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Review

Carcinoma-associated fibroblasts provide operational flexibility in metastasis

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ABSTRACT

Malignant cancer cells do not act as lone wolves to achieve metastasis, as they exist within a complex ecosystem consisting of an extracellular matrix scaffold populated by carcinoma-associated fibroblasts (CAFs), endothelial cells and immune cells. We recognize local (primary tumor) and distant ecosystems (metastasis). CAFs, also termed myofibroblasts, may have other functions in the primary tumor versus the metastasis. Cellular origin and tumor heterogeneity lead to the expression of specific markers. The molecular characteristics of a CAF remain in evolution since CAFs show operational flexibility. CAFs respond dynamically to a cancer cell's fluctuating demands by shifting profitable signals necessary in metastasis. Local, tissue-resident fibroblasts and mesenchymal stem cells (MSCs) coming from reservoir sites such as bone marrow and adipose tissue are the main progenitor cells of CAFs. CAFs may induce awakening from metastatic dormancy, a major cause of cancer-specific death. Cancer management protocols influence CAF precursor recruitment and CAF activation. Since CAF signatures represent early changes in metastasis, including formation of pre-metastatic niches, we discuss whether liquid biopsies, including exosomes, may detect and monitor CAF reactions allowing optimized prognosis of cancer patients.

1. Introduction

Malignant tumors consist of cancer cells and tumor-associated host cells. Spatial heterogeneity of cancer cells has long been recognized by pathologists and forms the basis of the Gleason score tumor grading prognostic classification system for prostate cancer. This morphological intratumoral heterogeneity of cancer cells has been confirmed at the genetic level using deep sequencing technologies [1–3]. It has long been recognized that genomic instability is a hallmark of cancer cells [4]. Campbell et al. [2] detected specific chromosomal fold-back inversions, which are duplicated chromosomal DNA regions following a breakage, in both the primary and metastatic lesion suggesting that fold-back inversions occur early in tumorigenesis and probably drive metastasis. However, cancer cells do not act as lone wolves, as they exist within a complex ecosystem consisting of an extracellular matrix (ECM) scaffold populated by stromal cells including fibroblast-like cells, endothelial cells, and immune cells, morphologically defined by desmoplasia, angiogenesis, and inflammation and immune response respectively. There is increasing histopathological and genetic evidence to suggest that tumor-associated stroma proportions or signatures may refine the prognostic assessment of tumors [5–7]. Stromal components, such as endothelial and immune cells are considered as emerging hallmarks indispensable for metastasis [4]. The specific role of the apparently innocuous-appearing desmoplasia (from the Greek word desmos, *fettering or restraining*; and plasis, *formation*) has received relatively little attention, primarily because of a lack of vital function being attributed to this accumulation of fibroblast-like cells and ECM proteins. This is particularly bemusing, since hard cancers amongst soft organs have been a critical diagnostic feature taught to all medical students [8]. Fibroblast-like cells contributing to desmoplasia often express α-smooth muscle actin (α-SMA), an important marker for myofibroblasts. Indeed, α-SMA expression in tumor-associated stroma is an independent prognostic marker in multiple cancer types including colon, pancreas, and breast [9–11]. In addition, gene expression signatures of tumor-associated fibroblast-like cells not only hold prognostic information [12] but also predict resistance to neo-adjuvant chemotherapy in breast cancer [13]. Clinical correlation data combined with experimental modeling suggests that tumor-associated fibroblast-like cells are not idle bystanders but rather corruptive drivers of metastatic progression. To avoid misunderstanding we shall use the term carcinoma-associated fibroblast (CAF) in this review which may refer to peritumoral fibroblasts, myofibroblasts, tumor-associated mesenchymal cells, tumor-associated fibroblast-like cells, mesenchymal stromal cells and other definitions described in literature.

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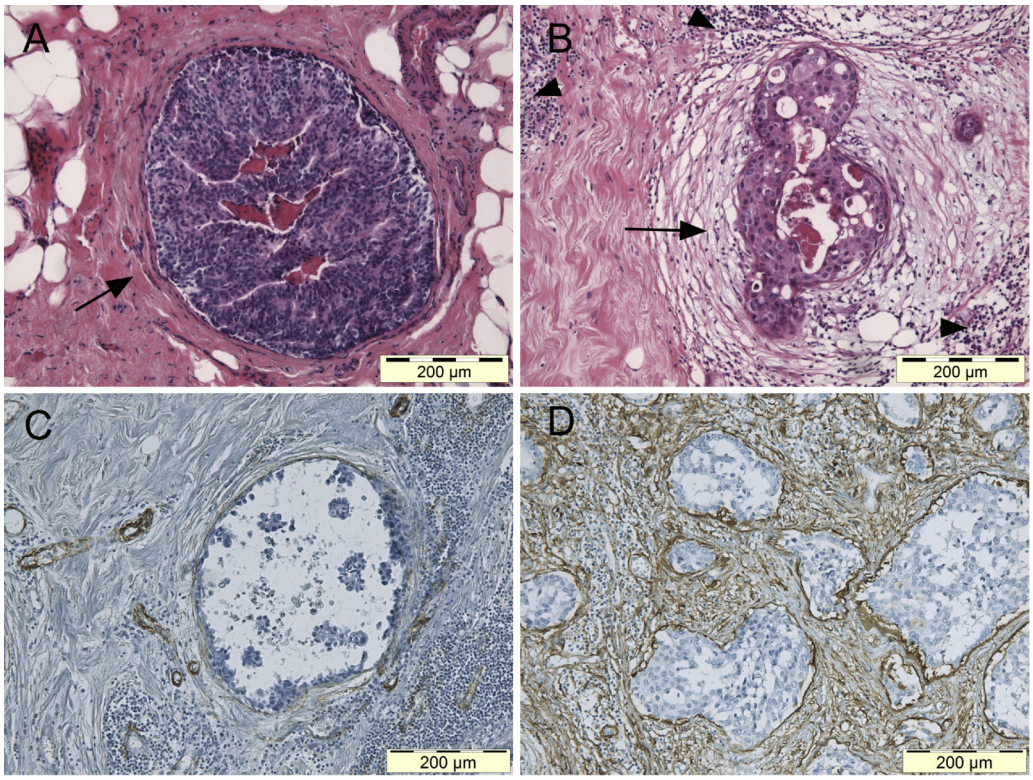

Fig. 1. Photomicrographs showing hematoxylin and eosin staining (A and B) and immunohistochemical staining of α-SMA (C and D) in DCIS lesions from the breast. Periductal sclerotic (S) stroma (A). Myxoid (M) stroma surrounding the affected ducts, areas with inflammatory cells are indicated by arrowhead (B). Periductal stroma with minor α-SMA expression, presumably pericytes of the blood vessels (C). Periductal stroma strongly positive for α-SMA expression (D).

Since previous reviews on the subject [14–18] many new findings have emerged to advance our understanding of the impact of CAFs on metastasis, but many questions remain unanswered and are important topics for further research. Key issues discussed in this review include: (i) Is there a molecular heterogeneity of CAFs in the primary tumor and at metastatic sites? (ii) What is the origin of corrupted CAFs? (iii) What are the instructive signals for CAF corruption? (iv) How do CAFs provide operational flexibility during metastasis? (v) Are CAFs implicated in metastatic dormancy? (vi) Do CAFs react to cancer management protocols? (Vii) Do liquid biopsies allow early detection of CAF?

2. Molecular heterogeneity of CAF

2.1. Within the primary tumor

Cancer cells within the primary tumor do not exist independently but interact dynamically with local and distant host cells, best conceptualized as a gradually evolving phenomenon termed microenvironment or ecosystem. Consequently, intratumoral heterogeneity of cancer cells will lead to heterogeneous local host reactions (Fig. 1). Indeed, some ductal carcinoma in situ (DCIS) lesions of the breast, which are considered to be a non-invasive precursor of invasive ductal carcinoma, have a mixed stromal architecture classified as either predominantly sclerotic or myxoid. Periductal myxoid stromal architecture is significantly associated with increased ipsilateral locoregional recurrence. Intriguingly, α-SMA expression is differentially expressed in periductal DCIS stroma but is not associated with the presence of myxoid stroma (unpublished data). In addition some DCIS regions contain an inflammatory component, others not. Even within one inflammatory component different subtypes of macrophages are described [19,20]. Macrophages are differentiated cells of the mononuclear phagocytic lineage and acquire a specific phenotype in response to signals present within individual microenvironments. The exact combination of such specific signals dictates both the differentiation and activation status of these cells. The diversity of macrophage subtypes raises the possibility that CAFs equally fall into different functional and molecular subtypes. CAFs closely resemble myofibroblasts, which are implicated in fibrosis and wound healing [16]. The most widely used molecular marker of CAFs in research and clinical diagnostics is the expression of α-SMA. The convenience of a unique molecular marker has fostered the misconception that a CAF must express α-SMA to be a CAF. However, the most important defining feature of CAFs compared to their precursors is their capacity to stimulate invasion and metastasis. In agreement, other common functional differences of CAFs compared to their cell of origin is an increased capacity to form colonies in soft agar [21], contract native type I collagen gels [22] and to acquire a nemosis response i.e. clustering of cells in culture leading to increased scatter factor/hepatocyte growth factor (SF/HGF) secretion [23]. Most importantly, we may consider CAFs, present at the primary tumor, as a heterogeneous population with different molecular identities that collaboratively stimulate metastasis (Fig. 2). Indeed, immunohistochemistry of prostate cancer CAFs showed mixed intensity of phosphorylated SMAD2, suggesting a heterogeneous transforming growth factor (TGF)-β responsiveness [24]. A computational model showed a 2-step mechanism in which proliferation and invasion of prostate cancer cells occur by distinct stromally derived paracrine signals produced by either TGF-β nonresponsive and responsive CAFs. In vitro cultures show that TGF-β nonresponsive CAFs secrete Wnt-3a which may cause epithelial hyperproliferation; the TGF-β responsive CAFs secrete CXCL12 (or stromal derived factor-1, SDF1) which support the invasion of proliferative epithelial cells through matrigel, a basement membrane mimic. In oral squamous cell carcinoma (OSCC)

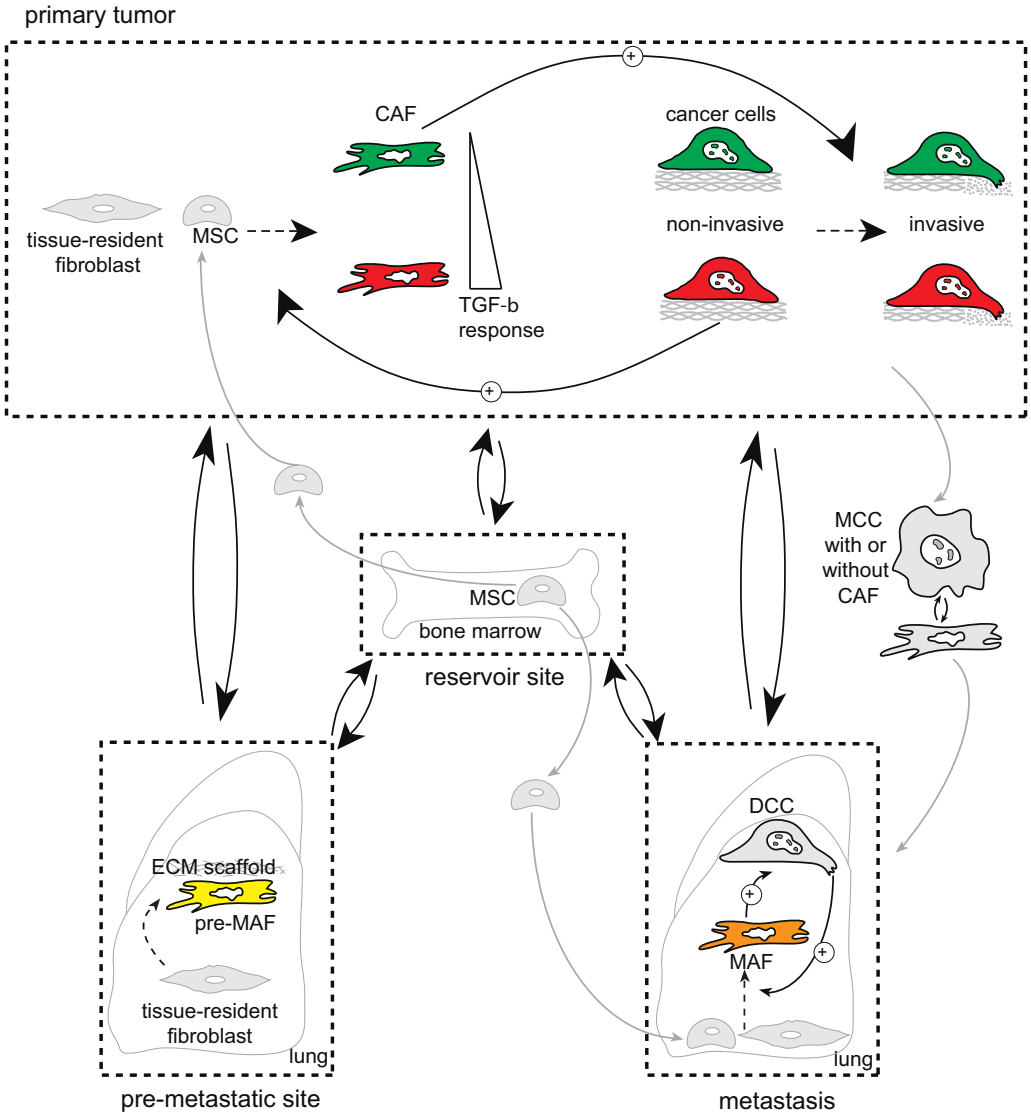

Fig. 2. Schematic representation showing the cross-signaling (arrows in opposite sense) within and between primary tumor and distant ecosystems. Gray arrows indicate displacement of cells between ecosystems, dashed black arrows indicate conversion from one cell type into another, positive arrows indicate stimulation. Colors indicate heterogeneity. CAF, carcinoma-associated fibroblast; DCC, disseminated cancer cell, ECM, extracellular matrix; MAF, metastasis-associated fibroblast; MCC, metastatic cancer cell; MSC, mesenchymal stem cell.

two distinct CAF subtypes were identified by combined-omics and functional assays. One subtype included more intrinsically motile fibroblasts maintained by high autocrine production of hyaluronan (termed CAF^{-HA}). The other subtype consisted of less motile CAFs but synthesized higher TGF-β1 levels (termed CAF$^{-TGF\text{-}\beta1}$) [25]. Both subtypes increased invasion of OSCC cells into type I collagen but CAF^{-HA} were more effective in supporting deeper invasion. Both subtypes also provided different pro-invasive cues. The CAF^{-HA} subtype created a network of tracks used by OSCC cells for invasion and deep invasion was supported by high SF/HGF secretion. In contrast the CAF$^{-TGF\text{-}\beta1}$ subtype stimulates epithelial to mesenchymal transition (EMT) of OSCC cells through excessive TGF-β1 secretion. Correlating transwell motility assay with secretome analysis and pro-invasive activity indicate that both CAF subtypes may actually be two different stages of CAF in OSCC progression with CAF^{-HA} representing an earlier stromal change than CAF$^{-TGF\text{-}\beta1}$ subtype. Both Kiskowski et al. [24] and Costea et al. [25] suggest a different degree in TGF-β responsiveness as essential in generation of distinct CAF phenotypes. Heterogeneity within the cancer cell population may be responsible for differential secretion of TGF-β modulating growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor, interferon-γ, interleukin (IL)-7 and tumor necrosis factor-α, and consequently initiating a balance of TGF-β responsive versus unresponsive CAFs all favoring reciprocal metastasis (Fig. 2) [15,25–27]. Interestingly, bFGF counteracts TGF-β induced α-SMA expression but not tenascin C and fibroblast activation protein expression in CAFs [27]. CAF heterogeneity in the signal reception of FGF from the epithelium has been suggested previously [28]. CAFs with a high fibroblast growth factor receptor (FGFR)1 expression exhibited a fibroblast-like morphology and did not express α-SMA. CAF with low FGFR1 exhibited α-SMA and smooth muscle cell-like morphology. CAFs from different primary colon tumors show a differential stimulation of LIM1215 and SW480 colon cancer cell migration. Enhanced pro-migratory capacity of CAFs correlates with their α-SMA expression and type I collagen gel contraction. A gene signature derived from CAFs with a 25-fold pro-migratory capacity is associated with poor disease-free survival in stage II and IV colon cancer patients [29].

2.2. Within the metastasis

Ample experimental and clinical evidence supports the metastasis-promoter role of CAFs in the primary tumor, whereas data about the presence and role of CAFs in lymph node and distant metastasis is scarce. Stromal reactions in metastatic lymph nodes, possibly containing metastasis-associated fibroblasts (MAFs), have been described as: reactive and fibrotic tissue with enhanced deposition of vitronectin and fibronectin [30]; desmoplasia [31]; nodal fibrosis [32]; and hyaline stroma [33]. Immunohistochemical characterization of CAFs was reported in only one of these publications, concerning a metastatic lymph node from a patient with an adenocarcinoma of the uterine cervix who received preoperative chemotherapy [31]. The glandular cancer cells were situated in the marginal and cortical sinus; they were surrounded by concentric fibrous connective tissue with spindle cells that were recognized by an antibody against α-SMA, but not against calponin, desmin or CD34. Fibrotic, desmoplastic reaction in metastatic lymph nodes is a factor of worse prognosis in cancer of the oral cavity [34], and in invasive ductal cancer of the breast [35]. In single, presumably early, lymph node metastases from gastric cancer a strong correlation was found between extracapsular invasion and fibrotic foci, both indicating a worse prognosis [36].

Is there an impact of the primary tumor on fibroblasts in target organs? Data from the Lyden lab show that tumor-secreted factors promote metastatic spread into specific distant organs [37]. Within days following primary tumor implantation, localized deposition of extra domain (ED)-A or ED-B spliced forms of fibronectin occurs by resident fibroblast within target organs that are conventional sites of metastasis, corresponding to the particular primary tumor. These primed sites characterized by pre-metastasis associated fibroblasts (pre-MAFs) are termed the pre-metastatic niche and are followed by accumulation of CD11b- and VEGFR1-positive myeloid derived cells. In addition, these pre-MAFs prepare the tissue for successful extravascular survival of cancer cells and macroscopic colonization by being a reservoir of soluble factors and matrix-associated factors that enhance metastatic efficiency. Indeed, lysyl oxidase (LOX) accumulates at pre-MAFs in target organs where it modifies the ECM by crosslinking collagen fibrils to make them more receptive for CD11b+ myeloid cell infiltration [38]. Involvement of CD11b+ myeloid cells has been shown in experimental hepatic metastasis from MC38 mouse colon cancer cells and in a subset of liver metastasis from colorectal cancer patients [39]. Since cellular forms of fibronectin and LOX are well known TGF-β target genes, it might well be that TGF-β responsiveness is equally important at the metastatic site as in the primary tumor. Indeed, phosphorylation of SMAD2 and expression of TGF-β-responsive genes, such as serpin-11, and IL-11, accumulate in stromal cells, presumably MAFs, surrounding hepatic metastasis from intrasplenically injected TGF-β hypersecreting KM12L4 colon cancer cells [40]. Unfortunately, it was not assessed whether SMAD2 phosphorylation of hepatic stroma was observed before the arrival of metastatic colon cancer cells. Increase of p-SMAD2 would have further supported the influence of metastatic TGF-β secretion on neighboring MAFs. In addition, the systemic role of TGF-β is confirmed in an orthotopic mouse model where TGF-β hypersecreting KM12L4 colon cancer cells develop lung and liver metastasis in 90% of the cases compared with three-fold less metastasis in animals bearing control KM12L4 tumors [40]. In another scenario put forward by Vidal-Vanaclocha [41] for the establishment of the pre-metastatic niche in the liver, MAFs appear in a later phase, after formation of an avascular micrometastasis. Endothelial cell migration toward avascular metastasis occurs only at high density of MAFs. The latter cells originate from three sources in response to paracrine factors from metastatic cancer cells (MCC): hepatic stellate cells transiting to MAFs, hepatic sinusoidal endothelial cells and Kuppfer cells. The specific recruitment of angiogenesis-promoting MAFs after the formation of micrometastasis may explain the puzzling clinical observation that metastasis from colon cancer occur less frequently in pre-existing fibrotic livers than in normal ones. Besides colon cancer, the liver is the main metastatic organ for melanoma, breast, pancreatic, and renal cancer. Fig. 3 shows a liver metastasis from uveal melanoma with a multilayer of α-SMA positive MAFs separating the metastasized melanoma cells and the liver parenchyma. Apparently, no direct cell–cell contacts occur between the melanoma cells and hepatocytes. Inflammatory cells and small capillaries are present at the desmoplastic edge. Nests of melanoma cells infiltrating the multilayered MAFs are often seen. Other metastatic growth patterns are the pushing growth patterns where hepatocytes are pushed aside by the metastases or the replacement growth pattern in which metastatic cells infiltrate the liver parenchyma without any disturbance of the preexisting liver structure at the interface. In liver metastasis of colon cancer patients all three growth patterns are observed. Clinical correlation studies suggest that the desmoplastic type of liver metastasis is associated with a better prognosis compared to pushing or replacement patterns [42]. The liver represents the third most frequent site for breast cancer metastasis, following the bone and lung. Despite the evidence that hepatic metastases are associated with poor clinical outcome in breast cancer patients little is known about the molecular mechanisms governing the spread and growth of breast cancer cells in the liver. Intriguingly, the majority of liver metastases from breast cancer have a replacement pattern [43]. Surgical resection of liver metastases represents an important treatment modality. However, experimental xenograft studies show that major liver resection protocols increase TGF-β release and stromal recruitment at wound site presumably responsible for increased metastasis of colon cancer cells [44]. In an alternative scenario, MCC can bring their own soil, including CAFs, from the primary tumor to the lung metastatic site. Efferent blood from tumors contains heterotypic cancer cell–CAF clusters. Passenger CAFs provide continuous prosurvival signals in the circulation leading to increased metastatic seeding and an early growth advantage leading to larger metastatic foci in the ectopic lung site [45].

Pre-metastatic niche preparation not only occurs through released messages by the primary tumor in the blood and lymphatic system but also through activation of sympathetic nerves, which is typical in chronic stress or depression. The bone, a major site of breast and prostate cancer metastasis is richly vascularized, but also abundantly innervated by sympathetic, sensory and glutaminergic nerves. Noradrenalin-releasing sympathetic nerves, activated by brainstem and hypothalamic centers, stimulate the formation of osteoclasts, thus favoring bone resorption. Sympathetic activation is triggered by prolonged or severe emotional stress. It does not affect overall tumor incidence, yet is associated with shorter patient survival and increased recurrence of breast cancer, which suggests that sympathetic activation may contribute to breast cancer metastasis. This hypothesis is supported by mouse models showing that sympathetic outflow stimulates RANKL secretion by osteoblasts and promotes bone colonization by breast cancer cells. In addition, breast cancer metastasis to bone, induced by increased chronic stress or pharmacologic sympathetic activation can be inhibited by the β-blocker propranolol or by knockdown of RANK expression in breast cancer cells [46].

3. Origins of corrupted CAFs

The precursor of pro-metastatic CAFs may be diverse and can be recruited from both local and distant reservoirs. Local tissue-resident precursors are fibroblasts, pericryptal myofibroblasts (gastrointestinal tract), smooth muscle cells, endothelial cells,

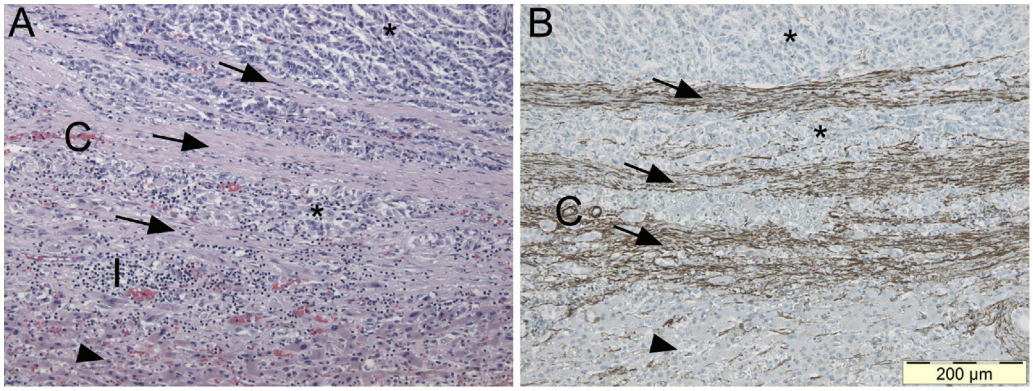

Fig. 3. Photomicrographs showing hematoxylin and eosin staining (A) and immunohistochemical staining of α-SMA (B) of consecutive sections of uveal melanoma metastasis in liver. Desmoplastic tissue (arrow) separates melanoma cells (star) and the liver parenchyma (arrowhead). Inflammatory cells and small capillaries are indicated by I and C respectively (A and B). α-SMA stains desmoplastic tissue, capillaries and Kupffer cells in liver parenchyma (B).

myoepithelial cells (breast), Kuppfer cells (liver) and stellate cells (liver, pancreas) [14]. Mobilization of the adipose tissue (AT) and bone marrow (BM) reservoir may lead to influxes of remote visitors like mesenchymal stem cells (MSCs). In addition, the BM may provide hematopoietic precursors of CAF by releasing CD34+ fibrocytes. Generally, local fibroblasts are considered the primary origin of CAFs and regulator of the desmoplastic reaction, but recent evidence suggests that BM- and AT derived MSCs may also significantly contribute. The stromal vascular fractions of visceral omental and breast AT are rich sources of MSCs [47]. Specifically, approximately 20% of α-SMA positive CAFs originate from BM, whereas non-BM tissues including AT contribute to the remainder [48,49]. In this review we will focus on AT- and BM-derived MSCs as precursors of CAFs. For detailed information about the role of other precursors we would like to refer to the following reviews [14,50].

GFP/RFP-positive BM transplantation models show recruitment of BM-MSC to developing tumors in xenograft, syngenic and transgenic tumor models [48,49,51]. By performing a series of multi-colored BM and AT transplantations prior to syngenic tumor establishment, Kidd et al. [49] were able to quantitate each contribution to CAF populations. BM-MSCs are dispersedly recruited at the periphery of tumors as CAFs expressing the calcium binding domain fibroblast specific protein (FSP or S100A4) and the dipeptidyl peptidase fibroblast activation protein (FAP), whereas the AT-MSCs were found in clusters, associated with vascular structures. These CAFs were defined by expression of α-SMA and the NG2 chondroitin sulfate proteoglycan. In conclusion, once resident in tumors, MSCs derived from AT sources or BM acquire a distinct, specific CAF phenotype in response to signals present within individual micro-environments. The exact combination of such specific cues dictates both the differentiation and activation status of these cells. Next to acting as sources for CAF precursors, AT is a substrate for cancer invasion. Colon cancer infiltration of pericolonic fat and breast cancer infiltration of neighboring AT are independent prognostic factors for disease free and overall survival in multivariate analysis [52,53].

In addition to transgenic/transplantation experiments, both AT- and BM-MSCs have been isolated, cultured and intravenously injected into mice to show that they possess both primary tumor tropism and pro-metastatic capacity [54–58]. Systemic injection of in vitro expanded BM-MSCs modified to express firefly luciferase (ffLuc-BM-MSCs) allows to analyze the biodistribution of MSCs in real time. In healthy mice tail vein-injected ffLuc-BM-MSCs initially reside in the lungs, then egress to the liver and spleen, and finally decrease in signal over time. In contrast, in syngenic and xenogenic breast carcinoma bearing mice, bioluminescence of intravenously delivered ffLuc-BM-MSCs revealed specific up to 29 days, detection at sites of primary tumor development [59]. Unfortunately, in these models it was not investigated if ffLuc-BM-MSCs accumulate at potential pre-metastatic niches.

In addition, human tissue from a male patient who had been transplanted with female BM and later developed esophageal adenocarcinoma allowed to track donor-derived cells into the tumor. Using X = Y fluorescent in situ hybridization (FISH), combined with specific lineage staining directed against epithelial, fibroblast, endothelial, and leukocyte markers, Hutchinson et al. [60] show that approximately 12% of CAF in esophageal adenocarcinoma were derived from female origin.

Complementary models provide substantial evidence that AT- or BM-MSCs are recruited to developing primary tumors and contribute to the genesis of pro-metastatic CAFs. However, are MSCs also recruited to pre-metastatic niches or metastasis? Shinagawa et al. [61] observed that tail-vein injected fluorescently labeled BM-MSCs migrate to liver metastasis induced after intrasplenic transplantation of KM12SM colon cancer cells. Recruited MSCs are dispersed in the tumor stroma and express CAF markers such as α-SMA, FAP and FSP. In agreement, ffLuc-BM-MSC intravenously injected traveled to lung metastases from 4T1 breast cancer cells, they persist and show an increased bioluminescence signal over time. There was no persistent bioluminescence signal in metastasis-free organs [62]. Interestingly, BM-MSCs engineered to overexpress biologicals to target cancer cells show efficiency to bone metastasis from prostate cancer [63], lung metastasis from melanoma [55] and renal cell carcinoma [64] further confirming the tropism of MSCs for established metastatic lesions.

4. Instructive signals for CAF corruption

CAF corruption is at least a three step process. Firstly recruitment of possible distant precursor cells occurs, secondly conversion of ostensibly normal cells into CAF take place. A third signal is the persistence of the CAF presence in the tumor environment. Recruitment of BM-MSCs to primary tumors is mediated through hypoxia [65] and chemokine gradients such as CXCL16 and SDF1 [66–68]. Maintenance of the SDF1 gradient by the primary tumor is independently controlled by both miR-126/miR-126*. This miRNA pair is considered a metastasis suppressor in breast cancer and shows a significantly lower expression in metastatic tissue compared to primary tumor. Metastatic 4T1 breast cancer cells ectopically overexpressing pri-miR-126 show reduced SDF1 secretion and impaired lung metastasis formation through suppression of BM-MSCs recruitment to the primary tumor [68]. SDF-1 synergistically acts with *Helicobacter*-induced inflammation to recruit BM-MSCs [67].

Table 1
Factors implicated in CAF or MAF corruption.

CAF or MAF	Factor	Reference
CAF	Act A	[69]
	MMP7	[70]
	PDGF	[73]
	Prograstin	[72]
	Sonic hedgehog	[74]
	TGF-β	[24,25]
	YAP	[76]
MAF	DDR2 deficiency	[79]
	Exosomal miR-494, miR-542-3p	[87]
	IQGAP-1 deficiency	[80]
	Met exosomes	[88]
	miR-31, miR-214, miR-155	[82]
	Osteopontin	[81]
	TGF-β exosomes	[85]

CAF conversion is mainly mediated through a differential response in TGF-β signaling (see Section 2) and TGF-β family members such as activin A [69] although other factors may play a role as well (Table 1). *Helicobacter pylori* infection causes release of matrix metalloproteinase (MMP)-7 by gastric epithelial cells, consequent degradation of insulin-like growth factor binding protein (IGFBP)-5 leading to a higher bioactivity of IGF-II and increased proliferation of pericryptal myofibroblasts [70]. In addition, IGF-receptor signaling signatures in CAFs are prognostic for breast and lung cancer patients [71]. Progastrin, a biologically active precursor of the hormone gastrin, increases colonic crypt hyperplasia and colon tumorigenesis through increased proliferation of pericryptal colon myofibroblasts in transgenic mice producing high circulating levels of progastrin [72]. PDGF is a potent mitogen for CAF in multiple tumor types [73] and sonic hedgehog signaling, including its Forkhead BoxF1 transcription regulates CAF accumulation in pancreas cancer and lung cancer [74,75].

Mechanotransduction and the YAP transcriptional regulator-dependent matrix remodeling is required for the generation and maintenance of CAFs [76]. Mechanical resistance of the ECM, in conjunction with the action of TGF-β1, is an amply documented stimulus for persistence of myofibroblast in fibrosis [16] and maybe CAFs. Proteoglycans are found abundantly in the ECM. They consist of a core protein which is covalently linked with glycosaminoglycans (GAG)[77]. Depending on their GAG composition, proteoglycans are able to bind different growth factors. Small leucin rich proteoglycans (SLRP) like decorin and biglycan differ from heparan sulfate proteoglycans in that they bind growth factors and other ECM proteins rather with their protein core instead of with their GAG side chains. Interestingly, both decorin and biglycan are able to bind TGF-β. By sequestering a wide range of growth factors, the ECM is able to function as a biological depository [15]. Proteolytic activity causes release of sequestered cytokines from this local reservoir and might create a self-sustaining loop, as TGF-β induced expression of MMPs promotes both the degradation of several ECM components and further release of sequestered growth factors. CAF persistence is also observed in profiling studies where specific signatures of CAF are retained during in vitro passages [12], suggesting a CAF memory that may be mediated by epigenetic modifications in the TGF-β signaling pathway. Methylation-specific digital karyotyping showed higher methylation of *CXorf12* in cultured breast CAFs compared to fibroblasts isolated at a distance from the tumor [78].

The mechanisms of CAF recruitment, conversion and persistence is well described in primary tumors but poorly at metastatic niches. A knock-out mouse model for the fibrillar collagen tyrosine kinase receptor discoidin domain receptor-2 shows more efficient liver metastasis upon intrasplenic injection of MCA38 colon cancer cells. Loss of DDR2 increases the density of α-SMA expressing MAFs in the liver [79]. Experimental metastasis that developed in the DDR2-deficient hepatic microenvironment preferentially grew through a replacement-type mechanism, and contained a sinusoidal-type angiogenesis, supported by hepatic stellate cell-derived MAFs. IQ motif containing GTPase activating protein 1 (IQGAP1) binds TGF-βRII and suppress MAF activation in the liver. IQGAP1 deficiency in hepatic stellate cells promotes MAF activation and metastasis after portal vein implantation of L3.6 human gastrointestinal cancer cells [80]. Orthotopic co-injection of MDA-MB-231 Luc breast cancer cells combined with GFP fluorescent BM-MSCs show an increased BM-MSCs migration to metastatic sites in lung and liver indicated by increased fluorescence activity; this mechanism is both dependent on the ECM protein osteopontin (OPN) and the chemokine CCL5. MSCs retrieved from sites of metastases exhibit OPN-dependent expression of the *a*-SMA, tenascin-c, SDF-1 and FSP-1 [81]. Decreased miR-31 and miR-214, and increased miR-155 collaboratively reprogram normal omental fibroblasts into omental MAFs from patients with serous ovarian cancer. CCL5 is a direct target of miR-214, and CCL5 is functionally implicated in HeyA8 ovarian cancer cell infiltration into Matrigel plugs containing MAFs [82]. HOXA9 homeobox transcription factor expression enables epithelial ovarian cancer cells to stimulate expression of TGF-β2 in peritoneal fibroblast and adipose tissue- and bone marrow-derived mesenchymal stem cells resulting in increased expression of α-SMA [83].

Over the last few years, a novel communication method based on extracellular vesicles (EVs) has been identified [84]. These EVs contain nucleic acids and proteins surrounded by a lipid bilayer, and are secreted by many cell types including cancer cells, CAFs and their precursors. EVs can travel to distant tissues to influence multiple aspects of cell behavior. EVs are defined and sub-grouped on size and on proposed origin. Exosomes are ~30–100 nm diameter and are derived from the endosomal compartment. In contrast, so called microvesicles (>100–1000 nm diameter) are derived from the plasma membrane via shedding. EV proteins, RNA molecules and lipids possess "footprints" resembling the cell from which they were secreted.

EVs produced by cancer cells can alter the function of CAF precursors, triggering a cellular response that promotes metastasis (Fig. 4) [85]. EVs from prostate and mesothelioma cancer cells contain TGF-β at the membrane surface in association with the transmembrane proteoglycan betaglycan. Although existing in a latent state, this complex was fully functional in eliciting SMAD-dependent signaling and α-SMA expression in human primary lung fibroblasts. Many classical TGF-β responses are triggered by TGF-β-positive exosomes in a manner similar to that achieved with soluble non-vesicular TGF-β. Importantly, however, there are some differences in the overall response, as exemplified by changes in FGF2 expression, in which exosomes exert a significantly more potent influence [85]. Also, proteins present on cancer cell-derived EVs stimulate secretion of several proangiogenic factors by CAFs to facilitate angiogenesis via the proliferation of endothelial cells [86].

EV messaging contributes to tumor environment interactions such as immune escape, thrombosis and CAF differentiation, thereby modulating metastatic niche preparation [84]. Exosomes, derived from metastatic rat adenocarcinoma cells BSp73ASML, are recovered in draining lymph nodes after subcutaneous injection, and are preferentially taken up by reticular fibroblasts and lung fibroblasts. Exosomal mRNA and miRNA are recovered in target cells, where transferred miRNA significantly affected mRNA translation. Exosomal miR-494 and miR-542-3p, target cadherin-17 and upregulate MMP transcription in reticular fibroblasts. Thus, cancer cell exosomes target non-transformed cells in pre-metastatic organs and modulate resident pre-MAFs through transferred miRNA. Fitting the demands of metastasizing cancer cells, transferred exosomal miRNA mostly affected

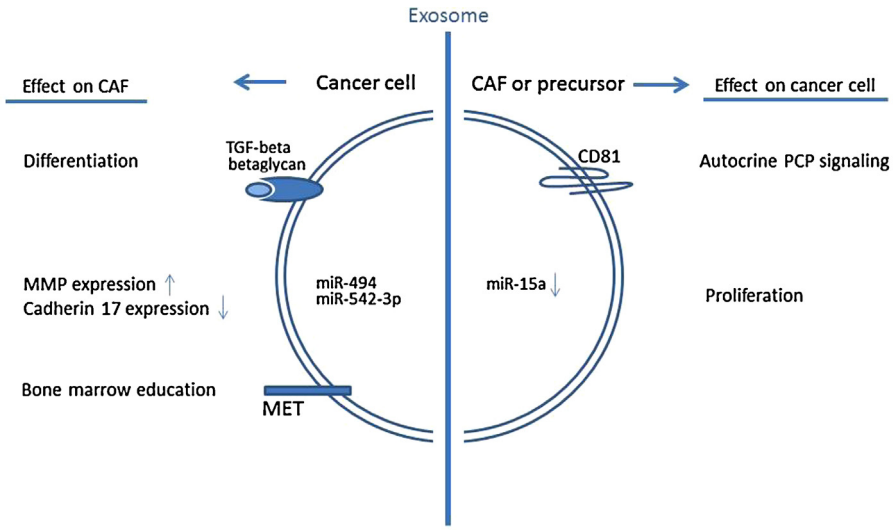

Fig. 4. Schematic representation of an exosome with content and surface molecules at the left side coming from the cancer cells; at the right side coming from the CAFs. MMP, matrix metalloproteinase; PCP, planar cell polarity.

proteases, adhesion molecules, chemokine ligands, cell cycle- and angiogenesis-promoting genes, and genes engaged in oxidative stress response [87].

Exosomes from highly metastatic mouse melanoma B16-F10 cells increased the metastatic behavior of primary tumors by permanently 'educating' bone marrow progenitors by upregulation of the receptor tyrosine kinase MET. Melanoma-derived exosomes induced vascular leakiness, an early event in pre-metastatic niche formation, and reprogrammed bone marrow progenitors toward a pro-vasculogenic phenotype that was positive for the receptor tyrosine kinases c-Kit (also Stem Cell Factor receptor), Tie2 (tyrosine kinase with immunoglobulin and EGF factor homology domains) and MET (also SF/HGF receptor). Reducing MET expression in exosomes by shRNA targeting of MET in melanoma cells, diminished the pro-metastatic behavior of bone marrow cells. Notably, MET expression was elevated in circulating CD45-c-Kit^{low}/TIE2+ bone marrow progenitors from individuals with metastatic melanoma. Rab1a, Rab5b, Rab7 and Rab27a, regulators of membrane trafficking and exosome formation, were highly expressed in melanoma cells. Rab27a RNA interference decreased exosome production, preventing bone marrow education and reducing metastasis [88].

Table 2
Factors implicated in CAF or MAF induced metastasis.

CAF or MAF	Factor	Reference
CAF	CCL2/FGF19	[104]
	CD81 exosomes	[122]
	EGF	[137]
	IGF-1	[137]
	LOX	[96]
	NRG1	[100]
	S100A4	[99]
	SDF-1	[66]
	SF/HGF	[101,102]
	Stanniocalcin	[91]
	TNC	[101]
	Track formation	[114,115]
	Type I collagen alignment	[95]
	Direct cell–cell contacts	[113]
MAF	CXCL10	[65]
	ED-A/ED-B fibronectin	[37]
	IL-11	[40]
	LOX	[38]
	NRG1	[100]
	Periostin	[110]
	SDF1	[80]
	SF/HGF	[80]
	TNC	[111]

5. Operational flexibility in metastasis

Unraveling the life history of a successful metastatic cell reveals a dynamic response of CAFs to a cancer cell fluctuating demands. CAFs continuously shift profitable signals translated as mechano-adhesive dialogs, soluble factors, packaged factors (vesicles), track generation and direct cell–cell contacts at the different steps of the metastatic cascade including invasion, ectopic survival, stimulation of angiogenesis, adhesion at the metastatic niche and macroscopic metastasis formation (Table 2). Induction of invasion by CAFs can be EMT dependent or independent. Key determinants of EMT are a reduction of epithelial cell–cell adhesion via the transcriptional repression of cadherins in combination with the acquisition of mesenchymal properties [89,90]. CAFs induce EMT and ultimately metastasis of the murine prostate cancer cell line RM1 through secretion of SDF1 [66]. PDGF-stimulated fibroblasts secrete stanniocalcin-1, a glycoprotein that was originally described to modulate mineral metabolism, and stimulate invasion of colorectal cancer cells through upregulation of vimentin. In an orthotopic mouse model of colorectal cancer, tumors formed in the presence of stanniocalcin-1-deficient fibroblasts displayed reduced intravasation of cancer cells along with fewer and smaller distant metastases formed in lymph nodes, peritoneum, diaphragm and liver and lungs [91]. CAF maintain the mesenchymal phenotype in breast cancer cells by producing linear bundles of ECM [92]. This radial alignment of type I collagen fibers relative to tumors facilitates local invasion and correlates with poor disease-free survival [93,94]. Interstitial flow and enzymes, such as LOX, modify the ECM [95,96]. LOX and LOX-like 2 (LOXL2) produced by CAFs [96,97] promote cross-linking of fibrillar type I collagen and results in matrix stiffening and consequent mechanotactic cues that support cancer cell invasion through focal adhesion maturation and actomyosin contractility [98]. Non-EMT induced invasion includes S100A4 produced by liver CAF in hepatocellular carcinoma [99] and soluble neuregulin-1 (NRG-1) produced by MSCs and CAFs in colon cancer [100]. SF/HGF stimulates stemness and invasion of colon cancer cells only when the appropriate context, e.g. suppression of RhoA activity by tenascin C, is provided by CAFs [101,102]. FAP-positive

CAF increase invasion of colon cancer cells through FGF1/FGFR3 signaling [103]. Colon CAFs secrete a combination of at least two complementary proteins, CCL2 and FGF19. CCL2 induces FGFR4 in RKO and HCT116 colon cancer cell causing FGF19 mediated EMT, local invasion and liver metastasis. Immunohistochemistry of consecutive sections in more than 30 cases revealed a significant positive correlation between epithelial FGFR4 and CCL2 expression in CAFs [104]. A differential secretome approach between BM-MSCs, TGF-β1-treated BM-MSCs and colon CAFs enabled us to identify multiple factors potentially implicated in cancer cell invasion, survival and adhesion including cytokines and chemokines such as growth and differentiation factor (GDF)-15, CXCL2, CXCL6 and CCL5 and matrix proteins such as secreted protein and acidic and rich in cysteine (SPARC), galectin-1, and ED-A fibronectin [105]. Some of these proteins are associated with disease progression, e.g. in patients with DCIS of the breast, stromal SPARC expression is associated with disease recurrence [106]. Angiogenesis at the primary and metastatic site may be induced through secretion of vascular endothelial growth factor (VEGF)-A, SDF-1 and thrombospondin-1 by CAFs or MAFs [22,107,108]. A successful invader must survive in an ectopic environment and MAFs provide advantageous signals to accomplish survival through AKT and signal transducer and activator of transcription (STAT)3 activation in the arriving MCCs. NRG-1 secreted by BM-MSCs increases colorectal cancer cell number through HER2/HER3-dependent activation of the PI3K/AKT/BAD pathway. Immunohistochemical data demonstrates that transmembrane NRG-1 is expressed in CAF in primary colorectal cancer and MAFs in liver metastasis, with the strongest expression in the immediate vicinity of HER2/HER3 expressing cancer cells. Moreover, high transmembrane NRG-1 expression was significantly associated with advanced tumor stage, invasion depth and decreased 5-year progression-free-survival [100]. Secretion of IL-11 by TGF-β-stimulated MAF triggers GP130/STAT3 signaling which suppresses apoptotic stimuli encountered during the colonization of the liver and lung metastatic sites [40]. Accumulation of STAT3 phosphorylation at tyrosine 705 in primary colorectal cancer samples was associated with a significantly higher colorectal cancer-specific mortality [109]. DDR2 deficiency fosters MAF expression of IL-10, TGF-β, VEGF, BMP-7 and syndecan-1, which is further exacerbated by soluble factors derived from MC38 colon cancer cells [79]. The DDR2 deficient MAF expression signature collaboratively impacts on survival, proliferation, angiogenesis, matrix production, and immune suppression. In anoikis assays the secretome of IQGAP1-knockdown hepatic stellate cells conferred a greater protection to MC38 mouse colon cancer cells and higher pro-migratory activity to HT29 human colon cancer cells most probably through increased expression of SF/HGF and SDF1 [80]. S100A4-positive MAFs in the lung provide tenascin C to facilitate metastatic colonization of 4T1 mouse breast cancer cells through protection from apoptotic stress [107]. Another ECM molecule, periostin, is deposited by α-SMA positive MAFs in the lungs of MMTV-PyMT transgenic mice. Periostin and MAFs are most probably upregulated by TGF-β2 or -β3 secretion by incoming metastatic breast cancer stem cells [110]. Blocking periostin by antibody treatment prevents metastasis. Both periostin and tenascin C may stimulate survival of incoming cancer stem cells by increasing Wnt signaling [110,111]. Pull-down assays confirmed interaction between periostin and Wnt1 and Wnt3A [110]. In the Rip1-Tag2 (RT2) model of pancreatic β-cell carcinogenesis a higher number of lung micrometastasis is observed upon overexpression of tenascin C most probably through tenascin C-mediated downregulation the Wnt inhibitor DKK1 (dickkopf1) [111]. Hypoxia-mediated bidirectional signaling between MSCs and breast cancer cells leads to the increased secretion of placental growth factor in breast cancer cells and increased secretion of CXCL10 in MSCs leading to a higher metastatic burden in lymph nodes and lungs [65]. Unidentified factors from liver MAFs stimulate Matrigel invasion of DLD1 colon cancer cells to a greater extent than matched primary CAFs suggesting that the metastatic microenvironment induces more efficiently aggressiveness than the primary microenvironment [112]

The CAF network may serve as a guidance structure to direct invasion by direct cell–cell contacts and induce long term epigenetic silencing through promotor methylation of the cysteine proteinase inhibitor cystatin M in breast cancer cells [113]. Individual CAF create tracks in the type I collagen rich meshwork through both proteolytic and force-mediated modifications [114,115]. Force-mediated track formation depends on α3- and α5-integrin, and Rho-mediated regulation of myosin light chain and LIM kinase activity [114,115]. Cytokine signaling through the Janus tyrosine kinase (JAK)1-STAT3 can generate actomyosin contractility in CAFs. Strikingly, actomyosin contractility itself positively modulates activity of the transcription factor STAT3 downstream of JAK1, demonstrating positive feedback within the signaling network [116]. Caveolin-1 (Cav-1), the major component of endocytic caveolae plasma membrane invaginations, is also implicated in regulation of p190RhoGAP and promotes Rho- and force-dependent matrix remodeling. Cav-1-deficient mouse embryonic fibroblast have disorganized ECM architecture and fail to support breast cancer cell invasion and metastasis in orthotopic mouse models. Stroma in human breast, kidney, and colon carcinomas and melanoma metastases are enriched in Cav-1-expressing CAFs, suggesting that stromal Cav-1 might be instrumental in both primary and secondary tumor niches [117]. In contrast to these impressive data is a study of Simpkins et al. [118] showing that loss of Cav-1 in breast cancer CAFs predicts poor outcome. At a functional level, Cav-1-deficient CAFs significantly increase the invasive capacity of breast cancer cells. Differences in model systems (transgenic mouse embryonic fibroblasts versus primary human CAFs) and the use of antibodies from different sources recognizing distinct epitopes may account for these contrasting results.

In vesiclepedia (http://www.microvesicles.org), a community compendium for extracellular vesicles, only two studies focused on the identification of the proteome of CAF precursor exosomes [119]. Comparative proteomic analysis identified several novel proteins for embryonic mouse NIH3T3 fibroblast exosomes including Serpin B6. Over 34 proteins including alpha 1 chain of type I collagen and milk fat globule EGF factor 8 (lactadherin) accumulated in EV's from Ras-transformed fibroblasts [120]. LC–MS/MS analysis of EVs from MSCs identified 730 proteins. The MSC-EV proteome included five positive and two variable known markers of MSCs, but no negative marker, as well as 43 surface receptors and signaling molecules controlling self-renewal and differentiation of MSCs. Functional enrichment analysis showed that cellular processes represented by the MSC-EV proteins include cell proliferation, adhesion, migration, and morphogenesis [121].

Exosomes secreted by fibroblasts promote breast cancer cell motility and metastasis by stimulating the autocrine Wnt-planar cell polarity (PCP) signaling pathway. Genetic screens in Drosophila pioneered the discovery of core PCP factors, which controls tissue polarity whereby cells orient themselves within a plane perpendicular to the apical-basal axis [122]. Conditioned medium of mouse AT-derived fibroblasts (L cell fibroblasts) stimulated protrusive activity and motility in multiple breast cancer cell lines including MDA-MB-231. Knock-down of PCP regulatory proteins such as the cytoplasmatic protein Prickle (Pk)1 in MDA-MB-231 cells counteracted fibroblast-induced effects. Co-injection of L cell fibroblasts with MDA-MB231 cells, stably knocked-down for Pk1, into the mammary fat pad of mice resulted in a decreased formation of lung metastatic foci. Pk1 knock-down however did not influence primary tumor growth suggesting that PCP signaling specifically regulates metastasis. Knock-down of Wnt11, a major regulator of the PCP signaling pathway, in MDA-MB-231 cells blocked

fibroblast-induced protrusive activity and motility. Fractionation of the fibroblast-activated conditioned medium by size exclusion chromatography followed by mass spectrometry, identified CD81-positive exosomes that were solely sufficient to stimulate breast cancer cell protrusive activity and motility. Tetraspanin CD81 is significantly enriched in CAFs associated with primary invasive breast cancer and CD81-positive exosomes are required to establish breast cancer metastasis. CAF-secreted CD81-positive exosomes are internalized by endocytosis in breast cancer cells and associate with autocrine Wnt11, which is present in endosomes for maturation and long-range signaling, in perinuclear endosomal structures to activate PCP signaling.

6. Awakening from metastatic dormancy

Late metastasis is a major cause of cancer-specific death as it occurs in malignant tumors such as mammary cancer and prostate cancer. Late metastasis is ascribed to awakening from metastatic dormancy. MCC escape from the primary tumor before its complete removal, they home at distant sites as disseminated cancer cells (DCC), or they establish micrometastases but in both cases fail to grow into overt metastases. Such isolated cells or small groups of cells may start growing years after successful treatment of the primary tumor, form overt metastases and kill their host. Awakening may be due to alterations of the environment (the soil) or of the cancer cells (the seed). At the time of awakening, cancer cells may be at the putative site of overt metastasis, termed pre-metastatic or metastatic niche, and start growing because of changes in this niche. Alternatively, they may move in from other sites. The latter scenario may implicate that DCCs initiate an overt metastasis because they are attracted to a niche that supports their growth. Wound healing tissue presents an example of such a chemo-attractant and growth-promoting niche [123]. Crucial mediators of awakening may be produced at the niche and act locally or elsewhere and act systemically. The clinically and mechanistically most relevant question is: which events are responsible for awakening? Possibilities include inflammation and tissue traumata because they sustain high levels of growth factors and cytokines [124].

CAFs and the ECM they produce are key components as they are present from the onset of niche formation [125]. They may intervene in the awakening of such dormant cancer cells directly, e.g. producing soluble or matricellular signals that activate pathways of proliferation, or indirectly through angiogenesis or immune suppression [126]. Dormant cells have a low extracellular regulated kinase (ERK) to p38 mitogen-activated protein kinase (MAPK) ratio, whereas in proliferating cells this ratio is high at the site of metastasis in nude mice and chick chorioallantois membrane models, ERK is activated by the environment [127]. ERK is part of the signaling pathway of many growth factors, some of which are produced by CAFs [125]. An example is SDF-1 that binds the CXCR4 receptor activating trimeric G proteins and consequently stimulating the Ras-ERK pathway.

There is little direct experimental or clinical evidence for a causal role of CAFs or MAFs in the awakening from metastatic dormancy. There is, however, abundant circumstantial evidence to accept that such a role does exist. For example, normal BM-stromal cells cause a dormant state in breast cancer cells infiltrating the bone. Several miRNAs including miR-127, -197, -222, -223, targeting the SDF1 chemokine are transmitted from BM stroma to breast cancer cells via gap junctions and exosomes [128]. Reduced SDF1 levels lead to cell cycle arrest in G_0. To further explore this circumstantial evidence, we have considered the molecules that are implicated in the interaction between CAFs and cancer cells as well as between CAFs and other tumor-associated host cells [129]. Each of these molecules "AND dormancy" were entered as search terms in PubMed. The more relevant data were found for TGF-β, and OPN.

At the early stages of tumor development, TGF-β acts as an inhibitor of invasiveness, whereas at later stages it acts as a promoter [130]. In the D2.OR/D2A1 mammary cell line model, the induction of fibrosis in the lung by an adenoviral vector expressing TGF-β1, induces dormant D2.OR cells to form proliferative metastatic lesions [131]. Fibrosis is associated with deposition of fibroblast-produced type I collagen in the metastatic environment to which the intravenously injected cancer cells are homing (experimental metastasis). The signaling pathway of type I collagen investigated using a 3D culture system for modeling dormancy and recapitulating the metastasis proliferation switch included β1-integrin mediated activation of SRC, focal adhesion kinase (FAK), ERK and Myosin Light Chain (MLC) phosphorylation by MLC-kinase and consequently actin stress fiber formation. When extrapolating these findings to awakening from dormancy in late metastasis it should be noted that in the model the environmental changes precede the colonization by the cancer cells, a situation that mimics DCCs staying dormant away from the site of overt metastasis at the time of awakening. Similarly, Cox et al. [132], using the orthotopic 4T1 mammary cancer model, described the pro-metastatic effect of radiation- or bleomycin-induced lung fibrosis upon LOX-mediated type I collagen crosslinking. The putative mechanism is cancer cell colonization and outgrowth rather than awakening of dormant metastasis. Dimethylnitrosamine-induced liver fibrosis also leads to increased metastasis from 4T1 mammary cancer. In both liver and lung, α-SMA-positive CAFs served as the major source of LOX.

By contrast but in agreement with the reputation of the TGF-β superfamily, their secreted antagonist Coco (also termed Dante) reactivates breast cancer cells at lung metastatic sites [133]. In the metastasis assay with four different cell types either noninvasive (67NR), metastatic to lymph nodes (168FARN), micrometastatic (4TO7) or macrometastatic (4T1), Coco overcomes the inhibitory action of bone matrix proteins (BMP) that 4TO7 cells encounter upon infiltrating the lung and that keeps these cells dormant. The activity of Coco is organ-specific as it fails to act in bone or brain. The multifunctional latent TGF-β binding protein (LTBP2) promotes dormancy in nasopharyngeal cancer cell lines presumably through decreased CAF adhesion to fibronectin [134].

Mc Allister et al. [135], using xenografts, showed that human breast carcinomas, called instigating tumors, secrete OPN (or bone sialoprotein, BSP) which induces the expression of granulin in a subpopulation of hematopoietic BM cells [136]. These BM cells are activated and mobilized into the secondary sites where the, otherwise indolent, responder cancer cells lay. There they facilitate growth by inducing α-SMA positive CAFs. CAFs provide an environment enriched for EGF and IGF-1 which enhances expression of transcription factors Oct-4 and Zeb-1 associated with pluripotency, proliferation, and EMT [137]. This does not explain awakening of dormancy at sites of metastasis in absence of an instigating tumor, but there exist other sources of OPN, a component of bone (osteoblasts). Hypocalcemia and hypophosphatemia stimulate secretion of OPN. It is part of the systemic inflammatory response after polytrauma (bone plus soft tissue) [138]. Osteoporotic vertebral fractures in postmenopausal women are associated with higher serum levels of OPN as well [139]. Interestingly, senescence of fibroblasts is associated with an increase in OPN secretion and this causes invasion of associated human mammary epithelial cells in vitro [140].

7. Impact of therapy-activated CAFs on metastasis

There are few direct experimental or clinical data for a causal role of therapy-activated CAFs or MAFs in metastasis. Abundant

circumstantial evidence indicates, however, that such a role does exist. Cancer management protocols comprise elements that influence recruitment of CAF precursors to tumors. Irradiated tumors, compared to unirradiated ones, show an increase in MSC recruitment [141,142]. This was demonstrated by bilateral hind leg breast tumor implants: one was left untreated, whereas the other was irradiated by 2 Gy before intravenous injection of MSCs. At 48 h postirradiation, more MSCs were detected in the irradiated than in the unirradiated limbs. In unirradiated tumors, MSCs were more commonly associated with intravascular or perivascular structures, whereas in irradiated tumors, MSCs were present in higher proportions in the tumor parenchyma [141]. MSC migration to irradiated tumors may result from a dynamic interplay in which cancer cells release cytokines or exosomes in response to radiation, leading to chemokine receptor upregulation on MSCs in the bone marrow, and ultimately resulting in enhanced attraction toward the tumor. The consequent production of MSC-derived factors may contribute to relapse and metastasis. Cancer treatment may also activate CAFs. Irradiation converts fibroblasts into α-SMA-positive myofibroblasts and stimulates pro-invasive activity [143]. Pancreatic cancer cells are more invasive into Matrigel in vitro or after orthotopic implantation in vivo when they are cocultured with 10 Gy irradiated as compared to non-irradiated pancreatic CAFs. Unfortunately, the authors did not investigate the potential impact on metastasis in their model. The secretome of 10 Gy irradiated CAFs contained higher levels of SF/HGF compared to non-irradiated CAFs. In addition, cellular scattering of Suit-2 pancreatic cancer cells is stimulated by the secretome of irradiated CAFs in a SF/HGF dependent way [144]. Irradiation of mouse embryo fibroblasts stimulates cancer cell repopulation in cell culture and in xenograft models [145]. This effect is lost when the fibroblast are deficient for caspase-3, a key executioner of programmed cell death. These data suggest a cell death-mediated tumor repopulation pathway in which caspase 3 regulated release of prostaglandin E2 plays a major role. Comparative analysis of matched colorectal cancer specimens shows that neoadjuvant chemotherapy results in increased presence of α-SMA-positive CAFs [146]. FOLFOX-treated colon cancer-derived CAFs promote self-renewal of cancer-initiating cells more than non-treated CAFs. FOLFOX-treated CAFs show a higher release of TGF-β and IL-17A. TGF-β is probably responsible for increased abundance of CAFs. IL-17A regulates self-renewal of cancer-initiating cells and increased expression in CAFs from neoadjuvant treated colon cancer patients is confirmed in three independent cohorts. Neutralizing IL-17A improves chemotherapy response of established xenografts from a coinjection of colon cancer initiating cells and CAFs. In conclusion, it is well known that breast conserving surgery followed by neo-adjuvant radiotherapy and adjuvant (chemo)radiotherapy in colorectal cancer minimizes the risk for local relapse. However, some experimental data point to the potential increased efficacy of cancer management protocols by combining c-met or caspase-3 inhibitors in radiotherapy and IL 17A antagonists in FOLFOX chemotherapy [146]. Although most of the experimental data are focused on primary tumor growth, further, research is certainly warranted to analyze whether or not some elements of cancer management protocols influence metastasis through an impact on CAFs or MAFs.

8. Liquid biopsies to detect CAF reactions

Needle aspirates of extracellular body fluids ("liquid biopsies") offer a minimally invasive approach to cellular and molecular witnesses of the involvement of CAFs in cancer progression. Furthermore, sequential liquid biopsies, mostly blood samples, allow dynamic monitoring of circulating tumor cells, exosomes and soluble protein markers. While literature data on these three elements mainly apply to the actual cancer cells, we will focus hereafter on the possible contribution of CAFs since the stromal response to a primary tumor or a metastasis in a given organ is probably more consistent among different patients.

Firstly, elevated numbers of circulating MSCs along with very small embryonic-like stem cells (VSELs) are observed in pancreatic cancer [147]. In colorectal cancer elevations of both circulating MSCs and CD34bright leukocytes (CD34b LCs) can be found [148]. Interestingly, circulating MSCs were more elevated in obese than in lean colorectal cancer patients. The authors suggest that these cells are mobilized from adipose tissue, and offer an explanation for the association between obesity and cancer progression. Based on the altogether very low levels of circulating stromal precursors in cancer, the interpretation of these cell concentrations in blood has been difficult. Recently, however, it was shown that circulating murine fibrocytes recruit secondary effector cells (Ly-6C$^+$ monocytes) to prepare pre-metastatic lung niches via the chemokine CCL2 [149]. These data indicate that the circulating fibrocytes are not necessarily the tissue-infiltrating cells.

Secondly, compared to circulating cancer cells the track record of circulating exosomes in cancer is much more recent. Yet, isolated reports on plasma membrane shedding via so-called koinozymes by cancer cells into the circulation were noticed as early as in the mid seventies of the previous century [150,151]. More recently, cancer cell-derived GFP-labeled exosomes were transplanted orthotopically in mice and depicted soon after in the circulation, which underpinned the rationale for exosome detection via liquid biopsies [152]. Until now, many reports have indeed dealt with elevated circulating levels of exosomes in the blood from patients suffering from colorectal [153], ovarian, lung carcinoma and glioblastoma (reviewed in [154]). Not only the enumeration of these exosomes, but also their miRNA-based profiling were correlated with tumor progression of prostate [155] and ovarian carcinoma [156], and their proteomic profiling with melanoma [88] and breast carcinoma [157]. No reports dealing with circulating CAF exosomes specifically are available, although early assessment of such exosomes containing MAF-specific miRNAs could be clinically relevant to monitor the development of metastatic niches.

Conceptually, the introduction of circulating exosomes into the clinic can profit from some intrinsic advantages, such as biological stability and the option of enrichment from liquid biopsies. These features, as a result of the technical amplification possibility of their mRNA and miRNA contents, push exosomes forward as exceptionally sensitive and specific circulating marker candidates for monitoring both cancer cell and CAF/MAF contributions to metastasis formation. In addition, exosomes have been detected not only in blood, but in urine, sperm, milk, pleural effusion, ascitic fluid and saliva as well, and their presence was correlated with locoregional cancers [154,158]. Much attention, however, has to be paid to the preanalytical variables of these liquid biopsies to obtain reliable and clinically relevant data. For this reason standardization is awaiting for collection and centrifugation of the samples [159], prevention of loss of exosome surface markers [160] and proper quality control of the vesicle size [161].

Thirdly, soluble metastasis markers that are related to stromal precursors, and which circulate in cancer patients, have been assessed in liquid biopsies. So, in a number of breast carcinoma studies elevated levels of circulating TGF-β have been confirmed to be associated with worse survival [162] and distant dissemination of circulating cancer cells [163]. This result could, however, not be reproduced in another breast cancer study [164]. When facing discrepant results in clinical TGF-β studies, it should be realized that pre-analytical protocols for plasma assays are very demanding. In locally advanced non-small cell lung cancer, decreasing circulating TGF-β levels under radiotherapy predict favorable outcome [165]. Stanniocalcin-1 appears to be a recent new marker molecule for

non-small cell lung cancer that circulates at higher levels in cancer than in patients with benign lesions and persons with no evidence of disease [166].

Type I Collagen turnover can be monitored in cancer patients via circulating propeptides, indicating collagen assembly (PICP, PINP), and circulating breakdown products (ICTP). In breast cancer patients elevated levels of circulating PINP were found and were related to metastasis [167]. It is, however, questionable whether these markers reflect collagen metabolism of CAFs, or chiefly assess bone remodeling at the metastatic sites [168]. As a circulating metastasis marker ICTP has proven to be superior to other type I collagen markers, as was shown for different cancer types: breast, prostate, lung and urinary bladder carcinoma [169]. Not only collagen type I markers have been studied in the circulation, other types were recently evaluated as well. Elevated collagen type IV concentrations were detected in patients with pancreatic carcinoma [170] and with colon carcinoma with liver metastases [171]. The source was traced back to the tumor-associated stroma and the hepatic desmoplastic regions respectively. Circulating type VI collagen is another stromal marker in pancreatic carcinoma [172] and melanoma [173].

Finally, a recently described patient-derived xenograft model ('xenopatients') closely mimicks disease response in humans and may provide a preclinical platform for additional biomarker discovery, for the generation of predictive classifiers for better patient stratification and for testing novel investigational therapies [174].

9. Conclusion and future perspectives

Accumulating evidence shows a crucial role for CAFs in metastasis. However, there still exist a significant ambiguity with respect to the identification of CAFs in cancer. Future work should therefore aim to define the function, identities and molecular profiles of distinct CAF subtypes in human cancer biopsies from primary tumors and from metastasis before, during, and after therapy. We have poor knowledge about the similarities or differences between CAF function in primary tumor, pre-metastatic niche and metastasis. Therefore animal models should be carefully designed to allow early identification and to elucidate the role of CAFs in metastatic niches (so called MAFs). The origin of CAFs remains controversial although fibroblasts and MSCs from BM and AT reservoirs are considered to be the main progenitor cells. Our current knowledge indicates a cacophony of mutual signals between cancer cells and CAFs which provide operational flexibility in metastasis. However, we must keep in mind that the oncogenic background of the cancer cell decides which of these multiple environmental signals become translated into a set of well-defined fate choices favoring metastasis. The implementation of systems biology will further learn us how these biological regulatory networks enable this information processing. Awakening from metastatic dormancy is a major cause of cancer-specific death. CAFs may induce this awakening through mechanisms which are poorly described. Whether cancer management protocols influence metastasis is an intriguing question. Specific animal models focusing on CAF-related activities may provide further clues. CAF-specific signatures present in exosomes may detect and monitor CAF reactions, and possibly MAF formation allowing optimized prognostic values. CAFs and their products may provide new therapeutic directions in metastasis focusing on ecosystem resilience.

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