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Bone marrow cells as precursors of the tumor stroma

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Abstract

Cancer is a systemic disease. Local and distant factors conspire to promote or inhibit tumorigenesis. The bone marrow is one important source of tumor promoting cells. These include the important mature and immature hematopoietic cells as well as circulating mesenchymal progenitors. Recruited bone marrow cells influence carcinogenesis at the primary site, within the lymphoreticular system and even presage metastasis through their recruitment to distant organs. In this review we focus on the origins and contribution of cancer-associated fibroblasts in tumorigenesis. Mesenchymal cells present an important opportunity for targeted cancer prevention and therapy.

Keywords

Cancer; Fibroblasts; Tumor microenvironment; Mesenchyme; Carcinogenesis

Introduction

Cancer is defined by changes within the neoplastic epithelium, however, it is the dynamic stroma that heralds many of the earliest events in carcinogenesis. Stromal cells in cancer arise from all germ layers. The mesoderm provides mesenchymal, hematopoietic and endothelial cells. The ectoderm contributes additional mesenchymal cells as well as cancer-associated nerves via the neural crest. Ultimately, the endoderm provides invading neoplastic cells in malignant solid tumors, many of which adopt a mesenchymal phenotype [1–3]. Like the epithelial compartment, the tumor microenvironment has disturbed differentiation. There is an expansion of stromal stem and progenitor cells with distinct functions compared to their more differentiated counterparts. Many stromal cells in cancer emerge from a tissue resident population, but a substantial portion of cancer stroma is recruited from distant sites [1,4,5]. In this review we focus on the organization, recruitment and contribution of mesenchymal cells to solid organ cancer.

Mesenchymal organization in normal tissues

Before considering mesenchymal stromal cells in cancer, it is worth exploring mesenchymal stromal cells in the normal tissues, in which the tumors develop, and the normal bone marrow, which is the quintessential mesenchymal organ. Bone marrow mesenchymal cells are heterogeneous [6,7]. They may be considered in terms of their functional mesenchymal lineages: chondrocyte, adipocyte, osteoblast and marrow stromal cells. But, even within these discrete lineages there is considerable diversity in terms of cellular differentiation that may be related to the specific cellular and extracellular context of these cells (referred to as their specific “niche”). These differences in differentiation affect both the multilineage potential of a given cell as well as its biological function [7,8].

The bone marrow is structurally organized to achieve its two chief responsibilities: hematopoiesis and skeletal integrity. In the bone marrow, an intricate network of small endothelial-lined fenestrated vessels, known as sinusoids, are woven into a lattice of trabecular bone. Niche specific microenvironmental gradients, involving calcium and oxygen tension, exist between these endosteal and perivascular zones to help regulate local hematopoietic stem cells (HSCs) [8]. The key niche cells contributing to the support of HSCs include osteolineage cells, endothelium, macrophages and perisinusoidal stromal cells, along with the sympathetic nerves that innervate them [8–10].

The perivascular bone marrow mesenchymal cells are believed to contain true mesenchymal stem cells (MSCs) [6–9]. Interestingly, perivascular stromal cells are also believed to contain MSC-like cells within extramedullary organs [11,12]. Given the lack of specific fibroblast and MSC markers the functional assays of clonogenicity (number of colony forming fibroblasts per cells plated) and multipotentiality *in vitro* are often used to stratify mesenchymal stromal cells. *In vitro* MSCs (those that exhibit multipotentiality and clonogenicity) may be enriched from the bone marrow through initial depletion of non-stromal populations (CD45 (hematopoietic), CD31 (endothelial), Ter119 (erythrocyte) triple negative) and then positively selected for using specific perivascular or stem cell markers such as, in the mouse, Sca1, CD105 or CD140a [6,7] or in humans CD105, CD73 and CD90 [13]. Even within these enriched populations of stromal cells only 2%–5% of cells prove to be clonogenic [6,7]. This suggests that true MSCs are extremely rare, which is likely, but also perhaps that our attempts to enrich for them and to cultivate them could be significantly improved. Nevertheless, the *Nestin*-GFP transgenic line is expressed in perisinusoidal cells and identifies a population enriched for clonogenic cells with multilineage potential *in vitro* [9]. A separate perisinusoidal transgenic line (*Lepr*-Cre), however, does not lineage trace osteoblasts during development [10]. These conflicting results suggest that there is heterogeneity even within similar cells in similar parts of the bone marrow. As proof of concept it is possible to transplant mesenchymal progenitors cells and achieve engraftment in the bone marrow and other organs [1,14] and tracing from some transgenic lines suggests the presence of a true MSC [9,15]. But the key issue remains the exact nature of the engrafted or the transgenically recombined cell. Which cells within an often heterogeneous population of transplanted or recombined cells are the true MSCs, which are lineage restricted mesenchymal progenitors and which are only differentiated cells that have lost the capacity for self renewal and multipotentiality *in vivo*? As newer, more specific transgenic

markers and methods develop we will ultimately be able to reconcile the evolution, function and specific potential of these cells.

Interestingly many of the fundamental features of mesenchymal organization in the bone are replicated in extra-medullary organs. In the intestine, for instance, the microenvironment also consists of an integrated community of stromal cells with extracellular matrix interspersed by pericyte-covered endothelium [1–3,16]. Whilst extramedullary organs lack the obvious osteoblasts and chondrocytes of the bone marrow, the intestinal myofibroblast sheath that rests just beneath the epithelial basement membrane is proposed to have an important role in intestinal stem cell function and throughout the mucosa perivascular stromal cells may also contribute important niche signals [1,4,5,16,17]. Extramedullary organs may even contain stromal cells with *in vitro* MSC capabilities [6,7,11]. In the gastrointestinal tract MSCs are recognized to be important cells in colonic wound healing, perhaps through the direct regulation of intestinal re-epithelialization [7,8,18,19]. It has been speculated that mesenchymal cells surrounding the intestinal crypts, vessels or within the intestinal serosa are the source of these MSCs [8,20]. Whether they also contribute to ongoing mesenchymal renewal in health or injury, however, is currently unknown. An interesting hypothesis is that stromal mesenchymal cells behave similarly in the tumor microenvironment: vital supportive cells nurturing the cancer stem cell niche [8–10,21].

Mesenchymal organization in cancer

In the tumor microenvironment, many of the mesenchymal subtypes remain the same, albeit that the number, distribution and even their compartment of origin are altered. Attempts to resolve stromal heterogeneity in cancer are complicated by the same lack of specific markers that have limited the classification of normal mesenchyme. Without the capacity to mark, measure and modify specific subsets of fibroblasts using discrete gene expression profiles, we are left with a more descriptive vocabulary. Thus the term cancer-associated fibroblast (CAF) is used for cells purely on the basis of their spindle-shaped morphology and their peritumoral context. Immunohistochemistry has been used to try and resolve CAF heterogeneity as well as to exclude other potential cell types such as inflammatory cells, endothelium, nerves and muscle. Several markers have been reported to help define distinct subpopulations of CAFs. The activated fibroblast markers of α SMA and Fibroblast Activation Protein (FAP) have been used extensively to examine the tumor promotion of mesenchyme in co-injection tumorigenicity studies [6–9,22,23]. Others have shown that certain markers such as FSP1+ label atypical CAFs that lack other “typical” stromal fibroblast markers, such as α SMA, vimentin, fibroblast-activation protein, fibroblast-associated antigen or prolyl 4-hydroxylase [11,12,24,25]. More recently, however, some groups have suggested that FSP1 is also a marker of activated macrophages that may explain some of these differences [6,7,26]. Another defining feature of CAFs compared to normal fibroblasts is widespread DNA hypomethylation [13,27]. Recent studies have also identified that an inflammatory gene expression signature is an important characteristic of CAFs relative to normal fibroblasts [1,6,7,28]. Ultimately CAF markers and populations based on distinct CAF biology will emerge to improve our understanding as well as present targets for prognostication and therapy.

In keeping with the concept of cancer stem cells within the neoplastic compartment [9,29], an interesting notion is whether there are also desmoplasia stem cells that sustain the biologically diverse and differentiated population of peritumoral mesenchymal cells? A perivascular mesenchymal cell expressing *Adam12* was recently reported as the non-MSc origin of myofibroblasts (MF) in fibrosis [10,30]. Interestingly, the *Adam12* model in which a non-MSc MF-progenitor is the immediate precursor to CAFs is in keeping with discoveries in the bone marrow that osteoblasts are maintained by osteolineage restricted progenitors rather than a true multipotential MSCs *in vivo* [1,7,14]. Clonogenicity and multipotentiality may be effective biological arbiters to help understand CAF heterogeneity as they have been in the bone marrow stroma.

Given that flow cytometry has helped clarify mesenchymal populations within the bone marrow, we have utilized flow cytometry to try and resolve mesenchymal heterogeneity and enrich for clonogenic fibroblasts in the tumor microenvironment. In an experiment designed for this review, we subcutaneously injected the C57BL/6 syngeneic colorectal cancer cell line (MC38, not fluorescent, 1×10^6 cells) into red fluorescent C57BL/6 mice (JAX stock number 7676, R26mT/mG). After 3 weeks the mice were sacrificed and the tumors harvested and digested to a single cell suspension. The cells were incubated with fluorescent antibodies against key cell surface markers EpCam (APC), CD45 (APC), CD31 (APC), Ter119 (APC), CD105 (PE-Cy7) and CD140a (indirectly conjugated to FITC) and then subjected to fluorescence activated cell sorting (Fig. 1). Using the MSC markers from a recent study [7,9,15], we analyzed the clonogenicity of red fluorescent stromal cells (i.e. recruited, non-neoplastic cells from the host that were CD45⁺/CD31⁺/Ter119⁺ triple negative) on the basis of whether they expressed CD105 and CD140a (Fig. 1). In keeping with the published findings from the bone marrow, we found that the CD45⁺/CD31⁺/Ter119⁺ CD105⁺/CD140a⁺ population had the greatest CFU-F efficiency (Fig. 1) [7]. This experiment proves that strategies employed in the bone marrow can be applied to the tumor microenvironment. But, it remains to be seen if clonogenic cells *in vitro* are truly the desmoplasia and tumor promoting stem cells *in vivo*.

Flow cytometry is an important tool in resolving heterogeneous cell populations on the basis of cell surface markers, but unfortunately it is blind to the cellular and extracellular context of a cell that may be as important as the cell surface markers that it expresses. Additionally, utilized marker sets used to define mesenchymal cell lineages are subject to alterations upon *in vitro* culture and potentially during isolation processes as well [1]. New techniques including those that allow single cell resolution of gene expression *in situ* will help clarify and complement flow cytometric approaches to define the tumor microenvironment.

The origin of cancer-associated mesenchyme

Developing mesenchyme in the bone marrow and gastrointestinal tract are likely to provide important insight for the origin of cancer-associated mesenchyme, which is, after all, a form of development in adulthood.

In long bone development early mesenchymal cells are attracted into and form condensations within the site of the future bone. In most of these condensations the cells

differentiate into chondrocytes where they secrete type II cartilage. Within the core of these mesenchymal aggregates chondrocytes differentiate into hypertrophied chondrocytes shifting from type II to type X collagen production [31]. These hypertrophied chondrocytes direct the subsequent mineralization of the bone. They secrete vascular endothelial growth factor and recruit chondroclasts, new blood vessels and attract other mesenchymal cell to differentiate into osteoblasts. Secondary ossification centers are established to allow ongoing bone lengthening during post-natal life, where again it is the chondrocytes that help direct vascular and osteoblastic invasion and further bone growth [31]. Many of the signaling pathways involved in bone development, including SHH, BMP and WNT, are also important in visceral mesodermal development in the gastrointestinal tract.

Gastrointestinal mesodermal development occurs in unison with the development of the endodermal gut tube. The visceral mesoderm gives rise to most of the non-epithelial components of the gut, except for the enteric nervous system that develops from the migrating neural crest (originally ectoderm) [32]. Many of the mucosal, submucosal and perivascular mesenchymal cells probably originate from serosal mesothelial cells [33,34]. These mesotheliocytes, perhaps analogous to the invading mesenchymal cells that develop into osteoblasts in the bone, ultimately give rise to α SMA positive vascular smooth muscle cells, as well as periglandular mesenchymal cells within the future mucosa [16,33]. The mesothelial cells do not, however, give rise to the muscularis propria [33]. Despite the mesothelium's presumed mesodermal origin, one report showed enteric ganglia, which is derived from the neural crest, labeled by a supposed mesothelial-tracing strategy (WT1-Cre line) [33]. It is possible that the adult intestinal mesenchyme has a contribution from both mesoderm and the neural crest. Interestingly, an analogous finding was reported for the bone where an early transient wave of MSCs was derived from the neuroepithelium, partly via the neural crest [35]. Such heterogeneity during development may have implications for lineage contributions to cancer-associated mesenchyme.

A number of studies have tried to determine the origin of CAFs, and have variously considered resident fibroblasts, smooth muscle cells, endothelial cells, epithelial cells (through EMT), and recruited bone marrow-derived cells such as MSCs and fibrocytes as candidates [1,2,14,36,37]. Several studies support the concept that cancer-associated mesenchyme is derived from multiple different sites. In a genetically-engineered model of pancreatic insulinoma Direkze et al. demonstrated that bone marrow-derived cells can give rise to CAFs and fibroblasts [36]. This finding has been confirmed in other cancer models [38] and we have also shown that in both mouse and human gastric cancer a significant population of CAFs are derived from bone marrow cells [1,5]. Undoubtedly, however, local tissue mesenchyme also contributes. A recent study used a variety of transplantation and fluorescent marker cell tracking techniques to show that the majority of FSP1 and FAP expressing CAFs originated from the bone marrow while most of the vascular and perivascular stroma was recruited from neighboring tissue primarily adipose tissue [4]. The notion that cellular origin leads to the expression of specific markers and results in discrete biology within the tumor microenvironment is very important in the translation of our mouse findings into human tumorigenesis.

Transgenic markers are potentially a very effective means to resolve heterogeneity. A limitation with many of the inducible Cre transgenic lines currently available, however, is that they are not sufficiently specific for mesenchymal lineage tracing *in vivo* [7,9]. The beauty of transgenic approaches is not limited to lineage tracing or the definition of origin. With suitable markers, one can also selectively and temporally ablate specific CAF subsets or genes in order to assign specific functional relevance to different groups of CAFs. One recent study found that by ablating FSP1 (S100A4) positive CAFs, the investigators were able to reduce metastasis, in part through a loss of Vegfa and Tenascin C production at the metastatic, or perhaps pre-metastatic, site [39,40]. In another notable example, the ablation of FAP-expressing cells, which include cancer-associated pericytes and fibroblasts, impaired tumor growth due to promotion of anti-tumor immunity [23].

This review has focused on mesenchymal cells in cancer, but it is worth considering fibrocytes (from the hematopoietic lineage) as another potential source of CAFs and of course a lineage derived from the bone marrow. Fibrocytes are circulating cells that have some of the proinflammatory functions of macrophages as well as the tissue remodeling capacity of fibroblasts. Their hematopoietic origin is defined by dual positivity for CD45 and LSP-1 [41]. In addition, they also express CD34, α SMA and collagen I. The relative contribution of fibrocytes versus recruited MSCs in the development of the bone marrow-derived peritumoral stroma is an important question, but one that is currently unresolved. Some groups report that fibrocytes can fulfill some of the *in vitro* criteria of MSCs [42], which further blurs the distinction.

The cancer-stromal partnership

What are the signals and mediators that promote the development of cancer-associated mesenchyme as described above? α SMA positive CAFs, perhaps the best-established marker of CAFs, are generated through a number of complementary mechanisms. Chemokines such as stromal cell-derived factor 1 (SDF-1/CXCL12) recruit fibroblast precursors expressing CXCR4, possibly MSCs and potentially fibrocytes, into the stroma from distant sites [1,43]. In the context of additional desmoplastic factors, including PDGF and TGF- β , these precursors differentiate into α SMA expressing CAFs. This is in addition to the local action of PDGF and TGF- β on resident mesenchymal cells. In culture, TGF- β and PDGF can transition normal fibroblasts into α SMA+ fibroblasts, as can treatment with the DNA demethylating agent decitabine [1,22,27]. The relationship between TGF- β , DNA hypomethylation and expression of α SMA, may be mediated in part through TGF- β related down regulation of the DNA methyltransferase *Dnmt1*. The resulting loss of DNA methylation alleviates the transcriptional silencing of the α SMA gene promoter [44].

Interestingly, many of the developmental signaling pathways important in organogenesis are recapitulated in the cancer mesenchyme. HH production by tumor cells act on the surrounding tumor stromal cells [45]. SHH signaling through the stroma is critical for tumor growth with key reciprocal feedback from the stroma to promote epithelial WNT and IGF signaling [45]. Potential SHH targets in the stromal cells include important stromal effectors such as forkhead transcription factors [46] and *Gremlin 1* [1,21]. *Gremlin 1* is a well recognized BMP antagonist in the cancer microenvironment and is another GLI1, and thus

possible HH signaling, target [47]. BMP antagonism can promote WNT signaling within adjacent epithelium and thus BMP antagonists have been postulated to be important stromal factors in the promotion of the pathological cancer stem cell niche. In normal tissues stromal BMP4 promotes epithelial differentiation, but its role in cancer is less clear [48]. BMP4 production by the cancer mesenchyme probably acts to limit tumor proliferation, but is balanced by concomitant BMP antagonism [48]. The interrelated SHH, WNT and BMP pathways in the tumor microenvironment are critical for tumor progression [49].

CAFs also produce a number of important inflammatory mediators, including matrix-metalloproteinases-2, -3 and -9, which can alter the stromal ECM and potentiate invasion, cell motility and metastasis [37]. Cancer-associated fibroblasts secrete chemokines and growth factors (SDF-1, VEGF, hepatocyte growth factor (HGF), insulin-like growth factor (IGF), nerve growth factor, epidermal growth factor (EGF) and FGF2) that directly promote growth in the adjacent epithelium and neovascularization within the stroma [37]. HGF may be of particular importance and recently it was demonstrated that stromal HGF signals were able to confer tumoral resistance to RAF inhibition therapy in BRAF mutant melanoma [50]. Additionally, CAFs are an important source of tumorigenic inflammatory cytokines including IL1 β [1,28].

One of the most interesting discoveries concerning the interaction between bone marrow cells and mesenchyme in tumor promotion occurs at the site of metastasis, or rather, premetastasis [39,51,52]. In a series of elegant studies the Lyden group has shown that cancer-specific exosomes are important in the recruitment and engraftment of Vegf1+ bone marrow cells, primarily hematopoietic progenitors, at sites of future metastasis defined by the upregulation of fibronectin by tissue resident fibroblasts.

The tumor promoting capability of CAFs relative to non-activated fibroblasts is clear. Breast CAFs are more likely than normal fibroblasts to enhance tumor growth, to express α SMA, to produce SDF-1, to enhance angiogenesis and to stimulate the CXCR4 receptor [24,25]. Local SDF-1 production by these cells enhances chemotaxis of CXCR4-expressing endothelial, hematopoietic and mesenchymal cells into the stroma [24]. CAFs from prostate cancer also show greater tumor potentiating effects *in vitro* and *in vivo* compared to normal prostatic fibroblasts [53]. Interestingly, in addition to the contribution from resident CAFs, bone marrow-derived stromal cells also promote tumorigenesis in a number of models [22]. In premalignant conditions, such as colorectal adenomas, there is already a significant expansion of the α SMA+ stromal fibroblasts compared to normal colorectal mucosa [54–56]. This suggests that stromal fibroblasts may play a role in driving pre-malignant lesions as well as in promoting advanced events such as invasion and metastasis. The role of stromal fibroblasts in the initiation of cancer was confirmed by a study in which conditional loss of fibroblast-specific Tgf β type II receptors caused fore-stomach squamous cell carcinoma and prostate neoplasia [57,58]. Similarly, genetic inactivation of PTEN in stromal fibroblasts of mouse mammary glands accelerated the development of mammary epithelial tumors [59]. This suggests that changes within the underlying mesenchyme can initiate as well as advance epithelial cancer.

Therapeutic targets in the cancer-associated mesenchyme

There are several new agents being developed to specifically antagonize CAF-related promotion of tissue invasion and metastasis. One rationale for adjuvant “desmoplasia-directed” therapy is that depletion of the physical stromal barrier will enhance the penetration of co-administered chemotherapy [60]. Inhibition of CAF development is also likely to have a broad range of direct benefits including the modulation of stromal mitogenic signals, [37] ECM remodeling, [61,62] immunological suppressive effects [23], reduction of proangiogenic signals [38,60] and perhaps even inhibition of the premetastatic niche [39]. One stromal target is SHH signaling and there are a number of SHH inhibitors being developed for cancer care [60,63–65]. Such agents may help to antagonize the important SHH–BMP–WNT signaling circuit between the neoplasia and stroma alluded to above as well as potentially directly inhibiting stromal development [45]. Several fascinating developments in stromal chemotherapy have originated from studies on tumor immunology. CD40 agonists were shown in both a human and a mouse model of pancreatic cancer to help disrupt desmoplasia through a reeducation of tumor-associated macrophages [66]. Agents that target both immunological and mesenchymal aspects of the tumor microenvironment are likely to play an increasingly important role in cancer therapy.

Conclusion

The mesenchymal contribution to cancer is diverse. New techniques such as specific mesenchymal transgenic lines and new discrete markers for flow cytometry, immunohistochemistry and single cell gene expression will all help to improve our understanding of the cancer-associated mesenchyme.

Stromal directed therapies are in early phase human trials with agents targeting many of the key biological processes in mesenchymal development. In the future therapeutic approaches will target local, bone marrow, and distant organ mesenchymal populations in order to help retard the initiation, progression and spread of cancer.

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Fig. 1.

1×10^6 Non-fluorescent MC38 cells were injected subcutaneously into red fluorescent C57BL/6 mice. The resulting tumor was harvested and digested and the single cell suspension stained for EpCam (APC), CD45 (APC), CD31 (APC), Ter119 (APC), CD105 (PE-Cy7) and CD140a (FITC). (A) CAFs were within the red fluorescent (recruited) CD31/CD45/Ter119 triple negative cell population 0.4%. The non-red and variably EpCam positive cancer cells made up the majority of the cells sorted (83%). The recruited hematopoietic and endothelial cells constituted about 12% of cells. (B) The CAF fraction (0.4%) was then sorted on the basis of CD105 and CD140a expression. (C) In keeping with a recent publication the stromal CD105+/CD140a+ population contained the greatest

proportion of clonogenic fibroblasts (1.4%). (D) This is an example of the colonies formed by these cancer-associated CFU-Fs.