Review Article
Understanding tumor-stroma interplays for targeted therapies by armed mesenchymal stromal progenitors: the Mesenkillers

Giulia Grisendi[1]*, Rita Bussolari[1]*, Elena Veronesi[1], Serena Piccinno[1,2], Jorge S Burns[1], Giorgio De Santis[3], Pietro Loschi[3], Marco Pignatti[3], Fabrizio Di Benedetto[4], Roberto Ballarin[4], Carmela Di Gregorio[5], Valentina Guarneri[1], Lino Piccinini[1], Edwin M Horwitz[6], Paolo Paolucci[2], PierFranco Conte[1], Massimo Dominici[1]

[1]Division of Oncology, Department of Oncology, Hematology and Respiratory Diseases, University-Hospital of Modena and Reggio Emilia, Modena, Italy; [2]Unit of Paediatric Oncology, Haematology and Marrow Transplantation, Department of Mother and Child, Modena; [3]Unit of Plastic Surgery, Department of Head & Neck Surgery, Modena; [4]Unit of Hepato-Pancreato-Biliary and Transplant Surgery, Department of General Surgery, Modena; [5]Section of Pathology, Department of Pathologic Anatomy and Forensic Medicine, Modena; [6]Division of Oncology/Blood and Marrow Transplantation, The Children's Hospital of Philadelphia and The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. *Equally contributing Authors

Abstract: A tumor represents a complex structure containing malignant cells strictly coupled with a large variety of surrounding cells constituting the tumor stroma (TS). In recent years, the importance of TS for cancer initiation, development, local invasion and metastases has become increasingly clear allowing the identification of TS as one of the possible ways to indirectly target tumors. Inside the heterogeneous stromal cell population, tumor associated fibroblasts (TAF) play a crucial role providing both functional and supportive environments. During both tumor and stroma development, several findings suggest that TAF could be recruited from different sources such as locally derived host fibroblasts, via epithelial/endothelial mesenchymal transitions or from circulating pools of fibroblasts deriving form mesenchymal progenitors, namely mesenchymal stem/stromal cells (MSC). These insights prompted scientists to identify multimodal approaches to target TS by biomolecules, monoclonal antibodies, and more recently via cell based strategies. These latter strategies appear extremely promising, although still associated with debated and unclear findings. This review discusses crosstalk between cancers and their stroma, dissecting specific tumor types, such as sarcoma, pancreatic and breast carcinoma, where stroma plays distinct paradigmatic roles. The recognition of these distinct stromal functions may help in planning effective and safer approaches aimed either to eradicate or to substitute TS by novel compounds and/or MSC having specific killing activities.

Keywords: Tumor stroma, TAF, mesenchymal stem/stromal cells, TRAIL

All around tumors

Physiologically, tissue specific cells interact with their surrounding stroma to maintain functionality, so that specialized cells can retain a precisely defined anatomical position, number and structural interactions with supportive cells and their extracellular matrix [1]. During cancer development, transformed cells loose these constraints through several types of changes. However, even in this context, the interplay between tumor and stroma seems reciprocal.

Tumor stroma (TS) consists of mesenchymal fibroblasts, vascular cells, extracellular matrix and immune cells that reside in normal connective tissue and/or derive from circulating blood [2]. Tumor cells (TC) maintain their interactions and influence surrounding non-malignant cells and, vice versa, the stroma itself can trigger tumors [3]. Several findings indicated that tumor progression is promoted by cross-talk between tumor and his surrounding supporting tissue either via cell-to-cell contacts or by secreted molecules [4,5].

The main TS-modulating growth factors produced by TC are represented by fibroblastic

Table 1. Tumor associated fibroblasts influence on tumor progression

Tumor Source	Impact	References
prostate carcinoma	↑	*Olumi AF et al.* Cancer Res. 1999 1;:5002-11
breast cancer	↑	*Chiavarina B et al.* Gene Ther. 2004 11:1155-64.
breast cancer	↑	*Akira Orimo et al.* Cell 2005 121:335–348
breast cancer	↓	*Sadlonova A et al.* Breast Cancer Res. 2005 7:R46-59
breast cancer	↑	*Liao D et al.* PLoS One. 2009 4:7965
squamous cell oral carcinoma	↑	*Kawashiri S et al.* Head Neck. 2009 31:1346-53
bone and soft tissue tumours	↑	*Dohi O et al.* Histopathology. 2009 55: 432-40
pancreatic adenocarcinoma	↑	*Hwang RF et al.* Cancer Res. 2008 68 ::918-26
several solid tumors	↓	*Francia G et al.* Cancer Cell. 2009 15:3-5
breast cancer	↑	*Spaeth EL et al.* PLoS One. 2009 4:4992
pancreatic adenocarcinoma	↑	*Spaeth EL et al.* PLoS One. 2009 4:4992
ovarian adenocarcinoma	↑	*Spaeth EL et al.* PLoS One. 2009 4:4992
melanoma	↑	*Balsamo M et al.* Proc Natl Acad Sci U S A. 2009 8::20847-52
squamous skin carcinoma	↑	*Erez N et al.* Cancer Cell. 2010 17:135-47

Inhibition (↓), growth (↑)

growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor receptor (EGFR)-ligand and transforming growth factor β (TGF-β) [4]. All these cytokines are able to alter the normal tissue homeostasis stimulating the formation of new blood vessels as well as recruiting and activating immune cells and stromal fibroblasts [6-8]. Inside the tumor burden,

fibroblasts may constitute a substantial volume so that in pancreatic and breast cancer (BC), they could account for up to 70% of the TS compartment playing a pivotal role in tumor maintenance, diffusion and, even drug resistance [9]. Based on their presence and trophic functions in a variety of tumors, these cells have also been referred as tumor associated fibroblasts (TAF) [10-12].

The real origin of TAF and the mechanisms by which they promote tumor progression is still debated, however there are reports demonstrating that, inside tumor burden, fibroblasts acquire an activated phenotype quite similar to one shown during wound repair in injured sites. This sort of shift from normal fibroblasts to activated TAF is driven to a great extent by cancer-derived cytokines, such as TGF-β [10].

Once transformed, activated fibroblasts present several peculiar features. TAF express smooth muscle cell markers desmin, α-smooth-muscle-actin (α-SMA), fibroblast activation protein (FAP) and vimentin [13-15]. TAF additionally represent the major producer of extracellular matrix (ECM), synthesizing type I, type III and type V collagen and fibronectin. This generatesa large net of fibrillar matrix inside the tumor that embeds TC like a supporting scaffold [16]. The high number of fibroblasts and their synthesized ECM components residing inside the reactive stroma gives rise to a pathological condition known as desmoplasia characterized by increased amount of collagen, fibronectin, proteoglycans, tenascin C and vessels. Beside this supporting action, TAF are also representing an important source of ECM-degrading proteases such as metalloproteinases that are fundamental to regulate the motility and invasiveness of cancer cells [14].

In addition to the mentioned mechanisms by which TS promotes cancer progression by stimulating proliferation, migration and invasion, there is also increasing evidences that TAF generally favour the initiation of neoplastic events from a transition of non-tumorigenic cells towards tumorigenic clones (**Table 1**) [17, 18]. This effect seems strictly related to changes in gene expression which may alter the physiological function of fibroblasts inducing an abnormal phenotype often combined with an overexpression of cytokines, such as TGF-β and hepatocyte growth factor (HGF) [19, 20]. Although non-
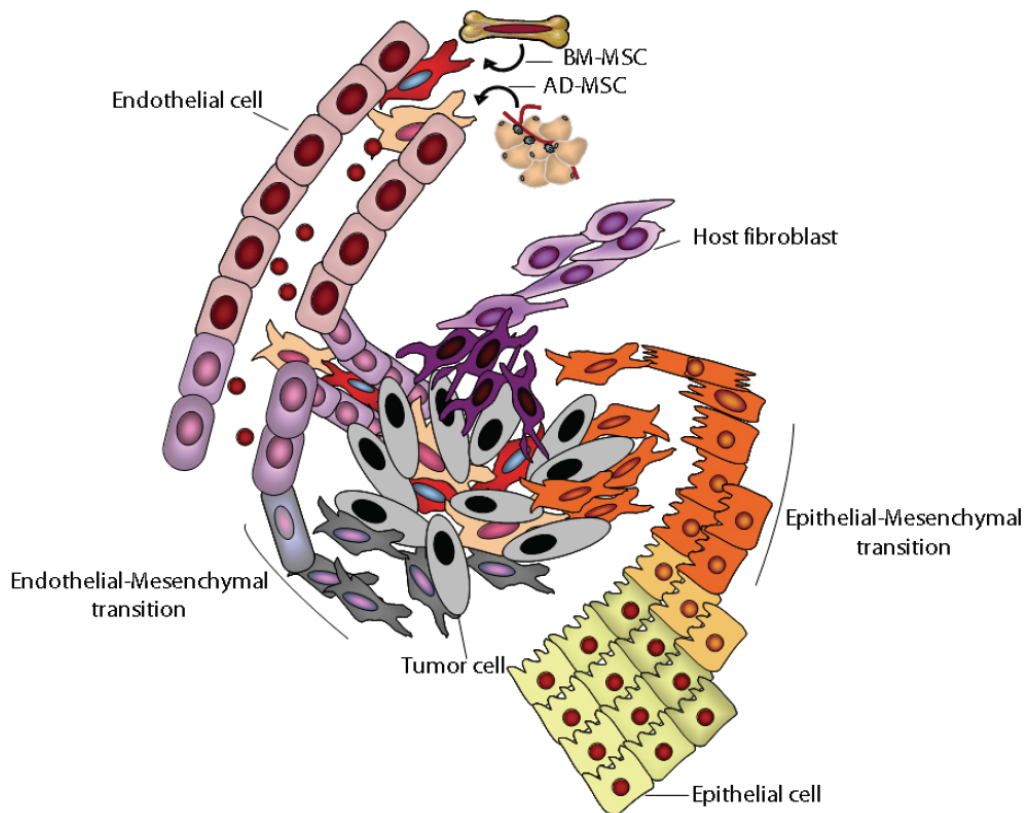
Figure 1. The origin of TAF. Tumor associated fibroblasts (TAF) may originate from different sources either by circulating mesenchymal progenitors derived from bone marrow (BM-MSC), adipose tissue (AD-MSC) or from locally recruited fibroblasts. In this latter case, TAF may derive from host normal mesenchymal fibroblasts and from cellular elements deriving form epithelial cells resulting from an epithelial-mesenchymal transition (EMT). Alternatively, TAF may be the result of another transition in which endothelial cells are the precursors: the endothelial mesenchymal transition (EndMT).

tumorigenic fibroblast may play a role enhancing proliferation of already malignant epithelial cells, they seem unable to initiate the malignant transformation of epithelial cells *de novo* [21]. On the contrary, TAF can induce neoplastic transformation in normal cells. Olumi *et al*. described that the co-culture of TAF with human prostatic epithelial cells resulted in a tumorigenic epithelial cell phenotype that may even persist in the absence of TAF [22]. To further underline the involvement of stromal TAF in cancerogenesis, several investigators demonstrated that cancer might also arise as consequence of inherited or acquired genetic mutations in stromal compartment [3,23]. Moreover, in patients with juvenile polyposis, deletions of chromosome 10 and 18 have been detected in stromal cells [24]. Moinfar *et al*. discovered genetic alteration and loss of heterozygosity in stromal cells close to primary BC [25], and very

interestingly, different scientists reported a tumor like phenotype in fibroblasts obtained from healthy relatives of patients with familiar breast disease [26, 27].

Tumor stroma ontogeny: resident transdifferentiating cells, circulating mesenchymal progenitors or both?

While large amounts of data are confirming the possible roles played by TAF in cancer initiation and progression, their ontogeny remains controversial (**Figure 1**). It has been shown that different tumor types may have specific TAF based on histology, organ localization and phase of tumor development (i.e. tumor initiating phase versus metastasis) [13]. Originally, resident normal stromal fibroblasts were considered to be the major source of TAF, nevertheless more recently several independent studies indicate a multi-

plicity of cell sources from which TAF may originate [28]. Accordingly to these data, TAF may also derive from transdifferentiating cells within the tumor. For instance TAF may be generated via an epithelial to mesenchymal transition (EMT) or from an endothelial to mesenchymal transition (EndMT) [29].

EMT is a complex cellular transformation that has been associated with molecular changes inducing an epithelial cell depolarization and the adoption of a mesenchymal phenotype characterized by an enhanced migratory ability and increased expression of extracellular matrix protein [30]. EMT is suggested to correlate with the malignant phenotype in epithelial derived tumors and in sarcomas suggesting that cells arising from EMT may be mediating the transition towards a metastastatic phenotype [31,32].

EndMT was first investigated in a case of tissue fibrosis but it has also been demonstrated to contribute up to 40% of TAF [33]. EndMT is often categorised as a specialised form of EMT, where endothelial cells represent the starting cell population. The molecular mechanisms of EndMT in tumors are not yet fully understood but is has been suggested to be mediated by TGF-β. EndMT may be initiated by autocrine and paracrine inflammatory signals originating the surrounding tissue. Alternatively, endothelium may undergo an indirect EndMT response to vascular injury. Transitioning endothelial cells loose their main phenotype and acquire a migratory phenotype, invade the basal membrane, and begin to express mesenchymal markers, as reported in other conditions [34].

Beside these two possible origins of TS generation by either differentiation or transdifferentiation, several findings indicate that TAF may originate from a pool of mesenchymal circulating cells [35], which could to be either bone-marrow (BM) or adipose tissue (AD) derived. The exact nature of these progenitors is still under investigation but there are clues that these may be sub-populations of mesenchymal stromal/stem cells (MSC). MSC are heterogeneous progenitors obtained from different tissues having robust regenerative potential [36]. While their role as regenerative tools has been studied for decades, only more recently have MSC been associated with tumors.

There is much evidences in support of this hypothesis: first human BM-MSC, exposed to tumor-conditioned medium over a prolonged period of time, assume a TAF-like myofibroblastic phenotype including sustained expression of stromal-derived factor-1 (SDF-1) and α-SMA [37,38], secondly BM-MSC are able to migrate into the tumor burden after systemic or local infusion [39-42]. Recently, Hall et al [43] have proposed that, during the stromagenesis, local tissue fibroblasts as well as circulating MSC coming from BM could equally be recruited into the tumor burden. They also have supposed that, once inside the tumor microenvironment, MSC could proliferate and acquire the main biological properties of activated TAF. Physiologically, BM-MSC play essential roles in maintaining adult tissues homeostasis as well as in wound healing [44]. Accumulating evidence demonstrated that, in healthy animals, systematically injected MSC, migrated preferentially in lung, liver and bone while they were found to a smaller extent in other tissues [45-47]. Most interestingly, in pathological conditions, MSC showed the tendency to preferentially migrate into injured sites irrespectively of the organ, probably driven by chemotactic gradients due to cytokines and chemokines released from the damage sites [48-53]. Once there, MSC take part to tissue remodelling providing structural support and secrete stimulatory factors for tissue repair [44,54]. Starting from these findings, one could assume a similar behaviour when in tumors that represent "wounds that never heal" [5]. In a tumor xenograft murine model, Ishii et al. demonstrated that, 28 days after implantation of pancreatic carcinoma cells, about 40% of TAF within the tumor burden were derived from BM [55]. More recently, others demonstrated a recruitment and engraftment of intravenously derived MSC into tumor sites of melanoma, breast, gastric and brain tumor xenografts [37-39,56].

Adipose tissue mesenchymal progenitors may also contribute to TS. As for BM-MSC, it has been shown that systemically administered AD-MSC can localize to sites of injury and contribute to revascularization [57]. Similarly to BM-MSC, AD-MSC migrate into tumor sites in response to discrete signals and become incorporated into the sites of disease serving as an extra TAF reserve [58]. Beside their possible role as TAF precursors, it has been proposed that AD-MSC become recruited for tumor vascu-
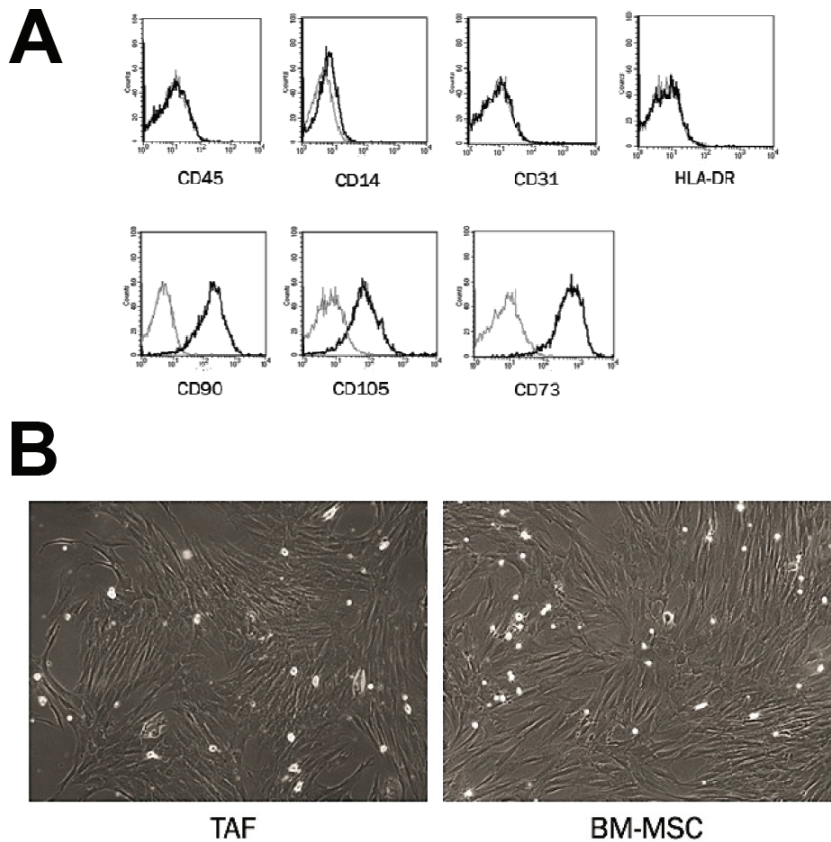
Figure 2. Morphological appearance of TAF from a primary lung cancer. Representative experiment of tumor associated fibroblasts (TAF) isolated from a patient with lung tumor by collagenase digestion, in vitro cultured and expanded. The FACS analyses (Panel A) reveal surprising similarities in TAF immunophenotype in comparison with mesenchymal stromal/stem cells (MSC). In addition, in vitro observations (Panel B; 100 x original magnification) indicate a spindle shape morphology of TAF analogous to MSC.

mesenchymal markers such as CD90, CD73 ad CD105 and lack of hematopoietic antigens. In addition, they were able to differentiate tumor stromal cells along the osteogenic lineage, suggesting some relevant similarities between TAF and MSC [60]. Similar data were also obtained by Zhao et al. who, comparing prostate derived TAF and BM-MSC, revealed antigens similarities, although associated with a different gene expression profile [61]. While the antigen similarities may indicate a common origin and being used to prospectively isolate TAF, more recent findings suggests that TS, just like it has been proposed for MSC [62], may exert an immunomodulatory function against lymphocytes and NK reducing their antitumor impact and favouring tumor progression [60]. All these data suggest TAF and MSC may share some relevant phenotypical and functional features.

logenesis, suggesting a dual and more complex contribution of AD-MSC: TAF generation and neoangiogenesis [60].

Even though much evidence show that MSC play a role in TS, extensive comparative analyses between MSC and TAF are lacking and identify the circulating derived component inside the population of TAF is extremely difficult. However, we originally observed that TAF isolated from different tumor histotypes (lung, colon and pancreas) share the same spindle shape morphology and similar antigen expression (**Figure 2A-B**). These data have been recently supported also by Johann et al. who demonstrated that tumor stromal cells, isolated from neuroblastoma and sarcomas, are positive for the main

Paradigms of tumor and mesenchymal stroma interdependence

During recent years, cross-talk between TC and stroma has been increasingly unveiled and we can now speculate that mesenchymal stromal cells may contribute to all the steps of tumorigenesis. A relevant amount of data suggests that stromal cell may be at the origins of tumors, this is the case of bone and soft tissue sarcomas where primitive precursors from different origins may play a fundamental role in initiating the neoplastic process. Regarding the role of stroma in cancer progression, pathologists recognize that tumors markedly differ from each other also in term of stromal composition

that deals with both the type and amount of TAF. In particular, in the so called desmoplastic tumors, such as pancreatic adenocarcinoma (PA) and some BC sub-types, up to 90% of the primary tumor burden may consist of stroma which it is also reported to promote tumor progression [63].

In addition, stromal cells may play a third fundamental role in tumorigenicity and namely metastasis and, in particular, for bone metastasis. BC cells are known for over 100 years to have a specific tropism for BM and for their stromal cells [64]. In this very frequent clinical scenario, we see TC going into marrow mesenchymal stroma and by this interaction, they can generate secondary lesions often associated with high levels of morbidity and mortality [65].

Here we are considering three different tumor types respectively as possible paradigms of fundamental aspects of tumor and stroma interactions to outline how mesenchymal stromal cells may determine initiation, proliferation and metastasis: sarcomas, pancreatic and BCs, .

Mesenchymal stromal cells as tumor initiators: the sarcomas

Sarcomas are malignancies deriving from mesenchymal tissues such as fat, bone, cartilage and muscle [66]. The relation between these tumors and the normal mesenchymal counterpart is unique: sarcomas are widely believed to develop as a result of genetic mutations in mesenchymal progenitor cells, nevertheless the exact cellular origin of most of these tumors remains unknown. A proper example of this is represented by Ewing's sarcoma (ES). The ontogeny of ES initiating cells is still controversial. While originally believed to be fully derived from primitive neuroectodermal cells by early neural markers expression, such as neuron specific enolase, S-100, and neurosecretory granules [67-69], some doubts regarding this origin are arising. In fact, it has been discovered that the typical ES translocation involving the EWS-FLI1 fusion gene, can itself induce neuroectodermal differentiation up-regulating a number of genes associated with early neural differentiation [70,71]. These findings suggested the possibility that neuroectodermal characteristics of ES might be a direct result of EWS-FLI1 expression and not necessarily linked to the cell origin and, as an alternative hypothesis, it may be that ES

may derive from MSC [72]. Since most cases of ES arise in bone, it seems plausible that the initiating cell could be resident in the bone. In addition, MSC themselves can exhibit some neuroectodermal features, for examples they can spontaneously express neural markers including S-100, neurofilament M, NGFR, CD56 and nestin [73,74]. In addition, MSCs can also be induced into different neural lineages [75]. The hypothesis of a mesenchymal origin of ES has been further strengthened in other experiments. ES cell lines with knockdown of EWS-FLI1 gene display human MSC features including induction of mesenchymal markers CD44, CD73 and the typical tri-lineage differentiation plasticity displayed by primary MSC [76]. Beside ES, there is evidence that other sarcomas may be initiated by MSC. That is the case for osteosarcoma, which seem to arise from BM-MSC like cells. Although with a weaker evidence than ES, these data suggest that MSC-like cells are capable of differentiation into fat and bone and recently, Berman et al. demonstrated that the combined loss of Rb and p53 in MSC is sufficient to induce osteosarcoma formation in mice [77]. Dealing with AD-MSC, various histologic types of liposarcoma have been associated with MSC. Matushansky *et al.* showed that liposarcomas have been linked with perturbation of MSC adipocytic differentiation suggesting a direct relationship between a MSC maturation arrest and sarcoma [78]. These authors hypothesized that, during differentiation, MSC could encounter an initial genetic assault that leads to differentiation arrest or altered differentiation potential and that secondary genetic changes may induce tumorigenesis.

Tumor-stroma interactions in local tumor progression: the pancreatic adenocarcinoma

PA is the most common pancreatic tumor and accounts for more than 85% of all malignant pancreatic neoplasms [79]. Its desmoplastic counterpart, unique among solid tumors, is a key feature of PA. This is characterized by a dense network of stromal elements considered to be originated from pancreatic stellate cells (PSC), endothelial cells, nerve cells and immune cells [80]. PSC, myofibroblasts-like cells identified as source of fibrosis in chronic pancreatitis, could be considered the normal counterpart of TAF in PA. PSC origin is still debated with mesenchymal, endodermal and neuroectodermal derivations as hypotheses. *In vitro*, PSC may

adopt spindle shape morphology with an activated phenotype characterized by expression of α-SMA and desmin [81]. PSC can produce also soluble factors and ECM that seem to stimulate proliferation and survival of PA cells by providing a physical scaffold and growth factor reservoir [3, 82]. TAF in PA have been also indicated to be originating from a circulating cell pool, and MSC recruitment from BM has been recently show to be associated with PA [55,83]. Since hypoxia influenced stromal cells as well as pancreatic cancer cells, the hypoxic environment is reported to play a key role in pancreatic cancer progression [84]. In this model, the severe hypoxia generated within the tumor resulted in a dramatic expression of several growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), representing pivotal chemotactic and mitogenic factors for MSC. Thus, MSC can migrate toward tumor blood vessels and promote vasculogenesis by an autonomous VEGF production and, therefore, further empower the pro-angiogenic potential of TC [85]. In PA, the role of TAF within the desmoplastic reaction is generally considered a sign of tumor progression, even if the great amount of matrix in PA may be, on the contrary, a barrier against tumor cell dissemination [86].

Tumor-stroma relationship in breast cancer: from local progression to metastatization

The interaction between TC and TS has been described in BC where TC influence stroma and *vice-versa* [87]. BC is a heterogeneous group of cancers originating from mammary glands. With different extent, BC is also characterized by a desmoplastic reaction, which involves the recruitment of a variety of stromal cells with pro-tumorigenic activities [88]. BC stroma contains increased number of TAF and immune cells associated with an increased collagen I, fibrin deposition and enhanced angiogenesis [3]. TGF-β, produced by BC, promotes a conversion of resident tissue fibroblasts into tumor-promoting TAF which, similarly for PA, facilitated tumor growth and progression. In turn, it is now recognized that the secretion of high levels of other molecules, such as stromal-derived factor-1 (SDF-1α or CXCL12), by TS can trigger specific receptors (i.e CXCR4) on BC cell surface and act as pro-tumorigenic factors.

This axis SDF-1α/ CXCR4 is also relevant in the interaction of BC cells with marrow MSC. BC have the ability to attract marrow derived MSC and in turn, BC preferentially metastasize to the bone marrow. In both cases, SDF-1α seems to be involved [89]. In addition, in animal models, MSC exhibit increased avidity to tumor sites when directly introduced into the peripheral blood [90]. Spaeth *et al.* investigated the possibility that MSC can be converted into TAF [35]. The observation that MSC can express an activated fibroblast phenotype, when exposed to xenograft models of BC, may demonstrate that microenvironmental stimuli can drive MSC into TAF. Karnoub et al. showed how MSC, mixed with BC cells before subcutaneous injection, can favour neoplastic cell dissemination by secreting the chemokine CCL5, suggesting that stromal cells in BC may not only facilitate local progression but may also enhance their motility, invasion and metastasis [89]. Dealing with BC metastasis, MSC have been additionally implicated in the interaction with BC cells inside the marrow via direct contacts as well as by secreted factors, there MSC, within specific niches, promote BC growth and invasion through multiple molecular mechanisms [35].

Tumor stroma as a novel but still controversial target for cancer therapy

Targeting tumor stroma by molecules as adjuvant treatment for cancer therapy

Thanks to these multiple evidences underlining the central role of TS in cancer formation and progression, it is reasonable to assume that a damage to TS may led to retardation or abrogation in tumor growth. Presumed advantages in targeting TS include that these cells are not as genetically unstable as cancer cells and are less likely to develop drug resistance. Inside the entire TS compartment, in this review we mostly focus on approaches targeting TAF, since these elements lead and influence more than others cell tumorigenesis [10]. The role of TAF in angiogenesis, TC proliferation and invasion thanks to the release of factors interfering with cell cycle, anti-cancer therapies and prevention of immune-surveillance has been discussed previously [91,92].

The cell surface molecule known as fibroblast activation protein (FAP) represents a promising candidate to specifically target TAF safely [15]. Mature somatic tissues, except for activated

fibroblasts during wound healing and within tumor stroma, do not express FAP [93]. A phase I study, using an anti-FAP antibody in patients with advanced colorectal carcinoma or non-small-lung carcinoma, showed high specificity for tumor sites without evident side effects [94].

Several other growth factors and hormones have also been used to decrease the number of activated fibroblast in cancer [95-97]. The hepatocyte growth factor (HGF)/Met pathway has been a target in several early pre-clinical studies targeting TAF, since it represents an essential molecule in epithelial-stromal crosstalk. HGF is primarily expressed by fibroblasts, while the cognate receptor, c-Met is expressed by epithelial cells [98]. Several reports have supported the transforming ability of HGF [99]. Ohuchida, by irradiating TS cells produced an activation of c-Met in carcinoma cells causing tumor progression. Interestingly, a specific antagonistic effect of HGF could block the enhanced invasiveness of pancreatic carcinoma due to irradiated stroma. More recent data reported the development of a soluble decoy c-Met receptor that interfered with HGF binding to c-Met significantly inhibiting the proliferation and metastasis in xenografts [100]. In addition, several studies using a competitive agonist of Met and anti-HGF-antibody obtained a remarkable decrease of metastatic potential and TC growth [101,102].

TAF also express various types of MMP that play a role in both epithelial-TAF signalling and in extracellular matrix remodelling. Increased expression of several MMPs (MMP-9, -2 and -3) or decreased expression of their inhibitors (i.e. TIMP2) has been associated with tumor progression or an invasive phenotype [103,104]. Recently, early clinical trials based on MMP inhibitors were performed to validate this approach on a large variety of tumor types [105]. However, their efficacy has been limited due to adverse effects, possibly due to an MMP role in normal tissue homeostasis, wound healing and angiogenesis [106].

As already mentioned for BC, the SDF1-α/CXCL2-CXCR4 pathway represents one of the possible targets against TS development. The CXCR4 antagonist, AMD3100 has been shown to be associated with tumor growth inhibitory effects in pre-clinical studies, blocking the TS-TC interactions within the tumor sites and in paral-lel reducing the intra-tumoral recruitment of marrow derived MSC [107,108].

Recently, Crawford et al. suggested that TAF may also mediate a resistance to anti-angiogenic therapies [91]. In a murine model, TAF extracted from an anti-VEGF treatment refractory lymphoma up-regulate PDGF-C mRNA levels of up to 200 fold compared to TAF isolated from non-refractory tumors. Those authors demonstrated that elevated levels of PDGF-C could inhibit the effect of VEGF inhibitors indicating that the use of anti-PDGF-C neutralizing antibody may overcome the ineffectiveness of the anti-VEGF compounds. In addition, in sensitive tumors, a combinatory treatment with anti-PDGF-C and anti-VEGF antibodies was revealed to be more effective than an anti-VEGF treatment alone.

Since pancreatic cancer represents a tumor with a robust desmoplastic reaction, several attempts have been established to target TAF. COX-2 is a key mediator of inflammation and recently this molecule has been shown to be expressed both by stromal and tumor epithelial cells [109]. Sato at al demonstrated that, in primary TAF from pancreatic cancer, COX-2 expression was markedly increased when TAF were co-cultured with cancer cells [110]. Additionally, it has been also suggested that COX-2 might promote tumor growth and invasiveness [111]. Thus, in this scenario, COX-2 inhibition could represent a suitable strategy to prevent cancer progression or invasion.

The inhibition of TS proliferation may also allow an additional indirect positive impact in oncology and in particular it can be associated with an improved delivery of cytotoxic agents to TC. Olive et al., using a specific inhibitor (IPI-926) of Hedgehog receptor expressed on TS and involved in pancreatic cancer desmoplastic reaction [112], were able to inhibit stromal proliferation and transiently increase tumor vascularization. This was followed by an increase delivery of gemcitabine to TC and, most importantly, by an increased mouse survival. This study also demonstrated that inhibition of the Hedgehog pathway can reduce tumor-associated stromal proliferation confirming a previous study by Yauch et al. who have shown that the effectiveness of HhAntag, an antagonist for Hedgehog pathway, was due to its ability to inhibit TS proliferation and this in turn led to inhibition of tu-

Table 2. Bone marrow or adipose derived (BM, AD) mesenchymal stromal/stem cells influence on different tumor types

Tumor Source	Impact	References
melanoma cells	↑	*Djouad F. et al.* Blood. 2003; 102: 3837-44
colon carcinoma	↓	*Ohlsson L. et al.* Exp Mol Pathol. 2003; 75 : 248-55
pancreatic adenocarcinoma	↑	*Ishii G. et al.* Biochem Biophys Res Commun. 2003; 309 : 232-40
breast cancer	↓	*Robinson S.C. et al.* Cancer Res. 2003; 63 : 8360-65
colon carcinoma	↑	*Zhu W. et al.* Exp Mol Pathol. 2006; 80 :267-74
Kaposi's sarcoma	↓	*Khakoo A.Y. et al.* Journ Exp Med. 2006; 203 :1235-47
breast cancer	↑	*Karnoub A.E. et al.* Nature 2007; 449 : 557-65
breast cancer	↑	*Mishra P.J. et al.* Cancer Res. 2008 ; 68 : 4331-39
pancreatic adenocarcinoma	↓	*Cousin B. et al.* Plos ONE 2009; 4: 1-10
ovarian cancer	↑	*Spaeth E.L. et al.* Plos ONE 2009; 4: 1-11
Kaposi's sarcoma, breast, lung , prostatic cancer	↑	*Zhang Y. et al.* Cancer Res. 2009; 69: 5259-66
melanoma glioblastoma	↑ ↓	*Kucerova L. et al.* Mol Cancer 2010; 28: 129

Inhibition (↓), growth (↑)

mor growth [113].

Altogether, these results indicate that, although TS may not necessarily be the primary target for defeating cancer, it can certainly be seen as an obstacle to be overcome to profoundly enhance anti-tumor effects in drug combinatory approaches.

Targeting tumor stroma by stromal cells: Trojan horses or food for tumors?

An alternative strategy to target TS is suggested by one of the possible TAF ontogenies: the circulating MSC pool. Accordingly to this approach, based on the powerful capacity of the tumor environment to recruit stromal progenitors, one can inject high doses of selected and purified MSC populations in the attempt to provide killer MSC rather than nourish TS progenitors. although this concept has been developed for years in our group and in several others, the results seem to be contradictory with without an unequivocal influence of wild-type MSC against tumors (**Table 2**).

Zipori et al. initially demonstrated a bi-modal action of both murine and human BM-MSC against several tumor cell lines, describing that *in vitro* MSC caused a dramatic increase in human lung and colon carcinoma cell lines growth. In contrast, MSC inhibited the *in vitro* cloning of both human and murine sarcoma cell lines. Thus, these opposite effects seem to depend on the tumor type [114].

In an elegant experimental model of Kaposi Sarcoma (KS), the authors have observed that intravenous injection of MSC potently inhibited tumor growth [115]. The inhibition was observed not only when MSC and KS cells were co-injected, but also when MSC were administered in mice bearing already established tumors. The *in vivo* tumor-suppressive effect of MSC against KS was found to be E-cadherin dependent and due to a yet unclear inhibition of Akt activation within TC. Confirming the pioneering data of Zipori, these *in vivo* data seem to be strictly tumor related, and inhibition was not reproduced for other TC types such as breast and prostate cancers tested.

Djouad and colleagues described an *in vitro* immunosuppressive effect of both mouse and human MSC on activated T lymphocytes, allogenic splenocytes and professional antigen presenting cells [116]. These findings were transferred into an immunocompetent mouse model of melanoma co-injected with MSC. In those experiments, MSC revealed the capacity to facilitate growth and progression of an allogenic melanoma cell line, suggesting how MSC can facilitate tumor escape from an allogeneic immune system.

In a different setting, Karnoub and colleagues suggested a critical role of MSC in the development of BC metastases. Distinct human BC cell lines showed an increased metastatic potential to the lungs when subcutaneously co-injected

with MSC. However, only one cell line showed accelerated primary tumor growth when co-injected with MSCs. Interestingly, the co-injection of TC with other types of mesenchymal cells, such as BJ fibroblasts, did not enhance tumor growth or metastasis. In search for factors differentially expressed in co-cultures of human BC cells and MSC, the same authors identified the chemokine CCL5-RANTES to be up-regulated up to 60-fold in co-cultures compared to monocultures. It seems that CCL5, released from MSC stimulated by TC, induces the recruitment of tumor-associated macrophages and endothelial cells in site of primary tumor growth enhancing cancer metastatization [117,118].

In addition, a toll-like receptor ligands known as LL-37 has been recently linked to MSC tumor homing. Several studies have uncovered how different tumor types, including ovarian, breast and lung cancer, show a high expression of LL-37 [119-121]. It has been demonstrated that LL-37 acts as proliferative signal, pro-angiogeneic factor and chemoattractant for various immune cells through activation of Formyl Peptide Receptor Like-1 (FPRL-1) as member of the toll like receptor family [122-124]. Coffelt et al. proposed that LL-37, expressed on ovarian cancer cells, could be also involved in MSC recruitment at tumor site [125], demonstrating LL-37 activates MSC migration in a dose dependent manner. Their in vivo studies have also shown a clear homing of MSCs into tumors demonstrating that an inhibition of MSC engraftment into TC resulted in disorganization of the fibroblast-vascular network as well as a reduction of tumor growth.

Beside the BM-MSC, AD-MSC have been also described to facilitate tumor progression after migration from subcutaneous injection into TS compartment promoting cancer cells proliferation [126]. On contrary, Cousin and colleagues shown that AD-MSC are able to strongly reduce pancreatic cancer cell viability and proliferation, inducing a cancer cell necrosis following G1-phase arrest. The authors were additionally able to reproduce the anti-tumorigenic effect of AD-MSC in vivo demonstrating a strong and long-lasting inhibition of tumor growth [127].

Transferring tumor killing activity to MSC: the Mesenkillers

MSC have been shown to preferentially inte-

grate and persist into TS. Despite both their pro- and anti-neoplastic attitudes are still under investigation, this feature prompted their use as delivery vehicles for various anti-cancer biological agents, in particular for that systemic administration is precluded due to their short half-life and excessive toxicity at the doses required for a therapeutic benefit [39]. In addition, the persistence of cellular vehicles delivering molecules into poorly accessible body sites, such as the brain, may by-pass the limits of systemic injections often associated with a limited bioavailability in those specific districts.

The application of MSC in the gene therapy settings is supported by their favourable biological properties suggesting their application as cellular vehicles. MSC have a relatively simple isolation methods and limited expression of alloantigens; also they retain the ability to be expanded in vitro, to be easily manipulated, to engraft in animal models and to express therapeutic proteins when systemically or locally injected [35,128].

There have been various approaches to introduce transgenes into MSC (**Table 3**). Gene deliveries were performed by retroviral, lentiviral and adenoviral vectors with different efficiencies. While adenoviral vectors have been associated by a transient transgene expression [129], the use of both Moloney-based and HIV-based retroviral vectors have been more stable and efficient [130,131]. In particular, the capacity of transducing non-dividing cells represents one of the advantages in lentiviral strategy since a subset of mesenchymal progenitors has been described to be quiescent [132]. The manipulation of MSC was also performed by modifying agents enabling a gene penetration through cell membrane. That is the case of physical (i.e. electroporation) and/or chemical (i.e. calcium phosphate and polycations) agents, still associated with a poor efficiency [133,134].

Based on these distinct gene modification techniques, MSC have been exploited to deliver genes encoding for a variety of biological agents impacting tumor growth. Generally, these biological agents are naturally produced anti-tumoral molecules that can be more efficiently and prominently synthetized by the gene modified MSC. In particular, molecules derived form immune effectors (i.e Natural Killer) and physiologically targeting tumors have been consid-

Table 3. Strategies to deliver therapeutic transgenes by vector engineered mesenchymal stromal/stem cells (MSC) from different sources

MSC Source	Tumor Source	Vector	Therapeutic Transgene	References
BM (human)	melanoma (human)	Adenoviral	INF-β	*Studeny M et al.* Cancer Res. 2002 1:3603-8
BM (rat)	brain glioma (rat)	Adenoviral	IL-2	*Nakamura K et al.* Gene Ther. 2004 11:1155-64
BM (human)	breast cancer, human melanoma (human)	Adenoviral	INF-β	*Studeny M et al.* J Natl Cancer Inst. 2004 3: 593-603
BM (human)	brain glioma (murine)	Adenoviral	INF-β	*Nakamizo A et al.* Cancer Res. 2005 15:3307-18.
BM (human)	melanoma (murine)	Retroviral	IL-12	*Elzaouk L* Exp Dermatol. 2006 15:865-74
BM (mouse)	colon-26 lung metastasis (murine)	Adenoviral	NK4	*Kanehira M et al* Cancer Gene Ther. 2007 14:894-903
AD (human)	colon adenocarcinoma (human)	Retroviral	Cytosine deaminase (CD)	*Kucerova L et al.* Cancer Res. 2007 1:6304-13
BM (mouse)	lung (murine)	Adenoviral	CX3CL1	*Xin H et al.* Stem Cells. 2007 25:1618-26
UCB (human)	glioma (human)	Adenoviral	stTRAIL	*Kim SM et al.* Cancer Res. 2008 1:9614-23
BM (mouse)	lung (murine)	Adenoviral	INF-β	*Ren C et al.* Gene Therapy 2008 1-8
BM (human)	lung (human)	Adenoviral	TRAIL	*Mohr A et al.* J Cell Mol Med. 2008;12:2628-43
AD (human)	prostate (human)	Retroviral	Cytosine deaminase (CD)	*Cavarretta IT et al.* Mol Ther. 2010 18:223-31
BM (human)	brain glioma (murine)	Adenoviral	S-TRAIL	*Menon LG et al.* Stem Cells. 2009 27:2320-30
BM (human)	glioma (human)	Adenoviral	Delta24-RGD	*Yong RL et al.* Cancer Res. 2009 1:8932-40
BM (human)	glioma (human)	Lentiviral	S-TRAIL	*Sasportas LS et al.* PNAS. 2009 Mar 24:4822-7
AD (human)	cervical carcinoma (human)	Retroviral	TRAIL	*Grisendi G et al.* Cancer Res. 2010 May 1:3718-29.
UCB (human)	glioma (human)	Adenoviral	IL-12	*Ryu CH et al.* Hum Gene Ther. 2011 Jan 24
BM (human)	breast - prostate cancer- (human) fibrosarcoma (murine)	Lentiviral	Timidina Kinase (TK)	*Song C et al.* Hum Gene Ther. 2011 22:439-49

BM: Bone Marrow, AD: Adipose tissue, UCB: umbilical cord blood.

ered, such as Interferons (INF) and death-ligands (DL). According to this, we are transferring the killing activity of immune cells to MSC, suggesting this approach as a mesenkiller–based therapy.

Studeny et al. have shown that IFN-β toxicity, after systemic administration, can be reduced by a more targeted delivery of MSC secreting-IFN-β directly into tumors [39]. By adenoviral vector modification, MSC were capable to deliver IFN-β both *in vitro* and in distinct xenograft models of breast and melanoma. The impact of infused of MSC producing IFN-β overcame the effect of human recombinant IFN-β injections with a prolonged survival of treated animals.

An additional approach adopted to fight melanoma, glioma and breast carcinomas [135] relies on the manipulation of MSC to express a pro-drug converting enzyme, such as cytosine deaminase, an enzyme that converts 5-fluorocytosine to a soluble toxic molecule that kills both MSC and the neighbouring cancer cells through a bystander effect.

The MSC based oncolytic viral therapy, by local delivery in brain tumors, constitutes a further example. Yong *et al* showed that a delivery of the Δ24-RGD virus, a tumor selective replication competent adenovirus with specific cellular infectivity to tumors, produces long term survival in an animal model of glioma. This underlines how MSC are suitable to delivery Δ24-RGD to the brain overcoming multiple barriers of intracerebral viral infection [136,137].

MSC can be also modified to mimic plasma cells producing monoclonal antibodies such as the scFv EGFRv III, which targets EGF receptor on glioma cells surface [138]. These MSC producing antibodies represent a paradigmatic strategy to more efficiently deliver therapeutic molecules that poorly penetrate the blood brain barrier acting as efficient therapeutic vehicles for malignant brain tumors

Recently, MSC from bone marrow, adipose tissue and umbilical cord have been modified with members of the DL family which includes tumor necrosis factor (TNF-alpha) and TNF-related apoptosis inducing ligands (TRAIL) as powerful anti-cancer molecules limited in their systemic delivery to patients. At present, although TNF-alpha variants have been recently produced by

modified MSC in a prostate cancer model [139], TRAIL has a significantly higher therapeutic profile against cancer: TRAIL signalling pathways, predominantly triggered by death receptors, could induce apoptosis in cancer cells without significant toxicity toward normal cells [140]. The recombinant TRAIL *in vivo* administration has been limited by short half-life in plasma and undesired toxicity against normal tissues [141]. For these reasons, several investigators have shown that MSC expressing both transmembrane and secreted TRAIL are able to infiltrate and abrogate tumors. Kim et al described that human umbilical cord blood mesenchymal stromal cells (UCB-MSC) are suitable cellular vectors for TRAIL delivery, since these cells were resistant to TRAIL-mediated apoptosis revealing a marked migratory ability and potent anti-tumoral activity toward glioma [142]. These data were supported by Menon *et al* using BM-MSC modified with a lentivirus expressing secretable form of TRAIL (S-TRAIL). That study confirmed that BM-MSC expressing S-TRAIL might provide a performing drug delivery system for intracranial glioma [143]. Other tumor types were tested, and Mohr *et al.* described the use of MSC expressing TRAIL against a lung cancer cell line. They genetically modified BM-MSC by an adenoviral vector expressing the full length human TRAIL and induced apoptosis by cell-to-cell contact both *in vitro* and *in vivo* [144]. More recently, others reported how murine BM-MSC may transport S-TRAIL to induce human pancreatic cancer death without the need of cell-to-cell contact. With the limitations to a xenogenic approach, this secretable TRAIL delivery by murine MSC could be more efficient to eradicate tumors without the need of cell proximity [145].

We have recently reported that also adipose AD-MSC may efficiently transport membrane-bound full-length TRAIL [90]. In this context, we showed that 3 different tumor cell lines representative of cervical carcinoma, pancreatic and colon cancer are susceptible to AD-MSC armed by TRAIL. More importantly, isolating and co-culturing primary lung cancer cells with AD-MSC armed by TRAIL, we were able to induce apoptosis *in vitro*, suggesting this strategy may be actually introduced as a therapeutic option for incurable cancers.

We also uncovered that this anti-tumor effect was essentially due to a cell to cell contact between AD-MSC expressing TRAIL and TC, medi-

ated by caspase-8 activation which rapidly activated and induce cell death. Our study also investigated the feasibility of associating the AD-MSC TRAIL approach with other therapeutic agents, such as Bortezomib, a well-known proteasome inhibitor. TRAIL refractory tumors were then treated *in vitro* in the attempt to sensitize them to our cell therapy approach. The obtained results demonstrated the synergic effect of AD-MSC TRAIL and Bortezomib against a BC tumor cell line, known to be resistant to TRAIL. Finally, by two different animal models, we demonstrated that TRAIL producing AD-MSC were able to migrate and persist in tumors without toxic effect and with a relevant benefit. These *in vivo* models revealed also more prominent HeLa growth in mice treated with AD-MSC only labelled with GFP in comparison with TC alone, as described for wild-type BM- and AD-MSC [126]. Interestingly, this trend decreased over time and in the late time points appeared totally reversible. The reasons behind this phenomenon are currently under investigation; it may be possible that the ratios between AD-MSC and TC can initially be in favour of a proliferative burst, however when cancer cells become prevalent, the amount of AD-MSC may not be adequate to feed TC. Nevertheless, the anti-proliferative effect exerted by mesenkillers producing TRAIL was able not only to counterbalance the tumor supportive capacity of AD-MSC, but also determine a powerful inhibitory effect opening, a novel promising approach for targeting cancer.

While the identification of supportive cancer stroma is a relatively old concept, the strategy to identify stromal cells as therapeutic targets is increasingly possible by the understanding of the complex interplays between tumor and stroma. It may be that, by this strategy, we will not be able to cure all the still incurable cancers but, certainly, one would be a great start.

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Address correspondence to: Massimo Dominici, MD, Dpt. Oncology, Hematology and Respiratory Diseases, University-Hospital of Modena and Reggio Emilia Via del Pozzo, 71; 41100, Modena, Italy Tel: 0039-059-422-2858, Fax: 0039-059-422-3341 E-mail: massimo.dominici@unimore.it

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