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REVIEW AND PERSPECTIVES

Cancer-associated fibroblasts as target and tool in cancer therapeutics and diagnostics

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Abstract Cancer-associated fibroblasts (CAFs) are drivers of tumour progression and are considered as a target and a tool in cancer diagnostic and therapeutic applications. An increased abundance of CAFs or CAF signatures are recognized as a bad prognostic marker in several cancer types. Tumour-environment biomimetics strongly improve our understanding of the communication between CAFs, cancer cells and other host cells. Several experimental drugs targeting CAFs are in clinical trials for multiple tumour entities; alternatively, CAFs can be exploited as a tool to characterize the functionality of circulating tumour cells or to capture them as a tool to prevent metastasis. The continuous interaction between tissue engineers, biomaterial experts and cancer researchers creates the possibility to biomimic the tumour-environment and provides new opportunities in cancer diagnostics and management.

Keywords CAFs · Tumour-environment · Biomimetics · Diagnostics · Therapy

Introduction

In Europe, cancer is the most important cause of death and morbidity after cardiovascular diseases [78]. In the majority of the cases death occurs after the primary tumour has metastasized. Treatment fails in these patients because metastasis is often resistance to chemo/radio therapy and difficult to resect. Metastasis is the culmination of persistent and systemic signal trafficking between genetically changed cells (the cancer cells) and their environment. Cancer cells recruit and corrupt various normal cell lineages of the host to assist them to colonize distant organs. Intriguingly, this absolute dependency of cancer cells upon collaboration with ostensibly normal stromal cells of specific organs reveals diagnostic and therapeutic opportunities.

Cancer cells are embedded in an extracellular matrix (ECM) scaffold populated by cancer-associated fibroblasts (CAFs), vascular space-related cells (endothelial cells, pericytes and smooth-muscle cells) and diverse innate and adaptive immune response cells (lymphocytes, macrophages and mast cells). CAFs, also referred to as peritumoural fibroblasts, activated fibroblasts, myofibroblasts or tumour-associated mesenchymal cells, contribute significantly to important hallmarks necessary for cancer progression such as invasion and metastasis, immune escape, inflammation, angiogenesis and sustained growth [102]. CAFs have pro-invasive and pro-metastatic activities which are different from their precursor cells, and attempts are made to use markers to discriminate CAFs from their precursors. Expression of α-SMA (α-smooth muscle actin), FAP (fibroblast activation protein) and podoplanin are commonly used as CAF markers but do not necessarily indicate the pro-invasive/pro-metastatic status of the CAFs [23]. However, using these markers CAFs are recognized as a bad prognostic factor in numerous tumour types from different origins (breast, colorectal etc. (see Table 1)). The formation of secondary tumours in distant organs requires the metastasis-competent cancer cells to recruit and interact with local stromal cells, including tissue resident fibroblasts. Fibroblasts associated with the metastasis may

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Table 1 Overview of immunohistochemistry studies with CAFs as a prognostic marker

CAF marker	Cancer	n	Quantification	Number of observers	Agreement (kappa value)	Cut-off	Results	Ref.
Lung carcinoma								
α−SMA	NSCLC	78	SQ	2	Not precised	Score defined by the multiplication between the positive cells % and intensity	High expression of αSMA associated with poor OS	[16]
FAP-α	NSCLC	59	SQ	2	Not precised	grade 0: <1 % grade 1: 1–10 % grade 2: 11–50 % grade 3: >50 %	High expression of FAP-α associated with poor OS	[64]
Podoplanin	NSCLC	106	SQ	2	Not precised	Negative: <10 % Positive: ≥10 %	Presence of podoplanin-positive CAFs associated with lower overall response rate to EGFR-TKIs	[119]
	NSCLC	87	SQ	2	Not precised	Negative: <50 % Positive: ≥50 %	Presence of podoplanin-positive CAFs associated with poor PFS	[58]
	NSCLC	142	SQ	2	Not precised	Negative: <50 % Positive: ≥50 %	High podoplanin expression in CAFs associated with poor OS and DFS	[77]
	NSCLC	304	SQ	2	Not precised	Negative: <10 % Positive: ≥ 10 %	Presence of podoplanin-positive CAFs associated with poor RFS	[49]
	NSCLC	211	SQ	not precised	Not precised	Score defined by the sum of positive cells % and intensity	Presence of podoplanin-positive CAFs associated with poor OS	[57]
	NSCLC	177	SQ	not precised	Not precised	Negative: <10 % Positive: ≥ 10 %	Presence of podoplanin-positive CAFs associated with poor OS	[53]
Breast carcinoma								
α−SMA	DC, LC, others	60	Q			Low: <8,48 % High: ≥8,48 %	High expression of αSMA associated with poor OS and RFS, higher expression of αSMA in the metastatic group compared to the non-metastatic	[116]
	DC	45	SQ	2	Not precised	Negative 1: <10 % 2: 10–30 % 3: >30 %	High expression of aSMA associated with poor OS and RFS	[99]
FAP-α	DC	112	SQ	2	2 discordant cases	Negative or weakly positive Focally positive: <10 % Positive: 11–50 % Highly positive: >50 %	High expression of FAP-α associated with long OS and RFS	[4]
Podoplanin	DC, LC	367	SQ	2	Not precised[£]	Negative: <10 % Positive: ≥ 10 %	Presence of podoplanin-positive CAFs associated with poor OS and RFS, correlation with higher histological grading	[92]
	DC	117	SQ	2	Not precised	Positive: >10 % Grade 1: 10–50 % Grade 2: >50 %	Correlation between podoplanin-positive CAF and tumour size, grade of malignancy, lymphovascular invasion, Ki67 in cancer cells	[81]
Esophageal carcinoma								
α−SMA	ADC	183	SQ	2	Not precised	Positive: ≥5 %	Presence of αSMA-positive CAFs associated with poor OS	[106]
α−SMA/FSP1/PDGFR	SqCC	116	SQ	2	Not precised	Negative Positive	Presence of CAFs associated with poor OS and DFS	[35]
Podoplanin	ADC	200	SQ	2	Not precised	Positive: ≥10 %	Presence of podoplanin-positive CAFs associated with poor OS and RFS, correlation with tumour stage, lymphovascular invasion, lymph node metastasis	[93]

Table 1 (continued)

CAF marker	Cancer	n	Quantification	Number of observers	Agreement (kappa value)	Cut-off	Results	Ref.
Colorectal carcinoma								
α−SMA/FAP/FSP1	ADC	289	SQ	2	Not precised	Low High	High expression of αSMA associated with poor OS and RFS	[40]
α−SMA	ADC	192	Q			Low: <5,55 % High:≥5,55 %	High expression of αSMA associated with poor RFS	[104]
	ADC	98	Q			Low: <50 % High: >50 %	High expression of αSMA associated with poor RFS	[110]
FAP-α	ADC	488	SQ	1	Not precised	Negative Low : 1 + Moderate :2 + High: 3 +	High expression of FAP-α (tumour centre) associated with poor OS	[114]
	ADC	138	SQ	2	Not precised	Negative: <1 % grade 1: 1–10 % grade 2: 11–50 % grade 3: >50 % and intensity (none, weak, intermediate, strong)	High expression of FAP-α associated with poor OS in metastatic patient	[39]
Podoplanin	ADC	120	SQ	2	Not precised	Negative: <30 % Positive: ≥30 %	Negative podoplanin expression associated with poor RFS	[115]
Vimentin	ADC	142	Q			Low: <8,8 % High: ≥8,8 %	High expression of vimentin associated with poor OS and RFS	[76]
Pancreatic carcinoma								
α−SMA	ADC	162	SQ	2	Not precised	Negative Weak Moderate strong	High α-SMA expression in tumour stroma associated with poor OS and RFS	[96]
FAP-α	ADC	70	SQ	2	>95 %	% positive cells intensity (0–3+)	High expression of FAP-α associated with poor OS, RFS and metastatic lymph node	[17]
Podoplanin	ADC	105	SQ	2	Not precised	Negative: ≤ 30 % Positive: >30 %	Presence of podoplanin-positive CAFs associated with poor OS and RFS	[95]
Head and neck carcinoma								
α−SMA	SqCC	50	SQ	not precised	Not precised	Poor Medium rich	High stromal αSMA expression associated with poor OS, correlation with metastatic lymph node	[27]
	SqCC	108	SQ	not precised	Not precised	Negative: 0 Scanty:1 Focal: 2 Abundant : 3	Grade 2 patients associated with a poor OS	[30]
	SqCC	282	SQ	2	95 %	Negative: <5 % Moderate: 5–50 % High: >50 %	High stromal αSMA expression associated with mortality regardless of disease stage	[69]
	SqCC	77	SQ	not precised	Not precised	Poor Medium Rich	Association between increased CAF density and mortality	[6]
	SqCC	50	SQ	not precised	Not precised	Low: 0/0.5/1 High: 2/3	Presence of CAF is an independent adverse effect of local recurrence	[109]
	SqCC	84	SQ	not precised	Not precised	Negative Positive	Presence αSMA associated with poor OS	[54]
	SqCC	83	SQ	3	Not precised	Negative: 0 Scanty:1 Abundant : 2	Abundant stromal αSMA expression in the tumour front associated with poor OS, correlation with N stage, vascular and lymphatic invasion	[55]

α-SMA α-smooth muscle actin, *FAP* fibroblast activation protein, *FSP1* fibroblast specific protein 1, *PDGFR* platelet-derived growth factor receptor, *NSCLC* non-small-cell lung carcinoma), *DC* ductal carcinoma, *LC* lobular carcinoma, *ADC* adenocarcinoma, *SqCC* squamous cell carcinoma, *SQ* semi quantitative, *Q* quantitative, *OS* overall survival, *PFS* progression-free survival, RFS relapse-free survival

have other functional activities compared to CAF present at the primary tumour.

The vast majority of in vitro studies in the tumour biology field fail to mimic the 3D environment of a tumour, and instead, most of the experiments are performed in two dimensions, in Petri dishes, multi-well plates or glass slides that in some conditions have been coated with ECM proteins to poorly mimic a tumour-environment. In contrast, engineered 3D matrices or scaffolds are tightly controlled microenvironments to study the complex interactions with cancer cells or other tumour-associated stromal cells. The use of CAF-engineered matrices with user defined properties as implantable microenvironments may be valuable tools in metastasis experiments using mouse models.

Fibroblasts are dynamic, and may respond to the fluctuating demands of cancer cells at the primary tumour site and at the metastasis. Both CAFs may provide mechano-adhesive signals, soluble factors (chemokines, growth factors, metabolic intermediates…), packaged factors (vesicles) and track generation to stimulate invasion, ectopic survival, adhesion and finally colonization of a metastatic site. Although CAFs are important players in metastasis, we still must consider that metastasis is a highly inefficient process and that cancer cells are not solely dependent on their relationships with fibroblasts. However, the dynamic response of CAFs to cancer cells suggests the use of CAFs, their gene signature or their derivative proteins in the molecular classification of tumours to improve the stratification and treatment of patients. Understanding the heterogeneity within fibroblast populations will be important to effectively target the metastasis-promoting activities. CAFs can be targeted at multiple levels including factors that promote or sustain CAF phenotype, CAF markers such as FAP or CAF secreted products such as matrix proteins, proteases, chemokines, growth factors and exosomes.

Since previous reviews on the subject, many new findings have emerged to advance our understanding of the use of CAFs (or their products) in diagnosis and therapy. Key issues discussed in this review include (i) the study of CAF-cancer cell interactions using 3D/biomimetic approaches, (ii) the use of CAF markers or secreted molecules as important diagnostic and therapeutic tools and (iii) the use of CAF biomimetic scaffolds as diagnostic or therapeutic tools.

Three-dimensional biomimetic approaches to better understand interaction between CAF and cancer cells

Biomimetics of the tumour-environment have been described by Ghajar et al. as the construction of complex culture models that recapitulate aspects of the in vivo tumour-environment to study the dynamics of tumour development, progression and therapy on multiple scales [33]. The implementation of tissue engineering is a tool cancer researchers can exploit to increase the relevance of in vitro and in vivo models [1, 47].

Infiltration of the type I collagen-rich ECM is an early event for local tissue infiltration. In a simplified model, this activity can be mimicked by adding a single cell suspension on top of a native, type I collagen gel (Fig. 1a). By adding the secretome of CAFs, cancer cells that have a non-invasive morphotype become elongated and invade the collagen matrix. This shows that CAFs stimulate cancer cell invasion without the need for direct heterotypic cell-cell contacts or track formation. [22]. Soluble factors can be added in a gradient by placing the type I collagen gel on top of a transwell filter and the soluble factors of interest in the lower compartment [26] (Fig. 1a). Similar models can be used as a surrogate to study metastatic activity, e.g. metastasis in the peritoneal cavity. The peritoneal wall can be mimicked by using a type I collagen gel imbedded with CAFs and seeded with mesothelial cells. The CAFs will further modulate the collagen matrix by contraction-mediated fibre reorganization, matrix deposition and the secretion of signalling intermediates. The mesothelial cells prevent a direct interaction of the cancer cells with the CAF (Fig. 1b). Together, these layers represent the peritoneal wall. A single cell suspension or multicellular spheroids of cancer cells are confronted with a peritoneal wall biomimetic to study the interactions occurring during peritoneal metastasis formation. Using this model, researchers showed that cancer cell adhesion is inhibited by normal mesothelial cells but stimulated when CAFs are included in the biomimetic model [56]. Mitra et al. used a similar model and discovered a downregulation of the tumour suppressing miR-193b in the regulation of early metastic colonization. [72]. CAFs deposit ECM proteins and this can be used as a CAF-derived biomimetic matrix. The setup makes use of gelatin gels on which CAFs are cultured, deposit their matrix which is stabilized in presence of vitamin C and subsequently, the CAFs are removed by an alkaline detergent treatment (Fig. 1c) [15]. Adhesion of breast cancer cells to this matrix occurs in a specific organized geometry, probably because of CAF specific patterning of the gelatin by local depositing of matrix or by track formation [107]. An alternative way to acquire the structural architecture of CAFs and the produced proteins into an in vitro model is to print CAFs on a polydimethylsiloxane (PDMS) substrate. Lee et al. presents a protocol where an intermediate poyvinylalcohol (PVA) film is used to transport architectural composition of the CAF environment. Glutaraldehyde fixed stromal cells and their matrix are covered by 5 % PVA solution. After evaporation a PVA film embeds the cells and their matrix. The PVA film, with the embedded cells and matrix, is pealed of the cell culture plate and transported to a PDMS matrix. Finally the PVA is dissolved, resulting in the transfer of the CAFs from a cell culture plate to a PDMS substrate (Fig. 1d). This transferred stroma is long-lasting and in adhesion assays this surface shows comparable results with living cultures [63]. Microfluidic devices are another tool to co-culture CAFs and cancer cells. Microfluidic devices consist

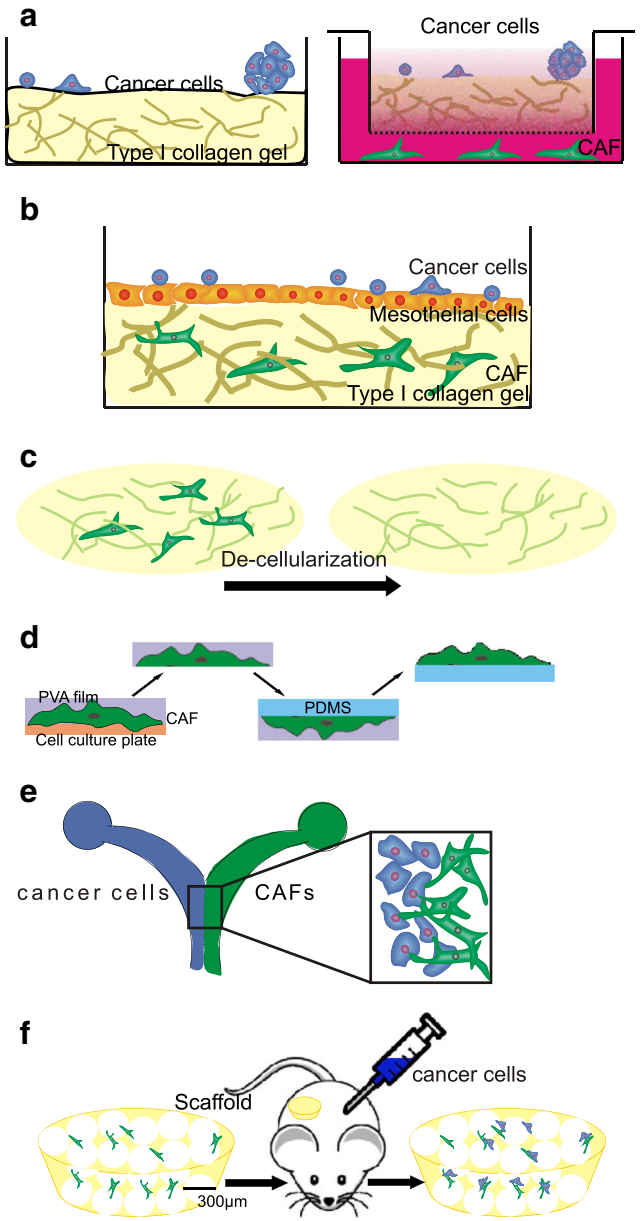

Fig. 1 3D models. **a** Hydrogels: single or spheroid cancer cells on top of a type I collagen gel (*left*). *Right*, the gel is present on top of a transwell filter, CAFs are in the bottom compartment. Soluble factors reach the cancer cells in a gradient. Type I collagen gel can be replaced by Matrigel, a mimic of the basal membrane. **b** Organotypic model: ECM of type I collagen and CAFs are separated from the cancer cells by a mesothelial monolayer. **c** CAF derived matrix: CAFs deposit their matrix on a gelatin coating and after de-cellularization a CAF derived matrix is left. **d** Printing the matrix: a PVA (polyvinylalcohol) film is used to transport the fixated CAFs and matrix to the PDMS (polydimethylsiloxane) substrate. **e** microfluidics: CAFs and cancer cells are seeded in a different channel and grow in a gradient towards each other. **f** Implantable scaffolds: scaffolds containing bone marrow-derived mesenchymal stem cells are subcutaneously implanted, intraveneously injected cancer cells are attracted to and populate the scaffold, which allows further ex vivo characterization

of micro-channels on a chip and are fit for high-throughput applications. These devices have been described as 'organs-on-a-chip' or 'lab-on-a-chip'. The channels can be coated with a matrix (like type I collagen) to improve cell adhesion and migration. Two, or more, channels are connected and debouch into one channel. The channels are subjected to continuous flow of cell culture medium that can be spiked with cytokines or inhibitors. CAFs and cancer cells are plated in separated channels and cell migration and proliferation are monitored (Fig. 1e). In another setup, two channels sprout in to multiple channels; in this way, a gradient is build up between the components in channel 1 and 2, mimicking the tumour-environment gradient [42, 87, 112]. Microfluidics reduce the sample volume, enabling upscaling for screening applications and control of the spatio-temporal dynamics of the environment. Confronting non-invasive breast cancer cells with CAF in this model has demonstrated that CAFs play a role in the transition of a non-invasive breast cancer cell to an invasive one. Soluble CAF-derived factors induce morphological changes in non-invasive breast cancer cells, but cell-cell contacts were necessary to transit them into an invasive phenotype [98]. Implantable scaffolds that mimic the tumour-environment have been designed to study cancer—host cell interactions in vivo and ex vivo. To mimic the bone marrow architecture, the group of Parekkadan et al. used a template based fabrication method by creating a polyacrylamide hydrogel scaffold. The polyacrylamide scaffold was polymerized around a template of crystals. Dissolving the crystals creates pores in the scaffold. These pores were coated with type I collagen and colonized by bone marrow-derived mesenchymal stem cells. These scaffolds are subcutaneously implanted in mice followed by tail vein injection of cancer cells. [62, 100]. Ex vivo imaging reveals that scaffolds populated by stem cells attract a higher number of metastasizing cancer cells than scaffolds populated with normal fibroblast. The invading cancer cells can be isolated for further downstream characterization [8]. In addition, silk scaffolds bio-functionalized with BMP2 (bone morphogenetic protein 2) are in vivo populated with bone forming host cells and biomimic bone tissue as early as 2 weeks after subcutaneous implantation. This newly formed bone tissue can be in situ manipulated to act as an ecological trap for metastatic breast and prostate cancer cells making it an interesting tool to understand tumour-environment interactions during metastasis [94].

Cancer-associated fibroblasts as target and tool in cancer diagnostics

CAFs as a target in cancer diagnostics

Transcriptome (RNA) signatures of tumours gain interest as a tool in the classification and prognosis of tumours [19, 68, 88]. However, the tumour transcriptome is complex since it does not only contain information about the cancer cells but also the neighbouring stroma. (Sub)groups with a 'EMT' or 'stem

cell-like' transcriptome are in reality tumours with a high stromal content and the signature corresponds with CAF or other stromal cell signatures. When these RNA signatures are validated in xenografted tumours, then almost 75 % of 'EMT/stem cell-like' genes are lost. Most of the 'lost' genes are recovered by a mouse specific array, confirming that these genes are expressed by the stromal cells instead of the cancer cells [48]. A large portion of the genes that classify patients as having a poor prognosis are CAF-expressed genes. Interestingly, there is a linear association between the expression of CAF-related genes and the risk of relapse after therapy [14]. This 'impurity' of the tumour signatures should not be considered a shortcoming but an asset in classifying the tumour prognosis. When genes expressed by the cancer cells and stromal cells are analysed separately, two profiles are created. One gives information about the cancer cells, the other about the activation status of the stroma. Stromal, including CAF, signatures have been developed and have prognostic value in colorectal cancer and non-small cell lung cancers [14, 41, 75]. A major drawback of gene profiling is that detailed spatial information is lost. Immunohistochemical-based profiling can distinguish between cancer cell and stromal expression and may also determine the ratio between cancer cells and stroma. Existing immunohistochemistry methods use antibodies tagged with fluorophores or enzyme reporters that generate coloured pigments. Because these reporters exhibit spectral and spatial overlap when used simultaneously, multiplexed immunohistochemistry to distinguish stromal or cancer phenotypes is not routinely used in clinical settings. Multiplexed ion beam imaging is a method that uses secondary ion mass spectrometry to image antibodies tagged with isotopically pure elemental metal reporters. Multiplexed ion beam imaging is capable of analysing up to 100 targets simultaneously over a five-log dynamic range allowing to quantify the relative involvement of cancer cells, CAFs, and immune subtypes in tumour biopsies or tumour resection specimens [3].

In breast cancer, several immunohistochemistry studies have demonstrated that a high proportion of stroma (≥50 % of the tumour) is related to a poor outcome [18, 25, 73]. This result was also observed in colorectal cancer [46], and other authors demonstrated that tumours with low proportion of cancer cells are associated with poor overall and disease-free survival [70, 113]. While some authors quantified the totality of the tumour stroma, others focused on the composition of the tumour stroma and, particularly, on CAFs. Table 1 summarizes immunohistochemical studies focusing on CAFs in different cancers and the impact of the presence of these CAFs on prognosis [4, 6, 16, 17, 27, 30, 35, 39, 40, 49, 53–55, 57, 58, 64, 69, 76, 77, 79, 81, 92, 93, 95, 96, 99, 104, 106, 109, 110, 114–116, 119]. Most of these studies showed a relationship of CAFs with poor outcome. Paulsson et al. meticulously described the methodological problems encountered with stromal quantification and to correlate stromal quantification with prognosis/outcome [79]. The majority of the studies are semi-quantitative. As Paulsson et al. underlined, semi-quantitative studies are observer-dependent and to have an idea about this inter-observer variation, a *kappa* value should be calculated. In those studies where a binary score is used, a good agreement between observers is generally obtained [18, 25, 46, 73, 82]. The main advantage of semi-quantitative analysis is its availability in every lab and its applicability in routine work. Furthermore, do we prefer to evaluate the quantity (using a general reactive marker like α-SMA) of the stroma or its quality (using for example a marker or substance that CAFs produce like neuregulin-1) if we want to consider the stroma in a prognostic setting? Perhaps a combination of these considerations should be used and the following three-step decision method may be proposed: first using H&E staining, tumours with a high- and a low-proportion of stroma are identified (using an agreed cut-off of 50 %) [18, 25, 46, 73]; secondly, a general CAF marker may determine the cellularity of the stroma and, finally, markers that stain the secreted proteins of CAFs may further refine the activation status. The overall conclusion of the studies presented in Table 1 is an overall correlation of a stromal reaction with a worse patient outcome. This seems to be universal across different tumour types. However, before we consider a stromal reaction as a clinically useful diagnostic tool a large multi-center study, with multiple-markers is needed. A quantitative study will help to determine the optimal cut-off value, through ROC-curve analysis. Now a cut-off of 50 % is accepted but the three quantitative studies in the table have determined their cut-off between 5.5 and 8.8 % [76, 104, 116], suggesting an optimal cut-off with a lower value. Multiple observers, from different centres, should score the same blind-coded samples in a semi-quantitative manner allowing to calculate a *kappa* value. Scoring the stromal reaction has to be standardized over multiple centres and demonstrated by a satisfactory *kappa* value, before demonstration of prognostic power and implementation into the clinic.

CAFs as a tool in cancer diagnostics

CAFs and other host cells may come to the rescue to fine-tune the characterization of circulating tumour cells (CTCs). CTCs are released from primary tumours or metastasis and are present at low concentration in the peripheral blood of patients. CTCs are of fundamental diagnostic interest because a subpopulation of CTCs may initiate metastatic colonization. Isolation of CTCs combined with ex vivo functional characterization of their metastatic potential may provide an opportunity to noninvasively monitor the risk of metastasis formation in individual patients. To adequately resolve the functional variance within a CTC population, it is important to evaluate each CTC individually. Single cell genomics allows to identify variations in CTCs but it is still elusive to link this genetic

diversity with a functional output, e.g. their ability to stay dormant or to colonize a foreign tissue. Functional characterization in tissue-like conditions will most probably refine the diagnostic potential of CTCs. Examples to mimic tissue-like conditions are extracellular matrix mimetics that allow to study the cell specific invasion and growth [11]. The hydrogels formed from peptide crosslinkers and polyethylene glycol monomers permit cell-controlled invasion and proteinase specific matrix peptides may further allow for selective control of invasion. Arrays of subnanoliter wells (nanowells) embedded with a fibroblast-derived matrices or a hydrogel containing fibroblasts or other organ-specific host cells allow the characterization of the functional diversity of CTCs [117]. In addition, microfluidic culture devices may further increase the realistic recapitulation of potential metastatic target organs such as the liver, brain and lung [10]. For example two cell types are cultured in direct juxtaposition by micropatterning ECM adhesive islands within the microfluidic chamber that preferentially support one cell population (e.g. hepatocyte), and the empty spaces are then filled with the second cell population (liver fibroblasts or hepatic stellate cells). Consequent addition of individual CTCs through the flow channel may allow to study interaction of the CTC with the hepatocytes/fibroblasts eventually followed by colonization of the microchannel. Furthermore, post-experiment recovery of CTCs allows further genomic characterization (Fig. 1e).

Cancer-associated fibroblasts as target and tool in cancer therapy

CAFs as a target in cancer therapy

Targeted therapy or precision medicine improved cancer management protocols but has raised some new issues. More and more therapies are developed for smaller groups of patients expressing the specific target, consequently these therapies are expensive. Unfortunately, the plasticity of cancer cells makes adaptation to these targeted treatments possible and most of the patients acquire resistance. In contrast, the interaction between cancer cells and their environment is most probably universal across different tumour types. Therapies targeting tumour-environment interactions should be applicable to a large group of patients and should be less likely to acquire drug resistance since the tumour-environment show less phenotypic drifts. The tumour-environment has been proposed as a promising target to develop new and effective cancer therapies [1, 91]. The tumour-environment offers a range of potential targets. Not only the CAFs are a possible target but also the interaction of CAFs with the cancer cell, metastatic niche, immune cells, extracellular matrix and endothelial cells [101]. Nintedanib is a good example of anti-tumour-environment drug. Nintedanib or BIBF1120 is a broad spectrum tyrosine kinase inhibitor, with the VEGFR, FGFR and PDGFR as target by binding the ATP pocket in a competitively and reversible manner. This inhibits downstream intercellular signalling of the receptor [43]. The VEGF, FGF and PDGF family proteins are abundantly present in the tumour-environment as they are secreted as well by cancer cells, CAFs and tumour-associated macrophages (TAMs) [21, 22, 24, 60]. The receptors are expressed on the cell membrane of cancer cells, CAFs, TAMs and endothelial cells. Activating these receptors stimulates cell proliferation, migration and survival through the AKT/MAPK pathways. Nintedanib inhibits the cross-talk between the different components of the tumour-environment (Fig. 2). At this moment Nintedanib is used as a second line therapy for non-small-cell lung cancer in combination with docetaxel [80] and in monotherapy for the treatment of idiopathic pulmonary fibrosis (IPF, a lung disease characterized by excessive fibroblast accumulation). IPF patients show an altered expression and release of tyrosine kinase ligands by activated fibroblast and immune cells stimulating proliferation of lung fibroblast. Inhibition of the tyrosine kinase receptors by Nintedanib reduces lung inflammation and fibrosis demonstrated by reduced deposition of type I collagen and the inhibition of fibroblast activation [34, 84, 105]. Interestingly, the mechanisms of fibroblast activation in fibrosis show a high similarity with fibroblast activation in cancer [44]. Furthermore Nintedanib is the subject of over 80 clinical trials (from which 60 in oncology for about 20 different tumour types). Pirfenidone (5-methyl- l-phenyl-2-(lH)-pyridone) is another anti-fibrotic and anti-inflammatory drug used for the treatment of IPF that is in clinical trials to improve cancer treatment (neurofibromatosis and fibrosis caused by radiotherapy) although the exact molecular mechanism of this small molecule is unknown [38, 105]. Anti-TGF-β therapies have also been presented as a way to interfere with the cross-talk between the different components of the tumour-environment. Cancer cells manipulate the environment through the secretion of TGF-β. TGF-β stimulates the activation of CAFs increasing ECM production, contractility and VEGF secretion leading to angiogenesis [14, 118]. Fresolimumab a monoclonal antibody against TGF-β has been tested in clinical trials for melanoma, renal cell carcinoma, mesothelioma and in combinational therapy for metastatic breast cancer [38, 74]. Unfortunately, anti-tumoural effects co-incided with the development of secondary tumours such cutaneous neoplasms [59, 97], causing an almost immediate stop in further clinical trials with this type of antagonists. A possible explanation is that TGF-β acts as a double-edged sword: a driver in the tumour-environment and a tumour-suppressor for normal epithelial cells [14, 118]. The TGF-β receptor type I small molecule inhibitor, LY2157299, currently in clinical trials phase II for liver, gastrointestinal and neurological tumours, does not boost growth of TGF-β responsive xenograft tumours and may be safer in patients [13, 14]. Therapy can also be targeted directly to the CAFs by targeting

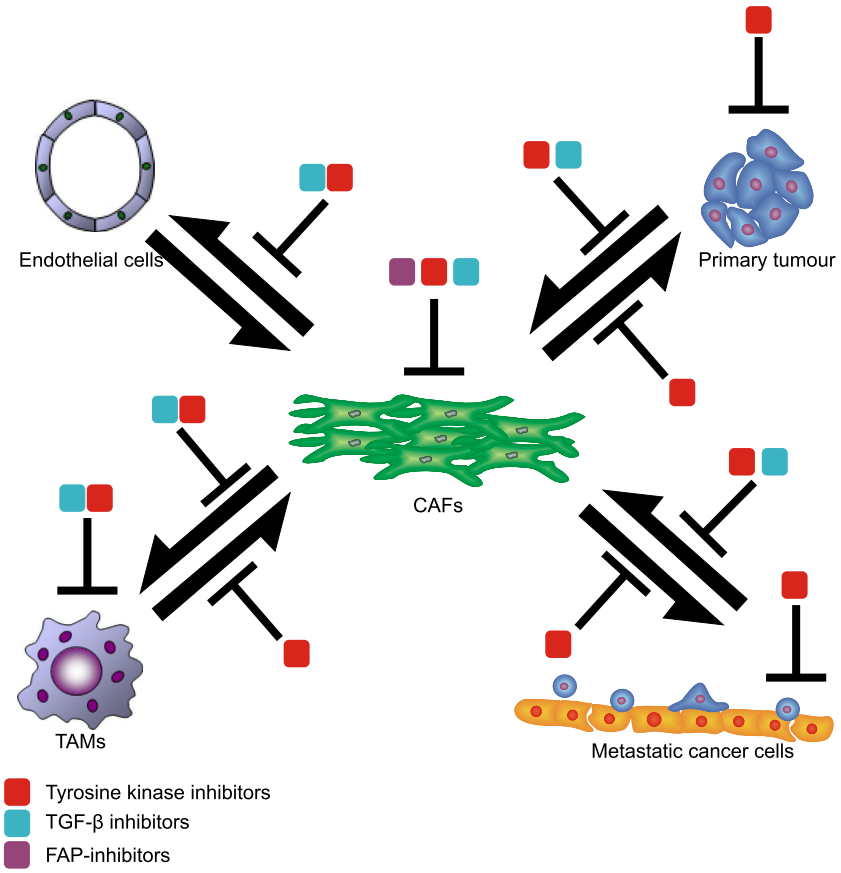

Fig. 2 CAF-targeted therapy. The tumour-environment is heterogeneous consisting of cancer cells (including cancer cells with metastatic potential), TAM (tumour-associated macrophages) and other innate and adaptive immune cells, endothelial cells and CAFs. The cross-talk between these cells are all potential therapeutic targets

a CAF marker like FAP (fibroblast activation protein) or by exploiting FAP as a drug delivery tool. FAP is an endopeptidase that cleaves the prodrug peptidyl-thapsigargin in the cytotoxic drug thapsigargin and produces a local therapeutic response in mice models for breast and prostate cancer [12]. A problem with FAP targeting and FAP based drug targeting is that FAP is not so specific for the tumour-environment as initially assumed. Besides the expression by CAFs, FAP is also expressed in fibroblasts during wound-healing (transiently), in fibrosis of the liver and lung, and Crohn's disease. But more importantly, FAP is expressed in bone marrow mesenchymal stem cells (BM-MSCs) and treating mice with genetically engineered FAP-reactive T cells resulted in severe cachexia and bone-toxicity [103]. However, other characteristics of the tumour-environment show less risk for side effects when exploited for controlled drug delivery by nanoparticles. Paclitaxel (PTX, a microtubule stabilizer causing mitotic arrest) is hydrophobic and water insoluble. The cremophors added to solubilize PTX give serious side effects [32, 66]. However, PTX stabilized by albumin (nab-paclitaxel or Abraxane) is soluble and has reduced systemic toxicity and increased efficacy against several cancer types [71]. Although, albumin increases the biocompatibility of PTX, it does not increase the specificity of the drug delivery. Nanoparticles containing the insoluble drug can improve drug delivery to the cancer cells. Nanoparticles smaller than 200 nm leak out of the vessels at the tumour site due to enhanced permeability and retention (EPR) effect. Specific uptake of the nanoparticles by the cancer cells can be obtained by tumour-environment biomimetics. Hyaluronic acid (HA), a structural molecule of the ECM and a ligand of the CD44 receptor, has been used to encapsulate PTX. HA based nanoparticles are specifically taken up by CD44 overexpressing ovarian and colon cancer cell and toxicity on CD44 negative cells is strongly decreased [52, 61, 85]. Gao et al. developed tumour environment-sensitive nanocarriers. Docetaxel was encapsulated in a micelle from polypeptides sensitive to the matrix metalloproteinases MMP2/9. Nanoparticles that arrived in the tumour-environment are cleaved by the MMP2/9 mostly produced by CAFs, locally releasing the chemotherapy. This results in a higher specificity and a lower systemic toxicity [31].

Targeting the stroma can also help overcome the negative effects of conventional therapy on the tumour-environment. The reaction of CAFs can be evaluated through α-SMA staining in patients that receive neo-adjuvant treatment, exemplified by rectal cancer, since in such cases material is available both before (diagnostic biopsy) and after (surgical resection) the neoadjuvant

chemoradiotherapy treatment (Fig. 3). Evaluation of therapy-induced changes of the CAFs should be carefully quantified using automated image analysis and statistically evaluated through multivariate analysis [110]. Chemotherapeutics activate CAFs to create a chemoresistant niche by the release of cytokines including interleukins and TGF-β [51, 65, 83]. Intriguingly, combinational treatment of nab-paclitaxel (Abraxane) and gemcitabine normalizes the amount of CAFs compared to gemcitabine alone in patients with pancreatic cancer [2]. Furthermore nab-paclitaxel compromises the integrity of the type I collagen fibres produced by CAFs. This combinational therapy significantly improved overall survival, progression-free survival and response rate in a phase 3 clinical trial [111]. Pancreatic cancer is characterized by a CAF-rich stroma which is suspected to create chemo-resistance. Indeed, the secretome of CAFs increases resistance of pancreatic cancer cells to chemotherapy. The mTOR/4E-BP1 pathway has been identified as critical player in the production and secretion of CAF-derived factors building up the chemoprotective stroma. Inhibition by SOM230 (somatostatin receptor inhibitor) prevents the activation of the mTOR/4E-BP-1 pathway and sensitizes pancreatic cancer to gemcitabine in vivo [28]. Oncologists are not only interested in the tumour-environment as a possible target but also to include the stromal reaction in the outcome measurements of clinical trials [38]. Stromal content and heterogeneity can be investigated on tissue biopsies or tumour resection specimens by immunohistochemistry (Table 2). Non-invasive evaluation of tumour cellularity and extracellular matrix composition can be assessed by diffusion-weighted magnetic resonance imaging (DWI) and tumour vascularity can be assessed by dynamic contrast enhanced magnetic resonance imaging (DCE-MRI). Finally, tumour hypoxia can be evaluated by T2* MRI and PET-CT, using the 18F-labelled hypoxic marker HX4 [38].

CAFs as a tool in cancer therapy

The tumour environment is an ecosystem, and the concept of an 'ecosystem trap or ecological trap' can be applied to target cancer cells. An ecological trap is an environment of low quality for survival that is preferred by an organism over a better available environment, often by mimicking factors from the high-quality environment. Ideally, a cancer cell trap as therapeutic device deceives and kills cancer cells in a two-step approach. Such a device has been developed for glioma: an implanted source chemo-attracts the cancer cells, followed by stereotactic radiation of the implantation zone [108].

The high avidity and affinity of disseminated cancer cell to the ECM makes this the perfect bait for an ecological trap. The ECM is composed of a wide range of adhesive substrate molecules. In this way, the heterogeneity of disseminated cancer cells is less problematic. For example detached cancer cells from primary colon or ovarian tumours survive in the peritoneal cavity and adhere to the peritoneal wall at the wound side where the protective mesothelial layer is removed and fibroblast are exposed and activated (Fig. 4a) [90], to support peritoneal metastasis. However, the artificial presence of particles

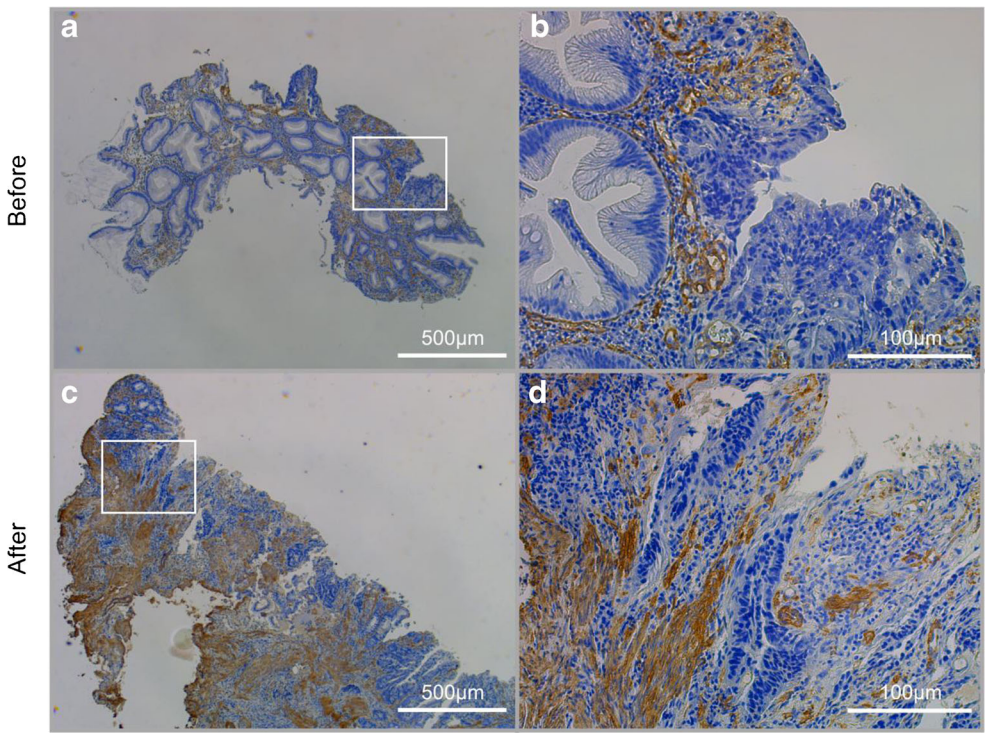

Fig. 3 Immunohistochemical staining of rectal cancers. α-SMA staining and haematoxylin counterstain of rectal tumour biopsy before treatment (**a**, **b**) and a resection specimen after chemo/radio therapy treatment (**c**, **d**). *Squares* indicates site of magnification

Table 2 Clinical trials with outcome 'stroma'

Clinical trials with outcome 'stroma'	Phase	Cancer	Stroma
The Role of the Tumour Microenvironment of Pancreatic Cancer to Predict Treatment Outcome (MIPA)	–	Pancreatic cancer	IHC of stroma SMA, collagen, SHH and measure: percentage of staining of the total tumour area
Rilimogene-Galvacirepvec in Preventing Disease Progression in Patients With Localized Prostate Cancer Undergoing Active Surveillance	2	Prostate cancer	Change of CD4+/CD8+ cells in stroma after treatment
Assessment Of Stromal Response To Nab-Paclitaxel In Combination With Gemcitabine In Pancreatic Cancer	–	Pancreatic cancer	IHC of SPARC, αSMA and collagen after treatment
Age-related Changes in the Immune System and Their Impact on Elderly Breast Cancer (IMAGE)	–	Breast cancer	Leucocyte infiltration of the tumour stroma
Pilot Study of Anti-oxidant Supplementation With N-Acetyl Cysteine in Stage 0/I Breast Cancer (NAC)	1	Breast cancer	Expression of caveolin −1 and MCT4 in CAFs
Studying Gene Expression in Samples From Younger Patients With Neuroblastoma	–	Neuroblastoma	Expression of TβRIII in the neuroblastic tumour and stroma

IHC immunohistochemistry, *SMA* smooth muscle actin, *SPARC* secreted protein, acidic and rich in cysteine, *MCT4* monocarboxylate transporter 4

that contain (Fig. 4b) ECM surface that is produced by CAF can act as an ecological trap. We recently demonstrated that alginate-gelatin microparticles with encapsulated CAFs (MP[CAF]) selectively deceive and redirect the adhesion of disseminated cancer cells [20](Fig. 4c). Encapsulated CAFs remain metabolically active and produce ECM proteins, which are adsorbed by a PSS/PAH coating (poly-styrenesulfonate/poly-allyhydrochloride) surrounding the

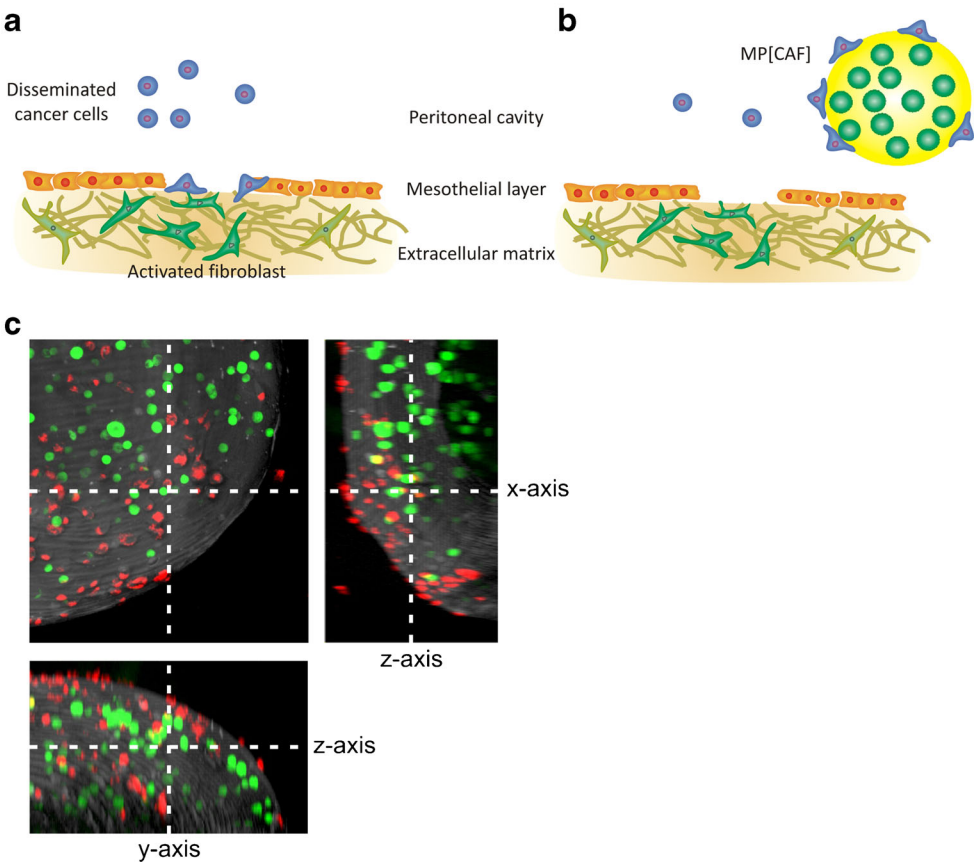

Fig. 4 Ecological trap. **a** At the wound site in the peritoneum, fibroblasts are activated and the protective mesothelial layer is disrupted. This is the ideal environment for free cancer cells to adhere. **b** Encapsulated CAFs (MP[CAF]) provide an alternative substrate and redirect adhesion from the wound site to the surface of the particle. **c** Confocal image of MP[CAF]; cancer cells (*red*) adhere to the coating surrounding the MP with the encapsulated CAFs (*green*)

microparticles. These polymers are non-biodegradable; the alternating negatively and positively charged layers retain the CAF-secreted proteins and prevent their leakage [20]. Release of CAF-derived proteins in the environment would stimulate cancer cell survival and metastasis. In addition, hazardous side effects from possible CAF exposure to the host are avoided because neither CAFs nor free secreted proteins are released from the microparticles. MP[CAF] can travel in the abdominal cavity after intraperitoneal injection just as the disseminated cancer cells do. MRI imaging shows distribution of MP[CAF] throughout the abdominal cavity without attachment to intestinal organs and without signs of inflammatory reaction. In contrast, a medical device implanted on the peritoneum would inherently damage the peritoneal wall and evoke an activated fibroblast reaction [9]. Myofibroblast, a component of the wound healing reaction, produces ECM and growth factors which stimulate cancer cells just as CAFs do. The presence of activated fibroblast, also called myofibroblast, at the implantation site will attract and stimulate cancer cells to adhere and invade the wound [29], potentially leading to an increased risk of metastatic colonization at the implant zone. However, the use of free floating particles in the peritoneal cavity avoids major surgical procedures and woundhealing reactions. In addition, the use of microparticles redirects adhesion of the cancer cells from an on-purpose-induced wound to the particle surface, most probably because the acute presence of a CAF-derived substrate creates a more favourable environment for adhesion (Fig. 4b). Iron-oxide nanoparticles (FeOX-NP) are added to the coating of the MP[CAF] allowing the easy, physical removal of MP[CAF] by a magnetic rod. This results in a reduction of the tumour load and a prolonged survival. Consequent prevention of peritoneal metastasis implicates peri-operative, magnetic field-assisted removal of the particles and reduction of disseminated cancer cell load. Alternatively, incorporation of FeOX-NP allows the application of AMF (alternating magnetic field). By applying an AMF to the abdomen of the patient the surface of the MP[CAF] will heat up [86]. When a local temperature of 48 °C can be reached the attached cancer cells will die due to thermal ablation [50]. Another approach is the incorporation of ^{10}Boron (non-radioactive isotope) into the coating of the MP which allows the application of boron neutron capturing therapy. By applying a low-dose neutron beam to the particles ^{10}B becomes ^{11}B but rapidly decays to lithium and α-particles. This event creates high but local (5-10 μm) ionization that will cause cell death on the MP[CAF] minimally harming healthy tissue [5].

Electrospinning [36, 37], cell encapsulation [7, 67, 89] and 3D printing [45] techniques are further improving the design of tumour-environment biomimetics. Without doubt, the interdisciplinary field on biomaterials, tumour-environment interactions and drug delivery will open new avenues for medical progress.

Conclusion

CAFs and the tumour-environment are important factors in cancer management. Incorporation of the tumour context improves the relevance of in vitro models, diagnostic and therapeutic strategies. Interdisciplinary collaboration between researchers in the field of biomaterials, tumour-environment interactions, pathology, engineers and clinicians gives rise to new opportunities and insights in cancer management.

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