.** Neoplastic cells interact with the ECM using a variety of cell surface receptors, of which integrins are the most studied. In a three-dimensional culture system of breast cancer, a  $\beta 1$ -integrin inhibitor induced apoptosis and inhibited growth of breast cancer cells, highlighting the therapeutic potential of blocking integrin interactions with the ECM (Park et al., 2006). Furthermore,  $\beta 1$ -integrin inhibitors synergized with radiation therapy to increase apoptosis and inhibit tumor growth in a mouse xenograft model (Park et al., 2008). Integrin antagonists have therefore been tested in clinical trials (Mullamitha et al., 2007; Reardon et al., 2008), and although they were well tolerated, clinical benefits have not yet been demonstrated. CD44 expressed by neoplastic cells allows an additional interacting point with the ECM. It binds to collagen and hyaluronic acid, and certain isoforms of CD44 provide survival signals in neoplastic cells (Bourguignon et al., 2003; Götte and Yip, 2006). Abrogating CD44–ECM interactions has been shown to inhibit tumor growth in xenograft models (Ahrens et al., 2001); however, this awaits clinical confirmation.

Soluble factors secreted from CAFs can also indirectly regulate adhesion of neoplastic cells. For example, CXCL12-induced integrin activation was found to increase adhesion of SCLC cells to fibronectin and collagen and thereby protects SCLC cells from chemotherapy-induced apoptosis (Hartmann et al., 2005).

**4. Targeting the CAFs.** Normalization of CAF behavior to change their properties to a more “non-myofibroblastic” phenotype can be achieved by multiple mechanisms. One way is by targeting epigenetic changes unique for CAFs. Many groups have recently shown that therapies aimed at reconstituting miR expression might be a successful strategy to deactivate CAFs (Musumeci et al., 2011; Bronisz et al., 2012; Mitra et al., 2012; Aprelikova et al., 2013). Inhibition of PDGF signaling, an important pathway in the activation of CAFs, can reverse CAFs to normal fibroblasts (Haubeiss et al., 2010). Using a clinically approved PDGFR kinase inhibitor (Imatinib) in a mouse model of cervical carcinogenesis, tumor proliferation was reduced and angiogenesis was inhibited. Furthermore, the progression of premalignant cervical lesions was attenuated and the growth of preexisting invasive carcinomas was impaired (Pietras et al., 2008). Because CAFs undergo changes in their metabolic activity, normalization of the metabolic phenotype and inhibition of metabolic pathways have also been suggested as a plausible way to target tumors (Witkiewicz et al., 2012; Doherty and Cleveland, 2013).

Another approach to target the CAFs would be to dedifferentiate them into a quiescent state. Hepatic stellate cells play a crucial role in liver fibrosis and in the desmoplastic reaction in liver cancer and are known to express several nuclear hormone receptors including the vitamin D receptor (VDR). Ding et al. (2013) demonstrated that VDR ligands promoted the dedifferentiation of stellate cells and abrogated fibrosis, stressing the potential use of VDR ligands to target CAFs. Taking advantage of existing epigenetic changes has also been suggested as a means to induce CAF arrest. Treatment of CAFs with hypomethylating drugs is hypothesized to

result in a “hypomethylation crisis” by causing further demethylation in the already hypomethylated CAF genomes (Gonda et al., 2010), resulting in growth arrest.

Finally, FAP $\alpha$  has been proposed as a potential therapeutic target (Brennen et al., 2012). This serine protease is expressed on most CAFs. Blocking FAP $\alpha$  was found to inhibit tumor growth, and to decrease myofibroblast content and blood vessel density in lung and colon tumors (Santos et al., 2009). Furthermore, a phase I trial showed that FAP $\alpha$  can be targeted without apparent side effects (Scott et al., 2003). However, no significant efficacy was shown for the treatment of metastatic colorectal cancer (Hofheinz et al., 2003).

FAP $\alpha$  can also be used to deliver cytotoxic drugs to the CAFs. An anti-FAP $\alpha$  antibody conjugated to an anti-mitotic agent induced long-lasting inhibition of tumor growth in xenograft models (Ostermann et al., 2008). Investigators have also used the proteolytic activity of FAP $\alpha$  to target CAFs. By delivering an inert pro-drug that contains the FAP $\alpha$  cleavage site, FAP $\alpha$ -positive cells in the tumor stroma can cleave and activate the drug, a method which has been tested both in vitro (Huang et al., 2011) and in xenograft models (LeBeau et al., 2009), resulting in a significant growth inhibition.

In addition, FAP $\alpha$  is a target selected for immunotherapy experiments. Cancer mouse models have been immunized against FAP $\alpha$ -positive cells, resulting in tumor growth inhibition (Lee et al., 2005). Also, an oral DNA vaccine directed against FAP $\alpha$ -positive cells caused CD8<sup>+</sup> T cell-mediated killing of CAFs and suppression of primary tumor growth and metastasis, accompanied by a greater intratumoral uptake of chemotherapeutic drugs and improved drug response (Loeffler et al., 2006). In spite of the successful preclinical results, no FAP $\alpha$ -directed agents are currently in clinical practice (Brennen et al., 2012).

### Concluding remarks

Based on the body of evidence now available, it is clear that the tumor microenvironment is involved in all stages of tumorigenesis, shaping how neoplastic cells respond to targeted therapies, chemotherapies, or radiotherapy. Thus, the tumor microenvironment has significant impact on clinical outcome. Unfortunately, preclinical drug testing has been largely restricted to two dimensional cell culture systems or subcutaneous xenograft murine models, without fully incorporating a relevant tumor microenvironment. This lack of tumor stroma in cancer models may reflect the lack of efficacy seen in later clinical trials of many drugs. Therefore, it is of high priority to transfer preclinical drug development to platforms that take stromal interactions into account.

CAF are tempting drug targets for many reasons. They are genetically more stable than neoplastic cells, making them less prone to develop resistance phenotypes as a result of high mutation rates and clonal selection. Additionally, CAFs show epigenetic changes that distinguish them from normal stromal cells, and furthermore, the composition of the cancer-associated stroma is distinct from normal stroma. This presents

opportunities to find unique drug targets that do not affect normal physiology. In addition, CAFs in some cases cause sensitization to certain drugs, giving a rationale for the use of inhibitors against pathways that are activated by CAFs in neoplastic cells. Moreover, CAFs have the potential to activate pathways that mediate resistance to targeted anti-neoplastic agents, and resistance may be avoided if CAF-derived stimuli are neutralized. Finally, CAFs accompany and support neoplastic cells during the whole spectrum of the disease, from preinvasive lesions all the way to the metastatic site. By targeting CAFs, different stages of the disease and also many different pathophysiological processes may become treatable, including angiogenesis, EMT, and immune evasion.

Developing combinatorial therapies that target both the CAFs and the neoplastic cells holds promise for synergistic effects. If neoplastic cells are dependent on a certain input from the CAFs, it is reasonable to assume that depleting this input would make the neoplastic cells more vulnerable and less resistant to a second line of treatment with conventional therapies. Rationale supporting this hypothesis was provided by a study where pretreatment with drugs targeting oncogenic signaling pathways sensitized breast carcinoma cells to conventional chemotherapy (Lee et al., 2012). In addition, some conventional therapies potentiate the pro-tumorigenic effects of CAFs, conferring an even more aggressive cancer phenotype. This effect may be avoided if the CAFs were targeted first. Finally, drug delivery in a second round of therapy would be improved in the absence of CAFs, the major constituent of the stroma barrier.

CAFs represent a very heterogeneous population of cells, and each subpopulation might have different pathophysiological functions. Some CAFs might also have tumor-suppressive roles by preventing neoplastic cell proliferation or migration. It is in no way excluded that certain CAFs, and also other components within the tumor-associated stroma, are induced as a result of a host-driven response to the neoplastic cells. In fact, cancer progression *in vivo* is a relatively slow process compared with what is observed *in vitro*. Autopsy studies show that many preneoplastic lesions, despite harboring dominant genetic alterations sufficient to promote tumorigenesis, remain dormant. Emerging data further reinforce this idea of CAFs as a double-edged sword in tumorigenesis by demonstrating that ablation of a single cue from epithelial cells to stromal cells (Rhim et al., 2014), or depletion of the entire myofibroblastic population (Özdemir et al., 2014), accelerates pancreatic cancer. These studies question the general dogma of the field and illustrate how components in the microenvironment actually inhibit cancer progression. These findings further enlighten the complex nature of the tumor stroma; however, the implications of these findings are yet to be determined in a broader perspective. Also, it is important to keep in mind that rapidly developing tumors in murine cancer models driven by one or a few defined mutations might not fully recapitulate the complexity of the tumor microenvironment seen in human tumors. This provides a probable explanation as to why some approaches resulting in a global depletion of the tumor-associated stroma can show promising results in mouse models but cause

increased tumor growth when applied to patients. It also raises the idea that the targets within the stroma must be specific and cannot cause a general and unselective depletion of the stroma or the CAFs. Another pitfall is that researchers often study a specific subpopulation of CAFs, based on one or a few expressed markers, but interpret the data as true for all populations of CAFs. The term “cancer-associated fibroblast” is therefore somewhat misleading, as it groups cells of different origin merely based on their location despite their heterogeneity and diverse roles in tumorigenesis. It is important to keep in mind that the only feature that unites the CAFs are their ability to alter the microenvironment and the behavior of neoplastic cells, but this can result in either a pro- or anti-tumorigenic effect. Efforts must therefore be put into resolving the complexity of the tumor stroma and understanding the unique roles of the different subpopulations of CAFs.

Traditionally, cancer is viewed as a result of clonal expansion, where neoplastic cells constantly undergo selection and the clones that constitute late-stage tumors are the ones most fit to avoid the host response and given therapies. However, when an initiating preneoplastic cell emerges, it does so in the context of its microenvironment, which then coevolves during the whole process of tumorigenesis such that both entities develop a symbiotic relationship that promotes tumor growth. Thus, what is seen in patients is a selected population of CAFs and neoplastic cells that collectively survives and thrives. It is vital for a neoplastic cell to achieve the ability to reprogram normal stromal cells into CAFs and by this influence to modulate the microenvironment. This process represents a fundamental and crucial hallmark of cancer. Neoplastic cells that fail to develop this capacity will not pass critical barriers in tumorigenesis and will remain dormant. Therefore, understanding the interplay between neoplastic cells and their microenvironment is critical, especially in the initiation of cancer. Further insights in this process have the potential to reveal relevant discoveries with subsequent clinical implications.

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