Bone Marrow–Derived Stromal Cells Express Lineage-Related Messenger RNA Species

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Abstract

Evidence has emerged that bone marrow cells have a greater degree of plasticity than previously thought. However, there has been a call to establish proof that these bone marrow–derived cells function appropriately in their new environment. We have already shown that the bone marrow contributes to myofibroblasts in multiple organs and that this is exacerbated by injury and occurs in a mouse tumor model. Here, we provide evidence that these cells are functioning appropriately by showing that bone marrow–derived myofibroblasts are expressing mRNA for the α_1 chain of type I (pro)collagen using a new customized technique. This provides evidence that the bone marrow-tumor stroma axis is functionally relevant and may therefore subsequently be exploited to develop new strategies for anticancer therapy. (Cancer Res 2006; 66(3): 1265-9)

Introduction

Myofibroblasts are cells with features of both smooth muscle cells and fibroblasts. They are widely distributed, have roles in growth and differentiation, as well as the inflammatory response, and are characterized by their cytoskeletal elements, with myofibroblasts typically expressing α-smooth muscle actin (α-SMA). Myofibroblasts are important in injury and contribute to the processes of fibrosis and scarring where they produce extracellular matrix proteins including collagen (reviewed in ref. 1). In cancer, myofibroblasts are a major component of the desmoplastic stroma, and as such have a major influence on tumor behavior. Thus, the source of tumor-associated myofibroblasts is of particular interest.

Bone marrow can apparently contribute to cell types in a variety of organs (reviewed in ref. 2). Excitement has been tempered as this contribution may not represent true transdifferentiation (reviewed in ref. 3) and there has been a call for more rigorous investigation and the establishment of proof that these donor-derived cells are functioning in their new environment (2, 4).

Our group and others have shown that the bone marrow contributes to myofibroblast populations throughout the body (5) using experiments where sex-mismatched or green fluorescent protein (GFP)–positive bone marrow is transplanted into female or GFP-negative mice, respectively. These experiments have shown that the bone marrow contribution is increased by diverse types of injury such as skin wounding (5, 6), radiation-induced lung injury (7), experimental colitis (8), and, in human, liver damage (9). There is increasing evidence that the bone marrow contributes to myofibroblast populations in tumor stroma in mouse models (10, 11).

However, if these cells are simply inactive cells residing in the tissues, then their role in tumor development and propagation is of less significance. In addition, there would be less potential in their exploitation for the development of new antitumor therapy. To answer the call for evidence that bone marrow–derived cells are functional, we have developed a new protocol to combine *in situ* hybridization for DNA and RNA with immunohistochemistry in the same section. Using this method, we can show that individual cells in our mouse model of tumor stroma (*a*) immunostain for a marker of myofibroblastic phenotype such as α-SMA; (*b*) have a Y chromosome (i.e., are male in a female recipient of a male bone marrow transplant and are of donor origin); and (*c*) are expressing mRNA for α_1 chain of type I (pro)collagen [pro(α1)I]. This provides clear evidence that individual bone marrow–derived myofibroblasts have appropriate gene expression.

Materials and Methods

Mice. All animal work was carried out under the British Home Office procedural and ethical guidelines. Recipient mice transgenic for the rat insulin promoter II gene linked to the large T antigen of SV40 (RIPTag) develop solid β-cell tumors of the pancreas (12). Donor bone marrow was obtained from transgenic mice that express enhanced GFP driven by a chicken β-actin promoter [TgN(GFPU)5Nagy, strain 003115, The Jackson Laboratory, Bar Harbor, ME]. Four female RIPTag mice were transplanted with male GFP bone marrow between 8 and 10 weeks of age.

Transplant protocol. Young adult female recipient RIPTag mice underwent whole-body irradiation with 12 Gy in a divided dose, 3 hours apart, to ablate the bone marrow. This was followed immediately by tail vein injection of 1 million male/GFP-positive whole-bone marrow cells as previously described (5). The mice were housed in sterile conditions. The animals were killed and pancreata were harvested at ~8 weeks after bone marrow transplant. The pancreata were fixed in 10% neutral buffered formalin before being embedded in paraffin wax for analysis.

Immunohistochemistry. To identify donor cells in transplant recipient mice, tissue sections were immunostained for GFP or *in situ* hybridization for detection of the Y chromosome was done. To identify functioning donor-derived myofibroblast cells, sections were immunostained for α-SMA in combination with *in situ* hybridization for the Y chromosome and *in situ* hybridization for mRNA for pro(α1)I. To distinguish the myofibroblasts from hematopoietic lineages such as macrophages, double immunohistochemistry for α-SMA with either F4/80 or CD45 was done.

Immunohistochemistry for α-SMA and GFP was done as previously described using a three-step immunodetection protocol (5). For α-SMA, the primary antibody (α-SMA; mouse monoclonal clone 1A4, A-2547; Sigma, Poole, United Kingdom) was applied at a dilution of 1:4,000. For GFP (GFP rabbit polyclonal, A-6455; Cambridge Bioscience, Cambridge, United

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Kingdom), extra antigen retrieval was required in the form of microwaving for 10 minutes in 0.01 mol/L trisodium citrate at pH 6 before antibody application at a dilution of 1:500. After the three-step immunodetection protocol, sections were washed in PBS and Vector Red substrate (SK 5100, Vector Laboratories, Peterborough, United Kingdom) or 3,3′-diaminobenzidine (DAB) in solution was applied to develop red or brown precipitates, respectively. Sections were again washed in PBS before the *in situ* hybridization protocol. With sections that were to proceed to an *in situ* hybridization for mRNA protocol, care was taken to keep the sections RNase-free and solutions were autoclaved where possible. Slides were then taken for *in situ* hybridization for both DNA and RNA.

For double immunohistochemistry, immunohistochemistry for CD45 (CD45 rat polyclonal, 550539; Becton Dickinson Biosciences, Oxford, United Kingdom) was done using extra antigen retrieval in the form of microwaving for 10 minutes in 0.01 mol/L trisodium citrate at pH 6 before antibody application at a dilution of 1:100 on zinc fixed tissue. Visualization of the stain using DAB was done before immunohistochemistry for α-SMA as described above, which was then visualized with Vector Red.

Immunohistochemistry for F4/80 (F4/80 rat polyclonal, MCA497; Serotec Ltd., Oxford, United Kingdom) was done without extra antigen retrieval on acid alcohol–fixed tissue. As with CD45, the F4/80 was visualized with DAB before immunohistochemistry for α-SMA that was visualized with Vector Red.

***In situ* hybridization for DNA.** After immunohistochemistry with Vector Red, sections requiring *in situ* hybridization for DNA to visualize the Y chromosome were subjected to a protocol that had been extensively modified (in comparison with ref. 5) so that subsequent *in situ* hybridization for mRNA would retain maximal sensitivity. As before, taking care to keep sections RNase-free, sections that had not previously been microwaved were microwaved for 10 minutes in 0.01 mol/L trisodium citrate buffer at pH 6. Sections were washed in PBS and then digested with proteinase K (50 μg/mL in PBS) for a range of digestion times from 15 to 55 minutes (a total of five sections per animal). Digestion was quenched in glycine (0.2%, w/v) in double-concentration PBS for 2 minutes. Sections were postfixed in paraformaldehyde (4%, w/v) in PBS for 2 minutes and dehydrated through graded alcohols before air-drying. A FITC-labeled Y-chromosome paint (Star-FISH, Cambio, Cambridge, United Kingdom) was used in the supplier's hybridization mix. The probe mixture was denatured by boiling for 2 minutes and then cooled on ice. The preboiled probe was added to the sections, sealed under glass with rubber cement, and incubated overnight at 55°C to hybridize. The slides were then washed in 0.5× SSC at 37°C for 5 minutes. All slides were then washed with PBS and incubated with 1:250 peroxidase-conjugated antifluorescein antibody (150 units/mL; Boehringer Mannhein, Indianapolis, IN; http://www.roche-applied-science.com) for 60 minutes at room temperature. Slides were developed in DAB (5 mg/mL PBS) plus hydrogen peroxide (20 μL). Sections were then subjected to the protocol for *in situ* hybridization for mRNA.

***In situ* hybridization for RNA.** Following the protocol for immunohistochemistry plus or minus that for *in situ* hybridization for DNA, slides were subjected to the protocol for *in situ* hybridization for RNA using previously outlined methods (13). Specific localization of mRNA for pro(α1)I was accomplished by *in situ* hybridization using an antisense riboprobe synthesized with T3 RNA polymerase using [^{3}H]UTP (~800 Ci/mmol; Amersham plc., Amersham, United Kingdom) and plasmid was prepared from IMAGE clone 335137 linearized with *Eco*RI to yield an antisense probe which was used without hydrolysis. The region of sequence used to produce the riboprobe did not show significant homology to any other known gene sequences in the database. [^{3}H]UTP decays, releasing low energy particles (0.018 MeV) and hence improves spatial resolution (0.5-1.0 μm) compared with ^{35}S. Slides were pretreated with proteinase K (Sigma P4914; 20 μg/mL in prewarmed PBS for 10 minutes). The presence of hybridizable mRNA in all compartments of the tissues studied was established in near serial sections using an antisense β-actin probe. Autoradiography was at 4°C before developing in Kodak D19 and counterstaining with hematoxylin. Sections were examined under conventional or reflected light dark-field conditions (Nikon ME 600 with epi-illumination) that allowed individual autoradiographic silver grains to be seen as bright objects on a dark background.

Results and Discussion

We have previously shown the bone marrow contribution to myofibroblast populations in tumor stroma (10) by transplanting male bone marrow into female mice that develop β-cell tumors of the pancreas (Fig. 1*A*). To show the contribution of bone marrow–derived cells to tumor tissue, we did *in situ* hybridization for the Y chromosome (Fig. 1*B*). Numerous male cells were seen in these female mice; however, these could be inflammatory cells or other cells of the hematopoietic lineage. To provide evidence that these bone marrow–derived cells were myofibroblasts, we combined *in situ* hybridization for the Y chromosome with immunohistochemistry for α-SMA. We found male α-SMA-positive cells in the stroma of these tumors (Fig. 1*C* and *D*). A particular difficulty of this technique is that even when a tissue abundantly expresses mRNA for pro(α1)I (Fig. 2*A*), a marked proportion of this message is lost when immunohistochemistry and *in situ* hybridization for DNA are done on the same tissue (Fig. 2*B*). In addition, not every cell with a Y chromosome, even in male tissue, is detected when *in situ* hybridization for the Y chromosome is done. This is because sectioning of tissue results in the loss of part of the nucleus, which may contain the Y chromosome. Even in male mice, in only 39% of myofibroblasts associated with RIPTag tumors could a Y chromosome be detected although 100% should be positive. Together, these factors mean that counts of triple positive cells would be a dramatic underestimate. In this study, we have aimed to show that bone marrow–derived cells express mRNA for pro(α1)I, but for the above stated reasons any quantification would be fraught with error. To show that these cells were functioning, we looked for expression of mRNA for pro(α1)I (Fig. 3*A*). We show

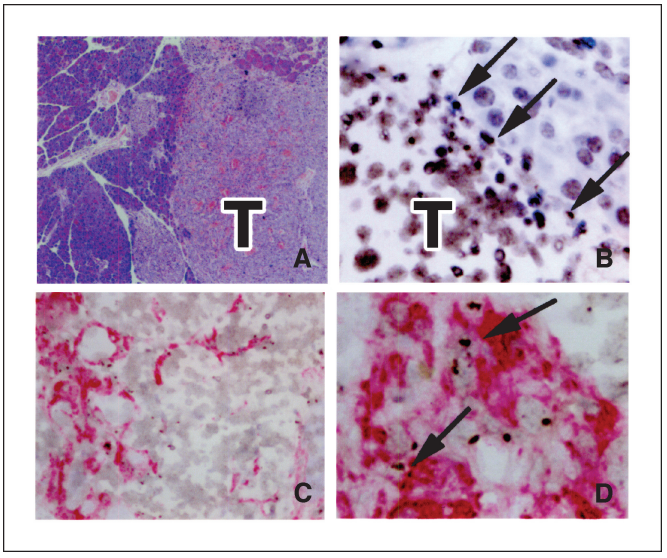

Figure 1. To show the contribution of bone marrow cells to tumor stroma. *A,* typical pancreatic β-cell tumor (*T*) that develops in the RIPTag mouse (×4). Donor-derived cells are identified following *in situ* hybridization for the Y chromosome. *B,* examination of the tumor border reveals numerous Y-positive cells (*brown dots, arrows*; ×60). The myofibroblast phenotype of male cells is illustrated by combining *in situ* hybridization for the Y chromosome with immunohistochemistry for α-SMA (*C* and *D*; ×20 and ×60); numerous donor-derived α-SMA-positive (red stain) cells are seen (*arrows*; *D*). Magnifications are objective magnifications.

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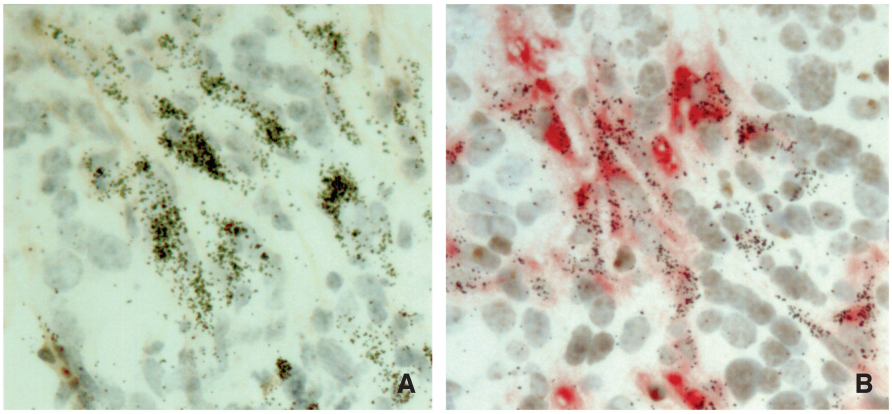

Figure 2. To show *in situ* hybridization for pro(α1)I on pancreatic tumor tissue with and without *in situ* hybridization for the Y chromosome. *A,* bright-field image of the tumor which has undergone the processes required for *in situ* hybridization for pro(α1)I (×40). A dense collection of silver grains is seen over almost all spindle cells totally obscuring their cytoplasm. *B,* a similar area is seen, which has been treated to allow detection of α-SMA, the Y chromosome, and pro(α1)I (×40). In this image, the grain density is greatly reduced, illustrating how much of the ribomessage has been lost in carrying out this combined technique. Magnifications are objective magnifications.

that myofibroblasts were the source of pro(α1)I in this model by combining immunohistochemistry for α-SMA with *in situ* hybridization for mRNA for pro(α1)I (Fig. 3*B*). Y chromosome–negative cells (Fig. 3*C*) provide evidence that apparently local myofibroblasts are one source of pro(α1)I. We were also able to show immunopositivity for α-SMA, binding of the isotopically labeled riboprobe for pro(α1)I mRNA, and positive *in situ* hybridization for the Y chromosome in the same cell, showing that bone marrow–derived cells are an additional source of collagen in the desmoplastic response (Fig. 3*D*).

Myofibroblasts are not the only source of collagen I; it can also be produced by cultured macrophages (14). To assess phenotypic overlap between myofibroblast populations and hematopoietic lineages, we did double immunohistochemistry for α-SMA in combination with CD45 and found that although CD45-positive cells may be located in close proximity to tumor tissue, CD45-positive cells seem to be a separate population. Figure 4*A* and *B* shows an insulinoma metastasis to a local pancreatic lymph node. As expected, the lymph node stained positive for CD45 whereas the tumor tissue did not. Myofibroblasts (α-SMA positive) are seen both within the lymph node and in tumor tissue but appear to be a separate population. To further delineate whether macrophages are a separate population to myofibroblasts, we combined immunohistochemistry for the mouse macrophage marker F4/80 with immunohistochemistry for α-SMA (Fig. 4*C* and *D*); these cell populations lie in close physical proximity to each other but appear distinct.

There is overwhelming evidence that tumor-associated myofibroblasts and fibroblasts influence cancer behavior. Myofibroblasts express a host of growth factors that modify tumor growth,

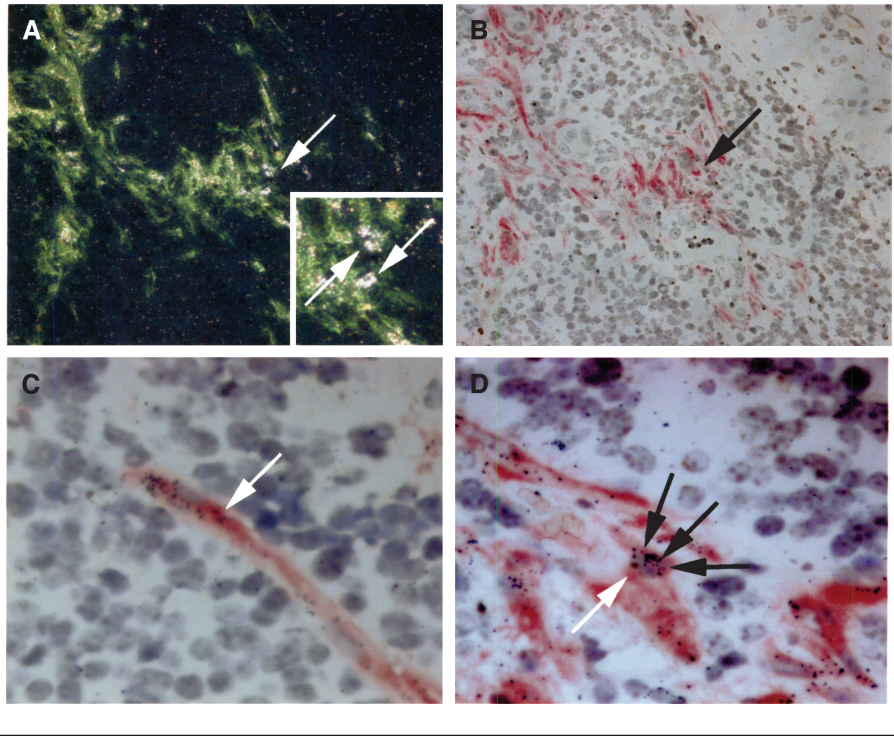

Figure 3. To show the functional activity of bone marrow–derived tumor stromal cells. *A,* dark-field image of a tumor section that has undergone a combination of *in situ* hybridization for DNA and RNA with immunohistochemistry for α-SMA to reveal functioning myofibroblasts (×20). The immunohistochemical stain shows spindle cells as green but white areas representing the autoradiographically visualized isotopically labeled riboprobe for pro(α1)I are present (*white arrows*); this area is magnified in the inset (×30). A bright-field image of the same area (*B*; ×20) shows the α-SMA-positive area (red stain). At higher power (*C*; ×50), α-SMA-positive cells (red stain) are clearly seen to be producing mRNA for pro(α1)I (*black grains, white arrow*). Functional donor-derived myofibroblasts are seen with all three markers in the same cell (*D*; ×50)—α-SMA red stain, along with expression of mRNA for pro(α1)I (*black grains, black arrows*) and detection of the Y chromosome (*brown dot, white arrow*). Magnifications are objective magnifications.

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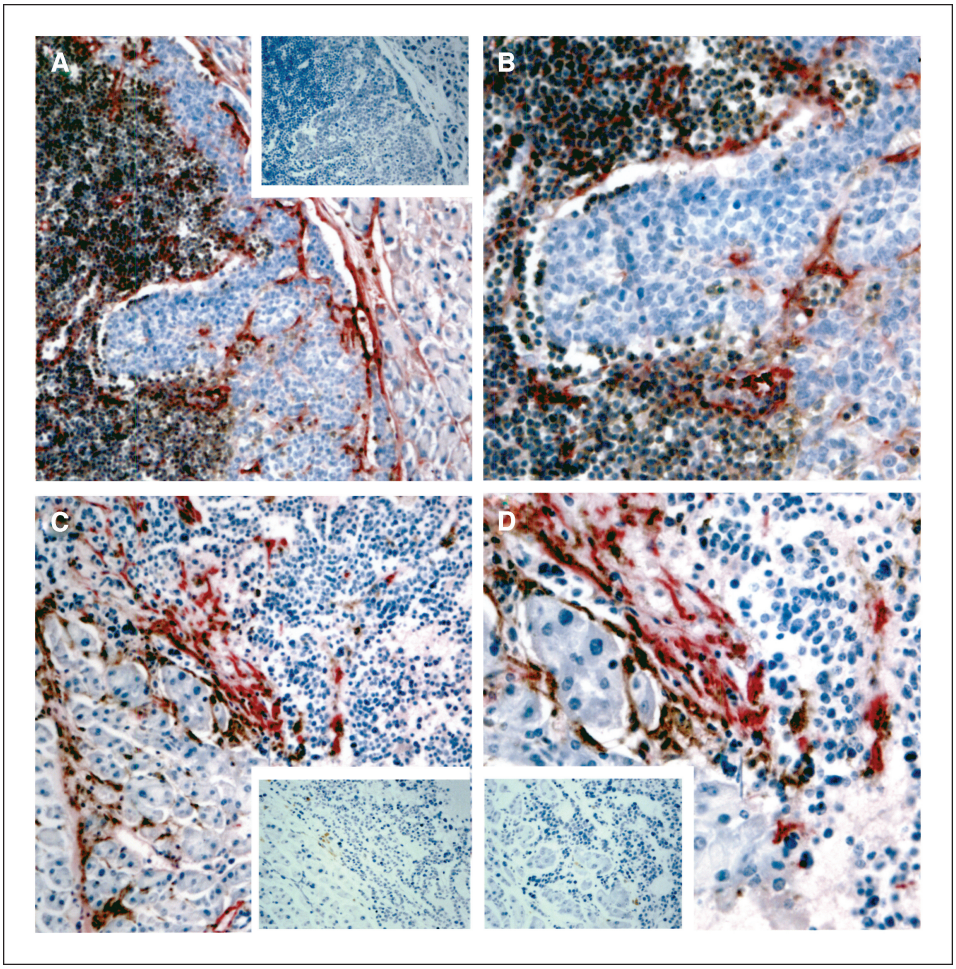

Figure 4. To show myofibroblast populations are distinct from cells of the hematopoietic lineage. *A* and *B,* tumor-involved pancreatic lymph node in a RIPTag mouse immunostained for CD45 (*brown*) and α-SMA (*red*; ×20). Although the two cell populations are in close proximity, they seem to be distinct. *Inset,* the same tumor infiltrated lymph node but omitting the primary antibody as a negative control for CD45 (×20). *C* and *D,* pancreas of a RIPTag mouse immunostained for F4/80, a mouse macrophage marker (*brown*), and α-SMA (*red*; ×20 and ×40). The tumor is seen to be invading the surrounding pancreas. The populations of F4/80-positive cells and α-SMA-positive cells are in close proximity but seem to be distinct. *C* and *D, insets,* the same tumor tissue but omitting the primary antibodies for F4/80 and α-SMA, respectively. All magnifications are objective magnifications.

such as transforming growth factor β and insulin-like growth factor (reviewed in ref. 1), and in human breast cancer, myofibroblast secretion of SDF-1 not only stimulates tumor growth but also aids in the recruitment of endothelial progenitor cells (15).

Our observations that the bone marrow contributes to tumor-associated stroma are less significant if these bone marrow–derived cells are not functional. One of the major functions of tumor-associated myofibroblasts is the production of extracellular matrix. In our experiment to show that bone marrow–derived cells are functioning, it was necessary to show that a single cell is α-SMA positive, is expressing mRNA for pro(α1)I, and is also male in a female recipient.

Evidence to support the functional activity of bone marrow–derived stromal cells has been more limited. Ishii et al. (16) have also suggested that bone marrow–derived fibroblasts express type I collagen. In their study, wild-type mice were transplanted with GFP-positive bone marrow. Bone marrow–derived cells were subsequently identified by virtue of GFP positivity after cancer implantation or skin wounding. These cells were identified as fibroblasts by histologic features. The bone marrow–derived cells were shown to express CD45, a finding we expect in cells of a hematopoietic lineage; this is at variance with our findings where the bone marrow–derived myofibroblasts are a separate population from the CD45-positive cells. To show that bone marrow–derived cells were expressing collagen I, double immunohistochemistry for GFP and collagen I was done by Ishii et al. However, as macrophages may also express collagen type I (14), there was potential for error. In our study, bone marrow derivation, phenotype, and collagen expression have been considered in single cells, thus avoiding this potential problem. Our results increase our understanding of the source of tumor stroma and may also lead to the development of new methods of cancer therapy. Clues to the source of exogenous stromogenic cells come from a number of groups. Abe et al. (17) found fibrocytes circulating in peripheral blood, which rapidly enter sites of injury and make collagen I, contract collagen gels, and express α-SMA. This builds on previous work by Friedenstein, who investigated the bone marrow as a source of nonhematopoietic fibroblastic cell types (reviewed in ref. 18). These fibrocytes may originate from the bone marrow, and further work by Prockop (18) suggests that marrow stromal cells repopulate the bone marrow after transplantation and subsequently act as a source of progenitors for a variety of mesenchymal tissues. Other groups have used bone marrow cells as delivery vehicles for anticancer agents: human mesenchymal stem cells were transduced with an adenoviral vector carrying the human IFN-β gene and were injected into immunodeficient mice with established xenografted human tumors, resulting in improved survival compared with controls (19). There is accumulating evidence that the stromal myofibroblast is a key player in the control of tumor cell behavior (20) and will become an important target for therapy.

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References

1. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. Am J Physiol 1999;277:C1–9.
2. Grove JE, Bruscia E, Krause DS. Plasticity of bone marrow-derived stem cells. Stem Cells 2004;22:487–500.
3. Alison MR, Poulsom R, Otto WR, et al. Recipes for adult stem cell plasticity: fusion cuisine or readymade? J Clin Pathol 2004;57:113–20.
4. Anderson DJ, Gage FH, Weissman IL. Can stem cells cross lineage boundaries? Nat Med 2001;7:393–5.
5. Direkze NC, Forbes SJ, Brittan M, et al. Multiple organ engraftment by bone-marrow-derived myofibroblasts and fibroblasts in bone-marrow-transplanted mice. Stem Cells 2003;21:514–20.
6. Mori L, Bellini A, Stacey MA, Schmidt M, Mattoli S. Fibrocytes contribute to the myofibroblast population in wounded skin and originate from the bone marrow. Exp Cell Res 2005;304:81–90.
7. Epperly MW, Guo H, Gretton JE, Greenberger JS. Bone marrow origin of myofibroblasts in irradiation pulmonary fibrosis. Am J Respir Cell Mol Biol 2003;29:213–24.
8. Brittan M, Chance V, Elia G, et al. A regenerative role for bone marrow following experimental colitis: contribution to neovasculogenesis and myofibroblasts. Gastroenterology 2005;128:1984–95.
9. Forbes SJ, Russo FP, Rey V, et al. A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. Gastroenterology 2004; 126:955–63.
10. Direkze NC, Hodivala-Dilke K, Jeffery R, et al. Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. Cancer Res 2004;64:8492–5.
11. Ishii G, Sangai T, Oda T, et al. Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. Biochem Biophys Res Commun 2003;309: 232–40.
12. Hanahan D. Heritable formation of pancreatic β-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature 1985;315: 115–22.
13. Poulsom R, Longcroft JM, Jeffery RE, Rogers LA, Steel JH. A robust method for isotopic riboprobe *in situ* hybridisation to localise mRNAs in routine pathology specimens. Eur J Histochem 1998;42:121–32.
14. Vaage J, Lindblad WJ. Production of collagen type I by mouse peritoneal macrophages. J Leukoc Biol 1990; 48:274–80.
15. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 2005;121:335–48.
16. Ishii G, Sangai T, Sugiyama K, et al. *In vivo* characterization of bone marrow-derived fibroblasts recruited into fibrotic lesions. Stem Cells 2005;23: 699–706.
17. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J Immunol 2001;166:7556–62.
18. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71–4.
19. Studeny M, Marini FC, Dembinski JL, et al. Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. J Natl Cancer Inst 2004;96:1593–603.
20. Desmouliere A, Guyot C, Gabbiani G. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int J Dev Biol 2004;48:509–17.

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